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Proteomics, Metabolomics, Interactomics and Systems Biology

Paul C. Guest *Editor*

Proteomic Methods in Neuropsychiatric Research

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Proteomics, Metabolomics, Interactomics and Systems Biology

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Preface

Hundreds of biomarker candidates for psychiatric diseases have been described in the scientific literature over the last 20 years. However, the rate of introduction of new tests into the clinical arena is much lower with less than a handful appearing on the marketplace. This disconnect is most likely due to inconsistencies at the discovery end including technical variations within and between proteomic platforms, a lack of validation of biomarker candidates as well as a lack of awareness within the research community on the criteria and regulatory matters for integrating biomarkers into the pipeline [1]. Another potential reason relates to the fact that psychiatric diseases are heterogeneous in nature and are comprised of different subtypes. This can cause difficulties in studies attempting to identify biomarkers since different investigators may analyse cohorts comprised of unique or even mixed subtypes of a particular disease. Furthermore, the use of patient and control groups in clinical studies which have not been stratified according to biomarker profiles is one of the biggest causes of failure in the development of new drugs [2–5].

Many of these problems could be due to the fact that the definition of psychiatric disorders has been based traditionally on symptoms alone. This may be a problem as different psychiatric disorders can display similar symptoms and the same disease may show symptom diversity. However, there are no empirical means of validating the symptom classification approach. There is now a scheme proposed by the National Institute of Mental Health (NIMH) that might be of use in improving classifications. This is called the Research Domain Criteria (RDoC) which is another way of classifying psychiatric diseases based on observable behaviours and neurobiological measurements [6]. Another major challenge for identification of biomarkers for psychiatric diseases is that proper development of tests requires a considerable number of validation steps, which means carrying out repeat studies at different sites from where the original test was developed and using larger cohorts. Also cost-effectiveness should be a priority when methods are translated into clinical use. Many existing tests for psychiatric disorders are simply too expensive [7–10]. This factor is related to the clinical utility of the test and avoidance of expenses related to misdiagnosis and disease-related burdens. Of course the ultimate goal of developing biomarker tests for psychiatric disorders is to aid

psychiatrists and clinicians in real-world settings. It is this last point where most if not all tests have fallen by the wayside.

One way of addressing these issues is through the increasing use of tests developed from proteomic techniques as these can provide a more complete picture of a disease. Proteomics allows the most real-time assessment of molecular phenotypes relative to other omic platforms [11]. Furthermore, most existing drugs for brain disorders target proteins such as G-protein-coupled receptors (GPCRs) and ion channels [12, 13]. Proteomic methods can simultaneously measure proteins in one experimental run on a single instrument as opposed to methods that measure only one analyte at a time such as single-plex immunoassays. This allows for lower sample and reagent requirements along with reduced processing times on a per assay basis. In contrast, testing for single analytes can be laborious, time consuming and expensive in cases where multiple parallel assays for different molecules are required. Most importantly, the use of a biomarker test comprised of multiple protein measurements allows for greater accuracy in the diagnosis of complex diseases like psychiatric disorders by providing more complete information about the perturbed physiological pathways in a shorter time period.

However, there are still challenges ahead. While some diseases are increasingly being treated according to biomarker profiling patterns, the one disease/one drug approach is still the norm. Psychiatric diseases can present difficult choices for clinicians when it comes to deciding on treatment options since multiple physiological parameters can be disrupted. Other variable such as age and gender can affect treatment options leading to even greater variability. In order to deal with this issue, collaborative research networks should be established for proteomic techniques to better integrate biomarker discovery in real time to targeted therapeutics. In 1988, the Clinical Laboratory Improved Amendments (CLIA) act was passed by the United States Congress as a way of integrating quality testing for all laboratories and to ensure accuracy, reproducibility and speed of patient testing results [14]. The Food and Drug Administration (FDA) is the responsible agency for applying these regulations to categorizing all biomarker-based tests. This is not straightforward as clinical validation will require participation of multiple laboratories and the resulting platforms are likely to need simplification stages and demonstration of increased robustness to merit widespread clinical use. Such tests may also require the use of an algorithm comprised of multiple clinical parameters as well as biomarker profiles for increased classification or diagnostic performance.

This book includes a series of reviews on general aspects of biomarker use in the study of psychiatric and neurodegenerative disorders as well as detailed protocols describing multiplex proteomic techniques such as multiplex immunoassay, two-dimensional gel electrophoresis, liquid chromatography tandem mass spectrometry (LC-MS/MS), selective reaction monitoring (SRM)-MS and isobaric tagging for relative and absolute quantitation (iTRAQ)-MS. It will also describe protocols for generation of preclinical models used in the study of certain aspects of psychiatric diseases, such as the maternal low-protein and phencyclidine-treated rat models, and cellular approaches including the use of neuronal precursor cells, MK-801-treated oligodendrocytes, peripheral blood mononuclear cells (PBMCs), fibroblasts

and analysis of whole pituitary extracts. Since clinical applications in point-of-care settings necessitate that platforms are small, user-friendly and fast, clinical procedures for setting up a biomarker test trial and the latest developments including lab-on-a-chip and mobile phone applications will also be described.

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Chapter 1
Application of Proteomic Techniques for Improved Stratification and Treatment of Schizophrenia Patients

Johann Steiner, Paul C. Guest, and Daniel Martins-de-Souza

1.1 Introduction

Schizophrenia is a debilitating and costly psychiatric disorder which appears to strike individuals in their late teen or early adulthood years and gravely impair health, quality of life, social and emotional well-being as well as productivity in the workplace and society in general [1]. The clinical presentation of this disease usually occurs with appearance of symptoms such as hallucinations, delusions, disorganized thoughts, cognitive impairment, deficits in social perception and anhedonia. Although this disease has been well recognized for more than 100 years, the diagnostic procedures have remained at a standstill and are still based on symptom manifestation. Furthermore, this procedure still relies on communications between the patient and a health worker, clinician or psychiatrist. This usually takes the form of an interview and can employ the Diagnostic and Statistical Manual of Mental

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Disorders (DSM) [2] or the International Classification of Diseases (ICD-10) [3] categorizations as guidelines. However, these can only be used to identify symptoms and have no input into pinpointing the underlying molecular physiological pathways that may be disrupted. One major problem of this is that classification of a person as having schizophrenia may be confounded by the common finding that individuals with other psychiatric disorders (such as organic psychoses, affective disorders or borderline personality disorder) may suffer from similar symptoms. This has led to concerted efforts by researchers and clinicians with the aim of identifying specific biomarker tests that can be used for predicting the onset of schizophrenia, improving diagnostic accuracy, monitoring disease progression and treatment response and even for guiding treatment options. To facilitate use in clinical settings, it would be important that these biomarker tests are developed for use in blood, serum or plasma. This is because blood-based biomarkers would be highly accessible and easy to standardize in clinical practice due to the low invasiveness and simplicity of the sampling procedure and the low associated costs.

The application of biomarker-based diagnostic tests that can accurately classify patients according to the type of disorder, or even the disease subtype, should help reduce the duration of untreated illness and improve patient responses by the simple fact that the right patients have been administered the best possible treatments for their specific situation, as early as possible. This is important since there is an undeviating correlation between longer periods that are devoid of treatment with poorer patient outcomes [4]. It is expected that incorporation of a biomarker-based approach will help revolutionize the current paradigm of reactive care to a new order involving optimized personalized treatments in psychiatry as well as in other fields of medicine (Fig. 1.1). In addition, by implementing earlier, more effective treatment, we should see a reduction in patient referrals to secondary services, including hospitals, community groups and crisis teams. Any decrease in the use of such expensive services will help to lower the overall financial burden associated with schizophrenia and other psychiatric disorders, which totalled more than 60 billion dollars per year in the 1990s, in the USA alone [5]. Most importantly for the patients,

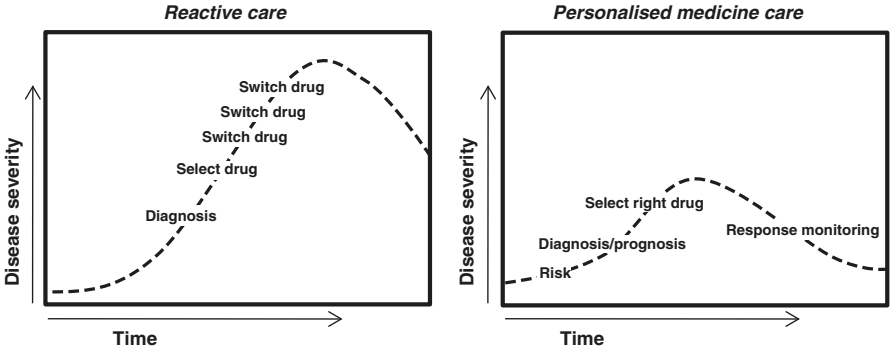

Fig. 1.1 Comparison of the old and new treatment paradigms in schizophrenia, distinguished by the use of biomarkers for improved patient stratification

an early efficacious intervention will help to reduce symptom severity. This is due to the fact that schizophrenia currently leads to decades of life disability [6], which surpasses the effects seen by other disorders such as cardiovascular disease [7].

The discovery of validated proteomic-based biomarker panels that can link clinical and molecular data is likely to advance future mental healthcare significantly. This is especially true if such tests can be incorporated into standard operating procedures as part of the clinical decision-making process and deployed as fast, user-friendly and cost-effective point-of-care devices. Although there are many ways of classifying biomarkers, the strictest system requires that new biomarker test results must be replicated multiple times in different laboratories and in different sites. This will be difficult to achieve for psychiatric disorders such as schizophrenia, since these conditions are only poorly understood at the molecular level and they are highly heterogeneous in the way that they are exhibited in different patients [8]. In this chapter, we discuss the challenges of developing and implementing proteomic biomarker tests for schizophrenia. We also focus on the use of biomarker tests for improved classification and management of patients with schizophrenia for improved treatment approaches and to help rekindle drug discovery efforts across the pharmaceutical companies in the area of psychiatric disorders.

1.2 Diagnosis

Many psychiatrists and clinicians now accept that schizophrenia is an all-purpose idiom for an assortment of psychiatric illnesses which have similar symptoms [8]. In other words, the variety of psychiatric manifestations labelled as "schizophrenia" do not necessarily represent a single disease entity. Instead, schizophrenia is a hypothetical construct created several decades ago by leading figures of the time and is now defined by international classification boards, which have marginally amended the inclusion criteria to fit with emerging hypothesis over time. It is not surprising that given this disease heterogeneity and plasticity in the classification systems that misdiagnosis can occur in psychiatric practice. One investigation found that more than 30% of patients who were initially diagnosed as having schizophrenia actually had bipolar disorder [9]. Another study challenged the foundations of the existing classification methods by highlighting the fact that there are no current means of validation which do not call upon the same concepts, in a circular manner [10]. In reality, most psychiatrists do not routinely use these classification systems for making their diagnosis. Instead, this is based on their training, experience and personal views, and therefore diagnosis is carried out in a more heuristic manner. Of course this has its own problems as it can result in mistakes caused by misconceptions, biases or selective memories.

The DSM and ICD-10 classification systems are actuated on the basis that psychiatric disorders such as schizophrenia are discrete diseases with common root causes and which can be defined by criteria based on signs and symptoms. In the real world, specific symptoms are not always linked to defined diseases. For

example, there are situations in which individuals can present with symptoms that occur in schizophrenia in instances of trauma or psychotropic drug use, or in cases of certain infectious diseases or metabolic conditions [11, 12]. In addition, a diagnosis can change over time. One study found significant changes in diagnosis involving a switch from major depressive disorder to either bipolar disorder or schizophrenia [13], and another investigation found that only half of the patients diagnosed initially with a particular psychiatric disorder stayed on this diagnosis [14].

1.3 What Causes Schizophrenia?

The concordance for diagnosis of schizophrenia across identical twins ranges from 10 to 70% [15–17]. This provides some evidence that there can be a genetic predisposition for schizophrenia, but it also highlights the important point that schizophrenia will not necessarily develop even when a causative genetic factor may be present. In reality, this indicates that environmental and other non-genetic factors may also be important. Precipitation of schizophrenia can be caused by pregnancy or delivery complications, such as infections, hypoxia or malnutrition [18, 19]. It can also result from non-biological factors such as social stress, experiencing a natural disaster, loss of a family member or chronic experience of an agonizing situation such as an intolerable work condition, a dysfunctional or destructive family life or an abusive relationship [20]. Although such situations are unfortunate, the role of environmental elements leads to the hope that disease prevention or minimization might be possible if such factors can be identified and avoided.

It is easy to envisage that environmental factors such as poor nutrition, social stress or physical trauma can affect a person's physiological state. Several research groups have now shown that metabolic abnormalities such as insulin resistance occur in some schizophrenia patients at their first clinical presentation [21–23], and others have found that perturbations in circulating inflammation-related molecules can occur [24, 25]. Whether these changes are a cause or effect of the disease has not been established, two recent studies have shown that such changes can occur months to years before full clinical manifestation of schizophrenia symptoms [26, 27]. Not only does this provide some evidence that perturbations in these molecular pathways may play a role in the aetiology, it gives some hope for identifying those individuals at risk of developing the disease. This is important as numerous reports have now described the benefits of early intervention therapeutics for individuals who have a high risk of developing schizophrenia [28–30]. Conversely, delayed diagnosis can lead to serious detrimental effects on the lives and health of the patients. For example, they may experience an unchecked full-blown psychosis which could lead to other problems such as substance abuse, alienation from friends and family, troubles in the workplace and the potential of self-harm [31, 32]. There is also the problem of misdiagnosis which can result in initiation of the wrong treatments, and these can either be ineffective or even cause harm to the patient.

Furthermore, this can have socioeconomic consequences, such as absence from work, harmful effects on family and relationships and inflated medical costs [33].

1.4 The Need for Blood-Based Proteomic Biomarkers

The European health authorities have shown their interest in the development and implementation of biomarkers in modern medicine by establishing agencies such as the Innovative Medicines Initiative (IMI) [34, 35]. This initiative began through a partnership between the European Commission and the European Federation of Pharmaceutical Companies and Associations (EFPIA) with the overall objective of improving health by speeding up the development of innovative medicines, particularly in areas where there is an unmet medical or social need and increasing patient access to these medications. A key objective is the discovery of biomarkers which can be incorporated into drug discovery pipelines for development of new medications and for use in human clinical studies. The European Commission contributed one billion euros to this project and this has been matched in kind by contributions from the participating companies. For the IMI 2 programme (2014–2024), the total budget is 3.28 billion euros, with 1.64 billion euros coming from European Union, 1.43 billion euros committed by EFPIA and 0.21 billion euros potentially being committed by scientific institutions who become involved as participants in projects.

Diagnostic biomarker tests in the USA are regulated by organizations such as the Clinical Laboratory Improved Amendments (CLIA) agency [36]. CLIA imposes regulatory standards that govern any tests are designated for use in a clinical setting for the purpose of diagnosis, disease prevention, treatment or health assessments. Commercially available tests marketed under CLIA are categorized by the Food and Drug Administration (FDA) according to potential health risks. The development of diagnostic biomarker tests for any disease requires repeated demonstrations of precise characteristics including performance scores such as correct identifications (sensitivity) and incorrect classifications of a control as a disease case (specificity). The latter is particularly important in the case of schizophrenia given the symptomatic and molecular overlap with other psychiatric disorders and even some somatic diseases. Excellent classification performance is an absolute requirement of developed tests since biomarker measurements can be affected by many variables including ethnicity, gender, environmental conditions, sample collection procedures and analytical variables. For example, the development of any multiplexed assay requires the testing and validation of each component assay as well as the combination of assays used in each multiplex to maximize repeatability, precision and accuracy. This includes ensuring that each individual assay has sufficient dynamic range and the required limits of detection [37].

Another criterion of biomarker tests is that they must be in a format that is robust and yet simple, user-friendly and fast to allow applications in the clinic by clinicians,

technicians and healthcare staff without the necessity of specialized training. Along these lines, we suggest that an automated system based on multiplexed immunoassay is a likely candidate as a clinically friendly platform as it has already shown some promise in this area. In addition, portable mass spectrometers are now in use in some airports which can detect hazardous substances such as bomb-making ingredients within seconds. Other devices which can be used to measure biomarkers in body fluids which have been collected on a swab or strip are also a possibility.

1.5 Biomarkers Identified for Schizophrenia

Although genomic studies have been used to identify risk genes for some diseases, these cannot be used to indicate that the disease is actually present. Instead, proteomic biomarkers are required as these can give a real-time readout of physiological function. Recent years have seen the increasing use of proteomics as a tool for the discovery of biomarkers for diagnosis, monitoring disease progression, treatment response and identification of novel therapeutic targets. However, analysis of central nervous system (CNS) disorders such as schizophrenia is difficult because the brain is not readily accessible for molecular diagnostic purposes. For this reason, sources such as serum and plasma have been undergoing increasing scrutiny in proteomic investigations of psychiatric disorders due to their higher utility in the clinic.

1.5.1 Biomarkers Associated with Inflammation

One multiplex immunoassay profiling study of cytokine profiles found increased levels of interleukin (IL)-1β in cerebrospinal fluid from first onset schizophrenia patients [38], consistent with the idea of an altered inflammation response in the brains of some patients (Fig. 1.2) [39, 40]. In addition, changes in inflammation have been linked to alterations in the glutamate system, the main excitatory neurotransmitter in the brain. In addition, transcriptomic and proteomic profiling studies of post-mortem brains from schizophrenia patients have identified increased levels of inflammation-related gene products in oligodendrocytes and endothelial cells in comparison to nonpsychiatric control subjects [41, 42]. However, it is possible that some of these effects may be related to prolonged drug treatment or an unhealthy lifestyle, as often occurs in the chronic or latter stages of individuals suffering from this disorder [43].

The finding of changes in circulating molecules such as inflammatory factors is what makes blood-based biomarker testing feasible in studies of psychiatric disorders [44]. A meta-study of circulating inflammation-related changes in schizophrenia patients showed that cytokines such as IL-12, soluble IL-2 receptor, interferon-γ and tumour necrosis factor-α may be useful as trait biomarkers, indicating that the

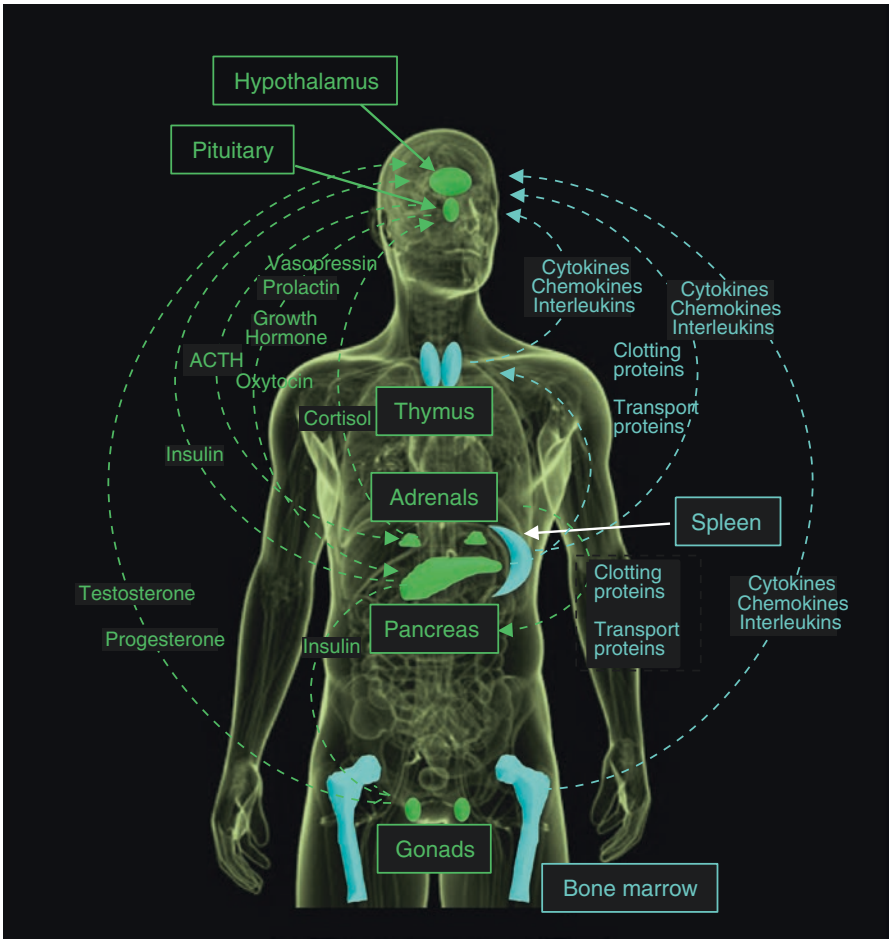

Fig. 1.2 Peripheral and central signalling molecules affected in schizophrenia with a focus on inflammation (*blue*) and hormonal/metabolic (*green*) pathways. The dashed arrows indicate connections via the bloodstream. ACTH = adrenocorticotrophic hormone. Note that the interleukins, cytokines, transport proteins and clotting factors are not listed individually for presentation reasons. See text for more detail

disease is present [44]. On the other hand, the same study showed that cytokines such as IL-1β, IL-6 and transforming growth factor-β may represent state biomarkers, which means that they could be used as readouts for acute changes in the disease [45]. In addition, there have been many reports on the discovery of blood-based biomarker signatures consisting of a high proportion of inflammation-related proteins, including some components of the clotting cascade and transport proteins in first onset schizophrenia patients [46, 47].

It is not too surprising that many biological pathways cross-react or act in concert with each other in carrying out their functions. Thus, effects on one pathway can

influence that of another. Inflammation in the periphery can affect brain function through effects on the hypothalamic-pituitary-adrenal (HPA) axis (see Fig. 1.2 above) [48, 49]. In this scenario, activated inflammatory pathways stimulate secretion of corticotropin-releasing factor from the hypothalamus, and this initiates a cycle involving release of adrenocorticotrophic hormone (ACTH) from the pituitary, which in turn stimulates cortisol release from the adrenal cortex [50]. Along with other effects in the periphery and brain, cortisol also exerts a negative feedback control on the HPA axis by binding to specific receptors in the brain and pituitary [51]. The association with psychiatric disorders comes from the fact that the HPA cycle is also involved in control of neurotransmitter systems throughout the brain, which are involved in regulation of mood and behaviour. Given this link, it is not surprising that some investigators have tested the use of anti-inflammatory drugs such as aspirin or cyclooxygenase-2 (COX-2) inhibitors in combination with traditional antipsychotics as a possible therapeutic approach to relieve some symptoms of schizophrenia, and the initial results have shown some promise [52–55]. However, these findings require validation in additional studies involving larger cohorts and different clinical sites.

1.5.2 Biomarkers Associated with Neuroendocrine Functions

A number of studies have now shown effects on a number of hormonal systems related to growth and metabolism in schizophrenia. Studies over the past decade have identified impaired fasting glucose tolerance, high insulin levels and insulin resistance in first onset [21, 22] and chronic schizophrenia patients [55–57]. Three multiplex immunoassay profiling studies found that first onset schizophrenia patients had increased levels of circulating insulin-related peptides compared to controls [23, 58, 59], and one of these also found that patients had high levels of chromogranin A, pancreatic polypeptide, prolactin, progesterone and cortisol, but lower levels of growth hormone [59]. Taken together, these findings indicated altered secretion from several neuroendocrine glands, including pancreatic β cells, pancreatic PP cells, anterior pituitary, adrenals and the sex organs (Fig. 1.2). All of these changes would be expected to have effects on brain function. Chronically high insulin levels have been found to increase brain inflammation, aberrant phosphorylation of synaptic structural proteins and amyloid plaque deposition [60–62]. High insulin levels can also cause alterations in neurotransmitter pathways [63] and perturb synaptic plasticity in brain regions such as the hippocampus [64]. The finding of increased cortisol levels is indicative of an activated HPA axis, which has been identified as a risk factor for schizophrenia in adolescents [65]. Changes in other hormones have also been found to occur in schizophrenia. For example, one study showed gender-specific effects on the sex hormones oestradiol and testosterone in schizophrenia patients [66]. Another study found decreased serum levels of thyroxine, triiodothyronine and thyroid-stimulating hormone [67], which may be associated with metabolism-related hormonal changes, as described above. It is likely that

some of these hormonal pathways are co-regulated in a feedforward-feedback relationship between different components of the diffuse neuroendocrine system. As an example, increased insulin secretion from pancreatic β cells has been associated with elevated prolactin secretion [68] and disrupted pulsatile release of growth hormone from the respective lactotrophic and somatotrophic cells in the anterior pituitary [69].

It has been known for decades that schizophrenia patients treated with antipsychotics can also exhibit high insulin levels. This is due to the fact that these drugs can induce metabolic side effects such as insulin resistance and weight gain. Interestingly, this gain in weight appears to be linked to antipsychotic therapeutic efficacy. One investigation found that changes in body weight, blood glucose and leptin levels were associated with improvement of both positive and negative schizophrenia symptoms [70]. However, it has now been suggested that such metabolic changes may not be an absolute requirement for antipsychotic efficacy. This was demonstrated through studies which co-administered antidiabetic and antipsychotic drugs which led to reduced weight gain and insulin resistance without impeding the psychiatric benefits [71]. Also, one study found that patients with mild Alzheimer's disease who were administered the antidiabetic drug pioglitazone showed improvements in cognition [72]. From these findings, it is clear that the relationship between metabolism and psychiatric symptoms requires further scrutiny.

Drugs targeting other hormonal systems have also been assessed as potential new treatments for schizophrenia symptoms. Dehydroepiandrosterone (DHEA) is an adrenal steroid-like compound, which has undergone testing as a potential add-on drug in combination with antipsychotics. One study found that this combination led to improvements in depression and anxiety symptoms in some schizophrenia patients [73]. Another study showed that treatment with raloxifene, a selective oestrogen receptor modulator, resulted in reduced negative symptoms in postmenopausal females with schizophrenia, in comparison with controls who did not receive the drug [74].

1.5.3 Proteomic Biomarkers for Prediction of Treatment Response

Biomarker tests that can be used for better classification of schizophrenia patients opens up possibilities of better treatment options. For example, biomarkers that can be used to predict response of schizophrenia patients to therapeutics would be an important step forward for the patients themselves as it could mean that they receive more effective treatments more rapidly. It could also assist the prescribing physicians as it could help to guide their decision on which drug to administer and to select the dosage. In addition, pharmaceutical companies could benefit in their conducting clinical trials by using biomarker test results to help in the stratification of patients prior to the trial and for monitoring responses throughout the actual study.

A genetic study has shown that polymorphisms in the histamine 2 receptor gene (*HRH2*) can be used to predict response to clozapine treatment in 76% of schizophrenia cases [75]. Other genetic studies have shown that variants in genes for dopamine receptors, serotonin receptors and enzymes involved in drug metabolism or neurotransmitter turnover can have influence of patient response to treatment, including the tendency to develop specific side effects [76]. Another way of potentially predicting response is through the use of physiometric measurements such as waist circumference, adipose composition and body mass index (BMI). Such measurements taken at the start of treatment have already been used to predict the development of side effects such as metabolic syndrome or insulin resistance with good sensitivity and specificity [77, 78]. In addition, some blood-based proteomic studies have been carried out with good results. One study showed that schizophrenia patients with higher levels of serum prolactin tend to have a better outcome following 5 years of antipsychotic treatment [79]. Two multiplex immunoassay serum profiling studies found that the levels of insulin were predictive of improvement in negative symptoms [80] and a panel composed of specific apolipoproteins, growth factors, hormones and cytokines could be used to predict weight gain [81] in first onset schizophrenia patients after 6 weeks of antipsychotic treatment (Table 1.1). Another multiplex immunoassay study showed that the levels of the heart form of fatty acid binding protein (H-FABP) could be used to predict response of first onset patients to olanzapine treatment [82]. It is important to note that these three multiplex immunoassay studies involved first or recent onset patients. Therefore, similar studies of more chronic patients might yield different results. Further studies aimed at retesting these prototype biomarker panels may lead to development of validated

Table 1.1 Significant associations between the levels of specific proteins measured at baseline with (**a**) psychiatric symptom scores (positive and negative syndrome scale – PANSS) and (**b**) body mass indices (BMI) after 6-week treatment with antipsychotics

(a)				
	Positive symptoms		**Negative symptoms**	
Protein	*P*-value	*R*	P-value	*R*
Insulin	NS	–	0.005	−0.37
(b)				
Protein			**ANCOVA**	***R***
Apolipoprotein CIII			0.019	−0.33
Apolipoprotein H			0.005	−0.33
Epidermal growth factor			0.025	−0.28
Follicle-stimulating hormone			0.043	−0.28
Interleukin 18			0.015	0.24
Interleukin 25			0.024	−0.26
Interleukin 6 receptor			0.031	−0.30
Matrix metalloproteinase 1			0.011	−0.24
Placental growth factor			0.016	−0.24
Thyroid-stimulating hormone			0.026	−0.23

R Spearman correlation coefficient, *NS* not significant, *ANCOVA* analysis of covariance [80, 81]

molecular tests that can be used to identify those patients who are more likely to respond to particular antipsychotic medications as well as those who are likely to benefit from an add-on compound that targets either the inflammatory or metabolic symptoms. This could also lead to the opportunity for clinicians to take decisive actions such as patient assessment, counselling or readjustment of drugs or dosages as guided by measured biomarker readouts.

1.6 Future Perspectives

For decades, psychiatrists have acted on good faith that psychiatric disorders such as schizophrenia are caused by defects in the brain. While this is undoubtedly true to some extent, developments over recent years have resulted in formation of a new concept that involves the whole body in precipitation or progression of these diseases. This is not too surprising since the brain is intimately linked in most fundamental biological functions of the body. Therefore, at least some functions of this organ can be monitored by determining whether or not any changes have occurred in the molecular composition of the blood [46, 47, 83]. This is useful since blood can be taken from living patients at different stages of the disease or during a course of treatment. In the foreseeable future, it is likely that increased biomarker testing by clinicians will lead to more extensive "bio-"signatures in individuals that reflect the physiological status of the patients more accurately than ever before. Blood serum and plasma samples contain many molecules such as hormones, growth factors and cytokines which can only be detected using methods that are highly sensitive. One of the best methods to achieve this is the sandwich format of immunoassay [84, 85], and this is the basis for the multiplex immunoassay platform described above.

Multiplex immunoassay biomarker tests have now been available for more than a decade on medium-sized laboratory equipment and with typical turnaround times of around 1 week for analysis of multiple samples from the sample preparation stages to the final results analysis. It is possible that single samples could be run against standards and quality controls and results returned in less than 1 day. More recently, multiplex methods have been developed using micro-fluidics in devices that are approximately the size of a credit card [86]. This offers the possibility of an inexpensive and rapid analysis using electrochemical or optical readouts on real time. These approaches are also user-friendly as no expertise is required for operation of the device or interpretation of the results. The protocol involves application of a blood drop to a slot in the card followed by insertion of the card into a book-sized analyser/reader and a diagnostic "score" can be read out in less than 15 min. The major benefit of this approach is the rapid turnover time, and this will help to minimize waiting periods for lab test results, which can often take several days or even weeks using standard methods. Furthermore, these devices can connect to a computer for transmission of data to a smartphone device. Companies such as Apple and Google are now showing interest in the diagnostic market and exploiting the potential of linking diagnostic test results with an app

driven by smart software. This would allow test results to be linked with mobile communication systems. This could be particularly useful in the field of mental disorders such as schizophrenia, since these are generally long-term conditions that require constant monitoring of both the disease and treatment effects. A review of clinical trials involving medical care interventions facilitated by smartphone monitoring showed that patient outcomes were improved in more than 60% of the cases [87]. Recently, miniature multiplex immunoassays have been developed on a hand-held smartphone-based colorimetric reader using a 3D-printed opto-mechanical interface [88]. This device has now been tested successfully in a clinical microbiology laboratory using mumps, measles and herpes simplex I and II virus immunoglobulin assays. It is not hard to imagine that similar tests for other diseases such as psychiatric disorders will be available in the not-so-distant future.

1.7 Conclusions

This chapter has described recent advances using proteomic-based biomarker tests which can be used for improved diagnosis and classification of individuals with schizophrenia. The ultimate goal is to provide more informed treatment options for improved patient outcomes. The use of miniaturized proteomics assays on hand-held devices like those described in this chapter would provide many potential benefits for patients. These include increasing our understanding of the array of the affected molecular pathways, facilitating identification of disease subtypes, helping to select the most appropriate treatments and monitoring treatment responses. This could include patient self-monitoring guided by feedback from the internet cloud and psychiatrists as required (Fig. 1.3). For example, many patients show distinct patterns of blood-based molecules which suggest the presence of perturbed inflammation- or metabolism-related pathways as described in this chapter. Thus, improved classification of such patients based on biomarker profiling would enable selection of better treatment options, including add-on therapeutics that target these pathways. Finally, the use of multiplex tests on hand-held devices capable of achieving some or all of these objectives would be an important breakthrough in point-of-care medicine. This would help to improve the lives of individuals suffering from this debilitating disorder, along with those of their friends and family, and have beneficial effects on society as well as significant cost savings for the healthcare services.

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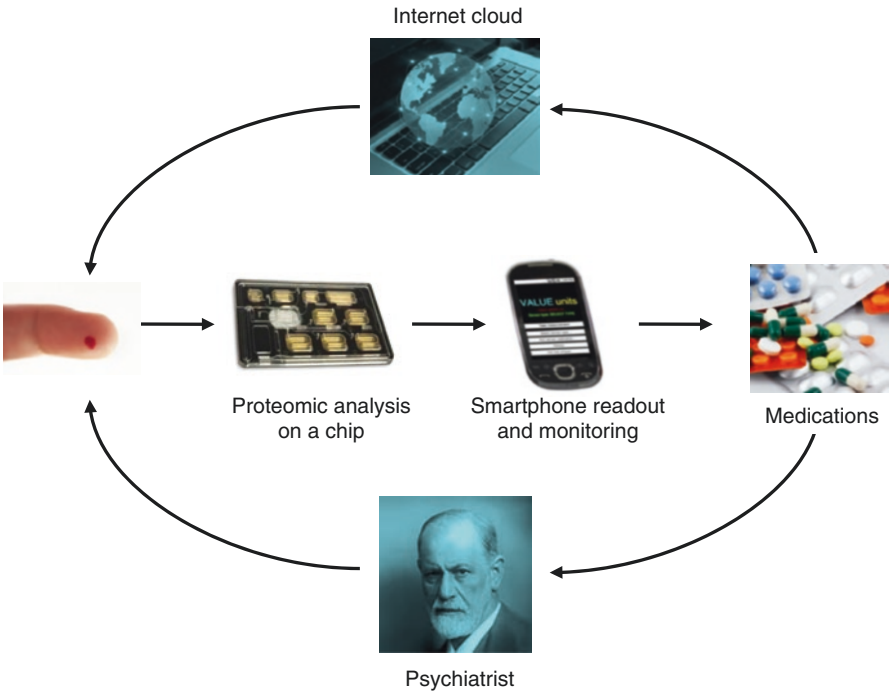

Fig. 1.3 Schematic diagram showing point-of-care monitoring of schizophrenia patients using lab-on-a-chip proteomic readouts

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Chapter 2
Multiplexing Biomarker Methods, Proteomics and Considerations for Alzheimer's Disease

Renã A.S. Robinson, Bushra Amin, and Paul C. Guest

2.1 Introduction

Recently, President Barack Obama signed a bill to provide a $122 million increase for Alzheimer's disease (AD) research, education, outreach and support for caregivers (www.alz.org). This bill came in response to the demands pushed by the Alzheimer's Association which, along with many other organizations and institutions, recognized that there is much work to be done to alleviate suffering and eliminate this devastating neurodegenerative disease. AD is projected to affect approximately 15 million persons in the United States [1] and more than 115 million persons worldwide [2] by the year 2050. It is the leading cause of dementia among persons aged 65 years and older and is the sixth leading cause of early death. The disease is characterized by progressive memory loss and impaired cognitive function both of which are irreversible physiological symptoms. In the early stages, psychiatric symptoms such as depression [3] can be evident, and the late stages of the disease can be accompanied by symptoms more commonly seen in schizophrenia, such as delusions and hallucinations [4]. Age is the biggest risk factor for AD, and with a growing elderly population, millions more will be affected by this disease. Other risk factors for AD include cardiovascular diseases, high blood pressure, diabetes, obesity, high cholesterol, kidney disease and psychiatric disorders [3–6]. A vast majority (>95%) of AD cases are sporadic. Genetic mutations in amyloid precursor protein, presenilin 1 and 2, are associated with familial forms of disease [7–13], and apolipoprotein E allele type 4 (APOE4) increases risk in most populations [14–16]. Other genes associated with AD include *SORL1*, *DAPK1*, *BIN1*,

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CR1, *CLU*, *CD33*, *ABCA7*, *PLD3*, *TREM2*, *UNC5C*, *AKAP9* and *ADAM10*, and some are associated with subpopulations of AD patients defined by ancestry or geographic location [17, 18].

The major pathological and physiological hallmarks of AD are the well-characterized amyloid-beta (Ab) peptides and neurofibrillary tangles (NFT), hypometabolism indicated by lower brain glucose levels, brain atrophy, mitochondrial dysfunction, neuronal loss and oxidative stress. Increased research efforts over the last few decades have helped to advance our understanding of the pathological events associated with AD. Aβ peptides are toxic species that oligomerize in the brain and form deposits known as senile plaques. The concentration of Aβ peptides, in particular Aβ (1–42), correlates with physiological and pathological symptoms [19, 20]. These peptides can circulate through the bloodstream and periphery and were the first established molecular biomarkers for AD. Tau protein hyperphosphorylated at threonine 181 (pTau) results in the formation of NFT. Total tau (tTau) protein and pTau levels present in cerebrospinal fluid (CSF) were the next biomarkers developed which have high sensitivity and specificity for AD diagnosis [21–24].

2.2 Clinical Diagnosis of AD

AD is not fully diagnosed until autopsy in which neuropathological analysis of brain tissues is performed. Clinical diagnosis of AD is based on noted changes in cognition and behaviour and can include information from the patient's family and medical histories [25–27]. Neuropsychological evaluations can also be performed including a panel of cognitive tests and psychiatric evaluations, such as in the mini-mental state examination (MMSE). The sensitivity of clinical diagnostic tests is 81% and the specificity is 70% [28]. While this has been useful for more than 30 years, there is a need for tests with improved diagnostic performance as there are closely related dementias and other disorders that present with similar symptoms in the clinic. Products for home screening of AD are also available such as the Alzheimer's Home Screening Test and Minnesota Cognitive Acuity Screen [29, 30], and there are genetic screening tests for APOE4 [31].The National Institute of Neurological and Communicative Disorders and Stroke and Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA) which was established in 1984 which set a number of standardized criteria for clinical diagnosis of AD [25, 26]. These criteria are important for distinguishing the three stages of AD: (1) pre-clinical AD, before symptoms occur but there are changes in the brain; (2) mild cognitive impairment (MCI), when initial symptoms occur and dementia is likely to occur within a few years; and (3) early- and late-onset AD, when dementia is diagnosed and there are significant neuropathological changes occurring [27]. One of the difficulties in diagnosing AD comes from the fact that changes can occur in the brain some 20 years before a clinical diagnosis is made [32, 33]. It is important to highlight the point that patients can convert from different disease stages. For example, some MCI patients can convert to AD, but in more rare cases, MCI patients can

convert back to a cognitively normal state. Such situations make it challenging to develop a diagnostic test that can account fully for all of these clinical stages of AD. With this in mind, in 2011, the Alzheimer's Association included the importance of developing molecular biomarker tests to help standardize clinical diagnoses [26].

2.3 Psychiatric Symptoms

Although decreased cognitive performance and decline in memory are the core features of AD, a variety of psychiatric symptoms can also occur over the different disease stages [34–37]. These symptoms include disturbances in motivation, mood and perception, as well as delusions and inappropriate behaviours. A 5-year follow-up study found that 97% of AD patients experienced one or more of these symptoms, with depression or anxiety having the highest frequency [35]. A recent meta-study showed that the most frequent psychiatric symptom in AD patients was apathy (49%), followed by depression (42%), aggression (40%), anxiety (39%), sleep disorder (39%), irritability (36%), appetite disorder (34%), aberrant motor behaviour (32%), delusions (31%), disinhibition (17%), hallucinations (16%) and euphoria (7%) [38].

Depression is a common occurrence in AD patients and may lead to a faster cognitive decline compared to disease progression in AD patients without psychiatric symptoms [3].The incidence of depression in other reports is approximately 40% in AD [34], and the signs of this are identical to those seen in major depressive disorder [36]. The typical symptoms include a low mood but irritability and anxiety may also be present. The cognitive decline in AD may be more severe in those patients with depression, but treatment with antidepressants first may be required to establish whether or not the depression is a cause or effect [37]. However, the onset of depression may be the first sign of AD in the aged population [39, 40].

Schizophrenia-like psychotic symptoms such as delusions and hallucinations occur in around 50% of AD patients, and this form of the disease is termed AD with psychosis [4, 41–45]. This is typically a more severe phenotype as cognitive decline occurs more rapidly than in AD patients without psychosis. Imaging studies have found that AD psychosis patients also have more severe synaptic impairments as shown by lower grey matter volume and reduced regional blood flow and glucose metabolism. In addition, neurological studies have reported consistently higher accumulation of pTau in AD patients with psychosis [4].

Correct identification of these apparent AD subtypes which exhibit either depressive or psychotic symptoms is now an important clinical need considering the potential detrimental effects on further accelerating cognitive decline. In addition the identification of biomarkers corresponding to these AD subtypes will aid in the early stratification of patients to initiate earlier and more appropriate treatment for improved long-term outcomes.

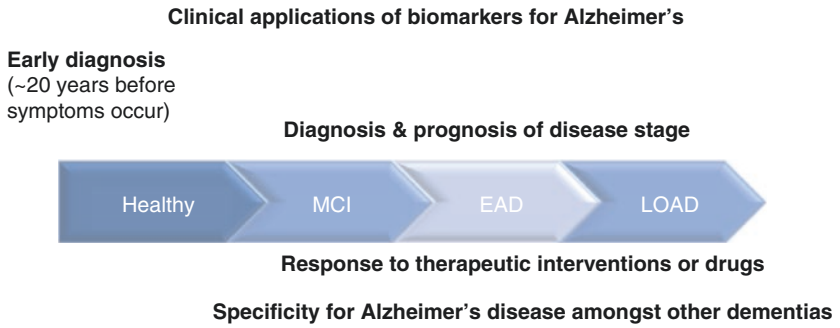

Fig. 2.1 Clinical applications for biomarkers in Alzheimer's disease research along the spectrum of cognitively normal to disease stage. MCI = mild cognitive impairment, EAD = early AD, LOAD = late-onset AD

2.4 Biomarkers of AD

Biomarkers can be used for both diagnostic and prognostic purposes in AD (Fig. 2.1). The requirements for a biomarker include the ability to measure a pathologic process, predict outcome, distinguish disease or measure a pharmacological response to a drug treatment or therapeutic intervention. Biomarkers should be easy to use, convenient, cost-effective and, more importantly, have high sensitivity (i.e. >80%) and specificity (i.e. >80%) [46]. With regard to AD, there are a number of promising biomarkers that are currently under investigation for clinical implementation. Because of the mixed pathology that is often found in dementia patients [28, 47–49], it is important that there are also biomarkers that have the ability to distinguish AD from other closely-related disorders such as frontotemporal dementia, dementia with Lewy bodies, Parkinson's disease, amyotrophic lateral sclerosis, psychiatric conditions and others. There is a high heterogeneity in patients across the AD spectrum, from MCI to late-stage onset of AD, and there is still considerable work to be done to fully standardize clinical diagnosis criteria across different clinical laboratories. Biomarkers are not limited in type and can be detected using various body fluids and sampling methods as will be discussed below.

2.4.1 Imaging Biomarkers

Magnetic resonance imaging (MRI) is used to measure whole brain atrophy, which is one of the hallmarks of AD [50–53]. Brain atrophy increases with disease progression and is associated with cognitive decline, as well as a potential risk factor for AD [50]. MRI equipment has become more widespread despite the high cost of carrying out these scans. In addition, some work still remains to standardize acquisition methods of MRI scans and to implement similar analysis

algorithms across laboratories [54]. Decreased cerebral uptake of glucose is another major pathological hallmark of AD and can be used as a diagnostic marker of disease even at potentially early stages and with high sensitivity [55–57]. Glucose uptake is measured by labelling with radioactive [^{18}F] glucose and using fluorodeoxyglucose positron emission tomography (FDG-PET) to determine which portions of the brain are hypometabolic. Typically, hypometabolism is localized to brain regions responsible for language and working memory and tracks with disease progression, age of onset and risk of disease [58, 59]. Aside from glucose, there have been advances in using amyloid PET ligands for detecting fibrillar Aβ deposition and in vivo monitoring of disease progression, notably through use of the Pittsburgh compound B (PIB) [56, 60]. High brain amyloid load as measured by PIB-PET is a potential diagnostic biomarker for AD and has promise as an early biomarker of the disease [61]. However, a number of challenges still remain regarding dynamic range, ligand selectivity and qualitative visual versus quantitative classifications, expensive costs and coverage [27, 60]. Although imaging biomarkers of brain processes are relevant in AD, it has become apparent that other biomarkers from the periphery may help to resolve some of the issues (Fig. 2.2). Fluid biomarkers would also be more widely accepted and appreciated by patients, especially if they are less invasive and cost-effective.

2.4.2 Cerebrospinal Fluid (CSF) Markers

CSF is obtained through a semi-invasive procedure from patients with lower lumbar puncture and is a rich medium for biomarker development and information about disease pathogenesis. CSF contains cargo proteins that arise from neuronal secretory vesicles; common markers such as Aβ, tTau and pTau; and numerous other proteins [27] that vary in cognitively impaired individuals [21, 54, 62, 63]. In particular, YKL-40, an astrocyte-derived inflammatory response protein, and other proteins, such as carnosinase I, chromogranin A and NCAM, may help with early disease diagnosis [64, 65]. Inflammatory markers in CSF are endophenotypic of different genetic variants in AD [66–68]. For example, TREM2 soluble forms are secreted at lower levels in AD patients compared to healthy controls [69]. The most promising biomarkers to date that come from CSF are Aβ (1–42) peptide, tTau and pTau (Thr181). Across a number of research groups, the associations of these markers with the disease have been consistent whereby Aβ (1–42) is present at lower levels and tTau and pTau levels are higher in AD patients compared to cognitively normal controls [70–72]. CSF Aβ peptide markers in particular have also been reflective of brain amyloid load [73, 74] and protein aggregation [70]. Total tau and pTau levels are characteristic phenotypes of patients with memory complaints [54], and pTau levels may help to distinguish AD from other forms of dementia [75]. It is important to highlight that Aβ (1–42), tTau and pTau markers when measured together provide better sensitivity and specificity compared to the performance of the individual markers [62] and can predict the conversion of MCI to AD. Other Aβ

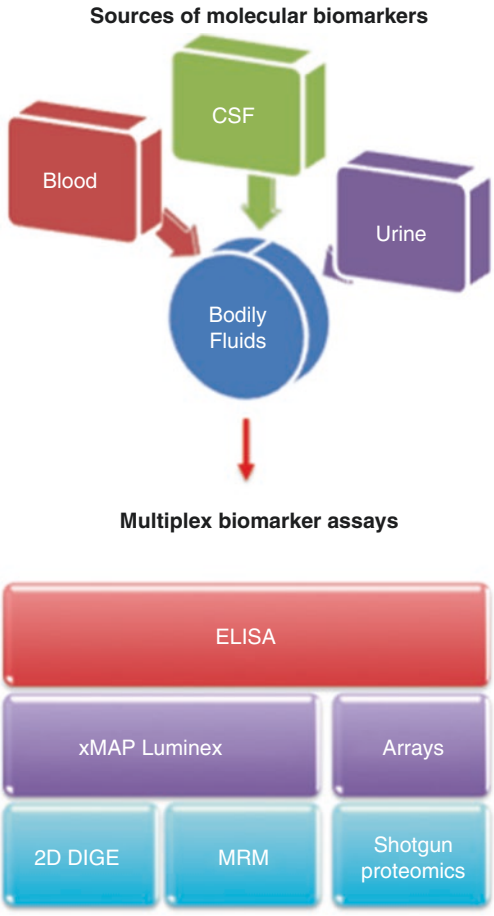

Fig. 2.2 Sources of body fluids that can be used for molecular biomarker analysis and most commonly used multiplexing biomarker assays

peptides found in CSF such as Aβ (1–37) and Aβ (1–38) have been shown to distinguish AD from other closely-related dementias [76].

However, there is inconsistency across laboratories regarding the concentrations that describe "low" Aβ (1–42) levels and "high" tau levels in CSF [54, 70–72, 77, 78]. One potential reason for this is the protocols for CSF collection, measurement assays and internal standard calibrators have not been standardized in the field [54]. Clinically, CSF collection in dementia and probable dementia cases is not common practice and requires some standardization. To date, CSF biomarkers are the closest to being developed as clinical biomarkers with moderate specificity and sensitivity for prediction of dementia in individuals with memory difficulties [26]. Another potential use of CSF biomarkers is as a marker of blood-brain-barrier (BBB) integrity using the ratio of CSF to serum albumin [70].

2.4.3 Urinary Biomarkers

Studies on identification of urinary biomarker candidates for AD have been limited. The most notable urinary marker is neuronal thread protein (NTP), a brain localized protein which is representative of AD pathology [79, 80]. High levels of AD-associated NTP (AD7c-NTP) in urine and CSF are indicators of AD but only in patients who already have AD [80–82]. Recent reports show that NTP in urine may also be diagnostic of MCI [83]. Because of the non-invasive nature in the collection of urine biomarkers, this may still be worth exploring further. Potential markers would not necessarily have to be proteins since metabolites are more readily extracted and give direct insight into metabolic pathways. For this reason, metabolomic studies of AD bodily fluids are underway, and there are recent reviews on the topic [84–86].

2.4.4 Blood-Based Biomarkers

The periphery has recently garnered a lot of attention as playing a critical role in Alzheimer's disease and for being a potential source of relevant biomarkers [46, 70, 87]. Ideally, a blood-based biomarker would be representative of biochemical and pathological changes in the brain that are diagnostic of disease, disease stage and progression. Because blood circulates through different organs including the brain, it contains a number of molecules that can give insight into pathophysiological changes in disease [88]. Furthermore, a number of regulatory proteins such as hormones, growth factors and inflammatory molecules are known to mediate two-way communication between the central nervous system and the periphery [89], and many of these have altered levels in AD and have been measured in blood [90]. Blood-based biomarkers can be used to measure response to therapeutic interventions or specific drug treatments [70]. Plasma levels of Aβ (1–42)/(1–40) in some cases are reflective of AD pathogenesis although there has been some conflicting information reported [54, 88]. For example, plasma Aβ levels have little correlation with CSF levels or PIB-PET imaging data and have a small predictive value for development of AD. This inconsistency makes CSF measures of these targets even more attractive. Other caveats to use plasma are that Aβ levels reflect both brain- and peripherally-derived versions of this peptide, the dynamics of circulation from the brain to periphery across a compromised BBB and binding to other plasma and membrane proteins [46]. Other studies have shown that decreased APOE4 levels in plasma are associated with neuronal degradation [16], and this therefore has some potential as a plasma biomarker. However, there is inconsistency in measurements of APOE4 plasma levels in AD which is likely to be due to the lack of standardized protocols for plasma collection, storage conditions, freeze/thaw cycles and variations in downstream analyses [e.g. antibody- or mass spectrometry (MS)-based techniques] [27, 54, 87, 88]. For example, study

participants are not required to fast overnight in some AD research centers but are in others [46].

Cytokines and chemokines have been shown to have variable levels in blood of AD patients [68], and it is likely that such molecules are taken up by the brain. System-wide dysregulation of the immune system in AD is reflected in plasma levels of cytokines such as interleukins, tumour necrosis factor and macrophage migration inhibitory factor [68, 91]. However, it is not clear if brain cytokines leak into the periphery or if peripheral cytokines cross the BBB and make it into the central nervous system or to what extent both of these situations occur. Cytokine arrays and multiplex assays have been helpful in identifying peripheral inflammatory processes in AD through study of potential markers including IL-8, TNFR-I, clusterin, IL-1, IL-7, IL-6, serum amyloid A, CCL15 and CXCL9 [92–95].

The search for blood-based biomarkers or those in the periphery presents some challenges. For example, aging, which is the highest risk factor for AD, will undoubtedly change the levels of different types of peripheral markers [70]. Factors such as diet, medication, stress and circadian rhythm [96] can influence peripheral Aβ levels as well as the levels of other plasma proteins. In addition, co-morbid conditions including hypertension, hypercholesterolemia, diabetes, obesity, vascular diseases and psychiatric disorders in AD patients may also manifest with differences in the plasma levels of specific proteins [70]. Gender [97] and genetics [98] are also factors that may have an influence on peripheral protein levels. Plasma is a complex matrix in which highly abundant protein species such as albumin, chymotrypsin, fibrinogen and immunoglobulins mask potential markers that are lower in abundance. The dynamic range issues of proteins in plasma are analytically challenging to tackle [99] and often require immunodepletion and fractionation strategies and the use of highly sensitive mass spectrometry instruments. Other challenges to use blood-based biomarkers include differences in the criteria used to select study participants for a given biomarker assay which can affect the study outcome. Despite these widely recognized challenges, there are still a growing number of researchers using plasma for biomarker discovery in AD, presumably for its increased accessibility over CSF as a biomarker source.

Aside from plasma, other blood fractions such as exosomes [100, 101], extracellular vesicles [102] or peripheral mononuclear lymphocytes [82] may also be useful as a source of potentially interesting biomarker candidates for AD. Exosomes are a smaller subset of microvesicles that circulate in body fluids and have sizes of around 100 nM in diameter. Blood-based exosomes have been implicated in AD [103] and may be reflective of dysregulations in cellular signalling and protein misfolding. Some microRNAs, which are small non-coding RNAs derived from various cell types and important in intracellular communication, have been isolated from CSF and blood with different levels in AD although issues related to sampling and time-points selected for analysis exist [104, 105].

2.5 Multiplex Biomarker Technologies

Single biomarker assays are straightforward and detect only a single species in clinical sample analysis. However, single biomarkers are not likely to be able to serve as the best diagnostic or prognostic markers for AD due to limited discriminatory power. On the other hand, biomarker panels comprised of multiple measured analytes provide both high sensitivity and specificity for distinguishing AD patients from cognitively healthy individuals [62, 90, 106]. Below, we focus attention on antibody and MS-based proteomic assays that have been used for biomarker development or biomarker discovery studies in AD (Fig. 2.2) and briefly highlight others.

2.5.1 Multiplex Immunoassay

Immunoassays can be multiplexed by using a standard 96-well plate in which each well plate is coated with a different antibody targeting a protein of interest. To date, there is only one multiplex immunoassay test that is used for AD diagnostics, called is the INNOTEST. This test measures pTau, tTau and Aβ(1–42) levels in CSF [107]. In addition, multiplex immunoassays have identified 16 serum molecules that can distinguish AD patients from controls [108]. There are some disadvantages with immunoassays in general. For example, the antibodies may have nonspecific interactions that limit overall sensitivity, and specific antibodies may have limited availability or require generation [46]. Immunoassays are limited further by the need for several wash steps, laboratory personnel errors resulting in intra- and inter-lab variability and a relatively high (20–30%) coefficient of variation [109] in comparison to other analytical platforms [88]. Recommendations for the optimal design of immunoassays have been recently put forth and may help minimize some of these issues [110].

Another format of the multiplex immunoassay uses color-coded microspheres with covalent attachment of monoclonal antibodies, peptides, receptors or other antigens on the surface. The clinical sample can be mixed with multiple surface coated beads to analyse several targets in a single analysis. This works because the beads can be discriminated in a flow cytometry-based analyzer. This is known as the x multi-analyte profiling (xMAP) assay and is employed on a commercially available Luminex analyzer. xMAP assays have already been used as a diagnostic and early indicator of AD [22, 84, 90, 106, 111, 112]. There are approximately 350 assays currently available in the xMAP panel (http://rbm.myriad.com/), and many of these have now been analyzed in AD plasma, representing a range of biological pathways [113, 114]. The Luminex analyzer is state-of-the-art technology but is relatively expensive in most laboratory settings and requires some optimization during initial stages in development of new assays. Inter-laboratory variability which is a limitation of standard immunoassays is also a limitation of xMAP and is likely to be due to issues related to non-standardization of fluid collection and storage as well

as data processing and analysis [88]. There is also the possibility of cross-reactivity or non-specificity across some of the antibodies in a given multiplex [115] although standardization can help to minimize some of these variations [27, 88, 116, 117].

After the initial demonstration by Ray et al. of the use of xMAP Luminex assay for AD diagnosis [90], there have been several reports of potential AD biomarkers identified in both CSF and plasma [54, 78, 116, 118–120]. Thus far, this has resulted in production of two commercially available AD diagnostic tests. The first is the INNO-BIA AlzBio3 kit (Innogenetics-Fujirebio, Ghent, Belgium) which is used to detect Aβ(1–42), tTau and pTau in CSF [117]. The sensitivity and specificity of this assay are >95% and >85%, respectively [121, 122], although these are dependent on cut-off levels set across different laboratories. Total analysis time of the three CSF biomarkers is less than a day, and the assay can measure as low as 10 pg/mL of these analytes in 150 μL of CSF [116]. The second kit is the INNO-BIA plasma Aβ kit which only measures Aβ (1–40) and (1–42) [123]. Efforts are underway to include other CSF proteins, such as YKL-40 and AD7c-NTP in the xMAP assays [27]. In addition, CSF proteins such as heart-type fatty acid-binding protein have been correlated with AD progression using these assays [124].

2.5.2 Other Proteomic Approaches in Biomarker Development for Alzheimer's Disease

Current state-of-the-art shotgun proteomics assays can measure up to ten thousand human proteins in a single analysis of tissues [125, 126] due to significant advances in MS instrumentation. There are a number of proteomics platforms that can be used in biomarker discovery; however, 2D gel electrophoresis methods, such as 2D difference gel electrophoresis (DIGE), multiple reaction monitoring [MRM, also known as selective reaction monitoring (SRM)] and quantitative shotgun proteomics, are among the most commonly used biomarker identification strategies, as discussed below.

2.5.2.1 Two-Dimensional Difference Gel Electrophoresis (2D-DIGE)

Based on 2D polyacrylamide gel electrophoresis (PAGE) approach, 2D-DIGE allows up to three samples to be multiplexed and quantified on each gel. 2D-DIGE is based on size- and charge-matched fluorescent Cy-dyes (Cy2, Cy3 and Cy5) that are attached to proteins prior to electrophoresis. Differential imaging at distinct excitation wavelengths of each Cy-dye allows one to monitor the differences in proteomic profiles between different functional states or disease stages more accurately. This is advantageous for overcoming non-biological variations such as gel-to-gel spatial spot intensities, field strength, pH gradient, inconsistencies of gel

composition and handling errors of the traditional PAGE system. Cy-dyes are N-hydroxy-succinimidyl ester derivatives that attach covalently with the ε-amino group of lysine residue of proteins and replace the ε-amino group positive charge with the positive charge of the dye. Since Cy-dyes are hydrophobic, the dyes label only one lysine residue per protein and prevent protein precipitation. Additionally, they also cause alterations of isoelectric point and dye-induced mass shifts of labelled proteins during electrophoresis. 2D-DIGE analysis of CSF from different research groups has led to the discovery of the potential biomarkers YKL-40 [64, 65], NCAM [65, 127], NPR [127, 128], α-1-β-glycoprotein [129, 130], β-trace [129, 130], DKK-3 [131] and proteins of the complement pathway [128, 130, 132, 133] (Table 2.1). In addition, analyses of plasma by 2D-DIGE have led to the incorporation of proteins such as APOE [138, 163], α-2-macroglobulin [165], complement factor H [165, 166], vitamin D-binding protein [166] and plasminogen [164] into the AD biomarker validation pipeline. However, technical limitations of the resolution of the 2D-DIGE approach at the extremes of pH and molecular weight have driven the increased use of shotgun proteomics techniques for identification of AD biomarker candidates.

2.5.2.2 Quantitative Shotgun Proteomics

Similar to gel-based proteomics, shotgun methods are untargeted and allow multiple novel candidates across a number of biological pathways to be investigated. Greater sample multiplexing is available with isobaric labelling techniques, such as tandem mass tags (TMT), isobaric tags for relative and absolute quantitation (iTRAQ), dileucine (diLeu) and stable isotope labelling of amino acids in cell culture (SILAC) [167, 180–183]. Each of these methods relies on the MS instrument to keep track of the sample origin from different groups and report on the relative concentrations of peptides from protein samples. TMT reagents offer 10-plex and diLeu reagents come with12-plex capability. This level of multiplexing can assist with experimental designs that compare across disease groups (healthy, AD), disease stages (healthy, MCI, preclinical AD, LOAD), longitudinal measures and drug outcomes in fewer analyses than with label-free untargeted approaches. Combinations of isobaric labelling approaches such as combined precursor isotopic labelling and isobaric tagging (cPILOT) developed by our laboratory [184–186] and others [187] can double or even triple the number of sample multiplexing channels. This would have high utility in large-scale screening of analytes and patient groups such that thousands of targets can be monitored in a single sample.

Shotgun proteomic analyses of CSF and plasma have added to the list of potential candidates for diagnostic markers of AD stages. CSF proteins such as amyloid precursor protein [134, 135], serotransferrin [135], complement cascade proteins [135, 136], T-cadherin [137], chromogranin B [137] and plasma proteins Apo-A1, AIV [142–144], Apo-B [142, 143], Apo-E [163, 164, 168, 169] and plasma protease

Table 2.1 Candidate markers of AD identified from proteomics techniques in 2000–2016

Candidate biomarker[a]	Disease stage[b]	Method[c]	Refs.
Cerebrospinal fluid			
Aβ(1–42), carnosinase I, chromogranin A, NCAM, tau and YKL-40	EAD	2D-PAGE/LC-MS	[65]
120KD isoform precursor of neuronal cell adhesion molecule 1 (NCAM-120), α-dystroglycan and neuronal pentraxin receptor-1 (NPR)	AD and PD	2D-PAGE/LC-MS	[127]
α-1-Antitrypsin, Apo-E/J, complement component 3, contactin, fibrin β, IgG chains NPR, plasminogen, proSAAS, retinol-binding protein (RBP), transthyretin (TTR) and vitamin-D-binding protein	AD	2D-PAGE/ MALDI-MS	[128]
α-1-β-Glycoprotein, α-1-antitrypsin, α-2-HS-glycoprotein, β-trace, Apo- A1/E/J, cell-cycle progression 8 protein, kininogen and RBP	AD	2D-PAGE/ MALDI-MS	[129]
α-1 β-Glycoprotein, β-trace, β-2-microglobulin, Apo-H, chitinase 3-like1, complement component 3, cystatin C and thioredoxin	MCI	2D-DIGE/ MALDI-MS & LC-MS/MS	[130]
Dickkopf homolog-3 (DKK-3)	MCI and AD	1D-PAGE/ MALDI-MS	[131]
α-1-Antichymotrypsin, α-2-macroglobulin, β-2-glycoprotein, complement C4B, fibulin 1 and gelsolin	AD	2D-DIGE/ MALDI-MS	[132]
Complement C3B/C4B and complement factor B/H	AD	2D-PAGE/ MALDI-MS	[133]
amyloid precursor protein (APP) and cathepsin B	AD	LC-MS/MS	[134]
APP, serotransferrin, complement C4A-B/5–6/8, afamin precursor, AMPA, calsyntenin 3, CD99 antigen, di-N-acetyl-chitobiase, glutamine receptor, hemopexin, NPR, phosphoprotein1, plasminogen and spondin-1	EAD and LOAD	LC-MS/MS	[135]
Apo-A1, APP and complement C4A/C4B	MCI and AD	LC-MS/MS	[136]
Apo-C1/H, ceruloplasmin, chromogranin B, fibrinogen β, haptoglobin, T-cadherin and vitamin-D BP	AD	LC-MS/MS	[137]
neuronal secretory protein VGF, integral membrane protein 2B, metalothionein-3	AD	LC-MS/MS	[138]
VGF and NPR	AD	LC-MS/MS	[139]
11.7 KD metal-binding protein (S100A7)	EAD	SELDI-MS	[140]
α-1-Acid glycoprotein and β-2-glycoprotein	AD	MALDI-MS	[141]
β-2-Macroglobulin, Apo-A1, cathepsin D, hemopexin, PEDX and TTR	AD	2D-PAGE/ MALDI-MS	[142]

Table 2.1 (continued)

Candidate biomarker[a]	Disease stage[b]	Method[c]	Refs.
Aβ(1–14)/(1–16)	AD	MALDI-MS	[143, 144]
Aβ(1–37)/(1–42)	AD	MALDI-MS	[145]
Aβ(1–38)/(1–42)	AD	MALDI-MS	[146]
Aβ(1–40)/(1–42)	AD	MALDI-MS	[147]
Aβ(1–40)/(1–42)	AD	1D-PAGE/ MALDI-MS	[148]
Aβ(1–40)/(1–42)	AD	LC-MS/MS	[149]
Aβ(1–40)	AD	SELDI-MS	[150]
Aβ(1–42) with metal ions and total/ phosphorylated tau	EAD and LOAD	ICP-MS	[151]
Aβ(1–42)	AD	LC-MS/MS	[152–154]
Aβ(1–42) and amyloid precursor-like protein-1-derived Aβ-like peptide (APL 1β)	PS1-AD	LC-MS/MS	[155]
Apo-J, chromogranin A, phospholemman, synaptic protein-like proSAAS and neuronal secretory protein VGF	FTD and AD	CE-MS	[156]
Apo-J, clusterin, Ig kappa chain C and osteopontin	AD	LC-MS/MS	[157]
Oxidized TTR	AD	LC-MS/MS	[158]
Cystatin C, VGF	AD	SELDI-MS	[159]
Monocyte differentiation antigen CD14 precursor	AD	2D-PAGE/LC-MS	[160]
Neurogranin	MCI and AD	MALDI-MS	[161]
Presynaptic protein SNAP-25	EAD	LC-MS/MS	[162]
Plasma			
Apo-E, glutathione S-transferase omega-1, monoamine oxidase B, tropomyosin-1,	MCI and AD	2D-DIGE/LC-MS	[163]
Albumin, Apo-E, complement component 3, IgG, haptoglobin and plasminogen	AD	2D-PAGE /LC-MS/ MS	[164]
α-2-Macroglobulin and complement factor H	AD	2D-PAGE/LC-MS	[165]
α-1-Antichymotrypsin, β-2-glycoprotein, complement component 6, C4B-binding protein, vitamin-D BP, complement factor H, hemopexin, lipocallin-1 and vitronectin	AD	2D-DIGE/LC-MS/ MS	[166]
α-1 antitrypsin and oxidized isoforms of fibrinogen γ-chain	AD	2D-PAGE/ MALDI-MS	[167]
α-1-Antichymotrypsin, Apo-A1/IV, Apo-B, gelsolin, plasma protease C1 inhibitor, Vitamin-D BP and others	AD	LC-MS/MS	[168]

(continued)

Table 2.1 (continued)

Candidate biomarker[a]	Disease stage[b]	Method[c]	Refs.
Apo-A1/B-100/C-III and Apo-E	AD	LC-MS/MS	[169]
Apo-AIV/ Apo-H/B-100, ceruloplasmin, complement factor H, complement regulator C1, fibronectin and vitamin-D BP	MCI and AD	LC-MS/MS	[170]
3370 Da, 3436 Da and 3586 Da peptides	MCI and AD	SELDI-MS	[171]
α-2-Macroglobulin	MCI	LC-MS/MS	[172]
Aβ1–17/28	AD	LC-MS/MS	[173]
Aβ1–40/42 and Aβ approximate peptides	MCI and AD	MALDI-MS	[174]
Complement 4a	AD	LC-MS/MS	[175]
Glycosylated IgG-Fc	MCI and AD	LC-MS/MS	[176]
TTR	AD	ICP-MS	[177]
Cerebrospinal fluid and plasma			
Aβ-42, total/phosphorylated tau protein and YLK-40	AD	2D-DIGE/LC-MS	[64]
Apo-E isoforms	AD	LC-MS/MS	[178]
Saliva			
Tau protein	EAD	MALDI-MS/LC-MS/MS	[179]

[a]Candidate biomarkers are proteins identified with differential levels in body fluids of AD patients. Proteins with long nomenclature are abbreviated: *AMPA* α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid, *YKL-40* astrocyte-derived inflammation response protein, *PEDX* pigment epithelium-derived factor

[b]Candidate biomarker identification studies accomplished with multiple stages/types of AD that include mild cognitive impairment (MCI), early AD (EAD), late-onset AD (LOAD), frontotemporal dementia (FTD) or presenilin-1-mutated familial AD (PS-1 AD)

[c]Proteomic method used. *ICP-MS* inductively coupled MS, *SELDI* surface-enhanced laser desorption/ionization, *MALDI* matrix-assisted laser desorption/ionization (MALDI), *LC-MS/MS* liquid chromatography-tandem MS, *2D-PAGE* two-dimensional polyacrylamide gel electrophoresis, *DIGE* difference gel electrophoresis

C1 inhibitor [170] are present at different levels in AD patients compared to control groups (Table 2.1). Because there are a variety of options for proteomic assays, there are differences in the proteins that are generated from each laboratory as characteristic signatures of AD (Table 2.1). Such inter- and intra-laboratory variations prevent some candidates from moving forwards in the pipeline. In general, untargeted proteomics assays can be used for screening and generating potential candidates for the biomarker pipeline. It has been suggested that blood-based markers, such as those highlighted in Fig. 2.3, may just be an initial step in discovery mode of disease diagnosis that then leads to more specific tests using CSF or imaging markers, as discussed above [87].

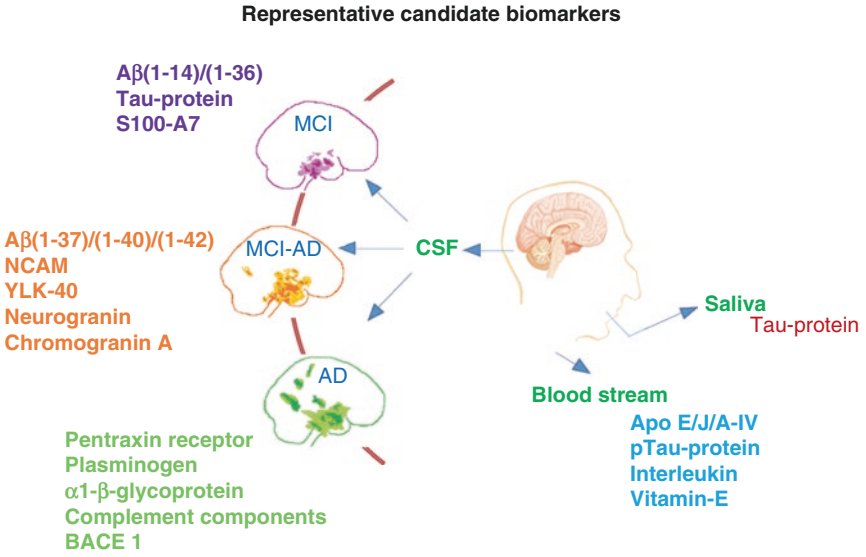

Fig. 2.3 Illustration of Alzheimer's disease (*AD*) progression with the formation of plaques in the hypothalamus, hippocampus and posterior cortex from mild cognitive impairment (*MCI*), MCI declining to AD (*MCI-AD*) and late-onset AD (*LOAD*). Examples of candidate biomarkers for each individual AD stage that require validation in larger cohort studies are listed

2.5.2.3 Selected Reaction/Multiple Reaction Monitoring (SRM/MRM)

Validation of potential biomarker candidates from shotgun proteomic experiments is often performed with MRM technology. MRM is a highly sensitive MS-based assay that measures multiple gas-phase transitions of well-characterized peptides from protein targets of interest. Because a large number of total candidates or differentially-expressed proteins are identified across such types of studies, validation of all of the proteins in the results list would have a prohibitive cost. It is important to highlight that study design is important in large-scale proteomic analyses, and it is critical that potential candidates that are identified in one population cohort are validated using another. This will help to eliminate false-positive hits and make it less costly to follow up candidates as there will be fewer proteins of interest in the final list. Four CSF biomarker candidates that predict progression of AD over a 12-month period were identified using a 39-protein biomarker panel [138], and biomarker leads that distinguish familial AD from healthy controls have been identified with MRM [135, 139]. MRM is a more sensitive MS technique than label-free or isobaric and isotopic labelling approaches. Due to the specificity of MRM, the assays can be of short duration depending on the sample complexity and number of transitions to be monitored. For example, 54 proteins were measured from 100 μL of CSF in a 10 min

multiplexed MRM assay, and this resulted in identification of ectonucleotide pyrophosphatase/phospodiesterase 2, lysosome-associated membrane protein 1, pro-orexin and transthyretin as potential CSF biomarker candidates for AD [188]. Furthermore, there is opportunity to obtain information about absolute protein abundances more readily with MRM as compared to relative differences that are obtained with shotgun proteomics.

There is considerable effort however to use proteomics strategies to find CSF and blood-based biomarkers for AD (i.e. a PUBMED search on July 7, 2016, using keywords "CSF OR blood AND biomarker AND Alzheimer's" returned over 235 entries). While most quantitative proteomics assays can lead to a higher number of potential biomarker candidates for discovery purposes, oftentimes these markers do not pan out when moving through the validation stages. It is not the scope of this review to discuss all of these studies in detail; however, it is worth mentioning example strategies of how proteomics can yield useful biomarker information for AD. For example, first an untargeted proteomics assay using a label-free differential mass spectrometry approach was used to identify CSF proteins that were present at different levels in samples from AD patients compared to controls [139]. From a number of candidates identified, two were selected for further validation in a separate cohort using the MRM approach. This resulted in validation of potential biomarker peptide candidates in CSF that correspond to the neuronal secretory protein VGF and neuronal pentraxin receptor-1 NPTXR as potential biomarkers for AD [139].

2.5.3 Other Multiplex Assays

Promising areas of technological advancement in multiplex biomarker analyses come from aptamer-based arrays, such as Slow Off-Rate Modified Aptamer-based capture array (SOMAscan, SomaLogic, Inc., Boulder, CO, USA), that can monitor a thousand human proteins simultaneously using protein to nucleotide signal transformation of fluorescent signals. SOMAscan requires minimal starting sample volumes [70]. For example, using as little as 8 μL of plasma from control, MCI and AD patients, a 13-plex panel that is predictive of AD has been established with SOMAscan assays [189]. While there are some inconsistencies with the proteins that fell out of the MCI and AD study, this is likely due to issues that are not unique to this technology, such as differences in the patient cohort, study design and sample treatment protocols. A platelet protein biochip has also been developed to measure blood platelets in MCI, AD and Parkinson's disease patients and is an alternative strategy for large-scale screening assays of a smaller number of protein targets [124].

2.6 Conclusions

Depending on the biological fluid to be analyzed and the multiplex assay used, there are a number of factors to consider for truly moving biomarkers forward that are specific to AD. Appropriate study design is necessary. When the aim is to characterize AD specific biomarkers among closely-related disorders such as other dementias, neurodegenerative disorders and psychosis, it is critical to have clear criteria for distinguishing symptoms and methods to categorize patient populations by their pathological state. Subsequently, it is also imperative that there are standardized criteria for sample collection and processing of study groups across laboratories and consortia. To identify real candidates that arise from blood or plasma proteomics, for example, candidates need to be validated in hundreds to thousands of samples that are well matched and come from multiple geographical locations with regard to AD research centers. Diagnostic candidates should be discovered in one cohort and validated in a separate cohort. Another area left to be explored is whether or not more accurate and sensitive tests can be developed that includes multiplexing across platforms. For example, biomarkers from various sources such as PET, MRI, clinical diagnosis, xMAP and mass spectrometry-derived analytes can be combined from a single patient using a complex algorithm to achieve the highest levels of sensitivity and specificity and even offer predictive value for AD. Early diagnostic biomarkers are urgent as these will facilitate earlier intervention for better treatment outcomes.

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Chapter 3
What Have Proteomic Studies Taught Us About Novel Drug Targets in Autism?

Paul C. Guest and Daniel Martins-de-Souza

3.1 Introduction

Autism spectrum disorders (ASDs) are heterogeneous neurodevelopmental conditions comprised predominantly of autism disorder, Asperger syndrome and pervasive developmental disorder not otherwise specified (PDD-NOS). There are also other conditions categorized as ASDs including the genetic disorders Rett's syndrome (RTT), fragile X syndrome (FXS) and tuberous sclerosis (TSC). ASD is characterized by impairments in social interaction and communication, often with restricted and repetitive behaviours [1]. Individuals with an ASD frequently have medical comorbidities such as epilepsy, gastrointestinal obstruction, sleep dysfunction, mental retardation and attention deficit hyperactivity disorder (ADHD) [2, 3].

The current prevalence of ASD has increased in recent years to approximately 1% of the population [4]. This could be caused by changing environmental factors or simply due to increased attention to diagnostic criteria or increased awareness of the disorder. A biased gender ratio has been found in ASD, with an approximate fourfold higher prevalence in males [5]. This disparity has led to the 'extreme male

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brain theory' which posits ASD as an extreme variant of male intelligence [6, 7]. This theory is driven by the finding that systemising quotient (SQ) questionnaire scores tend to be higher in 'normal' males compared to 'normal' females, and both males and females with ASD have higher SQ scores than normal males. In addition, empathy quotient (EQ) scores tend to be higher in normal females compared to males, and both ASD males and ASD females have lower scores than normal males [2].

Standard methods for diagnosis of ASD are the Autism Diagnostic Observation Schedule and the Revised Autism Diagnostic Interview, which are based on *Diagnostic and Statistical Manual of Mental Disorders* (DSM)-IV-R criteria [8]. However, these methods rely on human observations of behavioural symptoms and are therefore likely to be subjective or inconsistent [9]. There are two basic types of ASD termed symptomatic and idiopathic [10]. The symptomatic subtype comprises about 15% of the cases and is likely to be secondary to a specific cause or trigger. These individuals often display comorbidities and mental retardation [11, 12]. The idiopathic subtype of ASD constitutes about 85% of the cases and is often associated with genetic or neurological disorders such as FXS, TSC and RTT. Although idiopathic ASD individuals have abnormal behaviour, they tend to show similar cognitive levels as individuals with high-functioning autism or Asperger syndrome [13]. However, it is still not known whether these different clinical phenotypes are manifested through common or dissimilar pathways [14]. In addition, ASD may be manifested differently in males and females. Recent evidence has emerged which shows that males and females with Asperger syndrome have distinct molecular biomarker profiles in serum, which may reflect different underlying causes or differences in physiological responses [15, 16].

Most of the research in ASD has not attempted to identify which genes or biomarkers are associated with parameters such as ASD subtype, comorbidities or gender. Such approaches are critical for increasing our understanding of ASD, which is a prerequisite for developing novel treatment strategies. This is important as existing treatments for ASD do not alleviate core deficits, but instead treat the observed behavioural symptoms [17]. In this chapter, we describe the current status of biomarker-based research studies which have been aimed at increasing our understanding of ASD. This will be carried out with a view to introduce the latest research involving identification of potential novel treatment approaches.

3.2 Studies in the Brain

3.2.1 Imaging Analyses

A number of neuroimaging methods have been applied for elucidating the neurobiology of ASD. Structural imaging analyses of children with ASD have identified changes such as increased total brain size [18]. Although studies of brain

metabolism and blood flow have not yet yielded consistent findings, these investigations have demonstrated variability of cerebral synaptic activity in distinct brain regions in ASD patients [19]. Activation studies have identified changes in organization of brain regions involved in language and cognition, and positron emission tomography (PET) analyses have identified changes implicated in serotonergic and dopaminergic pathways [20]. Functional brain imaging techniques such as PET, single photon emission computed tomography (SPECT) and functional magnetic resonance imaging (fMRI) have been applied to the study of normal and pathological brain functions. Such studies have been performed at rest or during activation states and have found bilateral hypoperfusion of the temporal lobes [21]. Other studies have shown abnormal patterns of cortical activation during tasks involving perception and cognition, suggesting that different connections may occur in specific cortical regions of individuals with ASD. One study found that impairments in processing eyes and gaze could occur in ASD and may be central to the observed abnormal social cognition [22]. Studies using magnetic resonance spectroscopy (MRS) have also identified metabolic dysfunction in the frontal cortex and cerebellum of individuals with ASD [23].

fMRI studies in autism patients have found hypoactivation during social processing tasks in the prefrontal cortex, the posterior superior temporal sulcus, the amygdala and the fusiform gyrus [24]. The same approach has detected aberrant frontostriatal activation during cognitive control tasks [25–28], differential activation of language processing during communication tasks, anomalous mesolimbic responses to rewards and long-range functional hypoconnectivity and short-range hyperconnectivity in task-based paradigms [for a review, see [29]]. Similarly, it has now been established that the prefrontal cortex and cerebellum are altered in autism [30, 31], and impaired prefrontal cortex-cerebellar circuitry may be linked to autism symptoms [32]. Structural studies have demonstrated that the cerebellum receives inputs via afferent neurons from multiple brain areas such as the prefrontal cortex, best known for a role in cognition and mood regulation [33]. Similarly, efferent neurons from the cerebellum are routed through thalamic nuclei to both motor and nonmotor areas of the frontal cortex [34].

Increased myelination promotes and maintains axon integrity by increasing axonal calibre, which prevents axonal sprouting and synaptic plasticity [35]. Alterations in myelin thickness have been associated with disconnection of long-distance pathways, local connectivity and disruption of networks involved in emotions [36]. Several studies in autism have now found changes in connectivity and overgrowth of brain tissues [37] and altered white matter [38, 39]. However, changes in white matter differ depending on the brain area examined, the age of the subjects and the research methodologies used. This is interesting as a recent selected/multiple reaction monitoring-mass spectrometry (SRM-MS or MRM-MS) profiling study identified a difference in the levels of myelination-related proteins in the prefrontal cortex and cerebellum of *post-mortem* brain samples from autism patients compared to controls (see below, [40]).

3.2.2 Molecular Profiling

The imaging analyses described above indicate that changes in brain connectivity and synaptic function are common features in ASD [41, 42]. Specific genes which are now thought to be involved include *NLGN1* (neuroligin) and *NRXN1* (neurexin), which suggest an imbalance of neuronal excitation and inhibition [39, 43]. Proteomic studies have shown that brain-derived neurotrophic factor (BDNF) and glial fibrillary acidic protein (GFAP) are altered in individuals with ASD, consistent with the finding of increased brain volumes seen in some young children with autism [44]. Changes in proteins involved in inflammatory pathways have also been identified in the cerebral cortex, white matter and cerebellum of patients with autism [45]. In addition, alterations in mitochondrial energy pathways have been identified [46], although this could be a secondary affect in response to the neuronal changes [47].

A study which described a targeted SRM-MS analysis of the *post-mortem* prefrontal cortex and cerebellum from ASD patients and controls led to identification of altered levels of proteins related to myelination, synaptic vesicle regulation and energy metabolism [40]. These researchers found that the immature astrocyte marker vimentin was altered in both brain regions, which may indicate a decreased proportion of astrocyte precursors in some brain regions of autism patients. Interestingly, this study also found decreased levels of proteins associated with myelination and increased amounts of synapse- and energy-related proteins in the prefrontal cortex, which suggested that this brain region may have increased synaptic connectivity. Conversely, opposite directional changes were found for these same proteins in the cerebellum, suggesting that there was altered connectivity in the prefrontal cortex and cerebellum in autism patients, in support of the imaging studies described above [29]. In most other neurological conditions, proteomic studies have already contributed to the understanding of the affected biological pathways. However, progress along these same lines in ASD research has been poor.

3.3 Molecular Profiling Studies in Serum/Plasma

The reason for identifying biomarker candidates in serum or plasma for ASD is mainly due to the ease of use of these media in clinical studies. A recent study determined that a statistically selected panel of 15 biomarker candidates could discriminate newborns at risk for ASD from non-affected controls [48]. Examining circulating biomarker candidates may therefore give us ways to identify individuals who demonstrate specific developmental trajectories and aetiological factors related to ASD and, at the same time, offer avenues into potential novel treatment strategies.

In line with effects on brain function, several studies have identified changes in circulating levels of molecules related to neurotransmitter function in ASD. For example, the plasma concentration of the serotonin precursor tryptophan has been

found to be decreased in ASD patients compared to controls [49]. Another study showed that oxytocin and serotonin levels were negatively correlated with each other in children less than 11 years old [50]. In addition, Alabdali and co-workers found that the levels of the neurotransmitters GABA and oxytocin were associated with symptom severity of Social Responsiveness Scale (SRS) and Childhood Autism Rating Scale (CARS) test scores [51]. Receiver-operating characteristic analysis showed that these molecules could be used as predictive biomarkers of clinical symptoms.

One consistent finding of serum and plasma proteomic profiling analyses in ASD is alterations of proteins involved in lipid metabolism. Studies have found that lipid concentrations in individuals with ASD can be used to distinguish them from controls with good sensitivity and specificity [52]. Ngounou and co-workers found changes in apolipoprotein A1 (APOA1), APOA4 and serum paraoxonase [53], Steeb et al. showed that APOA1, APOC2, APOE and adiponectin were altered [16], Ramsey et al. found differences in APOA1 and leptin [54], and another study described the involvement of cholesterol [55]. Similarly, several lipid-related small molecules have been identified as being altered in ASD including 3-hydroxyisovaleric acid and serum b-OH-b [56], homocysteine, folate and vitamin B12 [57, 58], and a more recent study found that the increased lipid peroxidation found in ASD patients may be linked to elevated inflammatory responses [59].

Other serum molecules, which have been identified in association with ASD, include those associated with inflammation or the acute phase response [60]. One study showed that children with ASD had significantly lower levels of the iron transport protein ferritin compared with controls [61]. Another study found elevated levels of specific chemokines and interleukins in children with autism, and the levels of these molecules were significantly correlated with symptom severity [62, 63]. Potentially related to the inflammation, a number of molecules related to cell adhesion and vascular function have also been implicated in autism. These include changes in the levels of P-selectin [64] and platelet-endothelial adhesion molecule (PECAM-1) [65]. Changes in the former were correlated with impaired social development during early childhood, and changes in the latter were linked with head circumference at birth. Finally, several studies in autism have shown immune allergic responses in ASD patients [66]. In this regard, circulating autoantibodies to antigens such as myelin basic protein (MBP) and myelin-associated glycoprotein (MAG) [67] have been identified in ASD patients, as well as mitochondrial DNA [68] and antinuclear antibodies [69].

Other studies have identified changes in various hormones and growth factors in individuals with ASD. One study found increased levels of the anterior pituitary hormones adrenocorticotropic hormone (ACTH) and growth hormone, along with increased levels of adrenal cortisol in autism patients [70]. Another study found that ACTH levels were also higher in adults with Asperger syndrome [71]. In addition, amyloid-related peptides which have been implicated in Alzheimer's disease have also been found to be altered in serum from individuals with autism. One study showed that measurement of soluble amyloid precursor polypeptide-alpha (sAPP-alpha) in serum and human umbilical cord blood may have potential use in early diagnosis of autism [72]. Two other stud-

ies found that patients may have aberrant processing of APP in production of the $A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$ peptides [73, 74]. Finally, changes in the levels of vascular endothelial growth factor (VEGF) and its receptors [75] and BDNF [76] have been identified, consistent with theories on altered neuronal growth and survival in ASD. The latter study also found that increased levels of the growth factor neurotrophin 4 (NT4) could be linked to autism and mental retardation.

3.4 Sex-Specific Effects

Considering the higher prevalence of ASD in males compared to females, it is surprising that only a few biomarker profiling studies have attempted to determine whether or not this leads to sex-specific molecular profiles. This is important as this could mean that there either different aetiologies or that males and females with ASD activate different molecular pathways in response to a common aetiology. Three studies have now used multiplex immunoassay [15, 54] and mass spectrometry [16] analyses, which showed distinct molecular signatures in serum from males and females with Asperger syndrome. In a study by Schwarz and co-workers [15], males with Asperger syndrome had changes in 24 biomarkers, which included mainly increased levels of cytokines and other inflammation-related molecules. In contrast, Asperger syndrome females had altered levels of 17 biomarkers including growth factors and hormones such as androgens, growth hormone and insulin-related molecules (Fig. 3.1). Multivariate statistical classification of males using the panel of 24 male-specific analytes revealed a marked separation between patients and controls with a sensitivity of 0.86 and specificity of 0.88, whereas testing of females using this same panel did not

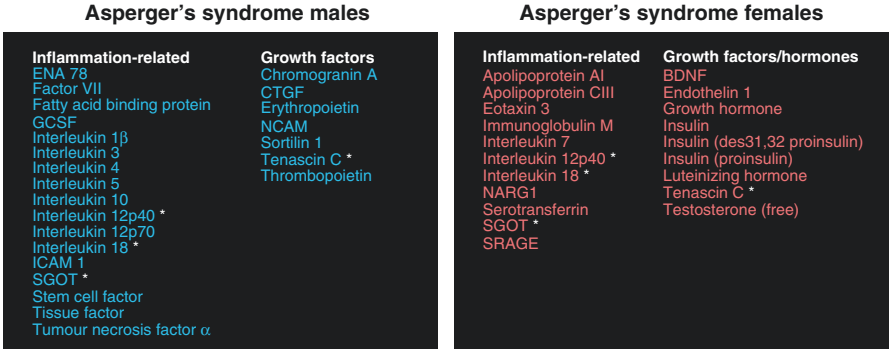

Fig. 3.1 Distinct serum biomarker signatures identified for males and females with Asperger syndrome. Common proteins are indicated with an asterisk. Abbreviations: *BDNF* brain-derived neurotrophic factor, *CTGF* connective tissue growth factor, *ENA 78* epithelial-derived neutrophil-activating peptide 78, *GCSF* granulocyte colony-stimulating factor, *ICAM 1* intracellular adhesion molecule 1, *NARG1* NMDA receptor-regulated protein 1, *NCAM* neuronal cell adhesion molecule, *SGOT* serum glutamic oxaloacetic transaminase, *SRAGE* soluble receptor for advanced glycation end products

result in any differentiation between the two groups. Conversely, classification of females using the panel of 17 female-specific analytes yielded sensitivities and specificities of 0.96 and 0.83, respectively, for separation of patients and controls with no differentiation observed for males. The finding of elevated testosterone in females with Asperger syndrome was consistent with the 'extreme male brain' theory of ASD. Ramsey et al. showed similar findings with Asperger syndrome males again having differences in immune and inflammatory markers and females showing changes in lipid metabolism, hormones and growth factors [54], and the combined multiplex immunoassay and mass spectrometry analysis of Steeb and co-workers [16] confirmed that these same changes occurred in Asperger syndrome subjects irrespective of any medications that they had received. These results provide evidence that the search for biomarkers or novel drug targets in ASD will require stratification of the tested individuals into male and female subgroups.

3.5 Current Treatment Approaches in ASD

There are a number of different classes of drugs which are in use for treatment of autism. Most of these are targeted towards management of symptoms seen in other psychiatric conditions, and others are directed against comorbidities.

3.5.1 Antipsychotics

Research on antipsychotic use in ASD has been limited due to concerns of extrapyramidal and metabolic side effects [77–80]. Risperidone has been approved for treatment of symptoms such as irritability, aggression, self-injurious behaviour, hyperactivity and repetitive behaviour symptoms in children and adolescents with ASD [81]. However, adverse effects have been apparent, including weight gain, increased appetite and somnolence [82–84]. Aripiprazole has also shown efficacy for reducing irritability, hyperactivity and stereotypies, although this drug produces similar side effects as risperidone [85–87].

3.5.2 Antidepressants

Children and adolescents with ASD showed improvements following treatment with the selective serotonin reuptake inhibitor (SSRI) fluoxetine [88, 89]. Also, improvements in anxiety, mood and irritability have been reported in studies using citalopram and escitalopram, although a recent meta-analysis found no evidence that SSRIs improved ASD symptoms and even suggested that they could be harmful [90, 91]. Clomipramine has been the most studied tricyclic antidepressant due to its

use in the treatment of obsessive compulsive disorder. Double-blind trials have found that this drug improves symptoms such as anger outbursts, repetitive behaviour, hyperactivity and irritability in ASD patients [92, 93], although its effect on hyperactivity has not been consistent [94].

3.5.3 Mood Stabilizers/Antiepileptics

Studies on the use of mood stabilizers and antiepileptic drugs for treatment of ASD have reported inconsistent results [95–97]. However, one investigation found that divalproex was helpful for improving symptoms of irritability and aggression in children and adolescents with ASD [98], and an open-label study with levetiracetam found improved symptoms of aggression, impulsivity, hyperkinesis and mood instability [99].

3.5.4 Treatments for ADHD

Methylphenidate is a stimulant used for treatment of ADHD symptoms. However, this compound has shown limited efficacy, resulting from adverse side effects in children with ASD and ADHD compared to children with ADHD alone [100, 101]. Three studies found that methylphenidate treatment led to improved ADHD symptoms in children with ASD [102–104]. The selective norepinephrine reuptake inhibitor atomoxetine has been approved for treatment of ADHD, although one study showed that it was only efficacious in individuals with lower symptom severity [105, 106]. Two alpha-2 adrenergic agonists (guanfacine and clonidine) have also been tested for treatment of hyperactivity, inattention and impulsivity symptoms in ASD patients with ADHD [107–109].

3.6 Novel Treatment Approaches in ASD

The lack of specific understanding of the neurobiology of ASD has stalled the development of novel treatment approaches. However, a number of drugs and alternative strategies have shown promise.

3.6.1 Melatonin

Melatonin is a neurohormone secreted by the pineal gland, involved in sleep regulation. It has now been tested in attempt to manage sleep disorders known to occur in children with ASD [4, 110]. A retrospective study on 107 children and adolescents with ASD resulted in an improvement in sleep in approximately 85% of the cases [111]. An open-label trial studied melatonin supplementation in 24 children with ASD

over 14 weeks, which led to improved sleep patterns in most children within 1 week [112]. Also, two small randomized, double-blind crossover trials testing melatonin supplementation in children with ASD found a significant increase in total sleep time and a decrease in latency compared with children who received placebo [113, 114].

3.6.2 Omega-3 Fatty Acids

Several studies have now reported low levels of omega-3 fatty acids in children with ASD [115]. Based on these findings, two studies were conducted in children diagnosed with ASD who received either omega-3 fatty acids or placebo [116]. However, there was no evidence that this had an effect on the outcome measures of social interaction, communication, stereotypy or hyperactivity. Larger trials are ongoing. A more recent study is planned which aims to test the co-administration of omega-3 fatty acids with vitamin D on primary outcome measures of a change in social-communicative functioning, sensory processing issues and problem behaviours between baseline and 12 months [117].

3.6.3 Glutamate Receptor-Related Medications

Effects on glutamatergic and GABAergic pathways have also been found in ASD [118, 119]. One study found that $GABA_A$ receptors were reduced in brain areas which have been associated with ASD [120]. Recently clinical studies have tested mGluR antagonists in small-scale phase II trials for potential therapeutic benefits [121] in the treatment of stereotypic behaviours [122, 123]. A double-blind clinical trial testing the chloride-importer antagonist bumetanide, which acts as a GABAergic inhibitor, found significant improvements in the CARS, Clinical Global Impressions and Autism Diagnostic Observation Schedule [124]. A retrospective open-label study of children and adolescents with ASD involved treatment with the NMDA receptor antagonist memantine. Out of 18 individuals tested, 11 showed improvements in social withdrawal and inattention behaviours [125]. However, some developed adverse effects, including sedation, irritability and increased seizure frequency. Another study which tested memantine found significant improvements in irritability, hyperactivity and inappropriate speech in children with ASD [126]. Considering these promising results, further work is warranted on the use of compounds targeting glutamatergic dysfunction ASD [127].

3.6.4 Oxytocin

Early studies tested the effects of oxytocin infusion in ASD. The main findings suggested that oxytocin treatment can reduce repetitive behaviours and improve affective speech comprehension [128, 129]. More recent studies found that intranasal

oxytocin administration in ASD subjects led to stronger personal interactions and increased eye gaze [130] as well as improved scores on the communication and social interaction domains of the Autism Diagnostic Observation Schedule-Generic (ADOS-G) [131]. Also, double-blind, randomized, placebo-controlled trials showed that intranasal oxytocin administration led to improved performance on the Reading the Mind in the Eyes Task and social cognition [132]. Another trial using intranasal oxytocin resulted in improved right amygdala response to facial stimuli in ASD compared to control subjects [133]. More recently, studies have shown that oxytocin may treat the social deficits in autism through a mechanism involving the serotonin 1B receptor, suggesting that this may be another future target [134]. However, some side effects of oxytocin treatment have been noted such as uterine contractions and lactation in females [135] and antisocial behaviour [136]. Furthermore, there are other unresolved issues involving development of oxytocin as a potential new therapeutic in ASD, including optimization of dose, duration of treatment and choice of suitable clinical endpoints for core symptoms [137]. Future studies should also involve optimization of the intranasal delivery approaches and address the matter of how different individuals respond to treatments.

3.6.5 mTOR Targeting

One of the latest targets to emerge in psychiatric research is the mammalian target of rapamycin (mTOR). mTOR is important in synaptic protein synthesis and integrates inputs from NMDA and metabotropic glutamate receptors. Abnormalities in mTOR signalling have been found in ASD [138]. Accordingly, mTOR inhibitors are currently being tested in treatment of ASD [139]. For example, a double-blind controlled trial is underway to test the effects of the mTOR inhibitor everolimus in children and adolescents with tuberous sclerosis, ASD and seizures for any improvements in cognition, ASD symptoms attenuation and seizure frequencies.

3.6.6 Other Approaches

Since a high prevalence of a variety of gastrointestinal symptoms has been reported in patients with ASD, a recent clinical study has set out to explore strategies which can restore normal gut microbiota by dietary supplementation with probiotics [140]. These researchers have set up a randomized controlled trial to determine the effects of supplementation with a probiotic mixture (Vivomixx®) in ASD children for effects on specific gastrointestinal symptoms and on the core deficits of cognitive and language development, as well as those on brain function and connectivity. A recent novel approach is the use of deep brain stimulation techniques of the amygdala in the treatment of severe cases of ASD [141]. Although the initial findings are promising, considerable further work is needed in this area.

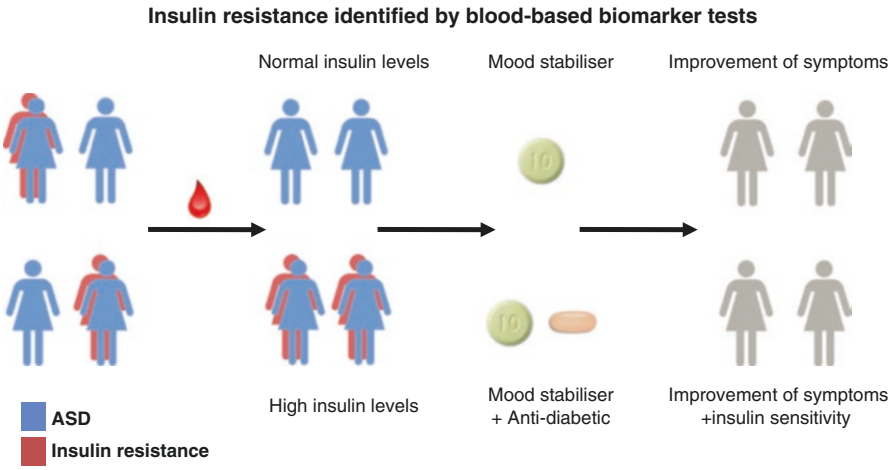

Fig. 3.2 Diagram showing personalized medicine strategy to treat ASD patients who have been stratified based on the presence or absence of insulin resistance through biomarker testing

3.7 Personalized Medicine Strategies in Autism

To date, none of the drug treatment trials in ASD have incorporated the use of biomarkers to stratify patient populations prior to the study. From the findings presented in this review, we suggest that this is essential to ensure that the correct patients are targeted with correct treatments. The sex-specific molecular profiles found in Asperger syndrome indicate that different compensatory mechanisms may occur in males and females with ASD or that these conditions may develop through distinct sex-specific molecular pathways [15, 16]. The finding that males showed greater changes in inflammatory molecules suggests that one possible treatment avenue could include anti-inflammatory agents (Fig. 3.2). This approach has already been undertaken in schizophrenia with some success at improvement of patient symptoms [142–145]. In this way proteomic biomarker testing of blood samples could be used for stratification of males based on whether they show distinct changes in key biological pathways such as immune factors. In addition, new adjunctive drug treatment strategies could be developed which target these comorbidities for combined treatments with either existing or newly developed therapeutics.

Schwarz et al. also showed that female Asperger syndrome patients had increased levels of free testosterone and insulin-related peptides [15], suggesting that at least some of these may have insulin resistance. Previous studies have shown that there is a link between hyperinsulinemia and hyperandrogenism in women with polycystic ovarian syndrome [146, 147]. Furthermore, treatment with insulin-sensitizing agents such as rosiglitazone has been shown to improve insulin sensitivity, leading to alleviation of hyperandrogenism and the associated symptoms [148]. This highlights the possibility of developing novel treatment approaches which target the biomarker-determined androgen or insulin signalling abnormalities in female

patients with ASD. We suggest that initial biomarker screening of male and female patients, followed by anti-inflammatory or antidiabetic treatments, potentially in combination with existing ASD treatments, could lead to improved patient outcomes (Fig. 3.2).

3.8 Future Perspectives

The current clinical practice in psychiatry focuses on the use of medications in ASD which target symptoms and not necessarily the underlying physiological changes. Recent research has now moved towards investigating the factors contributing to this complex spectrum of disorders by the use of genomic, transcriptomic, proteomic and metabonomic platforms, along with other targeted methods such as immunoassays and imaging technologies. Some studies have investigated the association between hormones, growth factors and immunological proteins and ASD. The paradigm shift towards increasing our understanding of the biological basis of ASD may lead to new clinical approaches in managing ASD. It will also allow development of novel drugs which target the physiological causes of ASD, and this could lead to better outcomes for these individuals [149]. In addition, the distinct patterns of serum biomarkers in male and female ASD patients suggest that a personalized medicine approach may be possible in the near future using more targeted treatments to elicit the best possible outcome. Although most of the findings described here require validation to account for potential variables such as age, gender, body mass index and ASD subtype, the results appear promising for the future development of new drugs for better therapeutic management of individuals with these conditions.

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Chapter 4
Application of Proteomic Approaches to Accelerate Drug Development for Psychiatric Disorders

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4.1 Introduction

Drug companies are under increasing pressure to improve their returns on investment in discovery and development projects. This is a difficult task and one that is almost impossible to forecast over time, considering that the average drug costs approximately one billion US dollars to develop and takes 10–15 years from initial discovery to the marketing phase [1]. This is made even more difficult by the fact that approximately 70% of drugs never recover their research and development costs and around 90% do not generate an adequate return on this investment. To compound matters, less than 10% of new compounds make it to the marketing stage, and some of those drugs have even been subject to withdrawal due to adverse reactions [2–5]. Pharmaceutical companies are now striving to minimise these risks by incorporating standard operating procedures, which will also help to meet the increasingly strict regulatory demands. In order to assist the drug companies in this challenging

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process, the regulatory authorities have encouraged the incorporation of biomarker-based tests into the drug discovery pipeline [6]. For example, the Food and Drug Administration (FDA) in the USA has initiated efforts to modernise and standardise drug development procedures to facilitate delivery of more effective and safer drugs. One important breakthrough would be the ability to predict the success or failure of drugs early on in the development process. The FDA has estimated that even a 10% improvement in the ability to predict failure of a drug before it reaches the clinical trial phases could save up to 100 million US dollars in development costs [7].

The development of new drugs for psychiatric disorders has proven to be one of the most challenging objectives in the pharmaceutical industry. This is due to the fact that the underlying causes of diseases such as schizophrenia, major depression and bipolar disorder are poorly understood [7, 8]. In addition, there is an overlap of symptoms across these disorders and heterogeneity in how they are manifested in different individuals [9]. However, the use of emerging multiplexed molecular profiling platforms has facilitated identification of biomarkers through the simultaneous measurement of hundreds and even thousands of molecules in a single experiment. This has led to benefits such as increased accuracy and lower amounts of sample required as well as reduced running costs.

4.2 The Need for Better Treatments

An important challenge for psychiatrists is to find the best possible drug treatment regime for their patients. However, double-blind randomised trials have shown response rates of 29–75% for schizophrenia patients after treatment with current antipsychotic medications [10–16]. This can result in switching of medications and a consequent delay in tempering of psychotic symptoms. In turn, this can cause a range of harmful consequences in the lives of patients, including those of a physiological nature, as well as increased costs to society and the healthcare systems. This highlights the urgent need to identify biomarkers for treatment response prediction, which could help to guide selection of more effective targeted treatment strategies and thereby improve prognoses.

Along similar lines, the treatment of major depressive disorder patients with antidepressants can be a costly process since the recovery periods are often lengthy. One reason for this is because current treatment protocols usually involve a waiting period, during which the clinician waits to see if a particular medication will be effective. Although over 20 antidepressants have been approved by the Food and Drug Administration (FDA) [17], the treatment response and remission rates have only been mediocre. In the best-case scenario, it can take at least 4 weeks to achieve a response and 6 weeks or more for remission [18, 19]. However, patients that do not respond are subjected to an additional trial and error period over which medication doses are increased and/or drugs are switched or tested in combination. This can lead to treatment periods spanning more than a year until adequate recovery is achieved. Other problems may also occur for approximately 75% of those patients who fail to improve with the first antidepressant treatment as these individuals may stop taking their medi-

cations [20, 21]. A meta-study of placebo-controlled trials showed that the response rate for antidepressants was only 53.8%, compared to 37.3% for placebo [22]. In addition, the Sequenced Treatment Alternatives to Relieve Depression (STAR*D) study found that only one-third of patients attained remission after 12 weeks of initial antidepressant treatment, and around two-thirds required up to four treatment attempts [23]. Another publication from the STAR*D group found that follow-up remission rates were inversely related and relapse rates positively related, with the number of successive treatment steps [24]. The consequences of a lengthy treatment can be serious since this may result in extended time periods spent in depressive episodes, leading to increased disability and increased costs [17]. Taken, together, these findings highlight the need to identify biomarkers that can be used to predict treatment response.

There is now significant interest in the discovery and integration of biomarkers by pharmaceutical companies and the regulatory agencies since these can be used to improve stratification of patients prior to clinical trials [25]. This could help to revolutionise treatment of individuals with psychiatric disorders by facilitating personalised medicine approaches such as those emerging in other areas of medicine, including oncology [26]. Multiple studies have now shown that patients with schizophrenia [27], major depressive disorder [28] and bipolar disorder [29] have perturbed serum concentrations of cytokines and other molecules associated with the inflammation response, and some patients with these disorders have also shown changes in circulating signalling molecules that can affect brain function including neuroendocrine hormones like insulin and growth factors such as brain-derived neurotrophic factor (BDNF) [30–34].

4.3 A Brief History of Failed Drugs

The need for biomarker tests to guide development in the pharmaceutical industry is perhaps best seen by recent failures in this process. Over the last 50 years or so, hundreds of drugs have been withdrawn, mainly as a result of liver or cardiac toxicity [35–43]. Many of these compounds targeted psychiatric disorders (Table 4.1). In other cases, many test compounds for psychiatric conditions failed during the later stages of clinical trials due to lack of efficacy. For example, a phase III study failed to show a significant improvement in Hamilton Depression (HamD) rating scores for major depression patients treated with the substance P antagonist aprepitant [44]. At least some of these disasters would not have occurred if procedures had been in place using biomarker assessments to guide dosing and/or predict efficacy or toxicity at an early stage in the drug discovery process.

4.4 Biomarker Impact in the Drug Discovery Process

It has been estimated that the total number of potential biomarkers exceeds one million, which is an awe-inspiring number. For this reason, it is typically only possible to study small to medium clusters of these molecules using proteomic methods at any one time. Many researchers and pharmaceutical companies have been investing

Table 4.1 List of psychiatric medications that were withdrawn due to toxicities

Disorder	Year	Country	Reason
Attention deficit hyperactivity disorder			
Pemoline	1997	Canada, UK	Liver toxicity [46, 48]
Proxibarbal	1998	Spain, France, Italy, Portugal, Turkey	Allergic response [46]
Anxiety			
Triazolam	1991	France, Netherlands, Finland, Argentina, UK, others	Psychiatric adverse reactions [46, 52]
Alpidem	1995	Worldwide	Liver toxicity [46, 47]
Chlormezanone	1996	European Union, USA, South Africa, Japan	Liver toxicity, epidermal necrolysis [46]
Temazepam	1999	Sweden, Norway	Abuse and overdose deaths
Tetrazepam	2013	European Union	Skin reactions [50]
Depression			
Phenoxypropazine	1966	UK	Liver toxicity, drug interaction [46]
Nialamide	1974	UK, USA	Liver toxicity, drug interaction [46]
Mebanazine	1975	UK	Liver toxicity, drug interaction [47]
Nomifensine	1981	France, Germany, Spain, UK, USA, others	Hemolytic anaemia, liver toxicity [45, 46]
Zimelidine	1983	Worldwide	Liver toxicity, hypersensitivity [46]
Minaprine	1996	France	Convulsions [46]
Nefazodone	2004	USA, others	Liver toxicity [40]
Schizophrenia			
Remoxipride	1993	UK, others	Aplastic anaemia [46]
Sertindole	1998	European Union	Arrhythmia, death [46, 49]
Thioridazine	2005	Germany, UK	Arrhythmia [51]

in multiplex proteomic techniques to help in the challenging task of sorting through this mass of molecules and to increase our understanding of diseases more deeply than ever before. All of these approaches offer the potential of identifying molecular fingerprints in clinical samples and translating this into information about health and disease. With the help of these multiplex proteomic approaches, we are now starting to categorise psychiatric diseases at the molecular level, rather than by symptoms alone. By finding biomarkers of a disease, early detection and diagnosis could be facilitated by testing for the presence or absence of a disease fingerprint. In line with the main aims of this review chapter, biomarkers could also assist pharmaceutical companies by allowing them to screen for drugs which help to normalise a disease signature. These could be used in the early stages of drug development such as in preclinical studies by determining the effect of test compounds in suitable disease models. They could also assist in looking for biomarkers of toxicity prior to

entry of the drug into clinical trials using representative models. In the latter stages of clinical development, proteomic biomarker tests could be used to help in stratification of patient groups to segregate those who are most likely to benefit or not benefit from treatment with the test compound. This is critical as many trials in the area of psychiatric medications may have failed due to inadequate selection procedures or because the wrong patients were included in the study. Improvements in this area alone could help to save millions of US dollars in costs since the phase II and phase III stages of clinical trials are typically the most expensive stages of the drug discovery and development pipeline.

4.5 Proteomic Biomarker Techniques

Biomarkers are physical characteristics that can be measured in bio-samples and used as an indication of physiological states such as good health, disease or toxicity or for predicting or monitoring drug responses [53]. For point-of-care use, it is important that biomarkers can be measured with high accuracy and reproducibility, within a short-time period and at an affordable focus proteomic approaches for use in blood samples. Since changes in physiological states are dynamic in nature, they are likely to cause changes in numerous proteins that converge on related pathways. For this reason, most researchers consider proteomic methods to be the most informative about physiological status. It is also becoming more accepted by researchers and clinicians that brain conditions such as psychiatric disorders can be investigated by looking in the blood. This is actually obvious when one considers the two-way communication system between the brain and the periphery in most bodily functions.

4.5.1 Multiplex Immunoassay Analysis

The bloodstream contains hundreds of bioactive and regulatory proteins including hormones, growth factors, transport proteins and cytokines. However, most of these proteins are present at very low concentrations. This means that biomarker measurement systems for serum and plasma should be highly sensitive. One way of achieving this is through the use of antibody-based approaches such as multiplex immunoassay [54]. These assays are constructed and carried out according to the following basic steps. First, microbeads are loaded with red and infrared dyes at different ratios such that each bead has a unique fluorescent signature. Then, specific capture antibodies are covalently attached to the surface of each signature-distinct bead. After this, the different antibody-bead conjugates are mixed together to form the multiplex. In the actual analysis, the sample is added, and the target molecules bind to their respective antibody-bead conjugates. After washing away the unbound material, fluorescently labelled detection antibodies are added in a

mixture, and each of these binds to their target molecules in a sandwich-like configuration. Finally, the samples are streamed through a reader, and the microbeads are analysed by two lasers for identification and quantification of the analyte present. In this step, the lasers identify which analytes are present using the unique signature of each dye-loaded microbead, and they determine the quantity by measuring the fluorescence intensity associated with the tags on the secondary antibodies.

4.5.2 Two-Dimensional Gel Electrophoresis

Two-dimensional gel electrophoresis (2DE) provides a means of studying intact protein chains, including any effects on post-translational modifications, such as phosphorylation or glycosylation. Such information is more difficult to obtain using other methods such as shotgun mass spectrometry (see below). In 2DE, protein mixtures in bio-samples are applied to a strip gel for separation in the first dimension according to their isoelectric points by isoelectric focusing. For the second-dimension separation, the proteins are separated by sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (PAGE) according to their apparent molecular weights, and the protein spots in the gels can be visualised with specific stains (e.g. Coomassie Blue R250 or Sypro Ruby) and then quantitated using an imaging software. Although this technique allows the study of many tissue types, there are some problems with analysis of blood serum or plasma samples. This occurs mainly due to the fact that blood contains a wide concentration range of proteins spanning at least 14 orders of magnitude [55]. Thus, highly abundant proteins such as albumin and immunoglobulin light and heavy chains appear as large poorly resolved blobs on the gels and thereby obscure the lower abundance proteins.

4.5.3 Mass Spectrometry

The emergence of shotgun mass spectrometry began towards the end of the Human Genome Project as a sensitive and medium throughput approach for proteomic biomarker identification [56]. The method is called “shotgun” due to the fact that proteins in bio-samples are cleaved with proteolytic enzymes to generate smaller peptides, which are the actual analytes. This is carried out as most intact proteins are too large and complex in their structure to be ionised or analysed directly in most types of mass spectrometry instruments. After proteolysis, the peptides are separated using liquid chromatography. As the peptides enter the mass spectrometer, they are ionised by a process such as electrospray, which is the application of an electric charge to render the peptides in a charged plasma state. After this, the peptide ions are accelerated magnetically towards a detector at a velocity that is inversely proportional to their specific mass to charge ratios. Quantitation is

achieved since the amount of each peptide that hits the detector per unit of time is proportional to the quantity of the peptide and, therefore, the corresponding parent protein. Simultaneously, peptide sequences are determined by streaming in a gas such as nitrogen, which breaks the peptides into smaller pieces. The mass of each piece can then be used to derive the amino acid sequences that make up the peptides which are used in database searches for identification of the corresponding proteins. The main advantage of this method is the ability to detect more difficult classes of proteins which cannot be detected by 2DE approaches, such as extremely basic or low molecular weight peptides. The disadvantages include the loss of intact protein information since the proteins are enzymatically digested prior to analysis.

4.6 Use of Proteomic Biomarker Profiling in the Drug Discovery Process

Biomarker profiling can be used at multiple stages of drug discovery process (Fig. 4.1). In the early phase, multiplex biomarker profiling could positively impact on target identification, target validation and lead compound screening and prioritisation. In addition, analytes could also be used in this phase as surrogate biomarkers of drug efficacy and for the validation of preclinical models of specific aspects of psychiatric diseases. Potentially of the most importance, any biomarker tests that arise from these early phase studies should be translated into rapid assays on hand-held point-of-care devices that can be used to identify disease signatures and monitor drug efficacy or toxicity in the latter clinical phases [57].

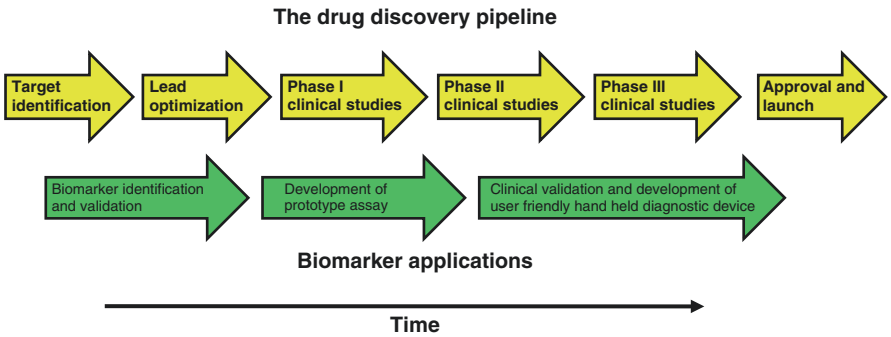

Fig. 4.1 Co-development of drugs with biomarker tests over the stages of drug discovery. The scenario is expected to lead to the development of more efficacious and safer drugs and reduce the overall process time, leading to greater returns on investment

4.6.1 Target Validation

Most drug targets are components of molecular complexes and networks [58]. However, many of these networks have not been fully elucidated in terms of the interacting proteins and other molecules or according to their relationship to other biological pathways. Therefore, there is a need for full mapping of these cell and whole-body networks further to identify new tractable drug targets. Identification of molecules that serve as a "switch" or a "weak link" in the disease process is usually the first stage of target validation. This can be tested by manipulating the expression of the target molecules using gain or loss of function methods in an attempt to cause or reverse the disease phenotype [59]. Increasing the function of the target molecule can often be achieved using agonists or through genetic overexpression approaches. In the opposite manner, function could be knocked down using antagonists, ribozymes, small interfering (si)RNAs or by genetic knock-down approaches. In all of these scenarios, a proteomic signature could be generated for monitoring purposes and for looking at the effects of test compounds on the phenotype.

For successful validation and prioritisation of novel drug targets, it is important to understand the relevant aspects of the disease at a functional level and confirm that the therapeutic concept works in preclinical models and clinical proof of principle experiments. Proteomic profiling studies can provide this information by identifying components of cellular networks that could be targeted for possible therapeutic intervention. One study showed that multiple proteomic approaches may be required, including identification of cancer cell membrane proteins by mass spectrometry and phenotypic antibody screening, for identification and validation of antibody tractable targets in cancer research [60]. In another investigation, a stable isotope-mass spectrometry metabolomic profiling approach was used to study the mechanism of action of d-cycloserine, an antibiotic used in the treatment of multidrug-resistant *Mycobacterium* tuberculosis infection [61]. The authors used labelled ^{13}C α-carbon-^{2}H-l-alanine for simultaneous tracking of alanine racemase and d-alanine/d-alanine ligase in *Mycobacterium* tuberculosis and found that the latter was more strongly inhibited than the former by d-cycloserine. In Alzheimer's disease, several clinical studies using antidiabetic compounds have now been performed which confirm a role of insulin resistance in the cognitive deficits [62, 63].

4.6.2 Lead Optimisation

Many compounds fail in the later stages of drug development because of an unanticipated toxicity or poor efficacy [64]. One example was the failure of the antidepressant compound nefazodone due to liver toxicity [40]. To overcome this calls for a greater understanding of drug properties at an earlier stage in the development pipeline. One approach would be through the incorporation of appropriate proteomic biomarker tests into this stage of the pipeline. Such tests can be used to

generate expression signatures from cells or tissues treated with new drugs for target identification and validation and for determining mechanism of action. Biomarker signatures can also be used in the identification and optimisation of lead compounds by looking for correlations of specific molecular patterns with efficacy or specific toxicities. For example, proteomic monitoring of the effects of developmental compounds in appropriate models of psychiatric diseases might provide an early prediction of efficacy or toxicity [65]. Compounds which induce the same signature of protein expression changes are presumed to share the same mode of action and toxicity effects. A recent study reported the development of a multiplex immunoassay for high-throughput screening of compounds in cell models using cytokines as physiologically relevant molecular fingerprints [66]. In addition, this multiplexed cytokine test can be used for profiling of bio-fluids such as blood serum and plasma for translational research.

In addition to animal studies, cellular models can provide useful screening platforms for drug profiling. This can be achieved using reporter systems for activation of cellular pathways such as receptor signalling or enzymatic cascades, which are known to be perturbed in the disease state or that respond to current effective medications. This approach has been called cytomics. By screening such cell models with currently used drugs and novel compound libraries, functional responses including calcium flux, phosphorylation, membrane potential changes, apoptosis, oxidative stress, proliferation and cell cycle status can be measured [67]. Thus, the cellular and biomarker reporter system would provide a means of looking for compounds which hit the appropriate target or achieve the desired effect. Then, compounds of the same class could be selected for additional studies and the most successful candidates taken forward for further clinical development.

4.6.3 Drug Toxicology Studies

To maximise chances of success, new drug candidates should be potent, specific for their targets and bioavailable with good pharmacokinetic profiles and low toxicity. Compounds lacking one or more of these qualities should be identified and weeded out during the early stages of the drug discovery pipeline so that only the most promising candidates are taken through to the later clinical trial phases. Toxicities normally only become apparent during the preclinical or clinical development stages when compound testing occurs in models and humans. However, there have been many cases in which toxicities have not been detected until the late stages of clinical trials or even after the marketing phase [68]. There are many reasons for this and the causes can be complex and on some occasions attributed to metabolism of the drug to toxic metabolites or to poor clearance from the body. Several drug companies are now incorporating screening approaches to identify proteomic toxicity signatures early in the development process so that only the best lead compounds get taken through to the clinical stages.

In collaborative approach, the EU Framework 6 Project: Predictive Toxicology (PredTox) studied the effects of 16 test compounds by applying a mixture of conventional toxicological measures and biomarker-based approaches [69]. This led to identification of three main classes of toxicity which were liver hypertrophy, bile duct necrosis/cholestasis and kidney proximal tubular damage. Multi-omic methods have been used in these efforts. DNA microarray studies have been carried out with known classes of toxicity-inducing compounds so that the resulting profiles can be used as references for novel development compounds [70, 71]. Another DNA microarray profiling study investigated the liver toxicity of ritodrine, a compound which has been used to prevent preterm labour [72]. They found a specific increase in the levels of serum amyloid A, which was more sensitive as a biomarker compared to the commonly measured liver enzymes aspartate aminotransferase and alanine aminotransferase. Anderson and colleagues analysed plasma samples from 134 patients using proteomic and metabolomic approaches, in order to uncover the mechanism of the liver toxicity induced by ximelagatran, a compound developed for treatment of thromboembolic conditions [73]. Schwarz and colleagues carried out multiplex immunoassay analysis of serum from schizophrenia patients taken before and after 6 weeks of treatment with antipsychotics to investigate molecular factors predisposing patients to the development of metabolic disturbances, a well-known side effect of these compounds [74]. Their analysis showed that the levels of ten serum proteins at baseline were significantly associated with increased body mass index, including cytokines, growth factors and hormones.

In addition, cellular models have been applied in preclinical toxicity screening efforts. Meneses-Lorente et al. used two-dimensional differential in-gel electrophoresis (2D-DIGE) and mass spectrometry profiling of hepatocytes to identify a proteomic signature associated with hepatocellular steatosis after dosing rodents with a developmental compound [75]. Within 6 h after dosing, the livers showed vacuolation, which increased over time. Proteomic profile changes were observed at the earliest time point, and many of the proteins were associated with liver steatosis, although changes in the standard alanine and aspartate aminotransferases were not detected until day 3.

4.6.4 Clinical Studies

Prognostic biomarker signatures can be used to aid in prediction of drug efficacy in patients and to identify which individuals are likely to benefit from treatment with specific drugs, in line with personalised medicine approaches [76–78]. The ultimate application of proteomics in drug discovery would be to identify biomarker signatures in accessible body fluids such as serum or plasma, which can be used as an early indication of efficacy or toxicity. This would aid in predicting the response of individuals to treatment and allow therapeutic adjustments in order to achieve the

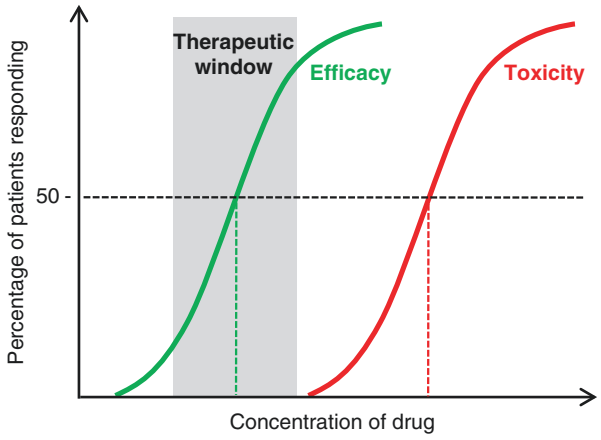

Fig. 4.2 Diagram showing the therapeutic window of a drug. This is the efficacious dosage range that does not lead to significant toxicity. This window could be identified and monitored in patients using proteomic screening of blood samples after dose range-finding studies

highest possible efficacy without reaching a level which elicits toxic side effects. The range of dosages that produce a therapeutic response without causing significant toxicity is known as the therapeutic window (Fig. 4.2). Likewise, this approach could be used to facilitate identification of patient classes who will respond favourably to the drug in clinical trials.

One study investigated the efficacy of multiple compounds in a phase II trial in patients by measuring changes in plasma cytokines and angiogenic factors using a multiplex immunoassay profiling approach [79]. They found that high baseline levels of interleukin 8 were associated with a shorter progression-free survival period, and changes in the levels of several growth factors were associated with angiogenesis and myeloid recruitment in the progressive disease phase. More recently, analysis of the biomarker results from a phase III trial of first-line bevacizumab plus docetaxel treatment for HER2-negative metastatic breast cancer showed that plasma levels of vascular endothelial growth factor (VEGF)-A and VEGF receptor-2 are potential predictive markers for efficacy [80].

In the treatment of psychiatric disorders two laboratory-based clinical studies were carried out to identify blood-based biomarkers for prediction of antipsychotic efficacy in schizophrenia patients. Schwarz et al. found that insulin levels measured at baseline could be used to predict efficacy following 6 weeks of treatment of first-episode schizophrenia patients with antipsychotics [81]. In addition, Tomasik and colleagues found that levels of the heart form of the fatty acid-binding protein could be used to predict response to olanzapine treatment [82]. Although these studies are promising, considerable further work is required in order to validate these findings and, if successful, translate these biomarker tests into user-friendly hand-held assays to facilitate use in point-of-care or clinical settings.

4.7 Conclusions and Future Perspectives

This chapter has described the emerging use of proteomic biomarker profiling techniques as enabling platforms for use in multiple stages of the drug discovery process. This is critical as current diagnostic procedures and strategies for developing novel psychiatric medications are in need of a paradigm change [83]. The regulatory health agencies are now on board with the concept that incorporation of biomarkers into the clinical pipeline is important for the future of drug discovery efforts, and they attempted to aid this process by introducing procedures to modernise methods, tools and techniques in the fields of drug discovery and development.

Multiplex proteomic tests have been available for more than two decades. For general laboratory use, most of these platforms are medium to large in size and require expert technicians in order to operate them. Another downside is that most of these methods require a turnaround time up to 2 days from the sample preparation stage to the final results output for each sample. Within the last 5 years, proteomic biomarker tests have been miniaturised using micro-fluidic approaches to yield devices which are approximately the size of a small pamphlet or a credit card [57]. Also, portable mass spectrometry devices are now in use in some airports and by emergency response units for detection of hazardous substances [84]. Most importantly, these devices are user-friendly since no expertise is required for operation and the results can be returned in less than 15 min. Such devices would meet the requirements of clinical studies in psychiatric medicine and slot smoothly into the pipeline in phase I–III clinical studies. Given their robustness, speed and user-friendly nature, these approaches should help to instil renewed drive into the pharmaceutical industry and most importantly help to improve the lives of individuals whose lives are affected by these debilitating disorders by enabling a personalised medicine approach for the first time in this field of medicine.

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Chapter 5
Proteomic Biomarker Identification in Cerebrospinal Fluid for Leptomeningeal Metastases with Neurological Complications

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5.1 Introduction

For decades, clinicians and scientists have investigated and treated neurological illnesses as diseases of the brain. However, the concept is becoming increasingly acknowledged that somatic causes can also be involved in the precipitation and course of these disorders. Brain function can be influenced by immunological or hormonal changes in either the brain itself or via the periphery, as well as by some physiological conditions and traumas. As an example, immunological changes that reach the brain can influence the function of neurotransmitter systems that affect movement, coordination, cognition and mood [1]. In addition, mental health disorders have been linked with inflammatory diseases such as asthma [2], and a variety of conditions such as Alzheimer's disease and severe depression have been found in approximately 40% of diabetes patients [3]. Likewise, dementia, psychosis and various anxiety and adjustment disorders can occur in some kidney and liver diseases [4, 5], and mental health problems such as depression can occur in patients infected with hepatitis C who have been treated with interferon-alpha [6]. Perhaps

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not too surprisingly, neurological defects can also occur in patients with brain tumours, which can complicate diagnosis and treatment options [7–9].

Brain cancers are a heterogeneous group of central nervous system (CNS) neoplasms that arise within or adjacent to brain. Metastatic brain cancers are a common neurological complication in oncology with an incidence of 9–17% and an extremely poor prognosis [10–13]. For these reasons, the development and deployment of biomarker tests that could enable the diagnosis of brain neoplasms and metastases or monitor therapeutic response are in high demand [14–16]. Primary CNS cancers and metastases are often located in close proximity to ventricular surfaces or cerebrospinal fluid (CSF) cisterns. Such tumours are diverse in their nature and typically comprised of a group of neoplasms derived from various different cell lineages. Much like those arising from other anatomical sites, tumours of the CNS have been classified historically on the basis of morphological and immunohistochemical features and more recently according to transcriptomic and proteomic profiling approaches [13, 17]. Leptomeningeal metastasis (LM) is a devastating complication of systemic cancer, reflecting multifocal seeding of the leptomeninges and CSF by malignant cells [18–20]. CSF is also a major route for seeding metastases of CNS malignancies. Therefore, the development of proteomic biomarker tests for this medium could be informative for diagnosis and risk stratification of certain brain cancers.

Deciphering the entire proteome of normal CSF would provide a critical standard to allow meaningful comparisons of samples from LM patients with those from individuals suffering from neurological disorders such as multiple sclerosis, Alzheimer's disease or severe psychiatric conditions. This would help to improve diagnostic accuracy of LM as well as these other conditions through the identification of disease-specific biomarker profiles. Since CSF contains both cellular and soluble components, the analysis of this body fluid could also help to provide insights into processes occurring in the CNS. This is mainly because 30–40% of the CSF is formed normally from the extracellular fluid of the brain and spinal cord.

5.2 Epidemiology

LM from solid tumours is also called leptomeningeal carcinomatosis or carcinomatosis meningitis. It is diagnosed in 4–15% of patients with solid tumours, in 5–15% of patients with leukaemia and lymphoma and in 5–8% of patients with primary brain tumours [21]. Postmortem studies have shown that 19% of patients with cancer and neurological signs and symptoms have evidence of meningeal involvement. Adenocarcinoma is the most frequent histological finding, and breast, lung and melanoma are the most common primary sites of metastasis to the leptomeninges [21, 22]. Although small-cell lung cancer and melanoma have the highest rates of metastatic spreading to the leptomeninges, at 11% and 26%, respectively [23], the frequency is greater for breast cancer due to its higher incidence [23, 24]. LM usually presents in patients with widely disseminated and progressive systemic cancer,

but it can also occur after a disease-free interval or even as the first manifestation of a cancer (5–10%), occasionally in the absence of other evidence of systemic disease [23]. Although any cancer can metastasize to the leptomeninges, breast cancer (12–35%), lung cancer (10–26%), melanoma (5–25%), gastrointestinal cancer (4–14%) and cancers of unknown primary origin (1–7%) are the most common causes of solid tumour-related LM.

5.3 Neurological Signs of LM

Effects on mental state can also occur in LM, and delirium is sometimes reported, highlighting the possibility that this disease can be masked as a neurological or psychiatric disorder. Trachman et al. described a case study of a 54-year-old man who was eventually diagnosed with LM who originally presented with a 1-week history of changes in his mental status and a past history of an "unknown psychiatric disorder" [25]. In the doctor's office, the patient could not describe the problem clearly although his wife stated that he had been well up to 1 week previously, but on the day of admission, he could not recognize familiar faces. However, his evaluation in the emergency room revealed no physical problems, and computed head tomography analysis showed that he had no gross signs of cerebral abnormalities. In addition, lab testing of his blood gave normal readings across the board, and toxicology and alcohol screens were negative.

Another case study described the admission of a 72-year-old male into the hospital because of hearing impairment, blurred vision, unilateral facial numbness and difficulties in walking [26]. Magnetic resonance imaging (MRI) analysis revealed two infiltrating lesions around the cranial nerves indicating either metastatic brain tumours or meningeal carcinomatosis. Finally, CSF cytological examination revealed the presence of malignant cells, and endoscopy of the upper gastrointestinal system identified a type 1 squamous cell carcinoma of the oesophagus. Together, these findings confirmed a diagnosis of LM. Another case study of a LM patient confirmed that similarities with psychiatric or neurodegenerative symptoms can occur such as confusion, changes in behaviour, hallucinations and deteriorating short-term memory [27].

A variety of neurologic symptoms may be seen in LM since multiple brain regions can be involved, such as the frontal cortex, cerebellum, spinal cord and cranial nerves. One effect of LM which was first described more than 100 years again is that of hearing loss [28, 29]. In addition, ocular symptoms may represent the initial manifestation of LM even in the absence of other symptoms [30]. The involvement of the CNS can also lead to generalized symptoms such as seizures, confusion, encephalopathy or intracranial hypertension, as well as symptoms focussed on specific brain regions including hemiparesis or aphasia. LM patients frequently present with neurological signs affecting the CNS, although it may be difficult to distinguish these symptoms from other disorders and from adverse cancer treatment effects [31]. The complete MRI of the neuroaxis and identification of

neoplastic cells in cytological examinations of the CSF are required for diagnosis. However, the sensitivity and specificity of these analyses are low. This is one reason why new methods such as CSF proteome profiling are now under development. In addition, biomarker tests that allow prediction or early detection of LM before profound deficits occur are desperately needed to improve prognosis, as this would allow initiation of more timely treatment. Currently, treatment options including radiation therapy and intrathecal administration of chemotherapeutics are mostly given only as palliative care with a mean expected patient survival time of only 2–6 months [32].

5.4 Mechanisms of LM

In LM, cancer cells reach the meninges by various routes, including haematogenous spreading through the venous plexus of Batson or arterial dissemination, direct extension from contiguous tumour deposits and centripetal migration from systemic tumours along perineural or perivascular spaces [19, 33]. Once cancer cells have entered the subarachnoid space, they can be transported by CSF flow, resulting in multifocal seeding of the leptomeninges. Tumour infiltration is most prominent in the base of the brain, specifically in the basilar cisterns, and on the dorsal surface of the spinal cord, particularly the cauda equina [18]. Hydrocephaly or impairment of CSF flow may occur due to the development of ependymal nodules or obstructing tumour deposits, mainly at the level of the fourth ventricle, basal cisterns, cerebral convexity or spinal subarachnoid space.

LM is a devastating complication of malignant cancers, characterized by the spread of the cancer to the CNS and the formation of secondary tumours within the thin leptomeningeal membranes surrounding the brain. Out of all patients who had cancer with neurologic symptoms and were autopsied, up to 20% showed evidence of leptomeningeal seeding [22]. The most common cancers that can lead to LM are lung cancer, breast cancer, gastrointestinal cancer, melanoma, acute lymphoblastic leukaemia and non-Hodgkin lymphoma. However, virtually any cancer can metastasize to the leptomeninges, and a definitive diagnosis of LM is based on finding cancer cells by means of CSF cytological examinations or by using convectional flow cytometry [34, 35]. However, up to 5% of patients who have had a lumbar puncture which revealed normal opening pressure, cell counts, protein levels, glucose concentration and no cytologically determined malignant cells can still have LM [36].

5.5 CSF as Biomarker Source

The CSF is formed mainly in the ventricular choroid plexus and distributed within the ventricular system and subarachnoid space [37–39]. It occupies the space between the arachnoid mater and pia mater and makes up the content of all

ventricles inside the brain and the central canal of the spinal cord. It contains 15–45 mg/dL protein, 50–80 mg/dL glucose and 0–5 mononuclear white blood cells/mL. Since the CSF is in direct contact with the CNS, it provides a sink function by the absorption of biologically active compounds or selective removal of various toxic compounds. This vital fluid also facilitates active regulation of the brain and other organs through the presence of circulating neuropeptides, hormones and other bioactive molecules. Thus, the CSF can also be an excellent source of biomarkers and serves as a window into CNS functions. As it is also moderately accessible, CSF has been widely targeted in biomarker discovery studies of brain cancers [36, 40–46] and a variety of neurological and psychiatric disorders, including Alzheimer's disease [47–50], Parkinson's disease [51, 52], multiple sclerosis [53–55], amyotrophic lateral sclerosis [56] and schizophrenia [57–60]. Up to 10 mL of CSF is usually collected for testing through a lumbar puncture, normally using a needle inserted between the third and fourth lumbar vertebrae.

5.6 Characterization of CSF by Proteomics Approaches

Bearing in mind the important role that proteins play in most of the physiological aspects of cellular life, it is not surprising that any dysregulation in protein expression can result in pathology [48]. Mass spectrometry (MS) has become one of the most powerful technologies in recent years, and different MS-based proteomic approaches have been used for the characterization of the human CSF proteome (Fig. 5.1) [61–64]. Several research groups have also combined two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) with matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) MS [65–67] and liquid chromatography-electrospray ionization (LC-ESI) MS [66, 68]. Other proteomic-based approaches

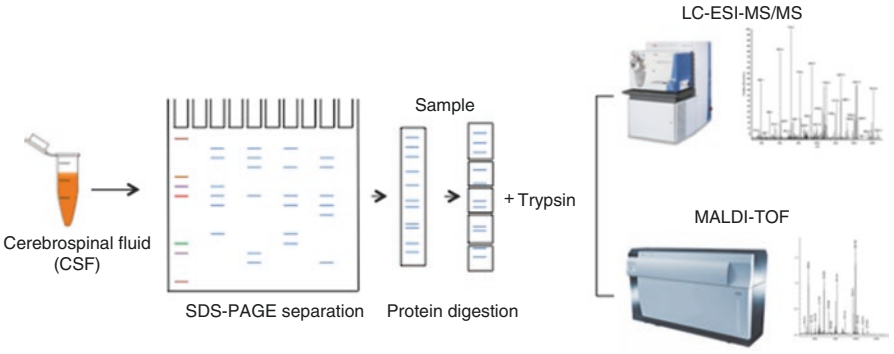

Fig. 5.1 Strategy to identity new biomarkers in CSF by proteomics approaches. Individual or pools of CSF samples were SDS-PAGE separation, digested, fractionated and analysed with MALDI-TOF or LC-ESI-MS/MS to generate new biomarkers

have been used to characterize the CSF proteome such as isobaric tagging for relative and absolute protein quantification (iTRAQ) MS [69] and multiplex immunoassay [70]. The first comprehensive study of the CSF proteome was published by Zougman et al. in 2008 [71]. In this study, both the CSF proteome and CSF peptidome were analysed using linear trap quadrupole-Orbitrap (LTQ-Orbitrap) and linear trap quadrupole-Fourier transform LTQ-FT-MS, which resulted in the identification of 798 proteins. From the peptidome dataset, 563 unique endogenous peptides originating from 91 unique proteins were identified. Interestingly, 46 of these proteins were not detected in the proteome dataset. This illustrates the importance of combining platforms to increase proteome coverage, given that some techniques have different capabilities and weaknesses. Some of these proteins are precursors of known neuropeptides, supporting the case that the CSF peptidome contains biologically important molecules as well as the ex vivo degradation products of high-abundance proteins.

Xu and coworkers used sodium dodecyl sulphate (SDS)-PAGE, strong cation exchange chromatography (SCX) and a combination of quadrupole ion trap liquid chromatography (LCQ)-MS and linear trap quadrupole-Fourier transform (LTQ-FT)-MS to map the CSF proteome of young individuals [72]. By combining the proteomes obtained using these different methods, they were able to identify 608 different CSF proteins. In 2007, Pan and coworkers [73] identified 2594 CSF proteins by combining their previously published data [69, 72, 73]. In this dataset, CSF proteins from healthy individuals [72], as well as those from patients with Alzheimer's disease, Parkinson's disease [69] and a glycoprotein database [73], were listed. The most extensive mapping of the CSF proteome to date was carried out by three studies which identified 3256 proteins [74] and 3081 proteins [75]. In addition, Schutzer and coworkers identified 2630 proteins from a group of healthy individuals using immunoaffinity depletion of abundant proteins, multiple liquid chromatography separations and label-free MS quantification, and Pan et al. identified 2594 proteins [61, 73]. In the Schutzer et al. study, 56% of the proteins were found to be CSF-specific [61]. A list of protein numbers reported in the proteomic characterization of human CSF is given in Table 5.1.

5.7 Proteomic Biomarkers of LM

The National Institute of Health (NIH) officially defines a biomarker as a "characteristic that is objectively measured and evaluated as an indicator of normal biologic process, pathologic processes, or pharmacologic responses to a therapeutic intervention" [76]. Currently, all available biomarkers for LM are based on CSF studies. However, the application of most of these has been limited by poor sensitivity and specificity [19]. Biomarker discovery through proteomic analysis of CSF has the potential for providing future clinical tests with a more sensitive and accurate means of detecting and monitoring these diseases [77]. Proteomic analyses using MS have been applied extensively in studies aimed at identifying biomarkers for complications

Table 5.1 List of proteins reported in the characterization of proteome of human CSF by proteomic and mass spectrometry

Study	Methodology	Proteins	Reference
Characterization of proteome of human cerebrospinal fluid	LTQ-FTMS/MS	608	[72]
A combined dataset of human cerebrospinal fluid proteins identified by multidimensional chromatography and tandem mass spectrometry	Multidimensional chromatography and tandem mass spectrometry (ESI-based IT and MALDI-TOF-TOF)	2594	[73]
Establishing the proteome of normal human cerebrospinal fluid	SCX chromatography and reversed-phase LC-MS/MS	2630	[61]
In-depth characterization of the cerebrospinal fluid (CSF) proteome displayed through the CSF proteome resource (CSF-PR)	(RP-AX) HPLC in combination with immunoaffinity depletion and LC-MS/MS	3081	[75]
A comprehensive map and functional annotation of the normal human cerebrospinal fluid proteome	High-pH reverse-phase liquid chromatography and LC-MS/MS	3256	[74]

of non-CNS primary malignancies such as LM as well as for CNS lymphoma and leukaemia [78, 79].

One approach for identifying biomarkers is through the comparison of differential protein profiles, such as those between CNS lymphoma and healthy control subjects. In the case of CNS lymphoma, these studies generated the first insights into the utility of proteomic analysis for biomarker identification and demonstrated that identifying specific proteins had much greater sensitivity for detecting metastases in comparison to standard CSF cytological protocols [79]. Proteomic studies of CSF in primary neurological malignancies using MS have focussed primarily on adult populations with some successes in the identification of putative biomarkers in meningiomas and astrocytic brain tumours [44, 46].

Two studies were carried out looking specifically at CSF protein expression profiles of LM patients with breast cancer [42, 43]. Dekker et al. analysed the protein patterns of 54 breast cancer patients who had LM in a comparison with 52 breast cancer patients without LM and 45 control subjects. On the basis of LM-specific protein expression patterns, they were able to employ a statistical method to build a prediction algorithm which had a sensitivity of 0.79 and specificity of 0.76 for distinguishing LM patients from the non-LM patients. This is similar to the sensitivity of magnetic resonance imaging (MRI) detection of LM. This suggests that the test may be useful to support the diagnosis of LM in patients with breast cancer [42]. In a follow-up study, Römpp et al. used three MS methods (MALDI-TOF, MALDI-FTICR and nano-LC-FTICR MS) which led to the identification of 17 peptides, corresponding to eight known proteins that were present at significantly different levels in the CSF of the breast cancer patients with LM (Table 5.2) [43]. Most of these had functions in molecular transport. Three of these proteins (apolipoprotein A1, haptoglobin and transferrin) have also been found to be correlated with poor

Table 5.2 Identification of eight CSF proteins that showed altered levels in breast cancer patients who developed LM

Protein	Function
Alpha1-antichymotrypsin	Protease
Apolipoprotein A-I	Transport
Apolipoprotein E	Transport
Haptoglobin	Transport
Hemopexin	Transport
Prostaglandin D2 synthase	Transport
Transthyretin	Transport
Serotransferrin	Transport

clinical outcome in serum of LM patients. The authors postulated that the identified proteins are not related to a blood-brain barrier disruption and are LM specific. Furthermore, antithrombin III was recently identified as a potential protein biomarker for distinguishing CNS lymphoma patients from those with benign focal brain lesions [78, 79].

5.8 Conclusions and Future Directions

LM is a consequence of solid peripheral tumours and primary brain tumours with an extremely poor prognosis. CSF proteome profiling has proven to be a valuable tool for identifying biomarkers for this devastating outcome of cancer. These approaches could lead to the development of clinical tests with improved sensitivity and specificity, which may allow more rapid detection and treatment of individuals in the earliest stages of LM before extensive pathophysiological changes have occurred. Despite the comprehensiveness of the CSF proteome database, there is still a need for continued developments, including integration with a CSF metabolomics database. Currently, the latter comprises approximately 500 molecules [80], which constitutes a wealth of additional potential biomarkers for LM. Integrating these databases will require the application of sophisticated bioinformatic approaches, considering that proteins, metabolites and other molecules are interactive as components of the same biological networks in a systems biology manner. This may even lead to the identification of tests which can be performed using blood serum or plasma as another means of diagnosing CNS disorders. As research in this area evolves from traditional clinical and biological investigations to incorporate multiomic technologies, the integration of the resulting data has emerged as an important next stage. Finally, it is most important that the resulting biomarker fingerprints of these endeavours are validated and then translated to user-friendly platforms to allow more rapid testing in clinical settings. This may allow clinicians to treat LM patients in a personalized medicine manner for the best possible therapeutic outcomes.

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Chapter 6
Connecting Brain Proteomics with Behavioural Neuroscience in Translational Animal Models of Neuropsychiatric Disorders

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6.1 Introduction

Neuropsychiatric disorders, including schizophrenia, major depressive disorder (MDD) and bipolar disorder (BD), are among the leading causes of disability throughout the world [1]. Despite the significant public health cost and personal suffering caused by psychiatric disorders, there has been relatively little progress made in their mechanistic understanding and development of novel drugs. Among the reasons for this lack of progress are the diverse and mostly ill-defined aetiology and considerable complexity of these disorders. However, mechanistic understanding of a human disease and the discovery of therapeutic agents cannot be achieved without good animal models. Unfortunately, most of the models used currently are limited in their ability to capture aetiology and neurobiological mechanisms and, therefore, to predict treatment efficacy in human neuropsychological disorders. In essence, animal models must meet certain validation criteria in order to be effective in this regard. These include construct, face and predictive validity which refer to parallels between the methods of how the model was constructed and the clinical disease, as well as similarities with the normalising effects of the pharmacological interventions in the latter [2].

Aetiological factors, whether genetic or environmental, are still largely unknown and hotly debated in psychiatry. Therefore, it is unlikely that animal models with high

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construct validity can be developed on the basis of our present understanding. Face validity signifies how an animal model recreates key features of a human disease. Due to the lack of well-established and widely replicated biomarkers for psychiatric disorders, animal models can only aim to replicate certain anatomical, neurochemical or behavioural features of the human disease, which will inherently limit their usefulness. Predictive validity refers to the utility of the model to predict effects of drugs, assuming that any similarities in effect are based on a shared mechanism of action. Achieving these levels of validity is less straightforward in psychiatry compared with many other diseases as most of the drugs used therapeutically were identified by studying the mechanisms of action of drugs discovered serendipitously.

One way forward is to search for biomarkers that can be objectively measured, compared and contrasted with the human disorders that we are attempting to model. Such biomarkers can help us to validate the animal models and can also be used to discover mechanistic changes related to the pathophysiology [3]. This is because changing expression patterns of proteins and protein networks in the brain might be related to functional outcomes such as behaviour. Ultimately, the aim is to be able to correlate changes in brain protein expression with behavioural alterations in order to better understand the brain processes that lead to psychopathology. In this chapter, we will review the recent advances in brain proteome research with respect to animal models of major psychiatric disorders, such as schizophrenia and major depression. Our aim is not to provide a comprehensive, systematic review of the field but rather to illustrate how brain proteomics can be used to better understand the pathophysiology of neuropsychiatric disorders using representative examples of well-validated animal models.

6.2 Rodent Models of Schizophrenia

There is a general agreement that schizophrenia is multifactorial with interacting genetic and environmental risk factors involved in the aetiology and development of the disease [2]. It has also been suggested on the basis of epidemiological studies and neurobiological findings that pre- and postnatal developmental events can be contributing factors [2]. Disrupted network connectivity due to abnormal functioning of neurotransmitter systems such as dopamine and glutamate may serve as a final common pathway for several psychiatric disorders [4]. Therefore, this section reviews animal models that have been developed to replicate the diverse aetiological factors of schizophrenia, including genetic susceptibility, early neurodevelopmental insults and pharmacological modification of neurotransmitter systems.

6.2.1 Genetic Models

During the last 20 years, more than two dozens of schizophrenia susceptibility genes have been identified, including neuregulin 1 (*NRG1*), reelin (*RELN*) and a number of genes within the 22q11.2 hemideletion region of chromosome 22 [5, 6].

6.2.1.1 Neuregulin 1 Hypomorph Mice

Polymorphisms within these genetic regions have been associated with an increased risk of schizophrenia. Mice that are hypomorphic for *Nrg1* (*Nrg1* HET mice) display schizophrenia-relevant behavioural phenotypes and aberrant expression of serotonin and glutamate receptors. *Nrg1* HET mice also display idiosyncratic responses to the main psychoactive drugs linked to schizophrenia, such as amphetamine and the constituent of cannabis, Δ(9)-tetrahydrocannabinol (THC) [7–9]. Spencer et al. investigated the hippocampal proteome changes in wild-type (WT) and *Nrg1* HET mice using a 2D gel-based proteomic approach [10]. This analysis led to identification of proteins linked to molecular changes in schizophrenia that have not been previously associated with *Nrg1*. These proteins are involved in vesicular release of neurotransmitters such as soluble N-ethylmaleimide-sensitive factor activating protein receptor (SNARE) proteins, enzymes impacting on serotonergic neurotransmission and proteins affecting growth factor pathways.

6.2.1.2 Heterozygous Reeler Mice

Associations have been identified between single-nucleotide polymorphisms of the *RELN* gene and working memory dysfunction along with the more severe positive and negative symptoms in schizophrenia, including sensorimotor gating deficits [11]. This gene encodes a neural extracellular glycoprotein that participates in control of pivotal processes during neurodevelopment, such as neuronal migration and positioning, as well as postnatal effects such as growth of dendrites and axons, synaptogenesis and neurotransmission. Studies on heterozygous reeler mice, which express approximately 50% of the normal level of reelin protein, found a variety of proteome changes in the brain (cerebellum, anterior cingulate cortex, dentate gyrus and CA3 region of the hippocampus) using 2D gel electrophoresis combined with matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF-MS) and Western blot analysis [12]. These changes showed considerable overlaps with protein level alterations found in individuals with schizophrenia. In the cerebellum, a number of proteins involved in energy metabolism (aldolase A, aldolase C, aspartate aminotransferase) and cell growth/maintenance (actin, spectrin) were decreased relative to the control levels. Furthermore, cell growth/maintenance proteins (actin, spectrin, tubulin) showed widespread lower levels, while proteins involved in energy metabolism (cytochrome b-c1 complex subunit 1, mitochondrial creatine kinase, vesicle fusion ATPase) were either increased or decreased in the anterior cingulate cortex of the heterozygous reeler mice, compared with the controls. Different forms of tubulin proteins were universally decreased, and glycolytic enzyme proteins were either increased or decreased in the dentate gyrus. Glycolytic enzyme proteins were increased, and the mitochondrial ATP synthase was decreased in the CA3 subregion of the hippocampus. In this region, cell growth/maintenance proteins were mostly decreased in the reeler mice compared to wild-type control mice. Finally, expression of heat shock protein a8 (Hsp8) was decreased in all brain regions studied of the heterozygous reeler mice.

The altered expression of proteins involved in neural energy metabolism and those associated with development and maintenance of the cytoskeleton showed good correspondence with findings from comparative studies of human control and schizophrenia post-mortem brain samples [12].

6.2.1.3 Chromosome 22q11.1 Deletion Syndrome Mice

Deletions on chromosome 22q11.2 are a strong genetic risk factor for development of schizophrenia and cognitive dysfunction [13]. A recent comprehensive proteomic/metabonomic study employed a shotgun liquid chromatography-tandem mass spectrometry (LC-MS/MS) profiling approach to investigate frontal cortex and hippocampal tissues from Df(16)A+/− mice, a model of the 22q11.2 hemideletion syndrome [14]. The proteomic brain profiling analysis revealed changes in proteins associated with various molecular pathways, including chromatin remodelling and RNA transcription in both brain regions, indicative of an epigenetic component of the 22q11.2DS. Furthermore, alterations in glycolysis/gluconeogenesis, mitochondrial function and lipid biosynthesis pathways were found. Consistent with this, in silico analysis using the Ingenuity Pathways Analysis (IPA®) software (Qiagen, Redwood City, CA, USA) revealed overlaps between the canonical pathways 'glycolysis I', 'gluconeogenesis' and 'mitochondrial dysfunction'. The proteomic findings were confirmed using selected reaction monitoring mass spectrometry (SRM-MS). This validated the decreased levels of several proteins encoded within 22q11.2 and confirmed increased levels of the computationally predicted putative miR-185 targets UDP-N-acetylglucosamine-peptide N-acetylglucosaminyltransferase 110 kDa subunit (OGT1) and kinesin heavy chain isoform 5A, along with alterations in the non-miR-185 targets serine/threonine-protein phosphatase 2B catalytic subunit gamma isoform, neurofilament light chain and vesicular glutamate transporter 1. Furthermore, changes in the levels of proteins associated with mammalian target of rapamycin (mTOR) signalling were detected in the frontal cortex, and those associated with glutamatergic signalling were found in the hippocampus. On the basis of the combination of different techniques to assess altered expression of protein networks, the authors concluded that the main effects were brain region specific and involved proteins associated with chromatin modulation pathways, along with alterations in lipid and energy metabolism pathways.

6.2.2 Neurodevelopmental Models

The neurodevelopmental hypothesis of schizophrenia is distinct from neurodegeneration and is supported by several pieces of evidence including (1) increased frequency of obstetric complications in patients; (2) the presence of neurological,

cognitive and behavioural dysfunctions that occur before illness onset; (3) a disease course and outcome that are mostly inconsistent with neurodegenerative illnesses; and (4) the absence of post-mortem evidence of neurodegeneration [15]. Animal models using the neurodevelopmental framework of schizophrenia have utilised several approaches including pharmacological lesion of the developing brain by application of specific neurotoxins or agents mimicking infections injected into pregnant dams. Such early insults can result in abnormal neurodevelopment and a schizophrenia-like behavioural phenotype that emerges in adulthood.

6.2.2.1 Neurodevelopmental Lesion Models

Neonatal, neurotoxin-induced ventral hippocampal lesion results in a widespread set of schizophrenia-like behaviours that emerge after puberty, including an abnormal hyperactivity to amphetamine, impaired social behaviour, altered prefrontal cortisol cognitive functions and impaired sensorimotor gating [16]. An organelle proteomic approach enabling the study of neurotransmission-related proteins in the frontal cortex of post-pubertal (postnatal day 60 [PD60]) neonatally ventral hippocampal (nVH)-lesioned rats was used by Vercauteren et al. [17]. This approach identified significantly altered levels of 18 protein spots on 2D gels of plasma membrane-enriched fractions from the lesioned rats compared to controls. MS analysis resulted in identification of nine of these proteins, including the finding of decreased levels of neurocalcin delta. Most of the identified dysregulated proteins were associated with various neurotransmitter systems with roles in plasma membrane receptor functions as well as synaptic vesicle exocytosis and recycling. These included clathrin light chain B, syntaxin binding protein 1b and visinin-like protein 1.

Administration of the DNA-alkylating agent methylazoxymethanol acetate (MAM) to rats on embryonic day 17 (E17) produces behavioural and anatomical brain abnormalities that resemble some aspects of schizophrenia [18]. Proteomic/metabonomic studies have shown that the MAM treatment on E17 results in deficits in hippocampal glutamatergic neurotransmission, as seen in some schizophrenia patients. Most importantly, these results were consistent with the findings of functional deficits in glutamatergic neurotransmission in another study of the MAM E17 rat model, as determined using electrophysiological recordings [19]. This latter study also found that many of the proteins found at altered levels in the hippocampus were components of an in silico-determined interaction network, suggesting significant effects of embryonic MAM treatment on neuronal signal transduction. The results also suggested that MAM treatment may alter synaptic neurotransmission by affecting the extent or innervation and/or postsynaptic signal transduction mechanisms through changes in α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor subunit expression or phosphorylation state, along with effects on clathrin-mediated receptor internalisation and calcium signalling.

6.2.2.2 Maternal Immune Activation Model

An increasing number of studies have found associations between pre- and perinatal immune activation and the development of schizophrenia. Consistently, a large number of heterogeneous abnormalities have been described in established maternal immune activation (MIA) rodent and primate models, which correlate with changes found in the human disease [20]. A recent study investigated the long-term effects of MIA on neocortical pre- and postsynaptic proteomes of adolescent rat offspring [21]. The model was lipopolysaccharide (LPS) administration on embryonic day 13.5, and a proteomic analysis was conducted via combinations of different gel-based proteomic techniques and LC-MS/MS. Interestingly, this study found changes in additional proteins beyond the synaptic ones. At the pathway level, the results implicated effects on synaptic vesicle recycling, cytoskeletal structure and energy metabolism in the presynaptic region in addition to alterations in vesicle trafficking, the cytoskeleton and signal transduction at the postsynaptic level in the MIA offspring. Differing levels of the prominent signalling regulator molecule calcium-/calmodulin-dependent protein kinase II were also found in the prefrontal cortex postsynaptic region. Taken together, these findings suggest that widespread changes occur in the synaptic machinery in MIA rats which might underlie the pathological cortical functions that are characteristic of schizophrenia.

6.2.2.3 Developmental Vitamin D Deficiency Model

Developmental vitamin D (DVD) deficiency is a candidate risk factor for schizophrenia [22], and animal models have found that this is associated with a range of altered genomic, proteomic, structural and behavioural outcomes in rat models [23]. Therefore, DVD deficiency may serve as a validated animal model for schizophrenia. McGrath et al. [24] used a combined 2D gel electrophoresis-MS approach and identified 35 spots, mapped to 33 unique proteins, which were present at significantly different levels in the nucleus accumbens between DVD and control animals. Of these, 22 proteins were decreased and 13 were increased. Among the significantly different proteins, three had calcium-binding functions (calbindin1, calbindin2 and hippocalcin). Other altered proteins associated with DVD deficiency included those with mitochondrial functions and dynamin-related proteins. These findings suggest that DVD deficiency is associated with disruption of pathways related to calcium-binding and mitochondrial functions in the nucleus accumbens, which may also be relevant in schizophrenia.

6.2.3 Pharmacological Models

The mechanism of action of drugs that provide symptomatic relief in psychiatric disorders has played a major role in the development of hypotheses to understand disease mechanisms. For example, the first-generation antipsychotics act

predominantly as dopamine (DA) receptor antagonists. Pharmacological stimulation of DA neurotransmission using indirect agonists such as the psychostimulant amphetamine induces a psychosis-like state in susceptible individuals. These findings led to formulation of the DA hypothesis of schizophrenia, which proposes that hyperactive DA neurotransmission in the mesolimbic system may underlie the psychotic features of the disease [25]. More recently, similar psychosis-inducing effects via inhibition of glutamatergic transmission using the N-methyl-D-aspartate (NMDA) glutamate receptor antagonists ketamine, phencyclidine (PCP) and MK-801 have given rise to the glutamate hypothesis. This hypothesis suggests that hypoactive glutamatergic neurotransmission is involved in development of the psychotic and cognitive symptoms of schizophrenia [4, 26].

6.2.3.1 Methamphetamine Model

Repeated exposure to methamphetamine (MAP) results in a progressively enhanced and enduring behavioural response. This phenomenon is known as behavioural sensitisation, and MAP-induced sensitisation has been suggested to underlie certain aspects of MAP psychosis and schizophrenia [27]. Iwazaki and co-workers examined protein expression profiles in the amygdala of acute MAP-treated and MAP-sensitised rats using a 2D gel electrophoresis-based proteomic approach and found changes in 64 and 43 protein spots, respectively [28]. A total of 48 and 34 proteins were identified in these two models, respectively, using matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF-MS). Comparison of the results between the acute and chronic MAP-treated groups showed that only nine proteins were identified in common. This suggested that the amygdala reacts differently to acute and repeated administration of MAP at the level of the proteome. A number of these proteins were associated with synaptic-, cytoskeletal-, oxidative stress-, apoptosis- and mitochondrial-related functions in sensitised animals. These effects could be associated with the mechanism underlying behavioural sensitisation and may have relevance to psychostimulant-induced psychosis in humans.

6.2.3.2 NMDA Receptor Antagonist Models

The NMDA-type glutamate receptor antagonist dizocilpine ([+]-5-methyl-10,11-dihydro-5H-dibenzo-[a,d]-cyclohepten-5,10-imine-hydrogen maleate; MK-801) induces a variety of behavioural changes, including hyperactivity, stereotyped behaviour, social withdrawal, working memory deficits and abnormal sensorimotor gating, which correspond with behavioural endophenotypes in schizophrenia [29–31].

In an early proteomic study using 2D gel electrophoresis in combination with MS, Paulson and colleagues studied the effects of 8 (short) and 18 (long) daily injections of MK-801 on the rat cerebral cortex proteome [32]. In the short MK-801 injection model, they identified decreased levels of proteins involved in energy

metabolism and oxidative stress, including ATP synthase, pyruvate dehydrogenase, γ-enolase and superoxide dismutase, as well as decreased stathmin and β-actin in the short MK-801 model. The long MK-801 injection model resulted in similarly decreased α-enolase, γ-enolase and stathmin levels. These results implicated alterations in energy metabolism and internal cellular transport mechanisms in the pathophysiology of schizophrenia which may be related to NMDA receptor function. These results were further supported by a follow-up study on the thalamic proteome by the same group [33]. This latter study found significant alterations in a partially overlapping set of proteins which included heat shock proteins 60 and 72, albumin, dihydropyrimidinase-related protein 2, aldolase C, malate dehydrogenase, pyruvate dehydrogenase complex E2, guanine deaminase, α-enolase, aconitase, ATP synthase and alpha-internexin.

One of the most widely used preclinical tools in schizophrenia research is the PCP rat model. PCP is a non-competitive NMDA receptor antagonist, which has been used to induce behavioural alterations in rodents, closely resembling specific symptoms and abnormalities observed in schizophrenia patients [29, 34]. In addition, it is known that PCP and other NMDA receptor antagonists can induce schizophrenia-like symptoms in humans, and this can be reversed by antipsychotic medications [35]. Although chronic administration of PCP to rodents appears to induce persistent alterations mimicking the long-term impairments of schizophrenia such as the cognitive deficits and the negative symptoms [36], the acute administration model resembles the clinical signs of first-episode schizophrenia [37]. A study of the acute PCP rat model found locomotor hyperactivity and enhanced stereotyped behaviour along with changes in 32 out of 449 proteins identified in an LC-MS/MS profiling analysis [38]. In silico analysis using IPA found that four of these proteins (excitatory amino acid transporter 1, microtubule-associated protein 6, dynamin 2 and calcium-/calmodulin-dependent protein kinase type II) were linked directly in an interaction network associated with molecular transport. In addition, this analysis showed that the top disease category associated with the altered proteins was neurological disorders, and the top canonical pathway was clathrin-mediated endocytosis signalling. Interestingly, transketolase, which links glycolysis to the pentose phosphate cycle, was increased by the acute PCP administration.

A proteomic study of the chronic PCP rat model (daily injection of 5 mg/kg PCP for 15 days) identified frontal cortex alterations in glutamate-mediated Ca^{2+} signalling (Ca^{2+}-/calmodulin-dependent protein kinase II, calmodulin-dependent calcineurin A subunit α-isoform [PPP3CA] and visinin-like protein 1 [VISL1]), energy metabolism and mitochondrial function (aspartate aminotransferase mitochondrial [GOT2] and pyruvate kinase isozymes R/L [PKLR]), as well as cytoskeletal remodelling (actin-related protein 3 [ARP3]) [39]. In a recent comprehensive study, the same research group carried out a head-to-head comparison of four different hypo-glutamatergic rodent models which have been well validated (treatment with acute PCP, chronic PCP and ketamine and the NMDA receptor knockdown model) in relation to human schizophrenia [40]. These researchers found that 47, 84 and 93 proteins were altered in the acute PCP, chronic

PCP and ketamine models, respectively, and 80 were altered in the knockdown model. In addition, in silico analysis found a partial overlap of protein-protein interaction networks across the four models. In the acute PCP model, these were cellular component assembly, transport, regulation of cellular metabolic process, negative regulation of biological process and regulation of signalling. For the chronic PCP model, phosphorus metabolic process, organic substance catabolic process, single-organism catabolic process, transport and cell projection organisation were identified. In the ketamine model, the main interaction networks were transport, regulation of localisation, cell communication, cell-cell signalling and regulation of cell communication, and for the NMDA receptor knockdown mice, these were phosphorus metabolic process, transport, establishment of localisation in cell, cellular component assembly and regulation of transport. The authors found that groups which clustered together and overlapped appeared to be involved in closely related biological processes, and this resulted in identification of five functional domains that were present in all four models: (1) intracellular signalling and regulation, (2) development and differentiation, (3) intracellular transport and organisation, (4) biosynthetic processes and energy metabolism and (5) nucleic acid metabolism and ATP/GTPase activity. They also found that the chronic PCP model represented human schizophrenia more closely than the other groups, on the basis that the frontal cortical protein expression profiles were linked to the latter four functional domains listed above.

Comparing and contrasting findings from a variety of animal models may allow us to identify common patterns of change, as highlighted above. A summary of the alterations in functional protein networks in different, aetiologically based animal models showing schizophrenia-like characteristics, indicating the existence of common patterns of change, is given in Table 6.1. For example, shared effects such as altered presynaptic transmission (synaptic vesicle recycling), abnormal energy metabolism (glycolysis and mitochondrial functions), cytoskeletal structure, intracellular transport and intracellular calcium signalling can appear across aetiologically diverse models. Such common patterns may indicate mechanisms that are not unique to a particular model and instead may underlie fundamental neurobiological changes of schizophrenia. From this, it is conceivable that abnormal glycolysis and mitochondrial energy metabolism may affect multiple processes in neurons that depend heavily on the availability of cellular energy in the form of ATP, such as presynaptic transmitter release, recycling and intracellular transport. In turn, this can lead to impaired information processing in networks of brain regions that give rise to abnormal behaviour. Since the same processes have been found to be impaired in post-mortem brains from individuals who suffered from schizophrenia [41], it can be proposed that the psychopathology (the collection of schizophrenia endophenotypes) is also driven by similar underlying neurobiological effects. The overlap of these common protein expression patterns with protein expression changes found in human schizophrenia brains further enhances the validity of the animal models. Furthermore, it can also validate the findings identified in diverse human sample cohorts through the power of converging evidence.

Table 6.1 Summary of changes in functional protein networks in different, aetiologically based animal models showing schizophrenia-like characteristics

Animal model	Brain region	Proteomics approach	Altered protein networks
Genetic models			
Neuregulin 1 hypomorph	Hippocampus	2D gel proteomics	Vesicular release of neurotransmitters such as SNARE growth factors
Heterozygous reeler	Cerebellum, anterior cingulate cortex, dentate gyrus and CA3 region of the hippocampus	Two-dimensional gel electrophoresis, matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry, and Western blot	Cell growth and maintenance, cytoskeleton and internal motility, glycolysis, energy metabolism, heat shock proteins
Chr. 22 g11.2 hemideletion (Df(16) A+/− mice)	Prefrontal cortex and hippocampus	Shotgun liquid chromatography-mass spectrometry (LC-MS) proteomics	Chromatin remodelling, RNA transcription, glycolysis/gluconeogenesis, mitochondrial function, lipid biosynthesis
NR1 knockdown	Prefrontal cortex	LC-MS proteomics	Phosphorus metabolic process, transport, establishment of localisation in cell, cellular component assembly, regulation of transport
Neurodevelopmental models			
Neonatal vHPC lesion	Prefrontal cortex	Two-dimensional gel electrophoresis organelle proteomic with mass spectrometry	Plasma membrane receptor, expression and recycling, synaptic vesicle exocytosis/ recycling
MAM (ED17) treatment	Hippocampus	LC-MS proteomics	Glutamate-glutamine cycle proteins, synaptic vesicle controlling proteins
Maternal immune activation	Neocortex	Combinations of different gel-based proteomic techniques and tandem mass spectrometry	Synaptic vesicle recycling, cytoskeletal structure, energy metabolism, vesicle trafficking, cytoskeleton, signal transduction
Developmental vitamin D deficiency	Nucleus accumbens	2D gel electrophoresis-based proteomics and mass spectroscopy	Calcium-binding proteins mitochondrial functions, dynamin-like proteins

Table 6.1 (continued)

Animal model	Brain region	Proteomics approach	Altered protein networks
Pharmacological models			
Chronic methamphetamine	Amygdala	2D gel electrophoresis-based proteomics and mass spectroscopy	Synaptic function, cytoskeletal structure, oxidative stress, apoptosis, mitochondrial function
Chronic MK-801	Neocortex and thalamus	2D gel electrophoresis-based proteomics and mass spectroscopy	Energy metabolism, oxidative stress, heat shock proteins, internal cellular transport
Acute PCP	Hippocampus	LC-MS profiling	Molecular transport, clathrin-mediated endocytosis signalling, glycolysis
Chronic PCP	Frontal cortex	LC-MS profiling	Glutamate-mediated Ca2+ signalling, glycolysis, energy metabolism, cytoskeletal remodelling
Acute PCP: chronic PCP, ketamine	Prefrontal cortex	LC-MS profiling	*Acute PCP:* cellular component assembly, transport, regulation of cellular metabolic process, negative regulation of biological process, regulation of signalling *Chronic PCP:* phosphorus metabolic process, organic substance catabolic process, single-organism catabolic process transport, cell projection organisation *Ketamine:* transport, regulation of localization, cell communication, cell-cell signalling, regulation of cell communication

6.3 Rodent Models of Depression

Due to known links in the literature, many models of depression in rodents have been generated through exposure to psychological stress, and these have been analysed by determining differences between the susceptible and non-susceptible animals. The main brain regions which have been implicated in depressive-like symptoms are the frontal cortex and the hippocampus [42].

6.3.1 Stress-Based Models

A study from 2012 used a chronic mild stress rat model of depression to identify hippocampal proteins which differed in their expression levels between stress-susceptible and stress-resilient rats using an isobaric tagging for relative and absolute quantitation mass spectrometry (iTRAQ-MS) approach [43]. This analysis showed that 73 out of 2000 proteins were differentially expressed. Interestingly, stress susceptibility was associated with increased levels of the SCN9A sodium channel protein, which is currently under investigation as a potential antidepressant target. Other proteins associated with stress susceptibility included SNCA, SYN-1 and AP-3, which are involved in synaptic vesicle release, and COX5A, NDUFB7, NDUFS8, COX5B and UQCRB, which are associated with energy metabolism pathways. Yang et al. attempted to identify differential protein expression in the frontal cortex using a chronic unpredictable mild stress rat model of depression in comparison to control rats [44]. For this, they analysed frontal cortices by 2D gel electrophoresis combined with matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF-MS). They found that 29 proteins were altered, and these were mainly involved in energy and glutathione metabolism functions.

Hodes and colleagues employed a social stress model in mice and found individual differences in the sensitivity of the peripheral immune system to social stress [45]. They used a multiplex immunoassay to assess cytokine profiles obtained 20 min after the first stress exposure, and this showed a significant elevation of IL-6 in susceptible mice. Interestingly, the levels of this cytokine remained high for at least 1 month after the stress exposure.

A study using the Flinders sensitive line and the Flinders resistant line rats were subjected to maternal separation stress during the early postnatal period and analysed at postnatal day 73 (consistent with rat adulthood) [46]. The analysis consisted of proteomic profiling of purified synaptosomes using 2D gel electrophoresis to display altered proteins and MS for identification purposes. This showed that proteins and molecular pathways related to energy metabolism and cellular remodelling were associated with the differential response to the stress, consistent with the other studies above. Along the same lines, Zhou and co-workers applied a chronic mild stress protocol to separate susceptible and unsusceptible rat subpopulations and then performed MS analysis to identify differential proteins in hippocampal synaptic junction preparations [47]. This resulted in identification of 89 membrane proteins that were present in differential amounts, and database searching confirmed that more than 91% of these had a synapse-specific localisation. Interestingly, proteins involved in membrane trafficking, membrane fusion and neurotransmitter release were altered in the non-susceptible rats. This suggested that increased neurotransmission in this brain region may be part of a stress-protection mechanism.

6.3.2 Inflammation Models

A number of previous studies have suggested an association between depression and inflammation. With this in mind, one research group systemically administered bacterial LPS to study inflammation-associated behavioural changes in rodents and attempted to link these with changes in proteomic fingerprints [48]. For the proteomic changes, they used 2D gel electrophoresis combined with MALDI-TOF MS to identify altered proteins in the frontal cortex. This led to identification of 26 proteins which showed changes in levels due to the treatment. Two of these were validated using Western blot analysis, confirming significant increases in the levels of creatine kinase B and dihydropyrimidinase-like 3 in the treated mice. Recapitulating the major pathways identified in the above studies, these findings implicated alterations in energy metabolism and neuronal growth or synaptogenesis in the mechanism of action. This is also consistent with the findings of previous human studies of depression [49, 50]. Putting all of this together, a recent study integrated results of proteomic profiling studies in depression models to identify the most reproducible molecular pathways which are likely to be involved in the disease [51]. This was carried out by comparison of overrepresented gene ontology (GO) biological process terms and pathways in each model. This led to identification of molecular processes such as the immune response as well as those involved in glutamatergic signalling, neurotrophic responses, energy metabolism and circadian rhythms. Effects on circadian rhythms and sleep pattern disturbances have been widely reported to occur in depression [52–54].

6.3.3 Chronic Antidepressant Treatment

The possibility that synaptic function is perturbed in depression models is supported by the possibility that antidepressants may work by increasing neurogenesis [55]. Others have postulated that synaptogenesis and reorganisation or reintegration of new neurons (rather than incorporation of new neurons by neurogenesis) may underlie the role of antidepressant action in alleviating symptoms of depression [56]. A proteomic study in guinea pigs in 2004 showed that antidepressant treatment resulted in frontal cortex changes in the levels of multiple heat shock protein 60 forms along with neurofilaments and related proteins that are critical determinants of synaptic structure and function [57]. Another proteomic study carried out in rats found that antidepressant treatment induced changes in proteins related to both neurogenesis and synaptic function, along with effects on proteins involved in energy metabolism and the oxidation-reduction cycle [58]. The finding of changes in proteins involved in all of these pathways is not surprising, given the high energy demands involved in processes such as neurogenesis and synaptic remodelling. This

also highlights the possibility of identifying novel drug targets for depression, based on one or more of these pathways.

6.4 Translating Brain Proteomic Findings from Animal Models to Understand the Neurobiology of Mental Illness

The major challenge of today's neuroscience is to translate preclinical findings to aid diagnosis and treatment of psychiatric disorders. It has been widely acknowledged, even by researchers working on the field of preclinical animal models, that no single animal model will be able to capture the complexity of a psychiatric disorder. With the advent of the Research Domain Criteria (RDoC) approach [59, 60], such an attempt will become obsolete. Instead, we should aim to focus on providing proteomic data for the RDoC matrices from aetiologically driven, behaviourally characterised animal models (Fig. 6.1). The protein expression changes from a particular

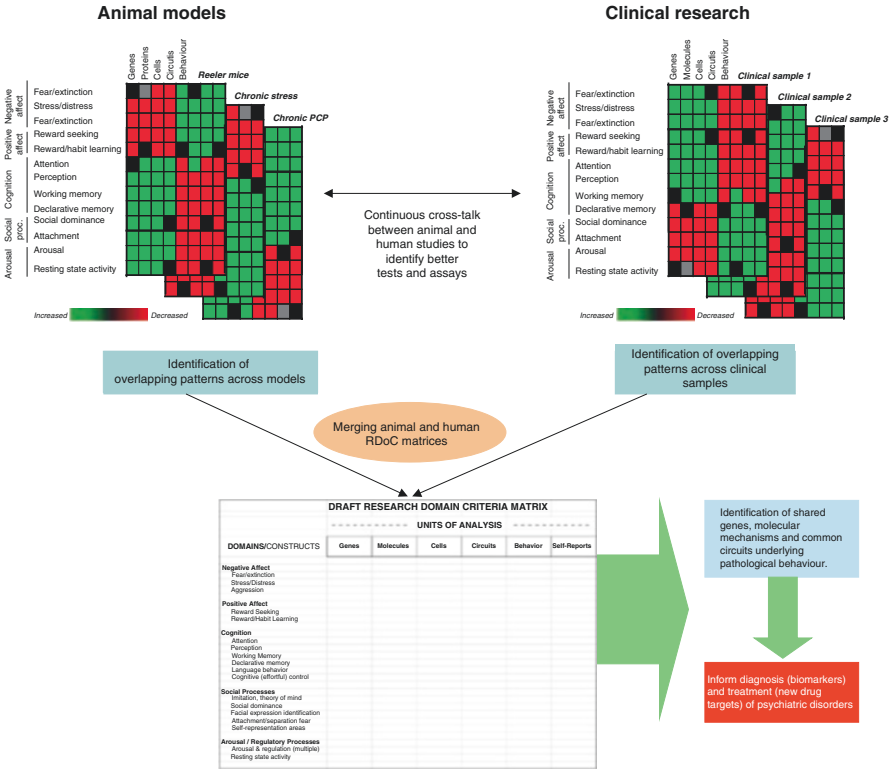

Fig. 6.1 Diagram showing the merging of RDoC matrices for animal models and the human disease

brain area can then be linked to the behavioural alterations associated with these models. Superimposing data from multiple, aetiologically driven models in this way will identify common protein expression patterns associated with particular RDoC behavioural domains. By doing so, we can then draw some inferences about the links between protein expression patterns in a network of brain areas with disease-relevant behavioural domains, such as the negative emotional domain or executive function. Merging these preclinical data with similarly populated RDoC matrices from human psychiatric disorders will allow direct translation to clinical scenarios regardless of the exact diagnostic entity. For example, we can analyse the protein expression networks in the dorsolateral prefrontal cortex of individuals with well-characterised neuropsychological profiles, such as impaired working memory, anhedonia and deficits in attentional functions. By superimposing this pattern in an RDoC matrix constructed by using the protein expression network profiles of animal models showing similar behavioural abnormalities determined by translationally validated behavioural tests, one will be able to achieve a previously unrealised translational precision of linking preclinical findings with clinical results. This can then offer mechanistically relevant protein expression patterns that are typical of a certain set of RDoC behavioural domains, which can be used for diagnostic purposes or to drive personalised approaches for treatment of individuals with the relevant set of behavioural abnormities, irrespective of the traditional psychiatric diagnosis.

6.5 Conclusions

This chapter has described the recent advances in connecting brain proteomic fingerprints and behavioural patterns in animal models of major psychiatric diseases. It is apparent that none of these models are capable of recapitulating the corresponding human disease. Instead, it is more likely that such models can only mimic certain aspects of these diseases. The most robust models of schizophrenia revolve around the disrupted neuronal connectivity related to abnormal functioning of neurotransmitter systems such as the dopaminergic and glutamatergic pathways. These include the acute and chronic PCP models, along with the ketamine-administration and the NMDA receptor knockdown models. Most of the animal models of depression have been produced by introduction of stress factors along or administration of inflammatory agents. We have also learned that most antidepressants may work by reversing the disruption of synaptogenesis and neurogenesis pathways in the disease state. Such findings leverage the Research Domain Criteria matrices by attempting to link molecular changes with behavioural endophenotypes. This approach should also lead to development of the most robust disease models for increasing our understanding of the underlying pathophysiologies of these devastating psychiatric disorders and pave the way for identification of novel biomarkers and therapeutic targets. It is also anticipated that this will help to guide much-needed novel treatment and drug discovery approaches.

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Chapter 7
LC-MS^E for Qualitative and Quantitative Proteomic Studies of Psychiatric Disorders

Mariana Fioramonte, Paul C. Guest, and Daniel Martins-de-Souza

7.1 Introduction

The proteomic research field experienced a revolution since the experimental approach named "shotgun proteomics" emerged combining analytical chromatography and mass spectrometry (MS) to analyse highly complex biological samples containing proteolytic digested proteins (bottom-up approach) [1]. The challenge behind this task lies on the large number of expressed proteins in the sample and their relative stoichiometry. The main goal in shotgun proteomics is to identify and quantify as many peptides as possible, ideally all peptides present in the sample.

The use of MS to detect and analyse these peptides requires the use of a separation technique since the presence of multiple peptides could lead to ionization suppression [1]. There are a wide variety of chromatography techniques such as capillary electrophoresis, solid-phase extraction, strong cation exchange, liquid chromatography (LC) and others that could be combined with matrix-assisted laser desorption/ionization (MALDI) or electrospray ionization (ESI) methods to perform MS experiments. From all of the combinations available, LC-ESI-MS has proved to be the most successful due to its sensitivity and reproducibility.

An important advancement in shotgun proteomics was the development of MS automated acquisition routines for tandem MS equipments, allowing high-throughput unattended peptide fragmentation during the LC elution of peptide

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mixtures. The data-dependent analyses (DDA), established in the early 1990s, consist of the acquisition of a full-scan MS spectrum followed by multiple product ion spectra (MS/MS) acquisition in an alternate fashion based on the most abundant precursor signals in a decreasing order of abundance [2]. After several MS/MS spectra are acquired (usually from three to ten, depending on the MS instrument and sample complexity), the mass spectrometer acquires a new full-scan spectrum, and a new acquisition cycle begins again. To avoid multiple fragmentation of the same precursor ion, a dynamic exclusion is usually employed during the length of the chromatographic peak, leading to fragmentation of the maximum number of unique peptides as possible using this methodology.

The DDA acquisition mode is mostly used in current proteomic studies for many purposes such as protein identification, characterization of post-translational modifications and protein quantitation. Although highly employed, DDA presents two main disadvantages: (i) as mentioned before, the precursor selection is based on relative abundance of ions in a specific elution time, making low-intensity ions unlikely to be selected; (ii) as precursor ion selection is a stochastic process, only a few ions are selected during their chromatographic apex, compromising data quality for peptide identification and quantitation [2, 3].

Data-independent analysis (DIA) is an alternative acquisition method for which there is no precursor ion selection in the MS/MS spectrum acquisition. This means that DIA does not depend on previous MS spectrum information for MS/MS acquisition. This kind of experiment is performed mostly on quadrupole time-of-flight (Q-TOF) instruments, and it is designed to maximize the acquisition time on the mass spectrometer [4]. Basically, after the full-scan spectrum is recorded, instead of selecting the precursor ions for MS/MS experiments one by one, the DIA mode can perform the MS/MS experiment in two different ways. The first way is by fragmenting all of the precursor ions. In other words, the whole mass-to-charge ratio (*m/z*) range is fragmented at once (called broadband DIA), leading to multiplexed MS/MS spectra containing fragments from all of the precursor ions [4]. The second way is by selecting several ions on predefined *m/z* windows (covering tens or hundreds of *m/z*), dividing the full range into small fixed ones, leading to several multiplexed MS/MS spectra containing fragments from multiple precursor ions [5, 6]. Compared to DDA, this approach increases the duty cycle on the mass spectrometer, enhances the dynamic range and acquires MS/MS spectra during the whole chromatographic peak for all ions [4, 7].

The challenge in DIA experiments is to deconvolute the multiplexed MS/MS spectrum into its components (i.e. to assign each fragment ion to its respective precursor ion). Two different computational approaches are currently used toward this end: (i) each multiplexed MS/MS spectrum is compared to a peptide spectral library, and matched spectra are sequentially depleted from the multiplexed one until no further matches are found. The peptide identification is therefore based on the peptide identity of the matched library spectrum [2, 5]; (ii) the fragment ions are assigned to their precursor ions by matching the irrespective chromatographic profile, as corresponding fragments and precursor ions must have exactly the same chromatographic profile, and a database search is then performed on these deconvoluted MS/MS spectra [3, 7].

Given this scenario, several DIA methodologies have been described combining these two different types of data processing with the two different types of acquisition [4, 5, 8–10]. The use of peptide spectral libraries relies on the presence of the specific peptide spectrum in the library for the organism of interest. On the other hand, deconvolution from the chromatographic alignment method is more advantageous as it only requires a protein sequence database. Broadband DIA acquisition leads to a higher mass spectrometer duty cycle, acquiring more MS and multiplexed MS/MS spectra per chromatographic peak, which is important especially for quantitation methods as this provides more points per peak. The MS^E methodology [4] was the pioneer of combining both, chromatographic alignment deconvolution and broadband DIA in one method.

7.2 MS^E Methodology

The MS^E methodology is performed on Q-TOF instruments cycling collision energy voltages between low (around 10 eV) and high (ramping from 25 to 35 eV) energy values to obtain full-scan MS spectra and MS/MS fragment spectra, respectively [4]. Consequently, precursor and fragment ion information is acquired at multiple points during the chromatographic elution.

The resulting file has three recorded functions. These are the low-energy function (full-scan MS spectra), the high-energy function (multiplexed MS/MS spectra) and the lock mass (calibrant acquisition all the time during the LC-MS). The algorithm for MS^E processing is called ion accounting [7], and the first step during data processing is to align the chromatographic profile of all precursor ions contained in the low-energy function with the chromatographic profile of the respective fragment ions from the high-energy function. As described above, the chromatographic profile of all ions associated with an eluting precursor ion should be exactly the same. The comparison of chromatographic profiles is based on the apex retention time, peak area, peak width at half maximum as well as the start and end of each peak [3] (Fig. 7.1).

After this step, a time-aligned inventory is obtained, and in addition to the chromatographic information, this inventory contains the monoisotopic accurate mass, the summed peak area for all isotopes for all charge states, a calculated area deviation and average charge state. Furthermore, all of this information is compiled into a table for each precursor/fragment ion set. For each precursor ion, multiple fragment ions are associated, and if a product ion matches multiple precursor ions, this fragment ion will be related to all precursors. Later, the algorithm will be able to decide which precursors this fragment belongs to, depleting product ions from securely identified peptides and proteins through an iterative search process. The data in the precursor/fragment ion tables pass through additional filtering processes to remove product ions which are not compatible with the assigned precursor (e.g. fragments with higher intensity than the precursor ion or fragments with mass higher than precursor ion). The final tables go through a pre-assessment peptide

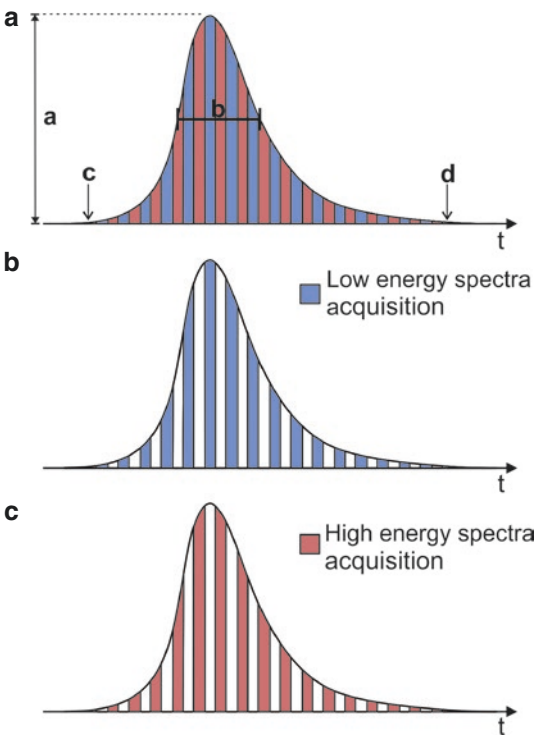

Fig. 7.1 (**a**) Time-aligned chromatographic profiles of the precursor and fragment ions and chromatographic characteristics: apex retention time *(a)*, peak width at half maximum *(b)*, start *(c)* and end *(d)* of chromatographic peak. (**b**) Chromatographic profile for the precursor ion. (**c**) Chromatographic profile for the fragment ions. Adapted from [3]

identification search, and based on the top 250 highest score peptides, the algorithm models some parameters related to the liquid and gas phase physicochemical properties, and these parameters will be employed as additional scoring terms in subsequent steps [3, 4, 7].

Each precursor/fragment ion table is then submitted to the protein sequence database search. The database search is a hierarchal process, testing many cycles as necessary until a minimum protein score cannot be reached or the specified false-positive rate of identification is ruptured (Fig. 7.2). The false discovery rate is calculated based on the precursor/fragment ion search against a reversed or scrambled protein sequence databank [4, 5]. The first iteration consists of querying each fragment/product ion table against the protein sequence database considering only completed cleaved tryptic peptides for identification. The score obtained through peptide matching for each identified peptide is then correlated and adjusted based on the pre-assessment model. Upon completion of the peptide score adjustment, these peptides are organized as tentative protein identifications. The initial protein score is also adjusted according to calculated physicochemical properties. For example, it would be adjusted according to the total number of product ions expected for a given protein or the total precursor intensity. After the protein scoring process is completed, the protein with the highest score is considered as correct, and all of the precursors and product ions associated with this protein with the highest scores are

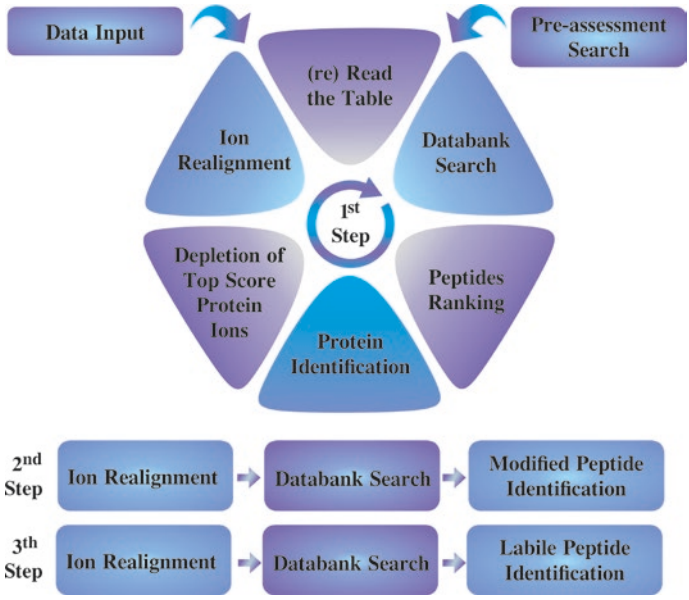

Fig. 7.2 Representation of ion accounting algorithm processing for MS^E protein identification

excluded from the precursor/fragment ion tables and thereby from the subsequent protein identifications. This cycle of ranking and scoring peptides and proteins is repeated until either no protein identification exceeds the minimum score or the proposed false-positive rate has been exceeded (Fig. 7.2).

The second step of protein identification consists of the identification of peptide modifications and nonspecific cleavage products. For this, the software performs a realignment of all precursor and product ions that were not identified in the previous step and uses a temporary database of previously identified proteins. This is also a hierarchal process with several iterations and score adjustments. A third and final step is performed with the remaining ions. In this case, the fragment/product ions are searched against the complete protein sequence databank. However, in this case, there is no restriction on product ion intensity, which could lead to identification of labile peptides that produce in-source fragments since these fragments could be more intense than the precursor ions [3, 7].

7.3 LC-MS^E and Label-Free Protein Quantitation

Mass spectrometry-based quantitative proteomics can be distinguished on the basis of absolute and relative quantitation [11]. Relative quantitation compares the fold change of protein abundance in different biological samples. In contrast, absolute quantitation measures the absolute amount (e.g. pg, ng, μg) of a specific protein in a sample. The main focus in this chapter is on relative quantitation [11, 12].

Relative quantitation can be performed through two main approaches: stable-isotope labelling and label-free quantitation. Some examples of labelling techniques involve modifying peptides with isobaric tags such as iTRAQ and TMT [13, 14] or through labelling of proteins by metabolic incorporation of stable isotopes with amino acids in cell cultures (SILAC) [15]. The isobaric methods are based on labelling of peptides derived from protein digests with distinct mass tags. These different isobaric tags will cause a constant mass shift in precursor peptides but will generate specific fragments with different masses. Thus, by measuring the relative intensity of these specific fragment reporter ions, one can measure the relative abundance of these peptides in the different conditions. Consequently, the relative abundance of the respective proteins in the original samples can be estimated. For the SILAC method, the cells are cultured in media containing different labelled amino acids, leading to metabolic incorporation of these heavy and light amino acid mixtures into newly synthesized proteins. After sample mixing and digestion, each peptide will present a pair of *m/z* in the MS spectrum representing the resulting heavy and light peptides. If the heavy peptide comes from the experimental sample and the light one from the control, the relative intensity of the heavy to light peptide which is correlated with the relative abundance of the respective peptide in the original sample and the protein abundance can be inferred from the peptide abundance. For both methodologies, samples can be analysed by MS using DDA acquisition mode. Although these methodologies offer good opportunities for large-scale relative quantification in biological samples, they present some limitations. For both methodologies, there is a limitation on the number of sample conditions that can be compared [11, 16], since this is limited by the availability of relatively expensive tagging reagents/amino acids. Furthermore, the SILAC method can only be used in cell culture studies [16], excluding applications for samples such as tissues and blood, for example. For the isobaric tags, the main disadvantage is the possibility that fragmentation of multiple precursor ions lies within the *m/z* window selection, generating a chimeric MS/MS spectrum. In this case, the resulting reporter ion intensities will be skewed [16–18], obscuring the quantitative information. The chimeracy is especially problematic for low-intensity ions in complex mixtures as the chances of multiple precursor isolation are higher. Another problem that can be seen in isobaric labelling studies is the compression of the reporter ion ratios [17–19]. In this case, accuracy is compromised as the ratios are compressed toward 1, leading to underestimation of the ratio [18]. On top of that, accuracy is usually limited to the order of magnitude [19]. On top of all these limitations and disadvantages, there are also the intrinsic limitations of DDA acquisition mode as mentioned before.

On the label-free quantitation side, as the name implies, there is no need of stable-isotope labelling, and there are also two different approaches called spectral counting and intensity-based label-free analysis (Table 7.1) [11]. The spectral counting method is based on counting the number of peptide spectra assigned to a protein after a DDA experiment. This works as more abundant peptides will be selected more frequently, leading to a higher number of MS/MS spectra than for a

Table 7.1 Examples of label-free quantitation methods

	Method	MS acquisition mode	Method of quantitation
Spectral counting	Spectral counting	DDA	Counting the number of peptide spectra
Intensity based	DDA intensity based	DDA	Integration of XIC
	MS^E	Broadband DIA	Integration of XIC
	SWATH	DIA on predefined *m/z* windows	Integration of XIC

less abundant peptide [11, 16]. The spectral counting methodology inherits the DDA acquisition problems mentioned before, being less reliable for trace and/or low-mass proteins and less responsive toward small fold changes [16].

The intensity-based label-free method is based on the peptide precursor ion response intensity. The samples from different conditions are acquired in parallel (it can be performed using both DDA and DIA acquisition modes), and the comparison is based on the integration of extracted ion chromatogram (XIC) peak for each peptide on each condition. An internal standard is usually spiked in to all samples for data normalization [11, 16].

As for data acquisition, the DDA method is not recommended as MS spectra are acquired in alternating mode, resulting in fewer points per chromatographic peaks for each peptide. This leads to inaccurate peak integration when compared to DIA acquisition mode. In this case, the use of the MS^E method is convenient, since MS^E fragments the whole *m/z* range at once, maximizing the duty cycle of acquisition and increasing the number of points per chromatographic peak for each peptide [3] (Fig. 7.1). Another advantage of using MS^E instead of DDA or even other DIA acquisition modes comes from the fact that MS^E processes all precursors from all charge states together, avoiding discrepancies in matching common ions detected in one run [7]. Also, when compared to the stable-isotope labelling methodologies, there are no restrictions about the number of different conditions that could be compared, and any soluble biological sample can be analysed, including tissues and fluids. The use of MS^E leads to a higher quantitative dynamic range (up to four orders of magnitude) [4] and eliminates the chimeric peptide problem.

To maximize the performance of intensity-based label-free quantitation using MS^E, a Q-TOF instrument combined with travelling wave ion mobility separation (TWIMS) can be used. A TWIMS device allows peptide separation based on ion mobility followed by measurement of all ions in the TOF analyser. Precursor/fragment ions can also be matched based on similarity of their mobility profiles. The use of ion mobility along with MS^E is referred to as HDMS^E and usually doubles the number of quantified proteins [20, 21]. One feature of this combination is the addition of a new separation dimension, resulting in separation of peptides with same *m/z* and retention time. HDMS^E increases the accuracy of quantitation for lower abundance peptides and also increases even more the dynamic range for peptide quantitation (over six orders of magnitude) [21].

7.4 LC-MS^E Analysis in Studies of Psychiatric Disorders

Several studies have now been carried out using LC-MS^E profiling in the investigation of psychiatric disorders, and more recently, a few papers have been published using (1D/2D)LC-HDMS^E. LC-MS^E has been most employed in the analysis of brain tissue, serum/plasma and peripheral blood mononucleocytes (PBMCs) as described in the sections below.

7.4.1 Brain and Neuroendocrine Tissues

Given that psychiatric disorders are diseases of the brain, it is not surprising that most LC-MS^E profiling studies have focussed on analysis of this tissue. One of the first LC-MS^E profiling studies examined post-mortem dorsolateral prefrontal cortex (DLPFC) tissue from schizophrenia patients and controls [22]. This resulted in identification of significant differences in the levels of 30 out of approximately 500 identifiable proteins, and these were involved mainly in synaptic function (e.g. myelin-associated glycoprotein precursor, myristoylated alanine-rich C-kinase substrate, neural cell adhesion molecule 1, neurofascin, neurofilament medium polypeptide, neuromodulin, synapsin-2, syntaxin-1A) and energy-/metabolism-related (e.g. glutamate dehydrogenase 1, glyceraldehyde-3-phosphate dehydrogenase, protein-arginine deiminase type 2, pyruvate dehydrogenase) pathways. Given the importance of the DLPFC in psychiatric disorders [23, 24], Martins-de-Souza and co-workers in 2011 used a combined gel electrophoresis/LC-MS^E approach and found 488 unique proteins, which were identified by a minimum of two distinct peptides and detected in at least 9 out of the 12 post-mortem samples [25]. These proteins were involved predominantly in cytoskeletal architecture, metabolism, transcription/translation and synaptic function. Combined with the above study, this resulted in a total of 755 unique proteins. These are modest numbers compared to the capacity that this technique possesses today. More recently, a total of 60,634 peptides corresponding to 3283 proteins were identified in the human corpus callosum proteome using a 1DLC-MS^E [26]. This provided the most extensive phosphoproteome analysis to date and also provided further support for the role of disrupted glial cell pathways in schizophrenia. The scenario can be much improved if 2DLC-HDMS^E is applied.

In 2012, LC-MS^E was also applied in a similar analysis of DLPFC tissue from major depressive disorder (MDD) patients, resulting in distinct proteome fingerprints between patients and controls [27]. The differentially expressed proteins were mainly involved in energy metabolism and synaptic function, consistent with the above studies of this tissue, and changes were also found in the histidine triad nucleotide-binding protein 1 (HINT1), which has been implicated in mood and behaviour regulation [28–30]. This same study also found distinct proteome profiles in samples from MDD patients with and without psychosis, with the former showing an overlap with changes seen in the analysis of the same brain region from

schizophrenia patients. This suggested that it may be possible to distinguish different subtypes of MDD based on LC-MS^E proteomic profiling.

Another study carried out a comparative phosphoproteome analysis of post-mortem DLPFC from MDD patients and control donors using LC-MS^E profiling [31]. This led to identification of more than 800 nonredundant proteins containing phosphorylated sequences. Interestingly, 90 of these proteins showed differential levels of phosphorylation in samples from MDD subjects compared to controls, and most of these were associated with synaptic transmission and cellular architecture. A more recent study investigated post-mortem prefrontal cortex tissue of patients with schizophrenia, bipolar disorder (BD) and MDD with and without psychotic features, compared to that from healthy controls using a combined LC-MS^E and selective reaction monitoring mass spectrometry (SRM-MS) approach [32]. In silico analyses of the resulting biological annotations showed that there were effects on common pathways across all of these disorders, associated mainly with presynaptic glutamatergic neurotransmission and energy metabolism. However, these appeared to be opposite in that schizophrenia patients showed changes linked to a hypoglutamatergic state and lower energy metabolism, whereas BD and MDD patients showed increased activation of these same pathways.

In LC-MS^E profiling studies of other brain regions which have been implicated in psychiatric disorders, Martins-de-Souza and colleagues characterized the human occipital lobe and cerebellum [33]. They identified 391 and 330 unique proteins in the occipital lobe and cerebellum, respectively, and 297 additional proteins which were present in both brain regions. Interestingly, the unique proteins found in the occipital lobe included growth hormone and several members of the Ca^{2+}-dependent calmodulin kinase and serine/threonine protein phosphatase families. The same group identified more than 100 differentially expressed proteins in the DLPFC, occipital lobe and cerebellum from frontotemporal lobar degeneration patients compared to controls [34]. The altered proteins were more strongly associated with distinct biological pathways in the different brain regions, with cellular transport affected more in the DLPFC, protein metabolism in the occipital lobe and energy metabolism in the cerebellum. By comparing their proteomic findings with other studies, the authors discussed the point that similar findings have been observed in LC-MS^E analyses of brain tissues from schizophrenia and Alzheimer's disease patients.

Two studies have also been carried out investigating post-mortem pituitaries from patients with schizophrenia [35], BD [36] and MDD [36]. Krishnamurthy et al. identified differentially expressed proteins in pituitaries from schizophrenia patients compared to controls [35]. This included changes in several hormones (e.g. agouti-related protein, arginine vasopressin, growth hormone, prolactin, pro-opiomelanocortin [POMC]) as well as proteins associated with lipid transport and metabolism (e.g. apolipoproteins, APO-A1, APO-A2, APO-C3, APO-H). Stelzhammer and colleagues found altered levels of the hormones galanin and POMC in pituitaries from BD patients as well as changes in proteins associated with gene transcription, stress response, lipid metabolism and growth pathways [36]. In contrast, pituitaries from the MDD patients had significantly decreased levels of

two prohormone-converting enzymes (carboxypeptidase E, prolyl-oligopeptidase convertase) and significant changes in proteins involved in intracellular transport and cytoskeletal functions. Given that the pituitary releases the major hormones and many other bioactive molecules directly into the bloodstream via regulated exocytosis [37], many of these may prove useful as potential proteomic biomarkers in blood serum or plasma. This would be advantages in clinical studies involving blood sampling of living patients [38].

7.4.2 Serum, Plasma and Peripheral Tissues

Although mainly considered as brain diseases, it is now clear that psychiatric disorders can also be manifested at the molecular level in blood serum and plasma, as well as in other peripheral tissues. This attribute can be explained by the fact that the body works as an integrated unit with two-way molecular communication between the brain and the periphery [39]. Levin and co-workers performed one of the first LC-MS^E studies of serum and identified a total of 217 proteins [40]. Ten of these proteins were found at significantly lower levels in samples from schizophrenia patients compared to controls. These were identified as five members of the apolipoprotein family (APO-A1, APO-A2, APO-A4, APO-C1 and APO-D), along with the inflammation-related markers CD5L and immunoglobulin M, the clotting factor XIII B and the transport protein serotransferrin. Considering that these represent some of the most abundant proteins in serum, the authors suggested that analyses on the lower abundance proteins in this biofluid are required. Such an analysis has been carried out using an immunoaffinity-based approach to deplete 20 most abundant proteins in human serum [41]. In this study, Koutroukides et al. found that this approach also resulted in removal of more than 120 additional proteins which may be associated with the major depleted components. These proteins had important functions which could report on physiological status such as the binding and transport of nucleotides, metal ions, carbohydrates and lipids. This raises the point that analysis of both the "depletome" and depleted fractions could provide useful information in biomarker-related profiling studies of serum or plasma.

Using LC-MS^E analysis of depleted serum, Stelzhammer et al. attempted to identify proteomic differences in first-onset, antidepressant drug-naive MDD patients [42]. This analysis led to identification of two proteins (ceruloplasmin- [CP] and haptoglobin-related protein [HP]) which could be used to distinguish patients from controls and four proteins (complement C4-B, CP, HP and plasminogen) which were correlated with symptom severity. These findings suggested that proteins involved in the inflammation response may be involved in at least the earliest stages of MDD. Another study found 13 sex-specific changes in serum from adults with Asperger syndrome using an LC-MS^E profiling approach [43]. Interestingly, 12 out of these 13 proteins were altered in females (APO-C2, APO-E, armadillo repeat-containing protein 3 [ARMC3], langerin [CLC4K], fetuin B (FETUB), glucuronyl C5-epimerase [GLCE], mitochondrial ribonuclease P protein 1 [MRRP1], protein

tyrosine phosphatase [PTPA], ring finger protein 149 [RN149], transducin-like enhancer protein 1 [TLE1], thyroid receptor-interacting protein 11 (TRIPB), zinc finger CCCH-type containing 14 [ZC3HE]), and one protein was altered in males (ranBP2-like and GRIP domain-containing protein 4 [RGPD4]). This suggested that females with Asperger syndrome had changes in proteins mostly associated with lipid transport, metabolism and transcriptional changes, while Asperger syndrome males may have more alterations in RNA transport pathways. From these findings, the authors suggested that the search for biomarkers or novel drug targets in studies of autism-related disorders may require stratification into males and females, and this could lead to the development of novel targeted treatment approaches based on gender.

Several studies have now been carried out using peripheral cells taken from psychiatric patients as potential cellular models for use in drug discovery. Peripheral blood mononuclear cells (PBMCs) have been used extensively for this reason considering that these cells contain many of the same G protein-coupled and ion channel receptors, along with the corresponding intracellular signalling pathways as those found in brain tissues [44–46]. Herberth and co-workers profiled PBMCs from antipsychotic-naive schizophrenia patients using an LC-MS^E protocol and found differences in the levels of 18 proteins compared to the levels of these same proteins in cells from controls [47]. Eight of these proteins were components of glycolysis, consistent with the findings of disturbances in this pathway in brain tissues from patients with psychiatric disorders, as described above. The authors pointed out that this glycolytic signature could be of diagnostic and prognostic value, and the combined cell and biomarker signature could be used as potential model for drug discovery. A similar approach using PBMCs from BD patients found changes mostly associated with cytoskeletal and stress response proteins [48]. Thus, there may be some differences in this model between schizophrenia and BD patients although studies involving a direct comparison are required to confirm this. Another LC-MS^E profiling study resulted in identification of 16 proteins that showed significant differences in expression levels in skin fibroblasts obtained from schizophrenia patients in comparison to those from control subjects [49]. These proteins were mostly associated with proliferation and cell growth pathways, and the authors validated this possibility at the functional level by confirming decreased proliferation of cells from the schizophrenia patients over an 8-day time course study. Interestingly, similar abnormalities in cell cycle and growth pathways have been reported to occur in the central nervous system in schizophrenia patients [50–52].

7.5 Conclusions

The label-free LC-MS^E approach has now been used widely in the study of numerous diseases, with a number of advantages over label-based approaches such as lower sample requirements, sample preparation and instrumental time. In the case of psychiatric disorders, LC-MS^E approaches have led to identification of both

common and unique proteomic differences across different psychiatric disorders, including schizophrenia, BD, MDD and autism spectrum disorders. In brain tissues, this has mainly revealed alterations in pathways related to synaptic function and energy/metabolism pathways. However, such studies may be of limited value considering the possibility of artefacts associated with post-mortem analyses as well as the fact that most patients would have been under potentially confounding drug treatments over their lifetimes. Thus, studies of living patients would be of potentially higher value, especially if these patients are investigated in the earliest stages of the various disorders. In this case, analysis of peripheral body fluids such as serum and plasma, as well as tissues such as PMBCs and fibroblasts, could be more informative. Such approaches have already proven successful through a number of similar findings as seen in the brain studies. This is important for translation of successful peripheral biomarker candidates into clinical use.

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Chapter 8
The Utility of Multiplex Assays for Identification of Proteomic Signatures in Psychiatry

Junfang Chen, Paul C. Guest, and Emanuel Schwarz

8.1 Introduction

Psychiatric disorders such as schizophrenia, major depression, and bipolar disorder are complex, multifactorial syndromes whose etiology is incompletely understood. There is a growing consensus that these illnesses are biologically heterogeneous and underlying biological determinants may not align with conventional diagnostic boundaries [1]. As a consequence, biological research on these illnesses is challenging since such heterogeneity limits the possibility to identify individual biological hallmarks with large effect sizes, and therefore in particular findings from smaller studies are frequently difficult to reproduce. While psychiatric disorders are typically considered being disorders of the brain, molecular investigations have also focused on peripheral tissues and body fluids. Such analyses are essential, not only to prevent confounding that frequently affects postmortem tissues but also because peripheral body fluids such as serum or plasma are easily accessible and would allow cost-effective testing in a clinical setting. However, analyses of individual biological markers have thus far not yielded biomarker candidates with sufficient effect sizes to be useful for clinical management of psychiatric illnesses. Technological advancements have allowed the non-hypothesis-driven screening of large numbers of proteomic markers [2–4], and this development has rapidly accelerated efforts to identify clinically useful markers of psychiatric illnesses [5, 6]. While the non-hypothesis-driven nature of such screening may lead to

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identification of individual markers with larger effect sizes, a fundamental advantage is the ability to apply machine learning methods on the derived data to identify combinations of markers that have higher predictive accuracy than any individual marker alone. One of the main challenges of such experiments is that they are typically affected by low throughput and can, therefore, only be performed on cohorts of limited size. From a machine learning perspective, it becomes increasingly difficult to identify useful predictive signatures when the number of predictors greatly exceeds the number of subjects the data was acquired on. This so-called curse of dimensionality is also known as the "Hughes phenomenon" and describes the problem that with increasing numbers of predictors, an exceedingly higher number of subjects is needed to cover the possible combinations of predictor values [7]. As a consequence, with fixed sample size, the data space becomes increasingly sparse and this often leads to a decrease in predictive accuracy. Despite the advantages of large-scale proteomics profiling, which is particularly useful in early stages of biomarker development, there is, therefore, a benefit of meaningfully reducing the numbers of investigated variables, relative to sample size. In this scenario, multiplex assays can have substantial utility since they can be used to facilitate the transition from a global biomarker screening tool to a dedicated measurement platform with low variability. Previous studies have found that the analytical performance of multiplex assays is comparable to that of the corresponding single-plex assays [8, 9]. However, this is dependent on the measured analytes and the experimental procedures [10].

Sometimes, it is possible to reduce the data space based on biological hypotheses about factors that have likely predictive qualities. In this scenario, multiplexed assays are particularly useful as they can be used to query important nodes of a given set of molecular networks and thus provide a systems biological fingerprint related to a prespecified biological hypothesis. For example, Surinova and coworkers used a phased mass-spectrometry approach to identify a biomarker signature for colorectal cancer [11]. Candidates derived from the initial profiling of approximately 300 secreted and cell surface candidate glycoproteins were translated into an 88-plex targeted selective reaction monitoring (SRM) mass spectrometry assay to measure the concentrations of these in blood samples from large populations. This was ultimately transferred into a five-protein signature with high accuracy. Another possibility is the aggregation of biological analytes into a multiplex system, which have previously been shown to be illness association but thus far only been analyzed in isolation. This provides the analytic basis for subsequent machine learning analyses that aim to identify optimally predictive combinations of such markers. An example of such application is the semi-hypothesis-driven multiplexed screening of schizophrenia patients, which led to an identification of a biological signature that could differentiate patients from healthy subjects [12, 13]. The signature was subsequently transferred a panel of 51 analytes part of a multiplex immunoassay assay system [12]. This system was then used to acquire serum protein concentration data on over 800 subjects for algorithm training, which yielded a receiver operating characteristic area under the curve (ROC-AUC) of 0.89, with a sensitivity of 83% and specificity of 83%. Further refinements of this algorithm consisting of a 26-plex

proteomic panel yielded a ROC-AUC of 0.97 for separation of first onset schizophrenia and control patients [14].

In addition to a reduction of potential predictors based on biological reasoning, a frequently employed method to address the curse of dimensionality is the data-driven preselection of predictors that lead to a reduced set of markers with performance optimized according to a given criterion [15, 16]. For example, biomarker screening in subjects with autism spectrum disorder employed regularized logistic regression to determine a subset of biomarkers that most strongly contributed to illness risk in children [17]. An interesting amalgamation of biological reasoning with data-driven selection of important predictors has been described as "knowledge-based variable selection" [18]. Here, a database on biological background information in the form of previously identified and validated proteomic biomarkers is used to enhance performance of a classifier derived from high-dimensional proteomic mass spectral data.

Issues arising from low sample sizes have received considerable attention in the field of psychiatry and neuroscience [19–21]. The lack of statistical power is frequently described from the perspective of univariate statistics, such as genetic association studies where markers are analyzed individually and where small sample sizes can lead to false-positive findings [22]. However, statistical power is an equally important issue from a machine learning perspective. On the one hand, it governs the generalizability of the performance of a signature identified in a given cohort. While there are certain theoretical methods to estimate the power to reproduce initial findings in independent cohorts, such methods typically give conservative bounds that are of limited utility in practice [23]. Similarly, researchers may be interested in the inverse question of "how many subjects are necessary to identify a biomarker signature" for a given clinical question. While this question is substantially more difficult to answer compared to the univariate case, it has, nevertheless, received very little attention in the current literature. Required sample sizes are affected by numerous factors including the expected effects in individual analytes, their correlation, the nature of the decision rule, and the total number of variables measured. Estimates of how many subjects are required vary broadly, from 75–100 to thousands of samples that are needed for robust prediction of patient outcomes in cancer studies [24, 25]. Similarly, a study in the cancer field has shown that gene profiles showed an overlap of only 16.5% when generated from independent cohorts of 600 subjects each [26]. Given the complexity of sample size estimation for machine learning application, a useful venue may be to numerically estimate the required sample size for a given classification task from an initial dataset with approximately 100 subjects [24].

On the other hand, low sample sizes give rise to phenomena that are frequently discussed in relation to univariate research but rarely within the machine learning context. One of such examples is the "winners curse," which describes the problem that if a study discovers a true effect in a given variable that the effect size of this finding is likely exaggerated [27]. For univariate statistics, this phenomenon typically occurs when effects are identified through thresholds of statistical significance

that in small cohorts can only be passed by markers with large effects. This becomes problematic when such effects are due to sampling variability in such cohorts and therefore not reproducible to the same extent in independent validation studies. In machine learning a similar problem arises when algorithm parameters are optimized in small training datasets. As the optimal thresholds are also affected by sampling variability, the chosen parameters may not be optimal in independent samples and therefore lead to reduced predictive performance [28]. In addition to these issues that are a direct consequence of low sample sizes, the required sample numbers are higher in practice, especially for investigation of psychiatric illnesses. On the one hand, this is due to the fact that the training data needs to reflect all properties of the target population for an algorithm to generalize well. This is often challenging due to differences in diagnostic assessments, technical variability across clinical sites [29], or differences in frequently unobserved biological parameters such as genetic background. On the other hand, since psychiatric disorders are inherently biologically heterogeneous, it is possible that machine learning approaches will have to go hand in hand with subgroup identification strategies that allow biological stratification of the patient cohort and subsequent prediction of individual subgroups using machine learning techniques. This is an active area of current research, for example, as part of the FP7 project IMAging GENetics of MENtal Disorders (IMAGEMEND) [30]. Here, multiple data modalities are used to identify patient subgroups that may span diagnostic boundaries, and machine learning tools are applied to derive the corresponding biological fingerprints as a basis for clinical tool development. A similar methodology can be applied for analysis of multiplexed data in psychiatric patients, in particular when multiplexes have been designed to cover multiple biological systems that may map to divergent biological signatures of patient subgroups. For example, we have previously used multiplex immunoassay data to show that schizophrenia patients could be separated into subgroups that showed predominant abnormalities in different biological systems. This approach was made possible by the multiplexed assay system that comprised important analytes within immune system and hormonal pathways [31]. The biological pathway-based stratification of psychiatric patients into subgroups is a multivariate extension of pathway stratified association, a concept previously described as "pathway-wide association study," which can aid in differentiating illness relevant variants from background [32, 33]. Substantial progress has been made in the development of subgroup identification strategies, and many of such methods are capable of integrating multiple data modalities that would allow multiplex data to be incorporated into a larger array of biological and other measures. While some have been specifically designed for such purpose [34], others are of more general nature, such as graph-based clustering procedures. This may allow the specific design of multiplex systems to capture biological measures that complement those from other analytical techniques or that are of known relevance for nonbiological, such as cognitive domains or neuroimaging data. In such multi-domain data, addition of biologically informed multiplex data may provide an important link between modalities that could facilitate application of novel subgroup identification and machine learning strategies on a combined multimodal database.

8.2 Technical Considerations Regarding Analysis of Multiplex Data

While the application of multiplexed assay systems holds substantial promise for identification of biomarker panels in psychiatry, several technological challenges should be considered that are of particular relevance when such data is analyzed using machine learning tools. One general issue is that the markers part of such assay systems can only give, by design, readouts on a limited spectrum of analytes present in a given sample. Therefore, it should not be expected that in aggregate the measures accurately quantify the entirety of analytes in the investigated sample. This becomes important when there are external factors of variability that influence analyte concentrations. For example, a given individual's hydration state may influence his or her plasma protein concentrations and such effects are typically of little experimental interest. To remove such factors of variability, a frequently applied procedure is normalization, where all measures in a given subject are normalized to, i.e., the total signal measured in this subject. However, if multiplexed assays measure only isolated biological systems, there may be no meaningful relation between the total signal and the influence of the external source of variability and other measures need to be used for meaningful normalization. In addition, some normalization strategies have been shown to bias the pairwise association between predictors [35], which may be a particular problem for downstream multivariate analyses. In addition, for multiplexed assays, a given factor of variability might simultaneously affect the different measured analytes and therefore lead to correlated measurement errors. Interestingly, such effects were recently found to more likely lead to false-negative rather than false-positive outcome associations [36].

Due to the large number of reagents required to perform multiplexed measurements, such measures are particularly prone to confounding and batch effects [37], which are generally difficult to prevent for high-throughput measurements and which may be difficult to remove from the data based on statistical techniques alone [38]. This is a particular problem for application of machine learning techniques where batch effects may not only lead to a loss of power but, if outcome associated, can lead to identification of at least partially artifactual sets of biomarkers [37, 39]. This problem is aggravated by the fact that frequently, multiplexed data are not acquired for machine learning analyses, but retrospectively analyzed using machine learning tools, and therefore study designs may not be suitable to exclude some confounding factors. In addition, such sources of variability are typically strong compared to the biological variability between subjects and may account for a large proportion of variance in the dataset. Therefore, subgroup identification strategies, such as clustering, typically pick up sources of strong variability before outcome-relevant data dimensions are found, making identification of patient subgroups substantially more difficult. As a consequence, such effects need to be removed from the data though normalization or other statistical methods, and this may alter the data such that the original biological signal is lost. It should be considered that even if batch effects are removed through statistical techniques,

residual bias may still impact on downstream analysis. Similarly, cofounding factors may remain undetected, and for classification, previous studies have found that such factors can significantly impact on classification performance [40]. For this reason, it is important that signatures underlying patient subgroups are validated in independent datasets and such efforts have received an increasing amount of attention in recent studies.

8.3 Conclusions

Here, we have discussed some challenges related to the multivariate analysis of proteomics data and highlighted various aspects where application of multiplexed assay systems can aid in the discovery process of biomarkers. In particular for psychiatric illnesses that are characterized by high biological heterogeneity, multiplex systems that can query different biological systems hold substantial promise to uncover etiologically relevant biological markers. However, application of these methods faces several challenges that may particularly impact on sensitive downstream machine learning analysis. Careful consideration of experimental design is required to ensure that potential confounding factors do not adversely affect the identification of biological signatures. Finally, given their analytical flexibility, multiplex assay systems may increasingly be combined with other data modalities to obtain a more comprehensive impression of disease etiology at a biological systems level.

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Part II
Protocols

Chapter 9
Blood Sampling and Preparation Procedures for Proteomic Biomarker Studies of Psychiatric Disorders

Paul C. Guest and Hassan Rahmoune

9.1 Introduction

Proteomic techniques have increased in use in recent years in the ongoing search for novel biomarkers for psychiatric diseases and other medical disorders. In clinical development, biomarker discovery, validation and translation of the findings into diagnostic tests require reproducible sample collection, handling and storage procedures. If possible, it is preferable that bio-fluids including serum or plasma are chosen for these studies since blood is easily accessible from most individuals by standardized venous puncture methods. Therefore, such samples are likely to result in production of biomarker tests with greater clinical utility [1, 2] which provide a sensible and rapid analysis platform in emergency room situations [3]. However, it is important that procedures for venipuncture, serum and plasma preparation and storage are standardized to yield accurate, reproducible and meaningful comparison of results across different laboratories or clinics in development studies.

Production of serum generally takes place by simply allowing clotting to occur at room temperature over a period of 90 min or by adding a clot activator to reduce the clotting time (Fig. 9.1). The resulting coagulated material, which is comprised mostly of cells, cell debris and clotting factors, is removed by centrifugation, fol-

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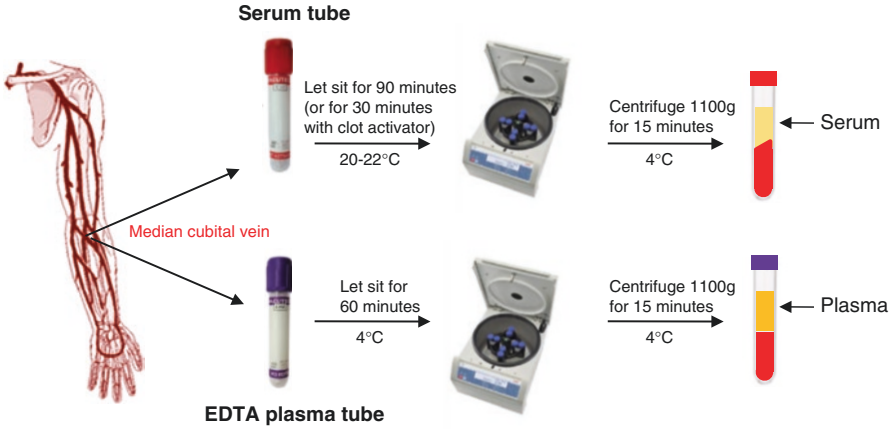

Fig. 9.1 Blood collection for plasma and serum preparation

lowed by aliquoting into low-binding tubes and storage at −80 °C for several months or even years [4]. Plasma is derived from blood by addition of an anticoagulant such as EDTA, heparin or sodium citrate to inhibit the clotting process, followed by removal of blood cells and cell debris by centrifugation, aliquoting and storage as above.

It is important to note that bio-sampling, processing, storage and handling can have an influence on biomarker study results. For this reason, compatibility between the preparation and analytical phases should be determined before initiating any biomarker study [5–7]. To ensure uniform and consistent bio-fluid processing, standard operating procedures (SOPs) should be established and applied strictly. Here, we present standard protocols for venipuncture along with plasma and serum preparation and storage techniques, which can be used in multiplex immunoassay [8], two-dimensional gel electrophoresis [9], tandem mass spectrometry (MS) [10], selected reaction monitoring mass spectrometry (SRM-MS) [11] and other biomarker profiling approaches.

9.2 Materials

9.2.1 Venipuncture

1. 14–20 gauge sterile blood draw needles
2. Holder/adapter (*see Note 1*)
3. Tourniquet
4. Alcohol wipes (70% isopropyl alcohol)
5. Latex, rubber or vinyl gloves

9.2.2 Serum and/or Plasma Preparation (See Note 2)

1. Evacuated collection tubes (*see Note 3*)
2. Sterile serological pipettes
3. Benchtop centrifuge (*see Note 4*)
4. Storage tubes or cryovials (*see Note 5*)

9.2.3 Serum Preparation

1. Tubes with no clotting agent or additive (*see Note 6*)
2. Tubes with clot activator +/−gel separator (*see Note 7*)

9.2.4 Plasma Preparation Only (See Note 8)

1. Tubes with chelating agent +/−gel separator (*see Note 9*)

9.3 Methods

9.3.1 Venipuncture (See Note 10)

1. Prior to blood draw, record the demographic details, physiological status and other metadata of each donor in a worksheet (Table 9.1) (*see Note 11*).
2. Draw from the most appropriate arm vein of the participant (*see Note 12*).
3. Clean the arm of the donor with alcohol in a circular fashion, beginning at the proposed draw site and working outward.
4. Allow to air-dry.
5. Insert the needle at an angle that is 20–30° of the vein to avoid trauma and excessive probing (*see Note 13*).
6. Draw 8–10 mL of whole blood for each 4–5 mL of serum or plasma needed.

9.3.2 Serum Preparation

1. Collect up to 8 mL of blood from each donor into serum tubes.
2. Invert the tube 8–10 times immediately.
3. Allow blood to clot by setting tubes vertically at room temperature for 90 min (*see Note 14*).

Table 9.1 Examples of metadata that may be collected for a biomarker study (*see Note 18*)

Samples	1. Type of sample (e.g., serum or plasma) (*see Note 19*) 2. Date and time of sample collection 3. Time in storage 4. Date of collection (*see Note 20*) 5. Additives (*see Note 21*)
Demographics	1. Gender 2. Ethnicity (*see Note 22*) 3. Height and weight 4. Hip and waist measurement 5. Fasting or non-fasting 6. Smoking (number cigarettes/day, duration) 7. Alcohol 8. Hormonal status (e.g., menstruation, menopause, hormone treatment) 9. Pregnancy or breastfeeding status 10. Age of disease onset, duration 11. Current medication and dosage 12. Comorbidities, including disease duration and regular medications taken
Physiological/biochemical analysis	1. Systolic/diastolic blood pressure (mm Hg) 2. Clinical laboratory measurements such as blood count, and levels of urea, creatinine, glucose and lipids 3. Total protein or albumin levels 4. Sodium and potassium levels 5. Liver enzymes 6. Glucose tolerance test results 7. C-reactive protein levels
Dietary and lifestyle	1. Type of food and beverages ingested before sample collection 2. If possible, participants should fast overnight before blood draw (*see Note 23*) 3. If applicable, all medications and dosages should be recorded 4. If possible, donors should avoid heavy exercise, alcohol, tobacco and nicotine use for 12 h prior to blood collection 5. Use and levels of illicit drugs should be recorded

4. Centrifuge at 1100 × g at 4 °C for 15 min.
5. Transfer 0.5 mL aliquots of the serum layer (top) to pre-labelled, pre-chilled 1.5 mL capacity Eppendorf LoBind tubes (*see Note 5*).
6. Discard samples which show signs of hemolysis, such as a red or pink tingeing, or lipemia, seen as a floating white substance (formed from accumulated lipoprotein particles).
7. Place aliquots on dry ice and then transfer to a −80 °C freezer prior to analysis (*see Note 15*).

9.3.3 Plasma Preparation

1. Collect up to 10 mL of blood from each donor in plasma tubes.
2. Invert the tube 8–10 times immediately after collection.

3. Place tubes on wet ice for 30 min (*see Note 16*).
4. Centrifuge at 1100 × g at 4 °C for 15 min.
5. Transfer 0.5 mL aliquots of the top plasma layer to pre-labelled, pre-chilled 1.5 mL capacity Eppendorf LoBind tubes (*see Notes 5 and 17*).
6. Discard samples which show signs of hemolysis or lipemia as above.
7. Place aliquots on dry ice and transfer to a −80 °C freezer as described above.

9.4 Notes

1. There are evacuated systems available with syringe, single draw or butterfly systems.
2. Blood proteome-based biomarker profiling results can be influenced by use of serum compared to plasma. This is mostly due to disparities in both content and stability of the resident molecules and to matrix differences [12].
3. The type of tube should be determined at the start of the study and kept constant throughout. This is important for comparison of results both within and across studies. As an example, a study identified significant differences in analyte levels after carrying out immunoassays of EDTA, heparin and citrate plasma and serum [13].
4. Centrifugation at different temperatures can affect the stability of blood-based biomarkers. For example, a temperature of 4 °C is better suited for platelet preparations.
5. It is important to pre-label all tubes to avoid sample mix-ups. It is recommended to use 1.5 mL capacity Eppendorf LoBind tubes when the volume of the sample is less than 1 mL and cryovials for larger volumes.
6. We normally use 10 mL Vacutainer Plus tubes with a clear cap. We suggest to avoid the use of tubes containing a gel separator as this can interfere with some assays, such as pharmacokinetic and pharmacodynamic analyses.
7. We normally use the 10 mL BD Vacutainer Plus plastic serum tubes with either a red or dappled red or grey cap.
8. The chelating agent can affect performance of specific platforms. For example, the use of EDTA tubes leads to more reproducible results compared to citrate or heparin tubes when using the Luminex multiplex immunoassay system. It should also be noted that protease inhibitors are not generally needed when collecting plasma in ETDA tubes since EDTA inhibits most proteases through chelation of specific metal ion cofactors.
9. We routinely use 10 mL Vacutainer K_2EDTA tubes from BD Bioscience or similar products. The choice is dependent on compatibility of the chelating reagent with the biomarker profiling platform, as described above.
10. Remember that safety comes first and all bio-samples and materials should be handled as if they are capable of transmitting infection, and risk assessments should be in place to help avoid contamination. In addition they should be disposed of with adequate precautions, according to state and local regulations at containment level 2 or level 3 as necessary.

11. It is important to maintain consistency. The operator should ensure that all measurements are carried out using the same systems and staff, if this is possible. Furthermore, consistent data should be collected from all participants to avoid missing information. This can be important to allow statistical corrections for potential confounding factors during the data analysis phase. Approximately half of all laboratory errors are associated with pre-analytic processing [14], which can lead to inaccurate readings of biomarker levels [15].
12. The median cubital vein is used commonly by phlebotomists due to its location and size (Fig. 9.1).
13. This should be performed by a trained phlebotomist.
14. Coagulation times can be reduced to 30 min when using tubes that contain a clotting activator.
15. Increased degradation or cryoprecipitation of the molecular content can occur at temperatures of −20 to 30 °C, which can affect biomarker profiling results [16–19]. Multiple freeze-thaw cycles should be minimized for similar reasons. Freezers should be monitored by an automated security system and back-up freezers should be put in place in case of loss of temperature control. Upon the first thaw of a sample, the use of additives such protease and phosphatase inhibitors may help to stabilize proteome profiles [19].
16. Times can vary but should be kept consistent.
17. When preparing plasma, care should be taken not to contact the cellular material in the white-coloured layer below.
18. This is a general guideline and the information required may vary from study to study according to the experimental objectives.
19. Samples should be collected from test and control cases in a random manner to avoid the possibility of a biased statistical outcome.
20. The readings of some biomarkers can be influenced by factors such as seasonal effects, comorbidities and medications.
21. Some additives can alter biomarker profiling results.
22. The distribution of the participants should be equally represented to maximize chances of successful validation or repeat analyses.
23. It is not always possible for all participants to be under fasting conditions at the time of blood draw, and this should be recorded with information regarding the time and nature of the last meal.

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Chapter 10
Multiplex Immunoassay Profiling of Serum in Psychiatric Disorders

Laurie Stephen, Emanuel Schwarz, and Paul C. Guest

10.1 Introduction

Schizophrenia patients have a decreased life expectancy due to increased incidence of comorbidities, including insulin resistance and type II diabetes [1]. Although these effects can be brought about by treatment with antipsychotics [2], there is emerging evidence that such metabolic disturbances can also occur in schizophrenia subjects prior to treatment at their first clinical presentation [3]. The bloodstream contains hundreds of bioactive and regulatory proteins including hormones such as insulin and growth hormone which are likely to be involved in these metabolic disturbances. However, most of the polypeptide hormones are present at very low concentrations in the circulation, which necessitates that the biomarker measurement systems used should be highly sensitive. One of the best means for achieving this is through the use of antibody-based approaches like the multiplex immunoassay technique [4]. We showed that this system can be used to simultaneously measure abundant serum proteins such as clotting factors, immunoglobulins and apolipoproteins as well as hormones such as the insulin-related peptides and growth factors, in first onset patients with schizophrenia prior to antipsychotic treatment [5, 6].

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Most immunoassays rely on antibodies to capture the protein of interest in a biological matrix such as serum or plasma. Traditional immunoassays are targeted at detecting a single protein and often rely on reactions associated with covalently linked enzymes. New technological developments over the past two decades have now led to the situation where multiple proteins can be detected in a single measurement vessel. The capability of measuring multiple proteins simultaneously maximizes the amount of information that can be obtained from single sample. At the same time, this reduces sample volume requirements and laboratory analysis time and decreases costs. However, multiplexed assays have potential problems that would not be encountered if single assays were used for each individual analyte, as described elsewhere [7]. Examples of these problems include the potential for cross-reactivity and false positives due to antibody interactions. These challenges, if not carefully addressed, can generate misleading results [8–13].

The chapter describes the development of a 5-plex immunoassay for the Luminex® system, although the same principles will apply to other similar platforms (Fig. 10.1) [14–17]. The samples used were serum samples from schizophrenia patients and controls in attempt to gain some insights into the circulating metabolism-related hormones which may be affected in this disorder.

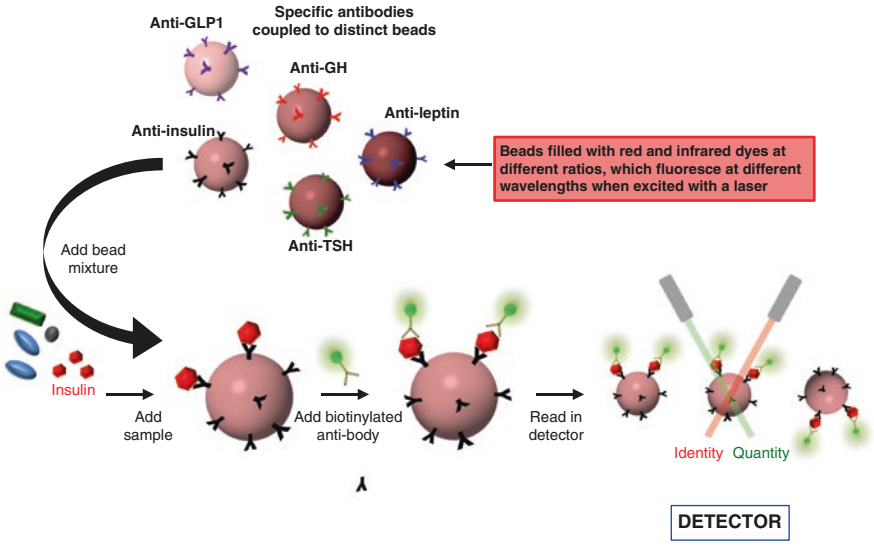

Fig. 10.1 Overview of the multiplex immunoassay protocol. Samples are added to dye-coded microbead-antibody conjugates that capture specific targets. Following incubation with a second antibody containing a biotinylated label to form a "sandwich" configuration, the mixtures are streamed through the Luminex instrument which uses lasers for identification of the antibody-microbead conjugates and quantitation of the bound hormones. The example shows a 5-plex assay capable of binding the targets glucagon-like peptide 1 (GLP1), growth hormone (GH), insulin, leptin and thyroid stimulating hormone (TSH) (although only the binding to the anti-insulin beads is shown in the flow chart)

Table 10.1 Demographic information associated with serum samples used in the study

	Schizophrenia patients	Controls
Male/female	161/75	152/78
Age (years)	31 ± 9	30 ± 9
Body mass index (kg/m^2)	24 ± 4	24 ± 4

10.2 Materials

10.2.1 Samples

1. Serum samples collected from 236 schizophrenia patients and 230 controls (Table 10.1) [6] (*see Note 1*)

10.2.2 Bead Conjugation

1. Magnetic separation device
2. Copolymer tubes and labels
3. 1–4 mL of magnetic 12.5 × 10^6 beads/mL Luminex microspheres/beads
4. 125 μg/mL monoclonal capture antibodies against glucagon-like peptide 1, growth hormone, insulin, leptin, and thyroid-stimulating hormone (*see Note 2*)
5. Sulfo-NHS, N-hydroxysulfosuccinimide
6. N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide (EDC)
7. Activation buffer: 100 mM NaH_2PO_4, pH 6.0
8. Coupling buffer: 0.05 M 2-morpholino-ethane-sulfonic acid monohydrate (MES) pH 5.0
9. Blocking/storage buffer: 10 mMNaH_2PO_4, pH 7.4, 150 mM NaCl, 0.02% Tween 20, 0.1% bovine serum albumin (BSA) and 0.05% NaN_3 (PBS-TBN)

10.2.3 Biotinylation of Detection Antibody

1. Epitope-distinct antibodies (from above) to glucagon-like peptide 1, growth hormone, insulin, leptin and thyroid-stimulating hormone (*see Note 2*)
2. Sulfo-NHS-LC biotin (ThermoFisher Scientific; Waltham, MA, USA)
3. PBS

10.2.4 Multiplex Development

1. Assay buffer: PBS, 1%BSA
2. Washing buffer: PBS, 0.02% Tween-20

3. 100 μg/mL streptavidin, R-phycoerythrin (SAPE)
4. 96-well plate
5. Magnetic separator (*see Note 3*)
6. Recombinant protein standards for growth hormone, insulin, leptin and thyroid-stimulating hormone
7. Hetorophilic blocking reagents (*see Note 4*)

10.3 Methods

10.3.1 Bead Conjugation

1. Place vials containing 1–4 mL of stock microspheres upright on a flat magnetic separator for 2 min so that the beads can settle (*see Note 5*).
2. Be careful not to disturb the beads and remove and discard 0.8 mL of buffer for every 1 mL bead stock.
3. Pool the remaining volume into a single copolymer tube.
4. Place tube in the separator for 30–60 s.
5. Remove and discard the supernatant leaving the tube in the separator.
6. Add 0.5 mL activation buffer and suspend the beads by vortexing and sonication.
7. Place in the magnetic separator for 30–60 s.
8. Remove and discard supernatant with the tube in the separator and resuspend in 0.4 mL of activation buffer as above.
9. Add activation buffer to Sulfo-NHS to give a final concentration of 50 mg/mL.
10. Add 50 uL of this solution to the tube and vortex.
11. Add activation buffer to EDC to give a final concentration of 10 mg/mL.
12. Add 50 uL of this solution to the tube and vortex.
13. Incubate 20 min in the dark while rotating at room temperature (*see Note 6*).
14. Place the tube in the magnetic separator for 30–60 s, remove the supernatant and add 0.5 mL of coupling buffer.
15. Repeat for a total of 2 washes and resuspend in 0.45 mL of coupling buffer.
16. Add 0.2 mL of each capture antibody to five sets of activated microspheres with immediate vortexing (*see Note 7*).
17. Incubate 2 h in the dark while rotating at room temperature.
18. Place tube in magnetic separator for 30–60 s, remove supernatant and add 1.0 mL of blocking/storage buffer.
19. Resuspend and incubate 30 min with rotation in the dark at room temperature.
20. Place the tube in magnetic separator for 30–60 s, remove the supernatant and wash twice as above with 0.25 mL blocking/storage buffer.
21. Resuspend in 0.25 mL blocking/storage buffer, count the beads with a haemocytometer, adjust to 50×10^6 beads/mL and store at 2–8 °C.

10.3.2 Biotinylation

1. Prepare a 10 mM solution of the biotin reagent immediately before use.
2. Add 10 mM biotin reagent to the 5 antibody solutions at a 20:1 biotin-antibody molar ratio (*see Note 8*).
3. Incubate on ice for 2 h (*see Note 9*).
4. Remove excess biotin by dialyzing the reaction mixture using a minimum of three exchanges into PBS.
5. Add BSA to a final concentration of 1% and a preservative for long-term stability.

10.3.3 General Protocol

1. Create a capture bead mini-pool by adding 5 µL of each bead solution to a final volume of 1.4 mL assay buffer.
2. Make an 8 standard (S8) mini-pool by adding 0.2 µg of each recombinant protein to a final volume of 0.2 mL assay buffer, and create seven tenfold serial dilutions to generate a standard curve.
3. Make a mini-pool mix of detection antibodies by adding 5 µg of each biotinylated antibody to a final 5 mL assay buffer.
4. Produce 1:5 and 1:10 serial dilutions of serum and plasma samples in assay buffer (*see Note 10*).
5. Add 30 µL of each standard or sample to different wells of the 96-well plate.
6. Add 10 µL of blocking solution and then add 10 µL of the 5-plex capture beads.
7. Incubate the plate for 1 h on a shaker at room temperature.
8. Wash 3 times with 100 µL wash buffer, add 40 µL of the detection mini-pool to each well and incubate the plate for 1 h on a shaker at room temperature.
9. Add 20 µL SAPE to each well plate, and mix for 30 min on a shaker at room temperature (*see Note 11*).
10. Wash 3 times with 100 µL wash buffer.
11. Add 100 µL assay buffer.
12. Incubate the plate for 2–5 min on a plate shaker at room temperature.
13. Analyse on the Luminex 100 analyser.

10.3.4 Data Analysis

1. Carry out data analyses and determine the levels of each hormone in each sample (*see Note 12–14*).
2. Identify significant differences ($P < 0.05$) between schizophrenia patients and controls using Student's t-test for each hormone measurement (Table 10.2).

Table 10.2 Schizophrenia/control (SCZ/CONT) hormone ratios as determined using the 5-plex assay. The results showed significantly higher levels of insulin and significantly lower levels of growth hormone and thyroid-stimulating hormone in patients

Hormone	SCZ/CONT ratio	*P*-value
Glucagon-like peptide 1	2.03	0.1491
Growth hormone	0.54	0.0200
Insulin	1.65	0.0002
Leptin	1.16	0.2658
Thyroid-stimulating hormone	0.83	0.0213

10.3.5 Packaging and Use

1. Package all reagents individually in assay buffer using the volumes listed in the general protocol above.
2. Store beads, SAPE, and assay buffer at 4 °C and all other components at −80 °C.
3. Make 2–3 levels of assay quality controls (QC) by spiking recombinant protein into serum samples, and package these individually and store at −80 °C.
4. Screen assay performance and determine assay acceptance criteria by testing a minimum of 20 of each QC in duplicate prior to the next analysis.

10.4 Notes

1. The protocols of the study were approved by ethical committees from the Universities of Cologne, Muenster, Magdeburg in Germany, and from Erasmus University in the Netherlands [6].
2. For each hormone, one antibody is needed for capture and another for detection, and these should recognize distinct epitopes on the protein. For best results, a monoclonal antibody of high affinity is needed for capture and either a monoclonal or purified polyclonal antibody should be used for detection.
3. A plate washer can also be used.
4. We use Tru-Block from Meridian Life Sciences (Memphis, TN, USA).
5. Prior to coupling or biotinylation of the antibodies, they should be free of any amines and other protein. If using a Tris-based buffer, dialyse the antibody into 1× PBS using a minimum of three buffer exchanges. If the antibody preparation contains stabilizer proteins such as BSA or gelatin, purify with Protein A or Protein G columns, followed by dialysis.
6. The antibodies can be prepared during this incubation.
7. 1 mL of microspheres is sufficient for ~40 plates of assays.
8. The molecular weight of immunoglobulin G (IgG) is 150,000 kDa, so the amount of biotin required for a 1 mL of a 1 mg/mL antibody solution could be calculated as follows: 1 mL IgG × 1 mg/mL × 20 mmol biotin/1 mmol

IgG × 1 mmol IgG/150,000 mg IgG × 1000 ul/mL= 0.133 mmol biotin = 13 uL of the 10 mM biotin solution.

9. The incubation can also be carried out at room temperature for 30 min.
10. The assay will be multiplex based on the dilution requirement of the samples. In general, if the expected levels are in the pg/mL or low ng/mL range, they will require the same dilution of approximately 1:5 or 1:10.
11. The SAPE concentration will vary with the number of analytes in the multiplex.
12. Carry out curve optimization by varying sample dilutions if required as described previously [7].
13. Carry out blocker optimization using different reagents if required as described previously [7].
14. Assess cross reactivity by comparison of the multiplex results with corresponding single-plex assays if required as described previously [7].

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Chapter 11
Sequential Immunoprecipitation of Secretory Vesicle Proteins from Biosynthetically Labelled Cells

Paul C. Guest

11.1 Introduction

Schizophrenia has been linked with insulin resistance [1, 2], and circulating insulin levels have been found to be elevated both in first onset patients [3, 4] and in patients treated with antipsychotic medications [5]. Thus, studies of the insulin-producing β cells of the endocrine pancreas are relevant as a source of biomarkers or potential drug targets in schizophrenia research. One way of investigating this is to study the effects of high glucose stress on the biosynthesis, intracellular processing and secretion of pancreatic islet secretory granule proteins, such as insulin and the prohormone-converting enzymes.

One approach is to use a pulse-chase radiolabelling protocol [6, 7]. In this method, a radiolabelled amino acid such as ^{35}S-methionine is added for a short period (the pulse) to cells in media for incorporation into newly synthesized proteins. Then the cells are incubated in non-radioactive media (the chase) to study the fate of the newly synthesized proteins over time. The effects on specific proteins can be studied by immunoprecipitation, followed by electrophoretic separation and fluorography for visualization and quantitation purposes [8, 9].

This chapter describes a 1 h pulse labelling of pancreatic islets with ^{35}S-methionine followed by a 7 h chase period (Fig. 11.1) to study the biosynthesis of insulin and prohormone convertase 2 (PC2), as described in previous studies [10–12]. Enzymological analyses have shown that production of mature insulin requires cleavage of proinsulin by a trio of processing enzymes [prohormone convertase 1 (PC1), PC2 and carboxypeptidase H], and this is initiated approximately 30 min

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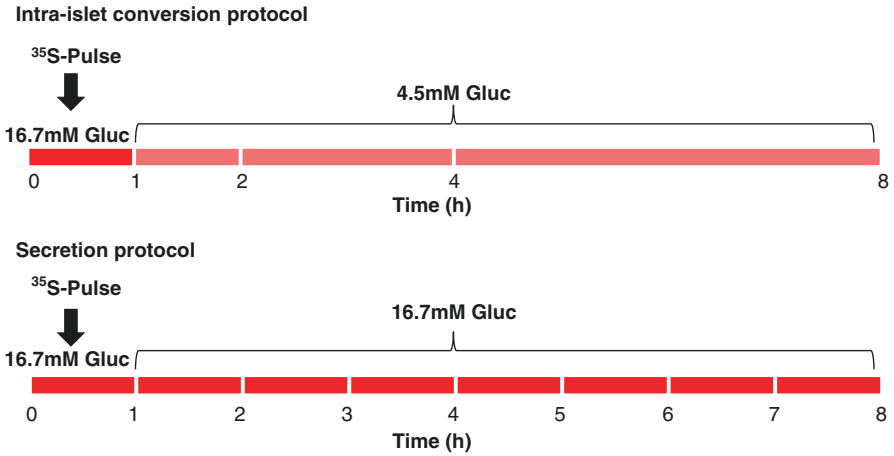

Fig. 11.1 Schematic diagram showing the ^{35}S-methionine pulse labelling and immunoprecipitation protocol. The top panel shows the protocol for measuring intra-islet conversion and the bottom panel shows the secretion protocol. Gluc = glucose

after synthesis during intracellular trafficking to nascent secretory granules [13]. In the presence of low glucose concentrations (<5.6 mM), most of the secretory granules and their protein cargo remain inside the cells. In the presence of high glucose levels (>8.3 mM), the secretory granules begin to fuse with the plasma membranes and release their contents into the circulation. Here, the preparation of immunoadsorbents, pulse-chase radiolabelling of pancreatic islets, the immunoprecipitation of insulin- and PC2-related proteins and subsequent gel-based analyses are presented. The same protocols can also be used to investigate other isolated neuroendocrine cells, providing that antibodies are available for immunoprecipitation of the target proteins.

11.2 Materials

1. Purified monoclonal antibody for proinsulin (*see Note 1*)
2. Polyconal antiserum against proPC2 (*see Note 2*)
3. CNBr-activated Sepharose 4 (GE Healthcare; Little Chalfont, Bucks, UK)
4. Immunoadsorbent activation solution: 1 mM HCl
5. Immunoadsorbent coupling buffer: 100 mM $NaHCO_3$ (pH 8.3), 500 mM NaCl
6. Immunoadsorbent quenching buffer: 100 mM Tris-HCl (pH 8.0)
7. Immunoadsorbent wash buffer 1: 100 mM Tris-HCl (pH 8.0), 500 mM NaCl
8. Immunoadsorbent wash buffer 2: 100 mM NaOAc (pH 4.0), 500 mM NaCl
9. Immunoadsorbent storage buffer: 10 mM NaH_2PO_4, 2 mM K_2HPO_4, 137 mM NaCl, 2.7 mM KCl

10. Modified Kreb's bicarbonate buffer: 25 mM $NaHCO_3$ (pH 7.4), 115 mM NaCl, 5.9 mM KCl, 1.2 mM $MgCl_2$, 1.2 mM NaH_2PO_4, 1.2 mM Na_2SO_4, 2.5 mM $CaCl_2$, 0.1% bovine serum albumin (BSA)
11. Low glucose chase media: Dulbecco's modified Eagle's medium (DMEM), containing 10% newborn calf serum and 4.5 mM glucose
12. High glucose chase media: DMEM, containing 10% newborn calf serum and 16.7 mM glucose
13. Pancreatic islet lysis buffer: 25 mM $Na_2B_4O_7$ (pH 9), 3% BSA, 1% Tween-20, 1 mM phenylmethanesulfonyl fluoride, 0.1 mM E-64, 1 mM EDTA, 0.1% NaN_3 (*see Note 3*)
14. Immunoprecipitation wash buffer: 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Triton X- 100, 1% deoxycholate, 0.1% SDS, 5 mM-EDTA
15. Insulin elution buffer: 25% acetic acid *(see Note 4*)
16. Protein A Sepharose (*see Note 5*)
17. Protein A Sepharose rehydration buffer: 20 mM NaH_2PO_4 (pH 8.0), 150 mM NaCl
18. Protein A Sepharose elution buffer: 20 mM HCl
19. ^{35}S-Methionine
20. Alkaline-urea electrophoresis gel: polymerized from 7.5% acrylamide, 0.2% NN′-methylenebisacrylamide, 12.5 mM Tris/80 mM glycine (pH 8.6), 8 M urea
21. Alkaline-urea gel electrophoresis buffer: 12.5 mM-Tris/80 mM glycine (pH 8.6)
22. Alkaline urea gel-loading buffer: 2.5 mMTris-HCl (pH 8.6), 8 M urea, 0.001% Bromophenol Blue
23. SDS polyacrylamide electrophoresis (PAGE): gels polymerized from 15% acrylamide and 0.08% NN′-methylenebisacrylamide in a Tris-glycine buffer system using the buffer system of Laemmli [14]
24. SDS PAGE loading buffer: 125 mM Tris-HCl (pH 6.8), 2% SDS, 0.25 M sucrose, 5 mM EDTA, 65 mM dithiothreitol, 0.005% Bromophenol Blue
25. Fluorography reagent: 20% 2,5-diphenyloxazole in acetic acid
26. MSE Sonifier and microprobe (Crawley, UK) (*see Note 6*)
27. UV spectrophotometer
28. 1.5 mL capacity microcentrifuge tubes
29. Immunoprecipitation preclearing reagent: 100 mg/mL suspension of Cowan-strain *Staphylococcus aureus* cells

11.3 Methods

11.3.1 Preparation of Insulin Immunoadsorbent

1. Dialyse the antibody using three changes of coupling buffer over a total of 6 h at 4 °C (*see Note 7*).
2. Measure the absorbance of the final antibody solution at 280 nm in the UV spectrophotometer and calculate the concentration (*see Note 8*).

3. Add 20 mL ice-cold activation solution to 1 g of dried resin, and gently mix for 2 h at 4 °C (*see Note 9*).
4. Centrifuge the resin at 1000 × g for 5 min in a swinging bucket rotor and discard the supernatant.
5. Add the dialysed antibody to the resin at a concentration of 10 mg antibody per 5 mL of swollen resin and mix overnight at 4 °C.
6. Centrifuge the resin at 1000 × g for 5 min, and retain the supernatant to determine the coupling efficiency (*see Note 10*).
7. Add 20 mL coupling buffer to the resin and mix with low agitation for 30 min at room temperature.
8. Centrifuge at 1000 × g for 5 min and discard the supernatant.
9. Add 20 mL quenching buffer and mix as above for 2 h at room temperature.
10. Centrifuge at 1000 × g for 5 min and discard the supernatant.
11. Add 20 mL wash buffer 1 to the resin, mix gently and centrifuge at 1000 × g for 5 min and discard the supernatant.
12. Add 20 mL wash buffer 2, centrifuge at 1000 × g for 5 min and discard the supernatant.
13. Repeat steps 11 and 12 twice and add 20 mL of storage buffer to the resin.
14. Centrifuge at 1000 × g for 5 min, discard the supernatant, add 20 mL of storage buffer to the final immunoadsorbent and store at 4 °C for up to 1 month if no preservatives are used (*see Note 11*).

11.3.2 Preparation of PC2 Immunoadsorbent

1. Add 20 mL of rehydration buffer to 4 g of dry Protein A Sepharose, and leave to mix gently for 30 min to achieve complete hydration.
2. Centrifuge at 1000 × g for 5 min, remove the supernatant, and suspend in 20 mL of the same buffer.
3. Repeat the centrifugation and washing steps twice and store in 16 mL of rehydration buffer at 4 °C for up to 1 month if no preservatives are used (*see Note 12*).
4. Add 200 μL of suspended Protein A Sepharose to microcentrifuge tubes containing 15 mL of PC2 antiserum and mix gently overnight at 4 °C (*see Note 13*).
5. Centrifuge at 1000 × g for 5 min, remove supernatant, and wash three times by centrifugation and resuspension in 500 mL of the rehydration buffer.
6. Centrifuge at 1000 × g for 5 min and remove the supernatant, leaving a packed gel of approximately 50 μL for immediate use.

11.3.3 Biosynthetic Radiolabelling of Pancreatic Islets

1. Preincubate 100 isolated islets for 30 min in 100 μL of Kreb's bicarbonate buffer containing 16.7 mM glucose at 37 °C in microcentrifuge tubes under 95% O_2/5% CO_2 (*see Note 14*).

2. Recover the islets gently by centrifugation at 100 × g for 10 s, and suspend the loose pellet in 100 µL of the same prewarmed medium containing 150 µCi of ^{35}S-methionine, and incubate for 1 h at 37 °C under 95% O_2/5% CO_2 (Fig. 11.1).
3. Recover the islets by centrifugation at 100 × g for 10 s, carefully remove and save the radioactive supernatant, and gently resuspend the islets in 100 µL of low or high glucose chase media for 7 h at 37 °C under 95% O_2/5% CO_2 (*see Note 15*).
4. For the low glucose chase, remove the media and collect the islets after 1, 3 and 7 h and leave on dry ice.
5. Add 200 µL lysis buffer and sonicate for 15 s at approximately ¼ power (*see Note 16*).
6. Centrifuge the lysates at 13,000 × g for 5 min, and retain the supernatants for immunoprecipitation.
7. For the high glucose chase, collect the media every 1 h interval for up to 7 h, and retain for immunoprecipitation.

11.3.4 Immunoprecipitation of Insulin and Other Pancreatic Islet Proteins

1. Incubate islet lysate and media samples for 1 h at room temperature in microcentrifuge tubes containing 50 µL of preclearing reagent (*see Note 17*).
2. Centrifuge the samples at 13,000 × g for 5 min and retain the supernatants.
3. Immunoprecipitate the insulin-related molecules by adding the lysates and media to the 50 µL packed gel of respective immunoadsorbent, and incubate overnight at 4 °C.
4. Centrifuge the anti-insulin immunoadsorbent in a swinging bucket rotor at 500 × g for 1 min and retain the supernatant for the PC2 immunoprecipitation.
5. Wash the anti-insulin immunoadsorbent by repeated centrifugation and resuspension in 4 x 1 mL lysis buffer, 2 × 1 mL immunoadsorbant wash buffer and 2 × 1 mL distilled water.
6. Elute the insulin-related peptides with 2 × 1 mL of insulin elution buffer, freeze dry and reconstitute in 50 µL of alkaline-urea gel-loading buffer.
7. Incubate the supernatant obtained after immunoprecipitation of the insulin-related molecules overnight at 4 °C with 50 µL packed gel of PC2 immunoadsorbent.
8. Wash the PC2 immunoadsorbent as above and retain the supernatant for future immunoprecipitation of other proteins.
9. Elute the PC2-related peptides with 2 × 100 µL Protein A Sepharose elution buffer.
10. Combine the two eluates, freeze dry and reconstitute in 50 µL of SDS PAGE loading buffer.

11.3.5 Electrophoresis and Fluorography of 3% S-Labelled Immunoprecipitates

1. Pre-run alkaline-urea gels in tank buffer for 600 Volt-hours, replace the upper tank buffer, load the insulin immunoprecipitates in alkaline-urea gel-loading buffer and subject to electrophoresis for 1000 Volt-hours (*see Note 18*).
2. Disassemble the gel plates and rock the gels gently for 2 × 5 min in acetic acid, 2 h in fluorography solution and then leave for 30 min under running cold water (*see Note 19*).
3. Vacuum dry and expose the gel to X-ray film for 6–72 h (*see Note 20*).
4. For the PC2 immunoprecipitates, heat the samples at 95 °C for 5 min and electrophorese to the point where the dye front just reaches the bottom of the gel.
5. Perform fluorography as above for 2–20 days (Figs. 11.2 and 11.3) (*see Note 21*).

11.4 Notes

1. We used a clone designated 3B7 which recognizes epitopes in mouse, rat and human proinsulin [13].
2. A polyclonal PC2 antiserum was raised in rabbits against a glutathione S-transferase fusion protein, incorporating amino acids 162–388 of rat PC2. The fusion protein was produced using a bacterial expression vector, as described by Bennett et al. [11].

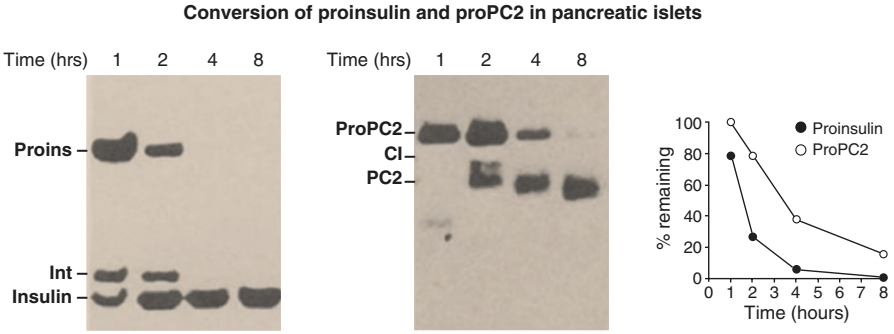

Fig. 11.2 Islet content ^{35}S-labelled proinsulin and proPC2 immunoprecipitate in pulse-chase-labelled islets. Islets were labelled for 1 h in Krebs incubation medium containing 16.7 mM glucose and 0.2 mCi ^{35}S-methionine and resuspended in non-radioactive. DMEM containing 4.5 mM glucose. The intra-islet forms of proinsulin and proPC2 were immunoprecipitated after the 1 h pulse and from the 1, 3 and 7 h chase incubation periods and subjected to PAGE and fluorography. The migration of proinsulin (proins), proinsulin conversion intermediates (Int′), insulin, proPC2, proPC2 conversion intermediates (CI) and mature PC2 (PC2) is indicated, and the graph on the right shows the % conversion of each precursor protein [15] (*see Note 23*)

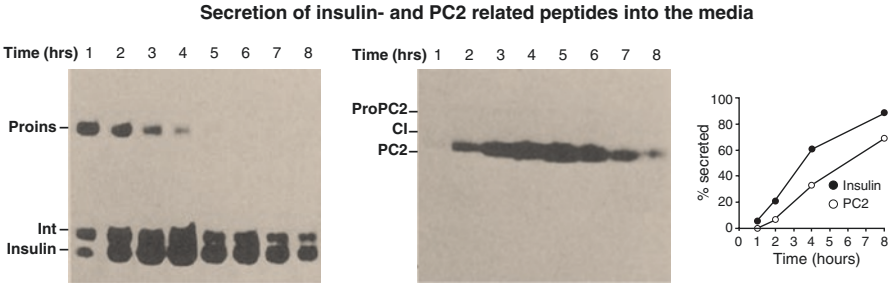

Fig. 11.3 Secretion of ^{35}S-labelled proinsulin- and proPC2-related peptides from pulse-chase-labelled islets. Islets were labelled for 1 h in Krebs incubation medium containing 16.7 mM glucose and 0.2 mCi ^{35}S-methionine and resuspended in non-radioactive. DMEM containing 16.7 mM glucose. The medium was replaced after each 1 h interval for up to 7 h of the chase incubation, and the molecular forms of proinsulin and proPC2 from each time window were immunoprecipitated and subjected to PAGE and fluorography [15] (*see Note 23*).

3. In general, BSA is added to prevent loss of protein on tube walls during immunoprecipitation, but very high levels are required when studying insulin due to its "sticky" nature.
4. Insulin elution requires strongly acidic conditions because of the high affinity of the 3B7 antibody and low solubility of the insulin molecule in more neutral pH buffers.
5. Protein A Sepharose is used commonly in indirect immunoprecipitation protocols, involving the use of polyclonal antisera (as is the case for PC2 in this study). In the indirect method, the antibody is bound non-covalently to the Protein A moieties on the Sepharose beads. This means that elution will generally result in release of the both the antibody and protein. In contrast, the direct method used for the insulin precipitation will lead to elution of only the protein and not the antibody, since the latter remains bound to the resin.
6. Other sonication devices can be used, but the probe should be able to fit inside a 1.5 mL capacity microcentrifuge tube, penetrating almost to the bottom.
7. All traces of Tris buffer should be removed as this will react with activated resin.
8. It is ideal to use 2 mg antibody per mL activated swollen resin in order to achieve a high-capacity immunoadsorbent.
9. 1 g of dried resin will swell to give a volume of approximately 4 mL.
10. The coupling efficiency of the antibody to the resin can be measured by reading the final optical density at 280 nm in a UV spectrophotometer compared to the starting optical density (before coupling). It is desirable to achieve an efficiency >80%.
11. The immunoadsorbent can be stored for longer if preservatives such as NaN3 are used. However, these must be removed prior to use by washing the desired amount of resin.
12. See note 11.

13. In this step, the immunoglobulin fraction in the serum is bound to the Protein A moieties on the resin.
14. We used isolated rat islets in this study prepared as described [9] although rat pancreatic beta cell lines (and other cells as appropriate) can also be used. If this is the case, 5×10^5 cells would be approximately equivalent to 100 islets since each islet contains approximately 5000 cells.
15. Use appropriate precautions when handling and disposing of the radioactive materials. The addition of ice-cold medium containing non-radioactive methionine stops the uptake of ^{35}S-methionine and halts metabolic activity of the islet cells.
16. Adjust power setting as appropriate using other sonication and probe devices.
17. This is to remove any immunoglobulin-like molecules that could interfere with immunoprecipitation experiments.
18. This is equivalent to approximately 1.75 lengths of the dye front migration. To measure this, halt the electrophoresis after the dye has run 1 gel length, add new dye to a blank well, and restart the electrophoresis for a further 0.75 length run.
19. The gel turns white during this final step.
20. We use Cronex 4 X-ray film (DuPont; Stevenage, Herts, UK) but other X-ray films can be used. It should be noted that obtaining the best exposure may require a few attempts and adjusting the times accordingly.
21. Longer exposure periods may be necessary as endogenous PC2 levels are much lower in abundance compared to insulin. However, the exposure period should not exceed the ^{35}S t ½ (87 days).
22. The biosynthesis of proinsulin, chromogranin A and PC1 was stimulated 10–30-fold at the higher glucose concentration, consistent with previous studies [9, 12, 15].
23. The image shows that proinsulin conversion and secretion is faster than those of proPC2. This could mean under prolonged stimulation conditions, both the islet and media contents of these two proteins could change with respect to each other, potentially reflecting the in vivo situation in cases of chronically stressed islets. Such a scenario may occur in first onset schizophrenia and antipsychotic-treated schizophrenia patients who are insulin resistant.

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Chapter 12
2D Gel Electrophoresis of Insulin Secretory Granule Proteins from Biosynthetically Labelled Pancreatic Islets

Paul C. Guest

12.1 Introduction

Some first-onset schizophrenia patients have been found to have insulin resistance and high circulating levels of insulin [1–4]. In addition, patients treated with antipsychotic medications can show the same effect which in turn can lead to adverse events such as weight gain and metabolic syndrome [5]. Thus, studies of the insulin-producing β cells of pancreatic islets may lead to identification of physiologically relevant biomarkers in schizophrenia research. One way of investigating this is to study the effects of high glucose stress on the biosynthetic rates of the resident hormones such as insulin as well as the accessory proteins involved in its packaging, processing and secretion from the storage granules [6].

In addition to insulin, these storage granules are estimated to contain more than 100 proteins [7], including the proteases involved in proinsulin-to-insulin conversion, proinsulin conversion intermediates, C-peptide, other polypeptide precursor proteins, membrane proteins involved in cell trafficking and secretion and ion-translocating proteins involved in regulation of the intragranular environment. In addition, the insulin-producing cells house neurotransmitter-containing synaptic vesicles and neurotransmitter-converting enzymes, along with many of the receptors that are found in the brain. Most of the storage granule proteins are likely to be synthesized, transported and packaged into nascent vesicles in a co-ordinated manner to ensure integrity of the system. The biosynthesis of insulin itself is known to be regulated by many circulating nutrients and other factors although glucose is the most important physiologically [8]. Several other granule constituents such as chromogranin A and prohormone convertase 1 appear to be regulated in a similar

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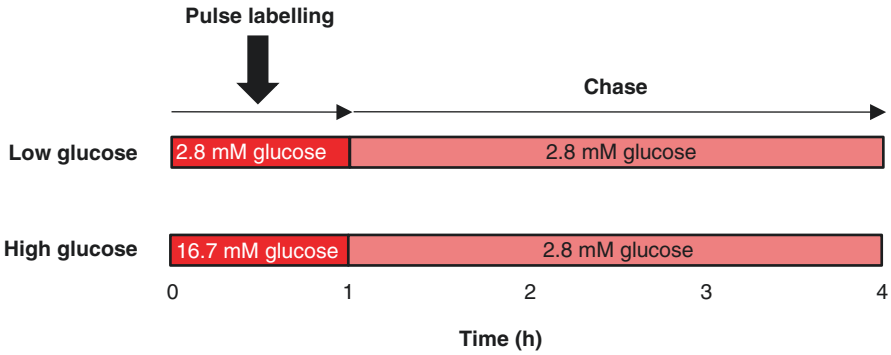

Fig. 12.1 Schematic diagram showing the ^{35}S-methionine pulse-chase labelling protocol

manner, consistent with the idea that they are released from the same storage vesicles. However, it is not known whether just some or all of the granule constituents are affected in a similar manner.

This chapter addresses this question by 2D gel electrophoresis (2DE) [9] of secretory granule subcellular fractions prepared from rat islets labelled with ^{35}S-methionine [10]. The protocol employed a 1 h ^{35}S-methionine pulse labelling of pancreatic islets in the presence of either basal (2.8 mM) or stimulatory (16.7 mM) glucose concentrations, followed by a chase period of 3 h in non-radioactive medium containing a low glucose concentration (Fig. 12.1). This procedure helps to ensure that the newly synthesized insulin granule proteins have sufficient time to reach the granule compartment and undergo processing to their mature forms [11, 12]. In addition, the chase under low glucose conditions minimizes secretion of these newly synthesized proteins thereby making sure that they are retained within the granule compartment. Here, a protocol for pulse-chase radiolabelling of islets, subcellular fractionation and 2DE analyses is presented.

12.2 Materials

1. 400 rat pancreatic islets per condition (*see Note 1*)
2. Post-nuclear density gradient fraction from 0.5 g of rat insulinoma tissue or similar (*see Note 2*)
3. High glucose Kreb's bicarbonate buffer: 25 mM $NaHCO_3$ (pH 7.4), 115 mM NaCl, 5.9 mM KCl, 1.2 mM $MgCl_2$, 1.2 mM NaH_2PO_4, 1.2 mM Na_2SO_4, 2.5 mM $CaCl_2$, 16.7 mM glucose, 0.1% bovine serum albumin (BSA)
4. Low glucose Kreb's bicarbonate buffer: 25 mM $NaHCO_3$ (pH 7.4), 115 mM NaCl, 5.9 mM KCl, 1.2 mM $MgCl_2$, 1.2 mM NaH_2PO_4, 1.2 mM Na_2SO_4, 2.5 mM $CaCl_2$, 2.8 mM glucose, 0.1% BSA
5. Low glucose chase incubation buffer: Dulbecco's modified Eagle's medium, containing 10% newborn calf serum and 2.8 mM glucose

6. ^{35}S-methionine (*see Note 3*)
7. Islet homogenization medium: 10 mM potassium 2-(N-morpholino) ethanesulfonic acid (KMes; pH 6.5) containing 0.3 M sucrose, 1 mM $MgSO_4$, 1 mM EGTA
8. 4.4, 8.8 and 17.7% Nycodenz solutions (Nyegaard Diagnostica; Oslo, Norway) in homogenization medium
9. Subcellular fractionation wash buffer: 10 mM KMes (pH 6.5), 0.25 M sucrose
10. Isoelectric focussing buffer: 9.5 M urea, 5% 2-mercaptoethanol, 0.40% pH 3–10 range Pharmalytes (Pharmacia Fine Chemicals; Uppsala, Sweden), 1.6% pH 5–7 range Ampholines (Pharmacia LKB Biotechnology, Bromma, Sweden), 0.001% bromophenol blue
11. Isoelectric focussing tube gel buffer: 8 M urea, 20% pH 3–10 range Pharmalytes and 1% pH 3.5–5.0, 5–7 and 7–9 Ampholines
12. Isoelectric focussing lower tank buffer: 0.085% H_3PO_4
13. Isoelectric focussing upper tank buffer: 0.2 M NaOH
14. Second dimension equilibration buffer: 50 mM Tris-Cl (pH 6.8), 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS, 0.01% bromophenol blue
15. Second dimension tank buffer: 25 mM Tris/192 mM glycine (pH 8.3), 0.1% SDS
16. Fluorography solution: 20% 2,5-diphenyloxazole in acetic acid
17. Nunc Cryotubes (Gibco; Paisley, Scotland, UK)
18. 1 mL capacity glass tube homogenizer (*see Note 4*)
19. 1.2 cm × 5.0 cm polypropylene centrifuge tubes (Beckman Instruments; Palo Alto, CA, USA) (*see Note 5*)
20. Swinging bucket rotor for ultracentrifugation (*see Note 6*)
21. MSE sonifier and microprobe (Crawley, UK) (*see Note 7*)
22. Vertical isoelectric focussing tube gel system using 15 cm × 0.15 cm (i.d.) glass tubes (*see Note 8*)
23. Second dimension gels cast in 15 cm × 15 cm × 0.15 cm glass plates: linear 5–20% acrylamide gradient, containing 0.065% N,N′-methylenebisacrylamide, 0.375 M Tris-HCl pH 8.8, 0.2% SDS (*see Note 9*)
24. Second dimension electrophoresis tank for running gels cast in 15 cm × 15 cm × 0.15 cm glass plates (*see Note 10*)

12.3 Methods

12.3.1 Biosynthetic Radiolabelling of Pancreatic Islets

1. Preincubate 400 isolated islets per condition for 60 min in 500 μL of modified Kreb's bicarbonate buffer containing 2.6 or 16.7 mM glucose at 37 °C in Cryotubes under 95% O_2/5% CO_2.
2. Recover the islets gently by centrifugation at 100 × g for 10 s and suspend in 200 μL of the same prewarmed medium containing 200 μCi of ^{35}S-methionine

and incubate for 1 h at 37 °C in Cryotubes under 95% O_2/5% CO_2 (Fig. 12.1) (*see Note 11*).
3. Recover the islets by centrifugation at 100 × g for 10 s, carefully remove the radioactive supernatant and gently suspend the islets in 500 μL of chase buffer and incubate for 3 h under 95% O_2/5% CO_2 (*see Note 12*).
4. Terminate the incubations by addition of 1 mL ice-cold low glucose incubation medium followed by centrifugation for 10 s at 3300 × g in a swinging bucket rotor and discard the media.
5. Wash the pellets using two additional cycles of resuspension and centrifugation as above and carry out subcellular fractionation immediately (*see Note 13*).

12.3.2 Subcellular Fractionation

1. Combine the radiolabelled islets with the insulinoma cell carrier, and homogenize using the glass tube homogenizer with ten strokes of a Teflon pestle at 600 revolutions per minute at 4 °C (*see Note 14*).
2. Centrifuge the homogenates for 5 min at 1700 × g at 4 °C to remove unbroken cells and nuclei.
3. Transfer the supernatants to the 1.2 × 5 cm centrifuge tubes, and add 1.3 mL portions of each Nycodenz solution in the order 4.4, 8.8 and 17.7% by loading from the bottom (Fig. 12.2) (*see Note 15*).

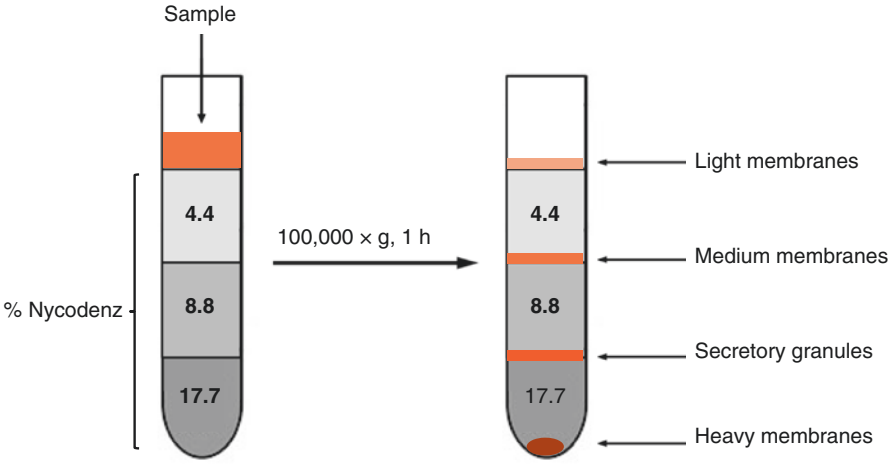

Fig. 12.2 Schematic diagram showing the subcellular fractionation protocol

4. Centrifuge the tubes for 1 h at 100,000 × g in the swinging bucket rotor in an ultracentrifuge.
5. Collect the material at the 8.8/17.7% Nycodenz interface with a 1 mL pipette and suspend this material in subcellular fractionation wash buffer (*see Note 16*).
6. Centrifuge for 20 min at 50,000 × g in the swinging bucket rotor.
7. Resuspend the particulate material containing enriched secretory granules in the subcellular fractionation wash buffer, repeat the centrifugation and store the final pellets at –80 °C (or proceed immediately to the 2DE step).

12.3.3 2DE of ^{35}S-Labelled Secretory Granule Proteins

1. Homogenize the secretory granule pellets by sonication in 100 uL of isoelectric focussing buffer.
2. Centrifuge at 13,000 × g, retain the supernatant and discard the particulate material.
3. Subject the samples to 2DE analysis by isoelectric focussing in the first dimension and SDS polyacrylamide gel electrophoresis in the second dimension (*see Note 17*).
4. For isoelectric focussing, prefocus the gels in the tube gel apparatus for 1 h at 200 volts.
5. Add up to 50 µL of sample above the bed of each tube gel using a Hamilton syringe, and carry out isoelectric focussing at 800 volts for approximately 6 h until the bromophenol blue has reached the bottom of the tube (*see Note 18*).
6. Using a syringe of the appropriate diameter, apply pressure to the top of the glass tubes to extrude the gels directly into second dimension equilibration buffer (*see Note 19*).
7. Incubate the gels for 5 min in the equilibration buffer by gently rocking and then load on to the top of second dimension gel using a 0.15 cm thick spacer to nudge the gels into position (*see Note 20*).
8. Carry out electrophoresis at 60 V for 1 h followed by 120 V until the dye front reaches the bottom of the gel.
9. Disassemble the gel plates and immerse the gels for 2 × 5 min in acetic acid, 2 h in fluorography solution and then leave for 30 min under cold running water (*see Note 21*).
10. Vacuum dry and expose the fluor-impregnated gel to X-ray film (Dupont; Stevenage, Herts, UK) for 2–8 days (Fig. 12.3) (*see Note 22*).

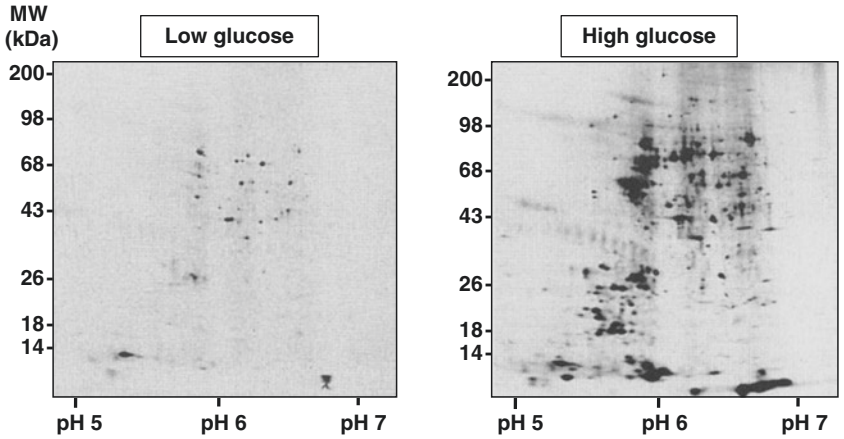

Fig. 12.3 Islets were labelled with ^{35}S-methionine for 1 h in either 2.8 mM glucose or 16.7 mM glucose and then chase incubated for 3 h in non-radioactive medium containing 2.8 mM glucose. Subcellular fractions were prepared, and those enriched in secretory granules were subjected to 2DE followed by fluorography, with a 2–8 day exposure to film. The image shows that the biosynthesis of the most of the proteins was stimulated 10–30-fold at the higher glucose concentration. Previous pulse-chase labelling and immunoprecipitation studies have shown that this occurs for the secretory granule proteins insulin [13], chromogranin A [13], secretory granule membrane protein 110 [15] and PC1 [16]

12.4 Notes

1. Rat islets can be obtained from 10- to12-week-old rats by collagenase digestion of the pancreas, as described by Guest et al. [13]. However, other protocols can be used, providing that these can yield the required number of large intact islets.
2. These cells were prepared as a carrier as described by Hutton et al. [7] for combination with radiolabelled rat islets prior to homogenization and density gradient centrifugation to facilitate efficient recovery. Other rat beta cell lines can be used as a substitute.
3. Use appropriate precautions when handling and disposing of radioactive materials.
4. A 1 mL capacity glass tube homogenizer and Teflon pestle from Jencons Scientific (Leighton Buzzard, Beds, UK) was used. Similar products are available from other suppliers.
5. Other tube and swinging bucket rotor combinations can be used, but it is important that these have appropriate specifications.
6. A Beckman SW 50.1 rotor was used in this study. Although other swinging bucket rotors can be used, ensure that these designated for use with the centrifuge tubes.
7. Any sonication/probe device can be used, but the probe should fit inside a 1.5 mL capacity microcentrifuge tube and therefore be no more than 2 mm in diameter at the tip.

8. A homemade device was used for this although tube gel systems are available from many suppliers. In addition, it is likely that the instruments designed for use of immobilized pH gradient strips could also be employed. The chosen device should be clearly labelled as being designated for use with radioactive materials.
9. The gels used for this study were cast in the lab. However, precast 5–20% gradient gels are also available from many suppliers and may be best in terms of delivering greater reproducibility when comparing large numbers of gels.
10. Many systems could be used for this although the size should be compatible with the isoelectric focussing stage in terms of tube gel or strip length.
11. When working with and disposing radioactive materials, use the institutional recommended precautions.
12. The addition of the chase medium containing non-radioactive amino acids stops the uptake of ^{35}S-methionine and halts metabolic activity of the islet cells.
13. Do not freeze the islets since this will disrupt membranes, rendering the density gradient subcellular fractionation step useless.
14. The clearance between the glass tube and pestle allows for disruption of cell plasma membranes but leaves the membranes on most intracellular organelles, such as the secretory granules, intact.
15. The under-layering approach helps to form sharper boundaries compared with over-layering.
16. All fractions and the pellet should be collected for further analyses but here only the results using the layer enriched in secretory granules are presented [10].
17. The method described by Anderson and coworkers was used [14].
18. This is a continuous isoelectric focussing approach as a non-immobilized pH gradient is used. Therefore, it is possible to electrophorese for too long, and some acidic proteins may run into the lower tank buffer. As a guide, bromophenol blue will turn green at around pH 4 and yellow at pH 3. Therefore, the run should be timed to finish when a green-yellow band reaches the bottom of the tube gel. It is best to run a time course ranging study to determine running time optima when loading a new kind of sample.
19. A 200 μL pipette tip works well for this as it creates a nice seal inside the glass tube.
20. Care should be taken not to damage the tube gel and to ensure there are no air bubbles between this and the second dimension gel.
21. The gel turns white during this step, which leaves the gel impregnated with the fluor.
22. Here, Cronex 4 X-ray film (Dupont; Stevenage, Herts, UK) was used although other suitable films are available. Please check the manufacturer's specifications. Obtaining the best exposure may require multiple attempts and adjusting the times accordingly.

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Chapter 13
Two-Dimensional Gel Electrophoresis: A Reference Protocol

Veronica M. Saia-Cereda, Adriano Aquino, Paul C. Guest, and Daniel Martins-de-Souza

13.1 Introduction

For around two decades, two-dimensional electrophoresis (2DE) was the most used method for large-scale proteomic analyses. Approximately half of the articles published from 2000 to 2010 in proteomics employed this technique [1]. Even though it has recently lost space to shotgun mass spectrometry, this a unique top-down method that still has some advantages [1]. The idea of separating complex mixtures of proteins was first presented in 1975 by O'Farrell [2] and then Klose [3]. Since these initial stages, this experimental technique has improved to the point that it is more highly reproducible, with a greater capacity and increased proteomic coverage [4].

2DE takes advantage of the physiochemical properties of proteins and separates them using an electrical field in two steps or dimensions. The first dimension employs isoelectric focusing (IEF), which separates proteins according to their isoelectric points (pI). During IEF, molecules migrate in acrylamide gel with the help of ampholytes or an immobilized pH gradient (IPG) until reaching the point at which their total net charge is zero. Next, the proteins are submitted to a second dimension of separation, generally employing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), which separates proteins according to

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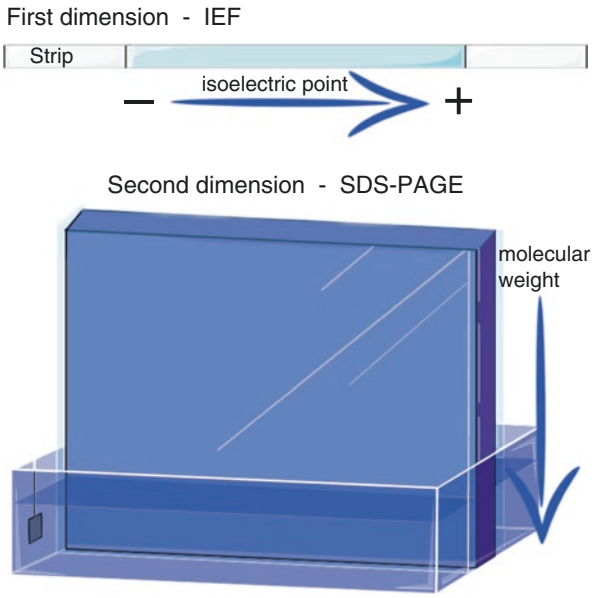

Fig. 13.1 Scheme of 2DE methodology

apparent molecular weight. SDS is an anionic detergent that keeps proteins unfolded and provides them with a net negative charge. This allows their migration toward the anode in a polyacrylamide gel. Larger proteins migrate slower, while the smaller ones migrate faster, promoting their separation. A scheme of this methodology can be seen in Fig. 13.1.

In the 1970s, IEF was performed in capillaries containing polyacrylamide gels and charged ampholytes. Under the influence of an electric current, the ampholytes generate a pH gradient. However, this technique lacked reproducibility. In the 1980s, this setup was largely replaced by the use of immobilized pH gradients (IPG) on polyacrylamide strips, nowadays commercially available [5]. In addition, the incorporation of reduction and alkylation steps prior to the second-dimension separation led to increased resolution and technical reproducibility [6]. Improvements in sample preparation also contributed to 2DE reproducibility and sensitivity. Proteins must be completely denatured and solubilized prior to 2DE, and this is a challenging task, considering the different classes of proteins [7–10].

After carrying out a 2DE separation, the next step is visualization of the separated proteins. These methods may vary and are usually chosen according to each experimental purpose. The most traditional methods are based on Coomassie Blue and silver nitrate staining. The former is more suitable for proteome quantitation and subsequent mass spectrometry (MS) analyses, but the latter is more sensitive and therefore favors identification of low abundance proteins [6]. After staining, the spots on 2DE gels are submitted to computational analysis, using softwares such as Image Master (GE Healthcare), Melanie (GeneBio), Delta2D (Decodon), and PDQuest (BioRad). Protein spots identified with different abundances across differ-

ent samples (such as disease versus control) can be submitted to MS analysis for protein identification purposes [8–10, 11].

The combination of 2DE-MS was the state-of-the-art for proteomics until shotgun-MS approaches emerged. Despite the technical advantages of MS-based shotgun proteomics, 2DE is still useful for specific purposes such as de novo protein sequencing of organisms whose genomes have not been characterized and for identification of posttranslational modifications and identification of protein isoforms.

Despite all of the technical benefits, the 2DE approach has some drawbacks. For example, the coverage of very acidic and basic proteins as well as proteins smaller than 10 kDa or larger than 120 kDa is low. However, some of these problems can be circumvented by employing narrow-range IPG strips in the first dimension and different acrylamide concentrations in the second dimension [9]. The reproducibility across gels has also been a problem, due to the need of comparing different samples on different gels. To overcome this, a new approach was developed in 1997 called 2D difference gel electrophoresis (2D–DIGE) in which two samples can be compared in one gel [12]. In 2D-DIGE, two proteomes under comparison are labeled prior to electrophoresis using two different fluorescent cyanine dyes (CyDyes). Later developments incorporated the use of a third CyDye which allowed in-gel comparison of a pool of the two samples as an internal standard [13]. After running the gels, the protein spots within the three different samples can be visualized though a scanner with laser/emission filters for each CyDye and quantitated using specific software. The downside of 2D-DIGE is the high experimental cost.

We present here a standard 2DE reference protocol since the basic technique is highly accessible at low cost in most laboratories. This method still occupies an important niche in proteomics and compliments traditional shotgun-MS approaches in biomarker identification studies.

13.2 Materials

13.2.1 First Dimension

1. Protein extracts of interest (*see Note 1*)
2. Extraction buffer: 7 M urea, 2 M thiourea, 4% 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate hydrate (CHAPS), 70 mM dithiothreitol (DTT), 2% Complete™ Protease Inhibitor Cocktail (Roche Diagnostics, Mannheim, Germany), and 2% IPG Buffer pH 3–10 (GE Healthcare; Little Chalfont, UK) (*see Notes 2 and 3*)
3. Ettan IPGphor Strip Holder (GE Healthcare)
4. Immobiline DryStrip Reswelling Tray (GE Healthcare)
5. Immobiline DryStrips pH 4–7 (GE Healthcare) (*see Note 3*)
6. Immobiline DryStrip Cover Fluid (GE Healthcare)
7. Isoelectric focusing (IEF), Ettan™ IPGphor™ 3 Isoelectric Focusing System (GE Healthcare)

13.2.2 Second Dimension

1. Polymerized 12.5% SDS-PAGE gels (*see Note 4*)
2. 10% ammonium persulfate (APS)
3. Tetramethylethylenediamine (TEMED)
4. Equilibration buffer: 6 M urea, 1.5 M Tris-HCl pH 8.8, 30% (v/v) glycerol, 10% (w/v) SDS, 0.1% DTT
5. Equilibration buffer: 6 M urea, 1.5 M Tris-HCl pH 8.8, 30% (v/v) glycerol, 10% (w/v) SDS, 0.04% iodoacetamide
6. SDS-PAGE running buffer: 25 mM Tris-HCl pH 8.3, 192 mM glycine, 0.1% SDS
7. Agarose
8. Gel electrophoresis tank and power supply (*see Note 5*)

13.2.3 Coomassie Blue Staining

1. Milli-Q water
2. Fix solution: 2% (v/v) phosphoric acid, 30% (v/v) ethanol
3. Stain solution: 10% (v/v) phosphoric acid, 10% (w/v) ammonium sulfate, 0.25% Coomassie Brilliant Blue G, 20% methanol
4. Destain solution: water
5. Image Scanner III (GE Healthcare)

13.3 Methods

13.3.1 First Dimension

1. Estimate the protein concentration in the samples using a technique of choice such as Lowry or Bradford and equipment such as a nanodrop or Qubit (*see Note 6*).
2. Add 500 ug protein to a final volume of extraction buffer according to the length of DryStrip (*see Note 7*) (Table 13.1).
3. Add the sample solution to the strip holder.
4. Place the strip with the gel side down in contact with the solution (*see Note 8*).
5. Cover the strip with Cover Fluid to prevent dehydration.
6. Place the strip holder with the electrode of the tapered portion in contact with the positive pole of the IPGphor and the opposite electrode to the negative pole.
7. Input the method in the IPGphor, adding 12 hours of hydration prior to the beginning of the voltage application as indicated in Table 13.2 (*see Note 9*).
8. After electrophoresis, remove the strip from the holder and freeze immediately at -80 °C.

Table 13.1 Rehydration solution volumes for the different sizes of IPG strips

IPG Strip length (cm)	Volume of solution per strip (µl)
7	125
11	200
13	250
18	340
24	450

Table 13.2 Guidelines for the Ettan IPGphor with rehydration loading/isoelectric focusing for Immobiline DryStrips (pH 4–7, 3–10, 3–10 nonlinear)

Immobile DryStrip		Rehydration loading			
Length (cm)	pH range	Step and voltage mode	Voltage (V)	Step duration (h:min)	Volt-hour (kVh)
7	03–10 3–10 NL 4–7	1 Step and hold 2 Step and hold 3 Step and hold Total	500 1000 5000	0:30 0:30 1:40 2:40	0.25 0.5 7.5 8.0
11	03–10 4–7	1 Step and hold 2 Step and hold 3 Step and hold Total	500 1000 8000*	1:00 1:00 1:50 3:50	0.5 1.0 12.5 14.0
13	03–10 3–10 NL 4–7	1 Step and hold 2 Step and hold 3 Step and hold Total	500 1000 8000*	1:00 1:00 2:00 4:00	0.5 1.0 14.5 16.0
18	03–10 3–10 NL 4–7	1 Step and hold 2 Step and hold 3 Step and hold Total	500 1000 8000*	1:00 1:00 4:00 6:00	0.5 1.0 30.5 32.0
	Narrow intervals	1 Step and hold 2 Step and hold 3 Step and hold Total	500 1000 8000*	1:00 1:00 7:30 9:30	0.5 1.0 58.5 60.0
24	03–10 3–10 NL 4–7	1 Step and hold 2 Step and hold 3 Step and hold Total	500 1000 8000*	1:00 1:00 8:20 10:20	0.5 1.0 62.5 64.0
	Narrow intervals	1 Step and hold 2 Step and hold 3 Step and hold Total	500 1000 8000*	1:00 1:00 10:30 12:30	0.5 1.0 94.5 96.0

* the indicated voltage may not be reached, depending on the sample.

13.3.2 Second Dimension

1. Place the strip in a channel of the reswelling tray, and leave on mild agitation for 12 min in equilibration buffer containing 0.1% DTT (*see Note 10*).

2. Replace the strip in a channel of the reswelling tray, and leave on mild agitation for 12 min in equilibration buffer containing 0.4% IAA and 0.002% Bromophenol Blue (*see Note 11*).
3. Add 200 uL 1 M Tris pH 6.8 on the strip and leave for 5 min.
4. After polymerization of the SDS-PAGE gel, position the strip in contact with the top of the gel (*see Note 12*).
5. Add 1% warm agarose solution (70 °C) for sealing the strip over the gel (*see Note 13*).
6. Add running buffer as appropriate to cover the cathode (top) and anode (bottom) reservoirs of the gel tank.
7. Subject to electrophoresis until the dye front just reaches the bottom of the gel (*see Note 14*). Use 60 V for the first hour and then 120 V for the rest of the run.

13.3.3 Coomassie Blue Staining

1. After electrophoresis, disassemble the equipment and place the gel in Milli-Q water twice for washing purposes and in the fix solution overnight.
2. Then place the gel solution for at least 5 h (*see Note 15*).
3. Bleach gel until the spots become visible in Milli-Q water (Fig. 13.2).
4. Image the gel using an appropriate scanner (*see Note 16*).

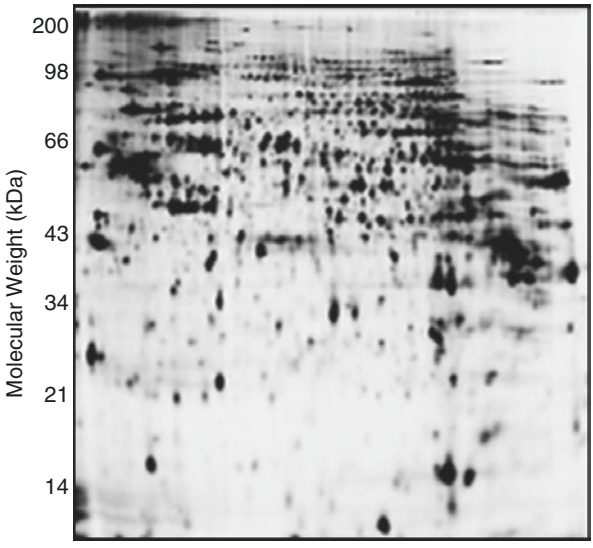

Fig. 13.2 2D-SDS-PAGE profile of mouse (*Mus musculus*) brain, as an example

13.4 Notes

1. The lysis buffer used in the extraction of the proteins must be compatible with the 2DE method. It should not contain salts or detergents since they impair the distribution of proteins in IEF.
2. This is added to the IEF buffer to give a final concentration of 2%.
3. Other pH ranges can be chosen depending on the experimental needs.
4. The acrylamide concentration is chosen according to experimental needs. Low percentage acrylamide gels (5–10%) favor separation of larger proteins, and high percentage gels (10–20%) allow resolution of proteins with lower molecular weights. The polymerization is achieved using APS and TEMED as a catalyst, based on a free radical mechanism.
5. The tank should be chosen to fit the size of the gels. Here we used the Ettan Dalt II system.
6. The DryStrip must be chosen taking into account the complexity of the mixture and the required resolution in the analysis.
7. To complete the final volume, the same buffer used in the extraction of proteins can also be added here. The amount of buffer needed is variable according to the size of the DryStrip (Table 13.1).
8. The positive pole should contact the tapered portion of the strip, with both ends contacting the electrodes. Care should be taken to avoid air bubbles as this can affect contact.
9. Use the voltages and specific times for each type of DryStrip, according to size and pH range (Table 13.2).
10. This step helps to denature the proteins prior to the second dimension electrophoresis step. The addition of DTT reduces disulfide bonds in proteins.
11. The addition of IAA alkylates the free sulfhydryl residues generated during the previous equilibration step.
12. Add PSA and TEMED to polymerize the gel. When placing the strip on top, be careful not to damage the top of the second dimension gel and attempt to avoid the introduction of air bubbles between the strip and the gel.
13. Agarose must be previously prepared and melted at a temperature of approximately 70 °C prior to use.
14. The dye front comes from the Bromophenol Blue in the second dimension equilibration buffer.
15. Adjust the staining time as required.
16. We use the Image Scanner III to acquire the image as a TIFF and analyze using a software such as Image Master 2D Platinum (GE Healthcare).

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Chapter 14
A Two-Dimensional Difference Gel Electrophoresis (2D-DIGE) Protocol for Studies of Neural Precursor Cells

Paul C. Guest

14.1 Introduction

Neural precursor cells (NPCs) are multipotent self-renewing cells which can generate neurons, astrocytes, and oligodendrocytes in the central nervous system (CNS) [1, 2]. The signalling molecules required for proliferation of NPCs include growth factors such as fibroblast growth factor (FGF) and epidermal growth factor (EGF) [3–6]. Identification of other factors is needed for increasing our understanding of adult neurogenesis and for developing new strategies for targeting endogenous stem cells in order to stimulate their proliferation and differentiation into the correct cell types needed for CNS repair and the generation of new synaptic connections [7]. NPCs have been isolated from fetal and adult brains in many mammalian species and cultured in the presence of growth factors such as FGF and EGF to form cell clusters called neurospheres [8–11]. All of the NPCs within each neurosphere can differentiate into neurons or glial cells following withdrawal of the growth factors, thus providing an in vitro model of neurogenesis [12]. By subjecting such neurospheres to different treatments or culturing them under different conditions, it might be possible to identify novel factors and mechanisms involved in NPC growth and differentiation [6, 13]. This is important because increasing our understanding of how to activate and differentiate endogenous NPCs in the CNS may offer a potential cell replacement therapy for neurological disorders [14, 15]. For example, repeated studies have now shown that neurogenesis may be required for antidepressant efficacy in the treatment of depression [16, 17].

Adult mouse subventricular zone (SVZ) cells can be expanded in culture as neurospheres, making these useful as a model for identifying compounds that act directly on NPCs. The large amounts of cellular material that can be obtained using

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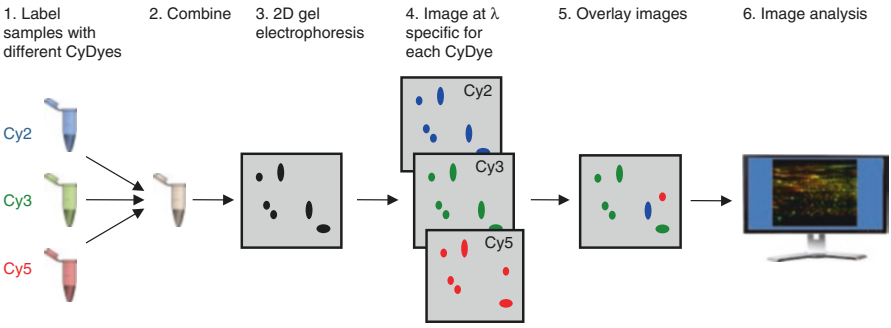

Fig. 14.1 Experimental flow of 2D-DIGE procedure incorporating the use of an internal standard

this approach also facilitates analysis using proteomic methods in attempts to identify new biomarkers and drug targets. As the most long-standing proteomic technique, two-dimensional gel electrophoresis (2DE) involves separation of proteins in two dimensions according to their isoelectric point and molecular size [18]. This enables simultaneous display of hundreds of proteins on one gel. Until the late 1990s, identification of statistically significant differences across two or more distinct proteomes required the running and analysis of many 2DE gels. This was difficult due to technical variations in sample preparation and gel running conditions [19]. In 1997, Unlu and colleagues presented an effective method of reducing gel-to-gel variation in a technique that they called 2D difference gel electrophoresis (DIGE) [20]. In a typical experiment, up to three protein extracts can be compared on a single gel by covalently labelling the proteins prior to electrophoresis with size- and charge-matched spectrally resolvable fluorescent dyes (Cy2, Cy3, and Cy5) [21]. After 2DE, fluorescent imaging of the gel at the wavelengths specific for each CyDye generates separate images for each proteome, and these can be overlaid directly for display of any differentially expressed proteins (Fig. 14.1). Differentially expressed proteins can then be identified by either matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) or liquid chromatography tandem mass spectrometry (LC-MS/MS) as described [22].

Here we present a typical 2D-DIGE protocol. The main objective was to characterize the proteomic changes in NPCs after 24 hours of growth factor withdrawal to induce differentiation.

14.2 Materials

14.2.1 Cell Culture

1. Purified mouse subventricular zone cells (*see Note 1*)
2. Growth medium: Dulbecco's Modified Eagle's Medium (DMEM)/F12 (1:1), penicillin/streptomycin/fungicide, B27, 20 ng/mL EGF, 20 ng/mL FGF-2 (*see Note 2*)

3. Differentiation medium: DMEM/F12 (1:1), penicillin/streptomycin/fungicide, B27 (*see Note 3*)
4. Wash solution: phosphate-buffered saline (PBS)

14.2.2 Subcellular Fractionation

1. Homogenization buffer 1: 20 mM Tris (pH 7.5), 250 mM sucrose, 1 mM EDTA, complete EDTA-free protease inhibitors (Roche Diagnostics, Mannheim, Germany)
2. Homogenization buffer 2: 20 mM Tris (pH 7.5), 2 M sucrose, 1 mM EDTA, complete EDTA-free protease inhibitors
3. 10 mL capacity glass tube homogenizer and Teflon pestle (Jencons Scientific; Leighton Buzzard, UK)
4. Swinging bucket rotor and matching tubes (*see Note 4*)

14.2.3 Protein Extraction and 2D-DIGE

1. Extraction buffer 1: 30 mM Tris (pH 8.0), complete EDTA-free protease inhibitors (*see Note 5*)
2. Extraction buffer 2: 30 mM Tris (pH 8), 7 M urea, 2 M thiourea, 4% 3-((3-cholamidopropyl) dimethylammonio)-1-propanesulfonate (CHAPS) (*see Note 6*)
3. CyDyes (GE Healthcare; Little Chalfont, UK): 25 nmol each of Cy2™, Cy3™ and Cy5™ DIGE Fluor minimal dyes
4. Anhydrous dimethylformamide (DMF)
5. Quench reagent: 10 mM lysine
6. Sonicator and microprobe
7. 24 cm IPG strips (pH 3–10) (*see Note 7*)
8. Strip rehydration buffer: 30 mM Tris (pH 8.0), 7 M urea, 2 M thiourea, 4% CHAPS, 2% dithiothreitol (DTT), 2% pH 3–10 IPG buffer
9. Equilibration buffer 1: 50 mM Tris-Cl, pH 8.8, 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS, 1% DTT, 0.01% bromophenol blue
10. Equilibration buffer 2: 50 mM Tris-Cl, pH 8.8, 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS, 2% iodoacetamide (IAA), 0.01% bromophenol blue
11. Strip equilibration buffer 1: SDS equilibration buffer with 1% DTT
12. Strip equilibration buffer 2: SDS equilibration buffer with 2% DTT, 0.01% and bromophenol blue (*see Note 8*)
13. 50 mM Tris-HCl, pH 6.8
14. IPGbox (GE Healthcare) or similar
15. First-dimension isoelectric focusing (IEF), Ettan™ IPGphor™ 3 Isoelectric Focusing System (GE Healthcare) or similar

16. 12.5% acrylamide, 0.33 N,N′-methylenebisacrylamide, 0.375 M Tris-HCl pH 8.8, 0.1% SDS, 0.1% (w/v) ammonium persulfate (APS), 0.025–0.09%(v/v) tetramethylethylenediamine (TEMED) (*see Note 9*)
17. 0.5% (w/v) agarose in Laemmli SDS-PAGE electrode buffer
18. Second-dimension Ettan DALT6 Electrophoresis System (GE Healthcare) or similar.
19. Typhoon FLA 9500 Imager (GE Healthcare)
20. DeCyder™ 2D Software (GE Healthcare)

14.3 Methods

14.3.1 Culturing and Differentiation of Neural Precursor Cells

1. Plate purified subventricular zone cells in growth medium.
2. Passage the resulting multipotent neurospheres to single-cell suspensions every 7 days for up to 14 days in growth medium (Fig. 14.2).
3. Split cells twice and plate at low density for a further 48 h growth.
4. Split cells again and plate at 2×10^5 cells/mL in growth media to recover from the passage procedure (*see Note 10*).
5. Culture cells for a further 24 h in either growth or differentiation medium to generate precursor and differentiate cells, respectively.
6. Centrifuge cells at 400 × g for 1 min and discard the supernatant.
7. Suspend cells in wash solution, repeat the centrifugation step three times and save the pellets on ice.

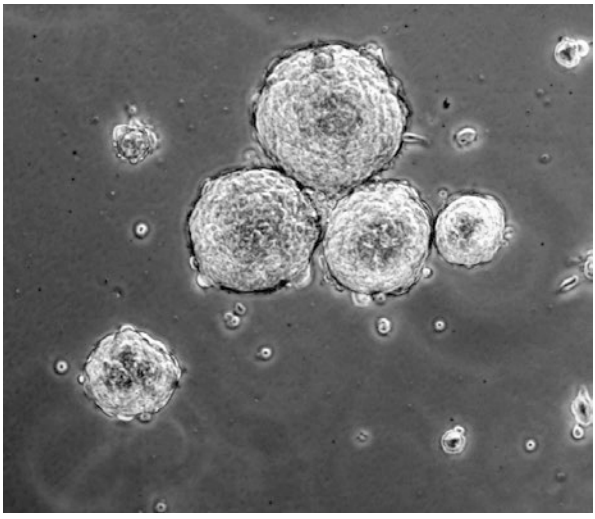

Fig. 14.2 Light microscopy showing neurospheres cultured in growth medium

14.3.2 Subcellular Fractionation

1. Suspend cells in homogenization buffer 1 in the glass tube, and homogenize using six strokes of the Teflon pestle driven at 600 rpm.
2. Centrifuge the homogenate at 750 × g for 10 min at 4 °C in the swinging bucket rotor to produce a heavy membrane fraction.
3. Resuspend the heavy membranes in homogenization buffer 2 in the glass tube and homogenize as above.
4. Centrifuge the homogenate at 40,000 × g for 30 min at 4 °C in the swinging bucket rotor.
5. Store the supernatant and pellets separately at -80 °C (*see Note 11*).

14.3.3 Protein Extraction and 2D-DIGE Analysis

1. Sonicate the pellets in 7:1 (w:v) extraction buffer, and centrifuge at 13,000 × g for 20 min to pellet insoluble debris.
2. Transfer supernatants to fresh 1.5 mL capacity microcentrifuge tubes.
8. Estimate the protein concentration of the samples using a standard protein measurement kit (*see Note 12*).
9. Dilute each CyDye with 25 uL DMF immediately prior to the labelling reaction to obtain a stock solution of 1 nmol (*see Note 13*).
10. Add 0.4 uL Cy2 to 50 ug of precursor cell membrane extract.
11. Add 0.4 uL Cy3 to 50 ug of differentiated cell membrane extract.
12. Add 0.4 uL Cy5 to 50 ug of a 1:1 mixture (25 ug each) of both samples above to serve as the internal standard (*see Note 14*).
13. Incubate samples 30 min in the dark on ice.
14. Add 1uL quench solution and leave 10 min in the dark on ice.
15. Complete the volume of each of the samples to 150 uL with rehydration buffer.
16. Incubate samples 15 min in the dark on ice.
17. Mix all three samples together (*see Note 15*).
18. Add 450 µL sample mixture to IPG strips and leave to hydrate at 20 °C for 12 hours in an IPG coffin.
19. Run the IPG strip on the IPGphor at 200 V for 1 h, 500 V for 1 h, 1000 V for 1 h and 8000 V for 8 h at 20 °C using a maximum current setting of 50 mA/strip (*see Note 16*).
20. Strips can be frozen at -80 °C or the next step can be carried out immediately.
21. Soak the IPG strip in 100 mL equilibration buffer 1 for 10 min.
22. Remove the buffer and soak the strip in 100 mL equilibration buffer 2 for 10 min (*see Note 17*).
23. Prepare resolving acrylamide gel solution as needed (*see Note 18*).
24. Add APS and TEMED to the solution, and pour the mixture immediately between assembled low-fluorescence glass plates to within 3 cm from the top (*see Note 19*).

25. Carefully layer butanol on top to help achieve a flat surface on top of the gel when it polymerizes.
26. Rinse the butanol of the gel surface with water, and apply the equilibrated IPG strip so it is sitting immediately on top of the gel.
27. Seal with 0.5% agarose in SDS running buffer on top (*see Note 20*).
28. Carry out electrophoresis at 60 V for 1 h followed by 30uA /gel until the dye front reaches the end of the gel.
29. Scan the gels directly between the glass plates (*see Notes 21 and 22*) using filters specific for the excitation and emission wavelengths of each CyDye (*see Note 23*).
30. Export the images as tagged image file format (TIFF) files for analysis.
31. Analyse images using the DeCyder Batch Processor and Differential In-Gel Analysis (DIA) software tools.
32. Compare the protein spot volumes on the Cy5-labelled pooled standard image with matching spots on the Cy2- or Cy3-labelled images (the precursor and differentiated cell extracts, respectively).
33. Match the images from each gel with the biological variation analysis (BVA) software using the pooled standard image to normalize each protein spot.
34. Use the software to identify protein spots with differences in spot volume (abundance) between the differentiated and precursor cells (Fig. 14.3) (*see Notes 24 and 25*).

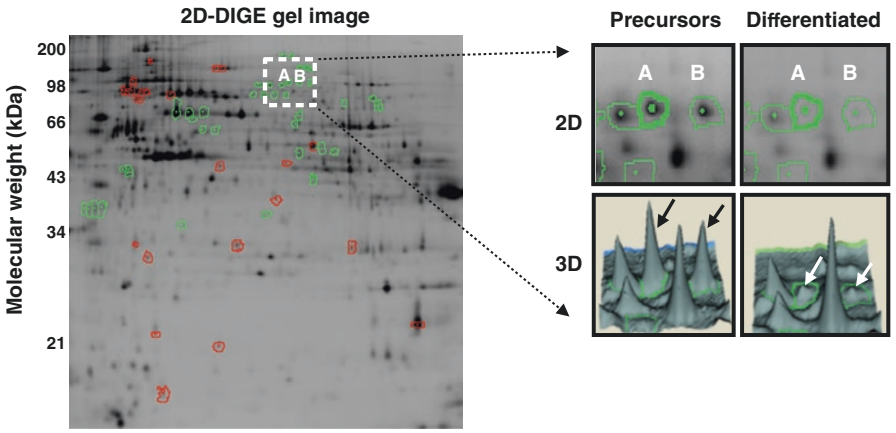

Fig. 14.3 2D-DIGE analysis showing decreased levels of a protein. The example shows the decreased expression of the nuclear cell cycle replication factor MCM7 in differentiated neural precursor cells. *(A)* 2D view of precursor cell, differentiated cell and pooled proteomes overlaid. The protein spots which are increased upon differentiation are marker in *red* and those which are decreased are marked in *green*. *(B)* Close-up 2D (*top*) and 3D (*bottom*) views of two spots showing a decrease in volume by more than fivefold in differentiated cells [22]

14.4 Notes

1. Here we have used mouse subventricular zone dissected from adult mice and enzymatically and mechanically dissociated as described previously [12].
2. B27 medium supports long-term growth of neuronal cells in culture.
3. The absence of growth factors halts proliferation and leads to differentiation.
4. Similar rotors can be used but be sure to convert g-forces properly according to the manufacturer's instructions.
5. This buffer favours isolation of soluble proteins.
6. This buffer releases less soluble material such as integral membrane proteins. However, this fraction was not analysed in the current study.
7. The IPG strip pH range should be chosen to maximize resolution of the proteins of interest. The pH 3–10 strips will provide resolution of acidic, neutral, and basic proteins. If only acidic proteins are desired, pH 4–7 strips should be selected.
8. The bromophenol blue is added to provide a visible dye front during electrophoresis.
9. The acrylamide and N,N′-methylenebisacrylamide concentrations should be chosen based on the desired molecular weight range separation (low percentages of acrylamide allow resolution of high molecular weight proteins, and high percentages resolve proteins in the lower molecular weight range). Ammonium persulfate and TEMED should be added just before use since these initiate the gel polymerization process.
10. Two weeks of culturing under these conditions should produce $1–4 \times 10^8$ cells, providing sufficient material for a proteomics study.
11. The pellet consists of cellular organelles.
12. The presence of urea in the extraction buffer might interfere with some assays, which could lead to false readings.
13. It is best to use DMF as fresh as possible after opening. We noticed decreased labelling efficiency even if the DMF was only 1 month old.
14. The use of an internal standard helps to minimize false positives and false negatives since it can serve as a control for each protein spot on all gels in the analysis. The standard is usually made by combining equal volumes of each extract.
15. It is possible that the CyDyes may show preferential labelling of some proteins although this can be accounted for by reversing the dye/extract combinations.
16. A step voltage gradient was used as we and others have noticed that this helps to avoid horizontal streaking of protein spots on the final 2D gel image.
17. This stage serves the same purpose as a stacking gel in 1D gel electrophoresis. The spots only begin to resolve once they reach the higher pH in the resolving gel.
18. Lower percentage gels (5–10%) favour resolution of high molecular weight proteins, and higher percentages (10–20%) resolve proteins of lower molecular weights. To maximize coverage, a 5–20% gradient gel can be used.

19. This will allow space to apply the equilibrated IPG strip.
20. Ensure that there are no air bubbles trapped between the strips and the gel tops to avoid distortion in the protein spot patterns.
21. The second-dimension gels should be poured between low fluorescent Pyrex glass plates to minimize background fluorescence during scanning. Furthermore, the Ettan Dalt II system allowed simultaneous running of multiple plates. This is important as it means that all second-dimension gels can be run under approximately the same conditions, which allows for better matching of the gel images in subsequent stages.
22. A key advantage of the 2D DIGE technique is that gels can be imaged after electrophoresis without disassembly of the low-fluorescence glass plates. This ensures that the gels are not deformed or damaged during imaging and also minimizes the possibility of contamination. Furthermore, gels can be scanned for different lengths of time to maximize detection of high- and low-abundance protein spots.
23. For Cy2, the excitation and emission optimal wavelengths are 480 nm and 530 nm, respectively. For Cy3, these are 540 nm and 590 nm, respectively. For Cy5, these are 620 nm and 680 nm, respectively.
24. Within gels, this is achieved by direct overlay of spots and across gels by landmarking, warping, and matching spots using the Biological Variation Analysis function of the DeCyder software.
25. Protein identification can be achieved using any kind of protein mass spectrometry. We suggest preparing a gel containing approximately 200 ug of the standard pool followed by colloidal Coomassie Blue staining for excision of spots. Spots must be digested in gel prior to mass spectrometry analysis for protein identification [23].

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Chapter 15
Identifying Biomarker Candidates in the Blood Plasma or Serum Proteome

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15.1 Introduction

The etiology and biological mechanisms of brain disorders such as neurodegenerative and neuropsychiatric disease still need to be understood. Moreover, there are no known biomarkers, which could help physicians in terms of diagnosis, patient stratification, choice of correct drug, or disease monitoring. In contrast, in cancer research there are at least 23 protein biomarkers approved by the US Food and Drug Administration (FDA) [1, 2].

For complex disorders, it is unlikely that one biomarker will be capable of determining disease and treatment states. A panel of molecules is potentially more precise and sensitive, and these should meet certain requirements such as being reproducible in clinical settings, inexpensive, and available in samples collected in the least invasive manner possible [3]. As body fluids such as plasma, serum, urine, and saliva are considered to be noninvasive, these sources have the highest potential for biomarker discovery investigations [4].

Schizophrenia is a mental, disable, and incurable psychiatric disorder that may affect 1% of worldwide population. One may argue that schizophrenia comprises several disorders, which are represented by a range of symptoms, classified as positive (e.g., hallucinations), negative (e.g., social withdrawal), and

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cognitive categories [5]. As with other psychiatric disorders, schizophrenia appears to be multifactorial in its etiology, with the combined influence of both environmental and genetic factors. Thus, finding a set of biomarkers for improving diagnosis or for monitoring drug treatment effects in clinical setting is still an open quest [2, 6].

The main way of treating schizophrenia is through the use of antipsychotics. Even though several antipsychotic medications are available, around 40% of the patients show a poor response to initial treatment with these drugs. Recently, a mass spectrometry-based shotgun proteomics study using blood plasma collected from schizophrenia patients before and 6 weeks after treatment revealed molecular differences between patients that responded well compared to those with a poor response to antipsychotics [7]. Other studies using mass spectrometry (MS) and multiplex immunoassay-based approaches have found that treatment with antipsychotics such as olanzapine can lead to changes in protein phosphorylation patterns [8] as well as the levels of cytokines [9] and metabolism-related proteins [10, 11].

The use of blood plasma or serum to study brain disorders using proteomics through mass spectrometry has been growing in recent decades. This is likely to be due to the fact that these disorders, despite being localized to the brain, affect the molecular composition throughout the blood. Studies on psychiatric disorders have shown that blood-based molecules can be disrupted, including those involved in neuroendocrine function [12], molecular transport [13], inflammatory response [14, 15], and oxidative stress response [16]. For blood plasma proteome analysis, sample preparation is a crucial step to guarantee the precise identification of biomarkers with the potential of clinical implementation. This includes the removal of highly abundant proteins (the depletome), since approximately only 30 proteins make up 99% of the blood protein mass [17]. These abundant proteins can obscure the lower abundance proteins, impairing their identification and precise quantitation. To remove the most abundant proteins from blood plasma and serum, methods can be employed such as immunoaffinity depletion [4, 18]. Depleted samples can be then submitted to MS-based shotgun proteomic analysis to identify and quantify candidate biomarkers [19].

Here we present a general protocol for protein biomarker identification in blood plasma or serum using MS-based proteomic analyses. This protocol focuses on depletion of the 14 most abundant proteins in plasma or serum using a high-performance liquid chromatography (HPLC) system. Subsequently, the depleted samples are applied to a two-dimensional liquid chromatography (2D-LC), which is connected online with an MS instrument operated in data independent analysis (DIA) mode, and the proteins are quantified by label-free analysis. These biomarker candidates can be combined in a mass spectrometry-based molecular assay based on selected/multiple reaction monitoring (SRM/MRM), which is a fast, sensitive, and robust technique with the potential of clinical implementation (Chapter 17 Reis-de-Oliveira et al.).

15.2 Materials

1. Human serum or plasma samples
2. Buffer solution A: 40 mM sodium phosphate (pH 7.4), 0.5 M NaCl, 0.02% sodium azide (*see Note 1*)
3. Buffer solution B: 2 M Urea, 0.5 M glycine (pH 2.25) (*see Note 1*)
4. MARS Hu14 column (4.6 mm inner diameter, length 100 mm, capacity of 40 µL of plasma or serum) (Agilent technologies)
5. HPLC system (*see Note 2*)
6. Empower Pro 2 software (Waters Corporation)
7. 0.5 and 1.5 mL Eppendorf LoBind tubes
8. 50 mM ammonium bicarbonate solution (pH 7.5)
9. Sample concentrator cater for 6 mL sample volume with 3000 Da molecular weight cutoff (MWCO)
10. 15 mL conical centrifuge tubes
11. Refrigerated bench-top centrifuge with rotor for 15 mL conical tubes
12. 100 mM dithiothreitol (DTT) in 50 mM ammonium bicarbonate solution (pH 7.5)
13. 300 mM iodoacetamide (IAA) in 50 mM ammonium bicarbonate solution (pH 7.5)
14. Sequencing grade modified trypsin (Promega)
15. Trypsin resuspension dilution buffer (Promega)
16. 5% trifluoroaceticacid (TFA)
17. Qubit® 3.0 Fluorometer (ThermoFisher Scientific)
18. Qubit Protein Assay Kit (Thermo Fisher Scientific)
19. Qubit Assay Tubes (Thermo Fisher Scientific)
20. Acquity UPLC M-Class (Waters Corporation)
21. Synapt G2-Si Mass Spectrometer (Waters Corporation)
22. Acquity UPLC M-Class Peptide BEH C18 Trap Column (130 Å, 5 µm, 300 µm × 50 mm) (Waters Corporation)
23. Acquity UPLC M-Class Symmetry C18 Trap Column, 2D (100 Å, 5 µm, 180 µm × 20 mm) (Waters Corporation)
24. Acquity UPLC M-Class HSS T3 Column (1.8 µm, 75 µm × 150 mm) (Waters Corporation)
25. NanoLock Spray dual electrospray ion source (Waters Corporation)
26. 100 fmol/uL human [Glu1]-fibrinopeptide B human (Glu-Fib)
27. Solvent C: 20 mM ammonium formate (pH 10)
28. Solvent D: 100% acetonitrile (ACN)
29. Solution E: 0.1% formic acid in water
30. Solvent F: 0.1% formic acid in ACN
31. UniProt human proteomic database
32. Progenesis® QI for Proteomics version 2.1 (Waters Corporation)
33. Protein Lynx Global Server® version 3.0.3 (Waters Corporation)
34. Ingenuity Pathway Analysis (IPA; Ingenuity Systems; Qiagen)

15.3 Methods

15.3.1 Sample Preparation (see Note 3)

1. Dilute 30 µL of human plasma sample using 90 µL buffer A.
2. Centrifuge the sample at 21,000 × *g* for 15 min (*see Note 4*).
3. Transfer the supernatants to 0.5 µL LoBind tubes and keep them on ice until experimental analysis.

15.3.2 Human Serum and Plasma Protein Depletion

1. Purge the whole HPLC system with water at flow rate of 1 mL/min for 5 min (*see Note 5*).
2. Prepare the buffer solutions A and B for the HPLC system.
3. Connect buffer A and buffer B flasks to pump inlets and purge the HPLC system at 1 mL/min for 5 min (without a column).
4. Attach the Agilent MARS Hu14 column in the HPLC system (*see Note 6*).
5. Equilibrate the column with buffer A and wash the sample loop with 3 volumes between the runs.
6. Set the depletion method using Empower Pro 2 Software (Table 15.1).
7. Before the first run, and after the last run, perform a blank method injecting 120 µL buffer A (*see Note 7*).
8. Inject 120 µL sample into HPLC loop and start the LC method.
9. Ensure that the volume of injected sample is suitable to the column capacity (*see Note 8*).
10. Run the chromatographic method for depletion, and collect eluted fractions as using 1.5 mL low-protein-binding tubes (*see Note 9*).
11. Store collected fractions at −80 °C until further analyses.
12. Equilibrate the column with buffer A for 11 min at 1 mL/min.

Table 15.1 Liquid chromatography method

Phase	Time	% Buffer B	Flow rate (mL/min)	Max pressure (bar)	Curve
Low-abundance fraction elution	0.00	0	0.125	60	6
	18.00	0	0.125	60	6
Washing	18.01	0	1.000	60	6
	20.00	0	1.000	60	6
High-abundance fraction elution	20.01	100	1.000	60	6
	27.00	100	1.000	60	6
Column regeneration	27.01	0	1.000	60	6
	38.00	0	1.000	60	6

13. Disconnect and store the depletion column at 4 °C.
14. Flush the HPLC system with water for 30 min at 1 mL/min (*see Note 10*).

15.3.3 Buffer Exchange

1. Wash sample concentration tubes with ultrapure water.
2. Add 3 mL 50 mM ammonium bicarbonate, and centrifuge at 6600 × g for 30 min or until the remaining volume is 300–600 µL (*see Note 11*).
3. For buffer exchange of low-abundance fraction, add 1.5 mL to the concentration tube, and centrifuge at 6600 × *g* for 30 min or until remaining volume in the upper chamber is 300–600 µL.
4. Remove assembly and discard the flow through (*see Note 12*).
5. For buffer exchange of the high-abundance fraction, add 6 mL of this sample to the concentration tube, and centrifuge at 6600 × *g* for 120 min or until remaining volume in upper chamber is 600–1000 µL.
6. Remove the assembly and discard the flow through.
7. Adjust the sample volume to 5.5 mL using 50 mM ammonium bicarbonate solution (*see Note 13*).
8. Centrifuge at 6600 × *g* for 120 min or until the remaining volume is 600–1000 µL.
9. Homogenize the concentrated sample several times, and transfer it to a 1.5 mL tube (Fig. 15.1).

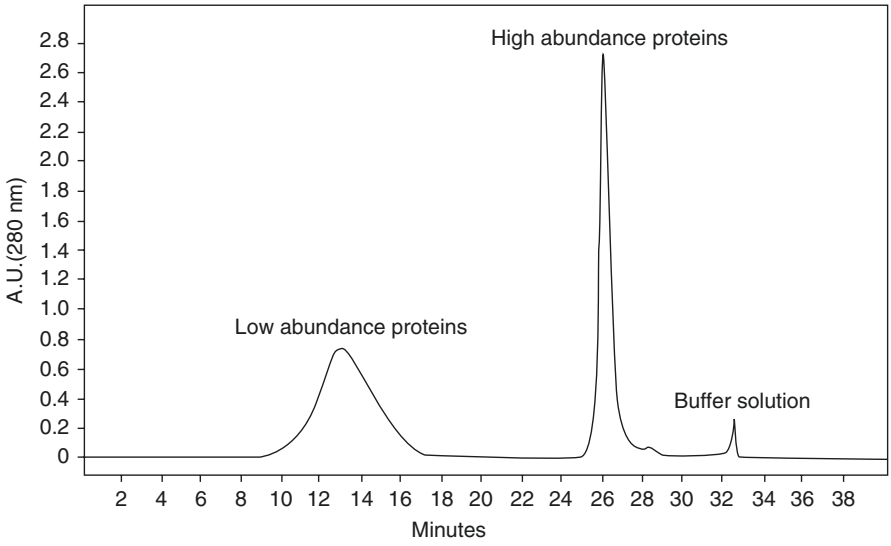

Fig. 15.1 Representative chromatogram of depletion runs on the MARS Hu14 column. The first peak contains the low-abundance proteins, the second peak contains the high-abundance proteins (bound proteins), and the last peak corresponds to the buffer solution

15.3.4 Quantification (See Note 14)

1. Prepare the working solution and standards from Qubit Protein Assay Kit for protein quantification according to manufacturer's instructions (*see Note 15*).
2. Add 1 μL of concentrated sample to 199 μL of working solution in Qubit Assay Tubes.
3. Vortex the tubes for 2 s.
4. Incubate the tubes for 15 min at room temperature.
5. Insert the tubes containing standards in the Qubit Fluorometer and press "Read Standards."
6. Insert samples tubes, set 1 μL as sample volume, and press "Read Samples" (*see Note 16*).

15.3.5 Tryptic Digestion

1. Add 50 μL of sample (1 μg/μL) in 1.5 mL microtubes.
2. Add 35 μL of 50 mM ammonium bicarbonate (pH 8.5) and homogenize.
3. Heat tubes at 80 °C for 15 min in a block heater.
4. Remove tube from the heater and centrifuge briefly.
5. Add 2.5 μL 100 mM DTT and homogenize (*see Note 17*).
6. Heat the samples at 60 °C for 30 min.
7. Remove the samples from the heater and cool them at room temperature.
8. Add 2.5 μL 300 mM IAA and vortex (*see Note 18*).
9. Incubate the samples for 30 min at room temperature in the dark.
10. Add 5 μL of trypsin solution and vortex (*see note 19*).
11. Incubate the samples at 37 °C overnight (12–16 h).
12. Add 10 μL of 5% TFA to stop trypsin digestion and homogenize.
13. Incubate samples at room temperature for 15 min.
14. Centrifuge samples at 20,800 × *g* at 6 °C for 30 min.
15. Transfer the supernatants to Waters Total Recovery vials (*see Note 20*).
16. Take an aliquot for peptide quantification.

15.3.6 Mass Spectrometry (See Note 21)

1. Set the column temperature as 45 °C in trap valve manager control.
2. Maintain the temperature at 8 °C in the sample manager.
3. Establish solutions C and D as mobile phases of the first-dimension chromatography.
4. Set up solutions E and F as mobile phases of the second-dimension chromatography.

Table 15.2 Nanoflow conditions

Synapt G2-Si HDMS	
Capillary (kV)	2.80
Sampling cone	30
Source offset	30
Source (°C)	70
Cone gas (L/h)	0
Nano flow gas (Bar)	0.5
Purge gas (L/h)	150

5. Calibrate the mass spectrometer using the Glu-Fib solution according to manufacturer's instructions.
6. Set the MS acquisition method able to alternate low and high energy, with no selection window and a continuum ion current.
7. Use CE ramp from 19 to 53 eV in transfer cell for the elevated energy MS scan.
8. Set nanoflow conditions at the tune page (Table 15.2).
9. Set the mass spectrometer to operate in resolution mode with an m/z ratio resolving power of 40,000 FWHM, using ion mobility with cross-sectional resolving power of 40 Ω/ΔΩ.
10. Adjust the first-dimension chromatography on ACQUITY UPLC M-Class Peptide BEH C18 Trap Column to perform the fractionation through discontinuous steps of acetonitrile (11.4%, 14.7%, 17.4%, 20.7%, and 50.0%) for 10 min at flow rate of 2 μL/min.
11. Adjust the second-dimension separation using ACQUITY UPLC M-Class HSS T3 Column using an acetonitrile gradient from 7 to 85% (v/v) for 36 min at a flow rate of 0.4 μL/min directly into the Synapt G2-Si HDMS.
12. Load samples of 0.5 μg into ACQUITY UPLC M-Class 2D System coupled to Synapt G2-Si HDMS, and perform LC-MS/MS acquisitions.

15.3.7 Proteome Quantification and Identification

1. Download the human proteome FASTA file from UniProt database, and create the human reversed-decoy database.
2. Load Progenesis QI for Proteomics version 2.0 software to process LC-MS/MS information (raw data).
3. Select the raw data and provide lock mass m/z as 785.8426 to perform the calibration.
4. Set up processing parameters for HDMSE acquisitions as shown in Table 15.3 and import the data.
5. After all files have been imported, click on "Start Automatic Processing," and select to assess all runs in the experiment for suitability.
6. Proceed with "Automatic Alignment" box and click "Next."

Table 15.3 Processing parameters for raw data

Thresholds	MS^E	$HDMS^E$
Low energy	500	150
Elev. energy	50	50
Intensity	750	750

7. In "Perform Peak Picking," click on "Set Parameters Up" in "Runs For Peak Picking" tab and check if all runs are selected.
8. Adjust the sensitivity of the peak picking to automatic in the "Peaking Limits" tab setting the "Noise Estimation Algorithm" to 4.
9. Set the "Maximum Charge" to 8.
10. Click "OK" to close the "Peak Picking Parameters."
11. Select the "Set Up An Experiment Design" box (*see Note 22*).
12. Select to "Run Groups by Conditions" in "Identify Peptides" tab, verify if the "Use MSE Data From My Runs To Identify Peptides" is selected, and click to set parameters.
13. Select human target-decoy database.
14. Verify default parameters and, if necessary, edit values (*see Note 23*).
15. At the "Protein Quantitation" tab, select "Relative Quantitation Using Hi-N" option, choose "3" as number of peptides to measure per protein, and use "Protein Grouping."
16. Click "Finish" to start automatic processing.

15.3.8 In Silico Analysis

1. Use Ingenuity Pathway Analysis to perform pathway and functional correlation analyses using differentially expressed proteins.

15.4 Notes

1. Refresh this buffer as required.
2. We use a Waters HPLC system with 2487 Dual λ Absorbance Detector and a manual injection system.
3. The procedures must be carried out at 4 °C, to prevent protein degradation. Keep the samples on ice until use. The chromatographic runs should be performed at room temperature.
4. Spin cartridges avoid the introduction of particulate matter to the column.
5. Data recording and processing were performed with Empower Pro 2 Software (Waters Corporation).
6. If the HPLC system is in ethanol or isopropanol, it is necessary to wash the system with water. To avoid precipitation within the system, do not change

from organic solvent to the buffer. The buffer solution does not need degassing.

7. This step allows the complete elution of residual proteins from the column. The sample loop volume should be at least twice the volume of the injected sample.
8. We use manual injection with a Hamilton syringe. This requires specific precautions to avoid entrance of air into the sample loop and cross-contamination. Before removing the syringe from the holder, switch the sample valve to the load position. Then, remove syringe, wash it with water, and buffer solution A. Place the syringe back in the syringe support and inject the sample from the syringe into the sample loop. Leave the syringe in the holder during the run. Repeat these steps for each sample.
9. The high-abundance proteins targeted for depletion will bind to the column, whereas the low-abundance proteins should flow through within the first 2.25 mL/18 min of the method. The high-abundance protein fraction should elute between 7.25 and 11.25 mL/23–27 min. Use low-protein-binding tubes to collect fractions. These help to avoid loss of sample by reducing sample binding to tube walls.
10. We recommend keeping a column record using a log book. Although the manufacturer guarantees unchanged chromatographic performance for 200 runs, we achieved more than 2000 runs with same column without loss of efficiency. The column efficiency is monitored by protein gel electrophoresis and visualization of each fraction.
11. Buffer exchange can take several hours to complete. Therefore, it should be done at 4 °C to minimize protein degradation. It is also important to check the specifications of the concentration tubes to grant the maximum allowed g-force. Higher g-forces might lead to leakage of proteins through the membrane filter.
12. To check the efficiency of concentration centrifuge tubes, a protein gel electrophoresis and visualization test can be carried out on the filtrate.
13. The steps 2 or 3 and 4 can be repeated twice to reduce the ionic strength and replace the buffer solutions A and B. For LC-MS systems fitted with a trap column, wash the sample as an additional desalting step before analysis. In our method, a single buffer exchange normally works well.
14. Make sure all assay reagents are at room temperature.
15. We use the Qubit Protein Assay Kit for protein quantification with Qubit 3.0 Fluorometer. Keep the reagents at room temperature 30 min before use.
16. The expected protein concentration is between 1–1.8 μg/μL for high-abundance fraction and 0.3–0.6 μg/μL for the low-abundance fraction.
17. DTT is used for reducing the disulfide bridge of cysteine residues in proteins.
18. IAA is used for alkylation of free sulfhydryl groups on cysteine residues.
19. We used 400 μL Sequencing Grade Modified Trypsin in 5 × 20 ug aliquots (0.05 μg/uL) and Trypsin Resuspension Buffer (Promega).
20. We used Waters Total Recovery vial preslit PTFE/silicone caps.

21. Quantitative and qualitative analyses were carried out in a bidimensional nanoUPLC tandem nanoESI-Q-IMS-TOF platform with the use of DIA with alternating low and high energy with ion mobility (HDMSE method) in positive mode nanoESI(+).
22. Create a comma-separated values (CSV) file with two columns. The first column corresponds to the sample name and must contain the same sample name as the raw file without the .raw extension. The second column corresponds to the group name and should have the name of biological condition used in the previous mass spectrometry run.
23. Common parameters must be set up as follows: trypsin as the digest reagent at a maximum of 1 missed cleavage; carbamidomethylation of C (cysteine) as fixed modification, oxidation of M (methoinine), and phosphorylation of STY (serine, threonine, tyrosine) as variable modifications; and 600 kDa as the maximum protein molecular weight. We set up the peptide and fragment tolerances as default and the false discovery rate at less than 4%. For ion matching requirements, we set up fragments/peptides at 1 or more, fragments/protein at 3 or more, and peptides/protein at 1 or more.

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Chapter 16
Selective Reaction Monitoring Mass Spectrometry for Quantitation of Glycolytic Enzymes in Postmortem Brain Samples

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16.1 Introduction

Patients with psychiatric disorders such as schizophrenia also suffer from a higher incidence of metabolic diseases compared to the general population [1]. This can occur at first onset as well as in more chronic patients receiving antipsychotic medications [2]. Molecular profiling studies have shown that this effect is reflected in the peripheral circulation by changes in the levels of hormones involved in metabolic regulation, such as insulin, prolactin, and cortisol [3–6]. In a similar manner, changes in proteins associated with glycolysis and energy metabolism pathways have been widely reported to occur in the central nervous system [7–10]. Like most cells of the body, neurons, astrocytes, and oligodendrocytes rely on tight metabolic regulation to drive normal synaptic function [9, 11]. Recent studies in cellular models of schizophrenia have even shown that such changes may be more prevalent in

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oligodendrocytes, which have an important role in myelination of neurons and, therefore, connectivity between different brain regions [12]. In this case, it is not surprising that perturbed metabolism can lead to the changes in mood, behavior, and cognition, which are the traditionally described symptoms of most psychiatric conditions.

Mass spectrometry-based proteomic methods have evolved rapidly over the last two decades to the point where identification and quantification of protein biomarker candidates can be achieved with good precision and high sensitivity. However, proteomes are dynamic and can differ significantly in composition and expression of entire molecular pathways under different physiological conditions [13]. Because of this, targeted proteomic approaches have now focused on precise quantitation of specific sets of proteins in multiple samples or time points. As one of the leading techniques for this, selective reaction monitoring mass spectrometry (SRM-MS) has been applied recently with increasing frequency in studies of many medical conditions for accurate quantitation of peptides and proteins [14]. SRM-MS is based on selecting and quantifying specific sets of peptides derived from a target list of proteins in a given study area, using liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis [15]. The method leverages the ion filtering capabilities of quadrupole-based MS instruments for selection and detection of precursor peptides and the corresponding fragment ions produced during the collision-induced dissociation stage of the process. Because of the rapid tandem quadrupole duty cycle and online chromatographic separation of peptides, multiple precursor-fragment transitions can be monitored simultaneously.

Here, a detailed protocol is presented for peptide selection and optimization in a quantitative SRM-MS analysis of three glycolytic enzymes in extracts from post-mortem brain tissue. The main emphasis is on creating potential biomarker tools for future studies in psychiatric disorders but the overall method can be applied in the investigation of other brain-related diseases marked by perturbations of metabolism in neuronal cells.

16.2 Materials

16.2.1 Sample Preparation Components for Targeted Proteomic Analysis

1. Extraction buffer: 7 M urea, 2 M thiourea, 4% 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), 70 mM dithiothreitol (DTT), 2% protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany)
2. Bradford protein quantitation kit (Bio-Rad, Hercules, CA, USA)
3. Denaturation buffer: 8 M urea, 0.15 M Tris-HCl (pH 8.5)

4. Trypsin solution: 100 ug/mL sequencing grade modified trypsin (Promega, Madison, WI, USA) in 100 mM ammonium bicarbonate (pH 8.0)
5. Reducing solution: 1% DTT in 0.15 M Tris-HCl (pH 8.5) (*see Note 1*)
6. Alkylation solution: 2% iodoacetamide (IAA) in 0.15 M Tris-HCl (pH 8.5) (*see Note 1*)
7. Dilution buffer: 0.15 mM Tris-HCl (pH 8.5)
8. Solid phase purification: OASIS HLB solid-phase extraction columns (Waters Corporation, Milford, MA, USA)
9. Equilibration solution: 5% acetonitrile, 0.1% formic acid
10. Elution solution: 50% acetonitrile, 0.1% formic acid
11. Reconstitution buffer: 3% acetonitrile, 0.1% formic acid

16.2.2 Solvents for Reverse-Phase Chromatography

1. Aqueous solvent (A): 94.9% MilliQ water, 5% acetonitrile, 0.1% formic acid
2. Organic solvent(B): 99.9% acetonitrile/ 0.1% formic acid

16.2.3 Equipment

1. SpeedVac concentrator
2. Tandem quadrupole Xevo-TQs mass spectrometer coupled to Class I Ultra Performance Chromatographic System (UPLC) (Waters Corporation)
3. ACQUITY UPLC HSS C18 Column, 1.8 µm particle size, 1 mm I.D. X 150 mm (Waters Corporation)

16.2.4 Software

1. Skyline® (v3.5) (https://skyline.ms/project/home/begin.view?)

16.3 Methods

16.3.1 Brain Tissue Preparation

1. Homogenize 20 mg brain tissue sample (*see Note 2*) with a sample grinding kit (GE Healthcare Life Sciences, Little Chalfont, UK) in 250 µL fresh extraction buffer.

2. Centrifuge the solution at 14,000 x g for 15 min at 4 °C.
3. Collect supernatant.

16.3.2 Sample Preparation for SRM Proteomic Analysis

1. Quantitate total proteins by Bradford protein assay and use 50 μg aliquots for SRM analysis (*see Note 3*).
2. Reduce protein cysteine residues by addition of 5 uL reduction solution and maintain at 37 °C for 1 h.
3. Alkylate protein sulfhydryl residues by addition of 10 uL alkylating solution and maintain at room temperature for 1 h.
4. Dilute samples so urea is approximately 0.6 M (*see Note 4*).
5. Perform in-solution trypsin digestion by addition of 10 uL trypsin solution (1:50 enzyme/protein ratio) and incubate for 2 h at 37 °C.
6. Add an additional 5 uL trypsin solution and incubate for a further 16 h or overnight at 37 °C.
7. Desalt samples using solid-phase extraction in OASIS columns as follows:
   (i) Condition column with 1 mL of acetonitrile.
   (ii) Equilibrate column with 1.6 mL equilibration solution.
   (iii) Apply sample through the column.
   (iv) Wash column with 1.6 mL of equilibration solution elute peptides with 1.2 mL of elution solution.
   (v) Dry eluted peptides in a SpeedVac concentrator (*see Note 5*).

16.3.3 SRM Method Development and Data Analysis

1. Select protein targets for SRM analysis and identify proteotypic peptides using the Skyline software (Table 16.1) (*see Notes 6 and 7*).
2. Perform a collision energy and chromatographic separation standardization for the selected set of proteotypic peptides (Table 16.1) (*see Note 8*).
3. Suspend samples in reconstitution buffer.
4. Vortex samples thoroughly, centrifuge 15 min at 12,000 x g, and transfer the supernatants (45 μL) to a mass spectrometry-compatible injection vial (*see Note 9*).
5. Inject 10 μL sample in triplicate to facilitate statistical analysis (Fig. 16.1) (*see Note 10*).
6. Analyze the data using the MS vendor or Skyline software (Fig. 16.1) (*see Note 11*).

Table 16.1 Proteotypic peptides and analytical parameters utilized to analyze brain tissue samples

Protein accession	Protein gene	Peptide sequence	Precursor M/z	Precursor charge	Collision energy	Transit 1	Transit 2	Transit 3
P09104	ENO2	FGANAILGVSLAVCK	760.4187	2	27.1	947.291	834.0	776.9
P09104	ENO2	SGETEDTFIADLVVGLCTGQIK	1177.8322	2	42.5	1374.6	1303.5	1188.4
P04406	GAPDH	GALQNIIPASTGAAK	706.3988	2	25.1	1043.2	815.9	702.8
P04406	GAPDH	VPTANVSVVDLTCR	765.9009	2	27.3	1049.2	950.1	863.0
P60174	TPI1	HVFGESDELIGQK	486.9123	3	16.2	802. 9	687.8	558.7
P60174	TPI1	IIYGGSVTGATCK	663.8397	2	23.5	938.0	880.9	823.9
P00338	LDHA	VTLTSEEEAR	567.7855	2	19.9	934.9	821.8	720.7

One peptide per protein was selected for monitoring. The selection of three SRM transitions per proteotypic peptide improved method reliability

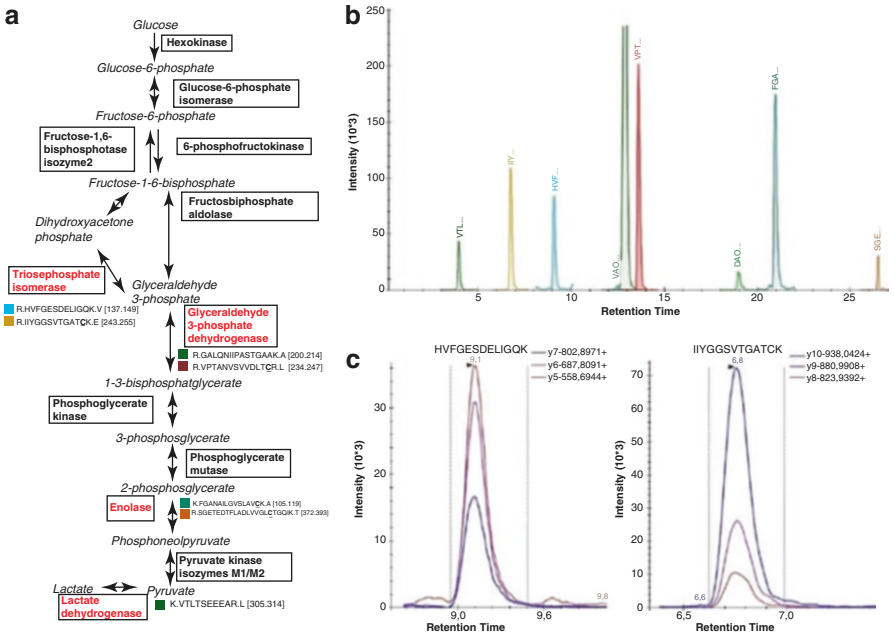

Fig. 16.1 SRM analysis of brain tissue samples for glycolytic enzymes. (**a**) Relevant proteins selected from previous data analysis and respective representative peptides to monitor glycolysis pathway. (**b**) Chromatogram showing eluted peptides monitored by MS. (**c**) Example of signal intensity from monitored transitions of peptides selected for triosephosphate isomerase

16.4 Notes

1. Always prepare fresh reducing and alkylation solutions to guarantee efficient reactions with cysteine residues. Do not store these solutions.
2. Remove brain tissue from the freezer −80 °C just before sectioning and keep the sample on ice throughout.
3. Quantitation of total protein is important to allow efficient reactions of reduction, alkylation, and enzymatic protein digestion.
4. Dilution of sample solution guarantees good trypsin activity, which is close to 100% in urea solutions at concentration less than 1 M, according the supplier.
5. Samples can be stored at −80 °C until ready for SRM analysis.
6. In order to develop a SRM method for target proteins, we use the following workflow:

   (i) Perform a virtual tryptic protein digestion, using, for example, the PeptideMass tool available in UNIPROT (www.uniprot.org).
   (ii) Select tryptic peptides containing from 10 to 20 amino acids, since these are easier to generate by solid-phase peptide synthesis.
   (iii) If possible, select peptides containing proline residues, which generate intense fragment peaks.

(iv) Avoid peptides containing methionine or N-terminal glutamine, which suffer from in-source modification during MS ionization.
(v) Check previous detection and predicted suitability for selected peptides using the SRMatlas databank (www.srmatlas.org) [16].

7. Although the synthetic peptides representing the target proteins are not obligatory, they are useful for method development and refinement. Peptides can be quickly synthesized in house using standard solid-phase Fmoc chemistry. Preselection of short peptides (< 20 amino acids) facilitates synthesis and provides a good yield. If an absolute quantitation is of interest, purify peptides by reverse-phase chromatography and use an accurate method to quantitate them (e.g., amino acid analysis). Also, the use of isotopically labeled peptides (e.g., ^{13}C-lysine) as internal standards allows more precise quantitation.
8. The study of ideal collision energy for SRM studies is essential for good sensitivity. Also, it is important to perform these studies in the local instrument, since several tuning parameters can affect fragmentation efficiency. The optimization of a chromatographic gradient with the specific set of peptides is also interesting to guarantee quick runs and good reproducibility. For these steps, consider using the software Skyline [17], since it is designed to facilitate method development as well as efficient data analysis across multiple platforms.
9. Chromatography in micro- or nanoscale is susceptible to microparticles originating from insoluble material or solid-phase extraction column leaking. This simple centrifugation step improves the column lifetime.
10. The injection of 10 μL corresponds to 10 μg of protein digest. This is kept below the loading capacity of the UPLC HSS C18 Column (1 mm I.D. X 150 mm long) in order to obtain the best chromatographic separations.
11. The multi-platform Skyline software can integrate, calibrate, and quantitate samples analyzed by SRM. This streamlines the development and application of the method.

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Chapter 17
A Selected Reaction Monitoring Mass Spectrometry Protocol for Validation of Proteomic Biomarker Candidates in Studies of Psychiatric Disorders

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17.1 Introduction

Hundreds of scientific publications claim to have discovered serum or plasma protein biomarkers for human disorders which could be used as a measure of prognosis, diagnosis, or drug response. But only a few dozen of these are being used clinically or have been approved for clinical trials. Among the protein biomarkers approved by regulatory agencies as FDA are carcinoembryonic antigen (CAE), thyroglobulin (Tg), and prostate-specific antigen (PSA), which monitor the progression of colon, thyroid, and breast cancer, respectively [1]. Moreover, troponin T, troponin I, and creatine kinase (CK) have been used to diagnose acute myocardial infarction, myocardial injury, and myocardial necrosis [2, 3], and brain natriuretic peptide (BNP) has been employed as an indicator of congestive heart failure and acute coronary syndrome diagnosis [4].

The development of a clinically applicable assay containing biomarkers discovered and validated in scientific laboratories is a long and complex process that includes basically four stages. The first stage is the discovery of protein candidates, generally using large-scale screening methods such as shotgun mass spectrometry

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and protein arrays in a limited number of samples. The next stage is an initial validation of the protein candidates using a different cohort of samples and utilizing targeted methods such as those based on use of antibodies (e.g., Western blotting, immunoassay) or mass spectrometry (e.g., selective/multiple monitoring mass spectrometry [SRM/MRM-MS]). After validation and optimization of the chosen method, a third stage should be launched which would take biomarker development to the next level. In this step, a well-established and optimized test – normally a targeted method – is employed for evaluation of biomarker candidates in a significant number of samples, ideally collected in multiple centers. Finally, the approval and adoption of biomarkers to the regulatory authorities and clinical activities should occur prior to clinical application [5].

For the targeted validation of protein candidates identified in serum or plasma, SRM-MS is being used increasingly for precise and fast protein quantitation [6]. This technique may use a high-performance liquid chromatography (HPLC) system coupled to mass spectrometer for peptide separation. In general, separated peptides are applied to a triple-quadrupole mass spectrometer (TQ-MS) and are ionized by eletrospray. Next, precursor peptide ions based on sequences within the targeted proteins are filtered, followed by fragmentation into product ions. Each one of these fragments will be filtered again and read by a detector, resulting in an MS peak associated with a specific chromatographic retention time and intensity value [7]. SRM confers high precision, accuracy, sensitivity, and reproducibility to analysis and could be used to confirmation and validation of potential biomarkers [8].

Here we describe a protocol for selection and optimization of peptides for use in studies involving validation of biomarkers arising from proteomic investigations of serum or plasma from patients with psychiatric disorders. The method is also applicable to the study of other disorders involving blood sampling.

17.2 Materials

1. Blood serum or plasma (*see Note 1*)
2. List of protein biomarker candidates from a proteomic study
3. Human proteome FASTA file from UniProt database
4. Skyline® version 3.5 software
5. NanoAcquity UPLC (Waters Corporation)
6. Solvent A: 0.1% formic acid in water (*see Note 2*)
7. Solvent B: 0.1% formic acid in acetonitrile (v/v) (*see Note 2*)
8. Xevo TQD® mass spectrometer (Waters Corporation)
9. Masslynx Mass Spectrometry Software®
10. NanoACQUITY Column BEH C18 (130 Å, 1.7 μm, 100 μm × 100 mm) (Waters Corporation)

17.3 Methods

17.3.1 SRM Method

1. Open Skyline and select "Blank Document" to create a SRM method.
2. Open "Peptide Settings" (under the menu "Settings") and go through all tabs to adjust the settings to the current project.
3. Go to the "Digestion" tab, select trypsin as the enzyme, zero as the number of max missed cleavages, and insert human proteome as background proteome.
4. In "Filter" tab, set the following parameters: peptides containing more than 8 and less than 25 amino acids; exclude peptides containing the N-terminal 25 amino acids of a given protein (*see Note 3*); exclude peptides containing methionine or tryptophan (*see Notes 4 and 5*); and remove peptides which are not unique (*see Note 6*).
5. In the "Modification" tab, set 1 as maximum neutral loss and 3 as number of variable modifications.
6. Click "OK" to confirm all peptide settings (*see Note 6*).
7. Insert the accession number of proteins of interest in blank document (*see Note 7*).
8. Open the "Transitions Settings" (also under the menu "Settings") and navigate over the tabs providing the settings to the current project.
9. In Prediction tab, select monoisotopic mass for precursor mass and product ion mass.
10. Use Waters Xevo for collision energy and none for declustering potential also for optimization library.
11. In filter tab, use the following criteria: precursor ions should contain two or three charges (*see Note 8*); product ions should contain one or two charges (*see Note 9*); enter "y" for ion types; for product ions, select "***From:*** m/z > precursor ***To:*** "4 ions"; leave "N-terminal to Proline" and "Auto-select all matching transitions" checked.
12. In the "Instrument" tab, set the mass/charge (m/z) range of precursor ions from 50 to 1500 Th (Thompson units) and 0.055 Th for mass tolerance.
13. Use 0.5 m/z as ion match tolerance and pick three product ions with the option "If a library spectrum is available" checked, in the "Library" tab (*see Note 10*).
14. Click "OK" to confirm all transitions settings.
15. Export the Skyline method to Masslynx Mass Spectrometry Software and set a run using 15 points per peak.
16. Select "Auto Dwell" and ensure it is higher than 0.025 s (*see Note 11*).
17. Establish a chromatographic method using a gradient and conditions from Table 17.1.
18. Establish nanoflow conditions and control as shown in Table 17.2 (*see Note 12*).

Table 17.1 Gradient of chromatographic method

Time	% Solvent B	Flow rate (μL/min)	Max pressure (psi)	Curve
0.00	3.0	0.300	10,000	6
1.66	3.0	0.300	10,000	6
29.00	40.0	0.300	10,000	6
30.65	85.0	0.300	10,000	6
33.97	85.0	0.300	10,000	6
35.62	3.0	0.300	10,000	6

Table 17.2 Xevo TQD instrument settings

Parameter	Value
Source voltages	
Capillary voltage	2.80 kV
Cone voltage	30 V
Source temperatures	
Source temp	70 °C
Source gas flow	
Purge	150 L/Hr
Cone	50 L/hr
Nanoflow	0.50 Bar
Analyzer	
LM resolution 1	10.8
HM resolution 1	14.8
Ion energy 1	0.1
LM resolution 2	9.4
HM resolution 2	14.9
Ion energy 2	1.3

19. Inject 0.5 μg of proteins into the NanoAcquity UPLC system coupled to the Xevo TQD.
20. Optimize the method establishing three best peptides for each protein and three transitions for each peptide (*see Note 13*), the correct collision energy, adding or decreasing voltage in the original collision energy based on signal intensities, and the retention time of each peptide allowing analysis of multiple proteins in the same run maintaining higher points per peak and dwell time.

17.3.2 Data Analysis

1. Import the results from Masslynx to Skyline in the method previously created.
2. Assign and integrate the signals of transitions in the same retention time range (Fig. 17.1a).

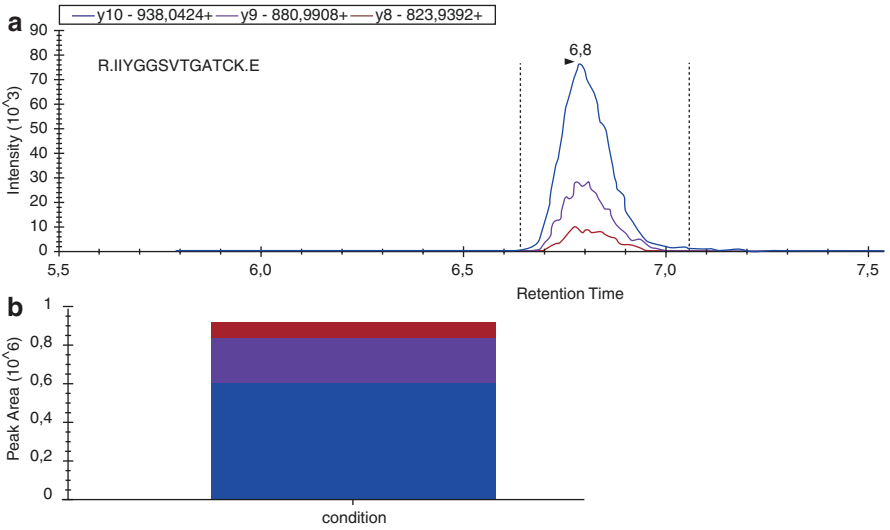

Fig. 17.1 Representative results of SRM assay. (**a**) Three transitions of peptide (R. IIYGGSVTGATCK.E) were selected in the same retention time range. (**b**) Peak areas used for quantification of such peptide

3. In absolute quantification, heavy and light peptides must be eluted in the same retention time range (*see Note 14*).
4. Choose three transitions of each peptide for quantification (Fig. 17.1b) (*see Note 15*).
5. Export the results as ".csv" files for further data analyses using other bioinformatic tools if desired.

17.4 Notes

1. Method development should involve the use of non-important clinical samples to avoid wastage.
2. Refresh the solutions every 30 days or where required.
3. This may be unavoidable for small proteins.
4. The side chains of methionine and tryptophan are prone to oxidation.
5. When a protein has a large tryptic peptide set, those containing cysteine (C), arginine-proline (RP), and lysine-proline (KP) can also be excluded because they are more prone to experimentally induced chemical modification. Furthermore, peptides containing glutamine (Q) or asparagine (N) may be unstable and convert to glutamate (E) or aspartate (D).
6. To verify the unique peptides, select the protein of interest and select the option "unique peptides" in edit table. Skyline compares the tryptic peptides across the entire proteome database and shows which peptides are unique or common.

7. Tryptic peptides, precursors, and transitions should appear on the left side of the file. In all SRM-MS studies, it is important that the peptides selected can identify the targeted protein uniquely (i.e., the sequence should only occur within one protein). In addition, peptides should be selected that can distinguish different splice forms or single nucleotide polymorphisms of the same protein. A search with the basic local alignment search tool (BLAST) can be used to confirm uniqueness in both situations.
8. Precursor ions with two or more changes are favorable as these confer a more measureable mass/charge (m/z) range. Also, for each histidine (H) present in the middle of peptide, one charge is added to precursor ion.
9. Selecting two or more y-fragment ions from multiply-charged precursor ions is favored to produce at least one transition with good performance.
10. Multiple transitions help to increase the specificity of the method.
11. If the value is lower, some peptides and/or transitions must be excluded or transferred to other methods.
12. These parameters may vary according to mass spectrometer specifications.
13. The best peptide or transitions are those with the most intense signals.
14. This only applies for quantitative comparisons between heavy and light isotope-labeled peptides.
15. Exclude the transitions out of retention time range and also those present interferences over the chromatography.

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Chapter 18
Application of iTRAQ Shotgun Proteomics for Measurement of Brain Proteins in Studies of Psychiatric Disorders

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18.1 Introduction

Psychiatric disorders are a leading cause of medical disability throughout the world, affecting one out of every three people at some point in their lifetime [1]. Despite the fact that years of intensive research have led to a better understanding of the biological pathways that are perturbed in people with these disorders, none of this information has led to newer and better approaches for patient management, particularly in the area of improved diagnosis and treatment. Thus far, this has been hindered by a poor understanding of the molecular pathways affected in these diseases and may also be a direct cause of disease complexity and overlap of symptoms across the supposedly different disorders [2, 3]. The current state-of-the-art diagnosis is based on evaluation of symptoms during clinical interviews, but this is only subjective and can vary depending on the experience, training, and methodology of the attending clinician and the validity of information given by the patient. Taken together, this can make effective disease management a difficult prospect.

The availability of biomarker tests based on the pathologies underlying psychiatric disorders would help to overcome some of these difficulties. Using biomarker tests targeting the affected pathways would give empirical readings, which could be used in combination with the standard methods for improved accuracy and timeliness of diagnosis. In turn, this would allow the access of patients to earlier and more efficient treatment options. In the initial discovery stages, it is important that brain tissue is analyzed in order to identify potential biomarkers, considering that this is likely to be the direct cause of symptoms due to effects on neuronal pathways [4].

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Furthermore, proteomic-based biomarkers are likely to be the most useful for development of diagnostic and prognostic tests considering the dynamic nature of proteins in general in response to changing environmental stimuli [5].

Mass spectrometry (MS) profiling is one of the key proteomic techniques in the study of disease biomarkers [6], and the incorporation of isobaric labeling methods in MS approaches, such as isobaric tags for relative and absolute quantitation (iTRAQ), has allowed measurement of multiple proteomes simultaneously [7]. Although the iTRAQ labels have an indistinguishable mass in MS mode, reporter fragment ions of different masses are released in MS/MS mode in the collision chamber. This enables peptides from different samples to be differentiated and quantitated based on the intensity of their specific reporter ions [8]. The iTRAQ approach has now been used for analysis of up to four [7] or eight samples [9]. In comparison to label-free MS techniques, the multiplexing potential of the iTRAQ approach allows a simultaneous analysis of different biological samples, decreases the inherent variations in chromatographic analysis between the samples, and requires fewer technical replicates to obtain a good accuracy in the measurement. In the iTRAQ 4-plex approach, the reagent contains an N-methylpiperazine reporter, carbonyl balance, and NHS-ester reactive groups. Each reagent has the same mass (145 Da) achieved by a combination of ^{13}C, ^{15}N, and ^{18}O in the reporter (114–117 Da) and balance groups (31–28 Da). The labeled peptides have identical retention times during chromatographic separation, and the peptides appear as a single peak with the same m/z in MS spectra. However, fragmentation of the precursor ion produces MS/MS spectra with reporter ion peaks at low mass region (114, 115, 116, and 117 Da) and peptide fragmentation ion peaks of higher masses. The intensity of the reporter ion peaks directly reflects the abundance of the peptide in each sample (Fig. 18.1).

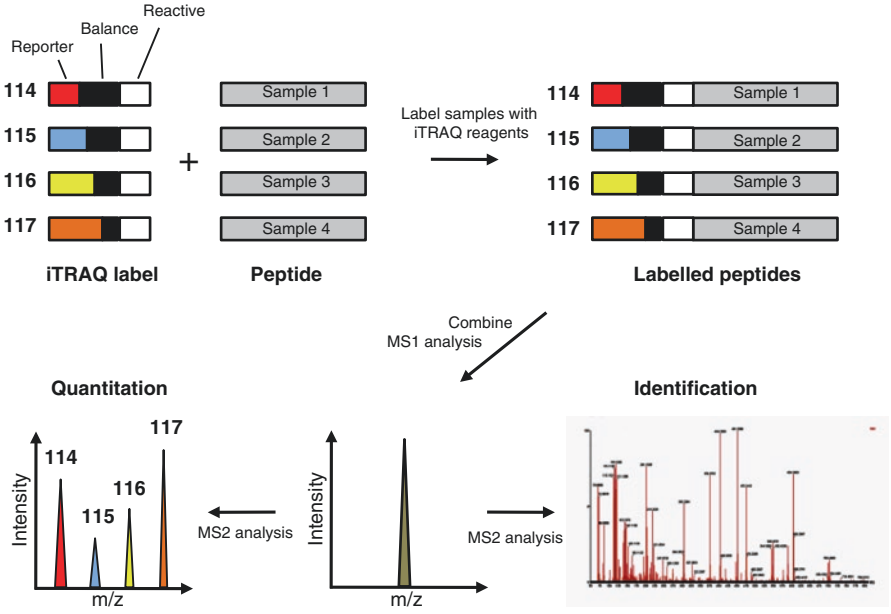

Fig. 18.1 Schematic diagram of the iTRAQ4-plex mass spectrometry approach

Here, we describe a detailed sample preparation and iTRAQ 4-plex labeling protocol for relative quantification of postmortem brain samples from patients with psychiatric disorders such as schizophrenia compared to those from controls. In addition, we outline a strategy for peptide fractionation after the iTRAQ-labeling procedure.

18.2 Materials (See Note 1)

18.2.1 Protein Extraction of Brain Tissues (See Note 2)

Procedure 1

1. RapiGest® (Waters Corporation; Milford, MA, USA)
2. 1 M triethylammonium bicarbonate (TEAB; Sigma-Aldrich; Sao Paulo, Brazil)

Procedure 2

1. Extraction solution: 7 M urea/2 M thiourea and 2% sodium deoxycholate (Sigma-Aldrich)
2. Cold acetone

18.2.2 Enzymatic Digestion

1. Qubit® 2.0 fluorometric assay kit for protein analysis (Invitrogen; Waltham, MA, USA)
2. Reducing solution: 100 mM dithiothreitol (DTT) or 50 mM tris(2-carboxyethyl) phosphine (TCEP) (*see Notes 3 and 4*)
3. Alkylating solution: 400 mM iodoacetamide (IAA) (*see Note 5*)
4. Sequencing grade modified trypsin (Promega; Madison, WI, USA)
5. Acetic acid
6. 10% trifluoroacetic acid (TFA)

18.2.3 iTRAQ Peptide Labeling

1. 0.1% TFA
2. 0.1% TFA, 50% acetonitrile (ACN)
3. 0.1% TFA, 70% ACN
4. iTRAQ reagent 4-plex kit (Applied Biosystems Sciex; Foster City, CA, USA)
5. C18 macro-spin column (Harvard Apparatus; Holliston, MA, USA) (*see Note 6*)
6. Strong cation exchange (SCX) macro-spin column (Harvard Apparatus) (*see Note 7*)
7. Buffer A: 5 mM KH_2PO_4, 25% ACN (pH 3)
8. Buffer B: 1 M KCl stock solution

9. LC-20AT high-performance liquid chromatography (HPLC) instrument for hydrophilic interaction chromatography (HILIC) (Shimadzu Corporation; Kyoto, Japan) (*see Note 8*)
10. 3 μm x 5 cm x 2 mm TSKgel® amide-80 column (Sigma-Aldrich) for use with the LC-20AT instrument (*see Note 9*)
11. Solvent A (HILIC-A): 90% ACN, 0.1% TFA
12. Solvent B (HILIC-B): 0.1% TFA

18.2.4 Labeled Peptide Analysis by Nano LC-MS/MS

1. Trap column: 2 cm length, 200 μm inner diameter
2. Analytical capillary column: 18 cm length, 100 μm inner diameter, 5 μm resin ReproSil-Pur C18 (Dr. Maisch GmbH; Ammerbuch, Germany)
3. Phase A: 0.1% formic acid, 5% ACN
4. Phase B: 0.1% formic acid, 95% CAN
5. Nano LC EASY II (Thermo Fisher Scientific; Waltham, MA, USA)
6. LTQ Orbitrap Velos mass spectrometer (Thermo Fisher Scientific)

18.2.5 Data Analysis

1. Data inspection: Xcalibur 2.1 software (Thermo Fisher Scientific)
2. Database searches: Proteome Discoverer 2.1 software (Thermo Fisher Scientific) with the SEQUEST algorithm
3. Databases: UniProt (www.uniprot.org/), NCBI (www.ncbi.nlm.nih.gov/), and neXtProt (www.nextprot.org/)

18.3 Methods

18.3.1 Protein Extraction in Brain Tissues

1. Pulverize and macerate the tissues in liquid nitrogen [10].
3. Add 0.1% RapiGest in 50 mM TEAB (*see Note 10*) or extraction solution.
2. Vortex the samples and centrifuge 30 min at 20,000 × g at 4 °C.
3. Transfer the supernatant to another tube and take one aliquot for protein quantification.
4. When using the extraction solution, precipitate the proteins with 4 volumes of cold acetone overnight and centrifuge at 20,000 × g at 4 °C for 30 min.
5. Wash the pellet three times with cold acetone followed by centrifugation at 20,000 × g at 4 °C for 10 min.

6. Remove the acetone excess and dry the pellet.
7. Solubilize the pellet in 7 M urea and 2 M thiourea solution.

18.3.2 Protein Digestion with Trypsin

1. Quantitate proteins using the Qubit 2.0 fluorometric assay kit according to the manufacturer's instructions.
2. Carry out reduction of disulfide bonds in proteins by incubating samples with DTT or TCEP solution at a final concentration of 10 mM for 1 h at 30 °C.
3. Alkylate thiol groups in proteins by incubating samples with IAA solution at a final concentration of 40 mM for 30 min at room temperature in the dark.
4. Add trypsin at a 1:50 enzyme/protein ratio and incubate 12–18 h at 37 °C (*see Note 11*).
5. Stop the reaction by adding 10% TFA to give a final concentration of 0.1% (*see Note 12*).

18.3.3 iTRAQ Peptide Labeling [11, 12]

1. For peptide cleaning, incubate C18 spin columns with 500 μL 100% ACN for 15 min and centrifuge at 2000 × g for 1 min.
2. Add the same amount of ACN and repeat the centrifugation step.
3. Equilibrate columns with 150 μL 0.1% TFA and centrifuge at 2000 × g as above.
4. Repeat this step three times.
5. Add 75–150 μL sample and centrifuge at 2000 × g as above.
6. Wash the columns using 0.1%TFA and centrifuge at 2000 × g as above.
7. Repeat the wash/centrifugation cycle three times.
8. Elute the peptides in two successive steps into the same collection tube by adding 0.1% TFA/50% ACN and 0.1% TFA/70% ACN followed by centrifugation at 2000 × g as above.
9. Dry the peptides by vacuum centrifugation.
10. Suspend peptides in 30 μL 20 mM TEAB (pH 8.5) (*see Note 13*) and quantify using the Qubit 2.0 fluorometric assay to normalize peptide amounts in each condition (20–100 μg).
11. Briefly centrifuge the iTRAQ reagent solution vial at room temperature to collect the content in the bottom of the tube and add 70 μL ethanol to each vial.
12. Vortex the vials and centrifuge briefly as above.
13. Transfer the contents of each vial to the specific sample tube, vortex, and centrifuge again (*see Note 14*).
14. Incubate samples at room temperature for 1 h.

15. Stop the reaction by adding formic acid at a final concentration of 1% and vortex and centrifuge as above (*see Note 15*).
16. Combine the contents of all samples labeled with different iTRAQ tags into one tube, vortex, and centrifuge.
17. Dry the contents in a vacuum centrifuge but stop before complete dryness is reached (*see Note 16*).

18.3.4 iTRAQ-Labeled Peptide Fractionation

1. SCX fractionation: suspend the semidry pellets in 100 μL of 5 mM KH_2PO_4, 25% ACN solution to give an approximate 1 μg peptides/μL concentration and vortex.
2. Incubate the SCX spin column with 500 μL of the same solution for 15 min at room temperature.
3. Centrifuge at 2000 × g until all solution has passed through the column and repeat this step.
4. Add the sample to the spin column, centrifuge at 2000 × g, and collect the column flow through.
5. Carry out four sequential elution steps using 150 μL of the 5 mM KH_2PO_4/25% ACN solution, containing 75, 150, 250, and 500 mMKCl, followed by centrifugation each time at 2000 × g, and collect the eluates in separate tubes.
6. Desalt the samples using the peptide cleaning step above (3.4.1–3.4.9).
7. Suspend the samples in 0.1% formic acid and quantify as above.
8. For HILIC fractionation [13, 14], suspend the samples in 100 μL of HILIC-A solution at approximately 1 μg/μL, vortex, centrifuge briefly, and collect the supernatant.
9. Load samples at a flow rate of 0.2 mL/min into the TSKgel Amide-80 column on the LC-20AT HPLC system.
10. Fractionate peptides by applying 100% HILIC-A (0% HILAC-B) for 10 min, 12% HILIC-B for 2 min, 20% HILIC-B for 30 min, 30% HILIC-B for 30 min, 100% HILIC-B for 5 min, and return into 100% HILIC-A for 5 min.

18.3.5 Labeled Peptide Analysis by Nano LC-MS/MS

1. Load 1 μg labeled peptides onto the trap and capillary columns on the nano LC system coupled online to a LTQ Orbitrap Velos mass spectrometer.
2. For peptide elution, apply a gradient from 100% phase A to 35% phase B over 120 min at a flow rate of 200 nL/min.
3. After each run, wash the column with 90% phase B and re-equilibrate with phase A.

4. Acquire spectra in positive mode applying a data-dependent automatic survey MS scan and MS/MS (*see Note 17*).
5. Set the resolution of the Orbitrap mass analyzer at 60,000 at m/z 400, automatic gain control target at 1×10^6, and maximum ion injection at 500 ms.
6. Acquire MS/MS spectra at a resolution of 7500 at 400 m/z, a signal threshold of 30,000, normalized collision energy of 40, and dynamic exclusion enabled for 30 s with a repeat count of 1.
7. Place an Eppendorf tube covered with 5% ammonia water solution under the nano ESI needle (*see Note 18*).

18.3.6 Data Analysis

1. Inspect raw data using the Xcalibur software.
2. Perform database searches against target and decoy (reverse) databases from UniProt, NCBI, and neXtProt using the following search parameters: MS accuracy = 10 ppm, MS/MS accuracy = 0.1 Da, trypsin digestion with two missed cleavages allowed, fixed carbamidomethyl modification of cysteine, and variable modification of oxidized methionine.
3. For identification of iTRAQ-labeled peptides, also include the iTRAQ 4-plex monoisotopic mass = 144.102 and variable modification for N-terminus, lysine, and tyrosine.
4. Accept false discovery rates of less than 1% and peptide rank = 1.

18.4 Notes

1. Reagents should be of analytical grade, solvents HPLC or LC-MS grade and solutions should be prepared with ultrapure water (18 MΩ-cm at 25 °C). LC-MS solutions should be made with LC-MS grade water.
2. It is advisable to use protease inhibitors in this step to prevent degradation of proteins caused by proteases in the sample. In addition, for phosphoproteomics, it is necessary to use phosphatase inhibitors to prevent dephosphorylation during preparation and handling of samples.
3. It is recommended to use a fresh DTT stock solution.
4. TCEP has the advantage of being a more powerful reducing agent than DTT, by providing an irreversible reaction. In addition, it is more hydrophilic, active in alkaline and acidic conditions, and more resistant to air oxidation. Also, it does not reduce metals and is significantly more stable than DTT in the absence of a metal chelator.
5. It is necessary to prepare the IAA solution immediately before use and keep it protected from light because it is unstable and light sensitive.

6. The C18 macro-spin column has a binding capacity of 30–300 μg of sample, accepting a sample volume of 70–150 μL. Review the manufacturer's specifications before use.
7. The SCX macro-spin column has an ion capacity of 0.18–0.25 mmol (Cl)/mL, has a binding capacity of 30–300 μg of protein sample, and accepts a sample volume of 70–150 μL. Review the manufacturer's specifications before use.
8. It is possible to use a HPLC instrument with an equivalent configuration.
9. HILIC is recommended to remove excess iTRAQ reagent from iTRAQ-labeled peptides to increase compatibility with mass spectrometry analysis. This eliminates an additional step of sample cleaning.
10. We recommend addition of 200 μL of 0.1% RapiGest per 100 mg of tissue.
11. Samples in 7 M urea, 2 M thiourea must be diluted to final concentrations lower than 1 M urea using 100 mM TEAB and heating must be avoided. Check the pH to ensure that it is close to 8.
12. For RapiGest samples, acidify the samples with TFA to a final concentration of 1% to stop the reaction and incubate 40 minutes at room temperature. Centrifuge for 30 min at 20,000 × g to remove the insoluble material.
13. Before peptide labeling, ensure that the pH is close to 8.5.
14. It is advisable to use commercial peptides (e.g., Glu-1-fibrinopeptide B) at a known concentration at the time of labeling to serve as an internal control and to facilitate data normalization.
15. It is advisable to analyze a peptide sample aliquot by mass spectrometry before making the final mix of all iTRAQ labels in order to confirm the presence of labeled peptides with the appropriate m/z peaks for each reporter ion. If the labeling process was not successful, repeat the labeling procedure.
16. The peptide pellets are easier to resuspend if they are not completely dry.
17. The data-dependent acquisition method consisted of the selection and fragmentation of the tenth most intense precursor ions by high-energy collision dissociation. The MS^2 spectra range must include the m/z of the reporter ions.
18. The presence of 5% ammonia during analysis avoids the supercharge effect of the iTRAQ 4-plex tag [15].

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Chapter 19
Co-immunoprecipitation for Deciphering Protein Interactomes

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19.1 Introduction

After the coining of the term "genome" back in 1920 [1], several other related terms have appeared, continuing on the theme of a library of cellular data. To name a few: transcriptome, a snapshot of expressed mRNA; proteome, a list of proteins a specific cell expresses under given conditions; and interactome, a visual map of the interacting factors with a biological molecule. Once digitally transcribed, these databases can be indispensable in the field of biological research.

While the amount of information that a genome of a specific organism can provide is massive, there are certain limitations, one being that some sequences of a genome can change during the lifetime of a cell due to environmental factors, diseases, and response to stimuli, although these somatic mutations affect individual cells, not the full genome [2]. This makes a genome an unsuitable tool for certain realms of disease research, as it cannot represent the overall changes that a multicellular organism experiences in the development of a disease.

In contrast, a proteome is more fluid and reactive to stimuli over a wider scale, making it a perfect study tool for novel biomarker and drug target identification, especially with the advent of new, quantitative techniques [3]. Obtaining a proteome is a relatively straightforward process, starting with the collection of cells. This can

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be from a biopsy, autopsy, cultured cell, or other tissue source. The cells must be lysed to solubilize the maximum number of proteins for proteomic analysis. An optional step to enrich lesser-expressed proteins is to deplete the more abundant proteins found in a specific cell type through affinity chromatography [4]. However, this reduces the overall protein yield and can also lead to other artifacts so the benefits must be weighed against the negatives. The next step is to separate the proteins via a method such as sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and run the resulting protein bands through a mass spectrometry machine for identification and quantitation. By comparing proteomes between individuals, or even the same individual over time, it is possible to glean information regarding relationships between specific proteins and the development and/or progression of a disease. Sometimes this leads to a breakthrough, identifying a dysregulated protein. However the initial data are not always so easily and immediately conclusive.

Interactions between proteins leading to regulatory, activating/deactivating, physical binding and degradation, within or across different cells, are complex and numerous [5–7], potentially leading to multiple downstream effects from a single change. Thus, sometimes multiple proteins showing perturbed expression patterns can be traced back to a more concise source of dysregulation. This can lead to a better understanding of what causes an illness in question, instead of just seeing its downstream effects. Such protein networks have been termed interactomes, which can link proteomic studies back on themselves and expand the usefulness of pre-existing data, even from other studies. After selecting a protein of interest (POI), usually through a proteomic study, it becomes important to map out its role within the organism, tissue, or cell under investigation. Interactome analysis is carried out in a similar manner as in the isolation of a proteome, with the main exception that it is carried out using an immunoprecipitation approach (Fig. 19.1). This leads to the isolation of the POI and all associated proteins.

Creating the interactome is achieved by binding the POI to any of several resins, most often using cross-linked antibodies. All interactors with the POI are also retained on the resin, and the rest of the proteome is washed away. The POI and the bound proteins are then eluted from the resin and run through a liquid chromatography tandem mass spectrometry (LC-MS/MS) system to identify the protein subset, as described previously [8–11]. Several computational steps are then performed to eliminate nonspecific interactors from the dataset.

Here we present a standard protocol for isolation and characterization of an interactome from cell lines.

19.2 Materials

19.2.1 Laboratory Equipment

1. Microcentrifuge for 1.5 mL Eppendorf tubes capable of 13,000 × *g*
2. Microcentrifuge for 15 mL Falcon tubes for cell pelleting

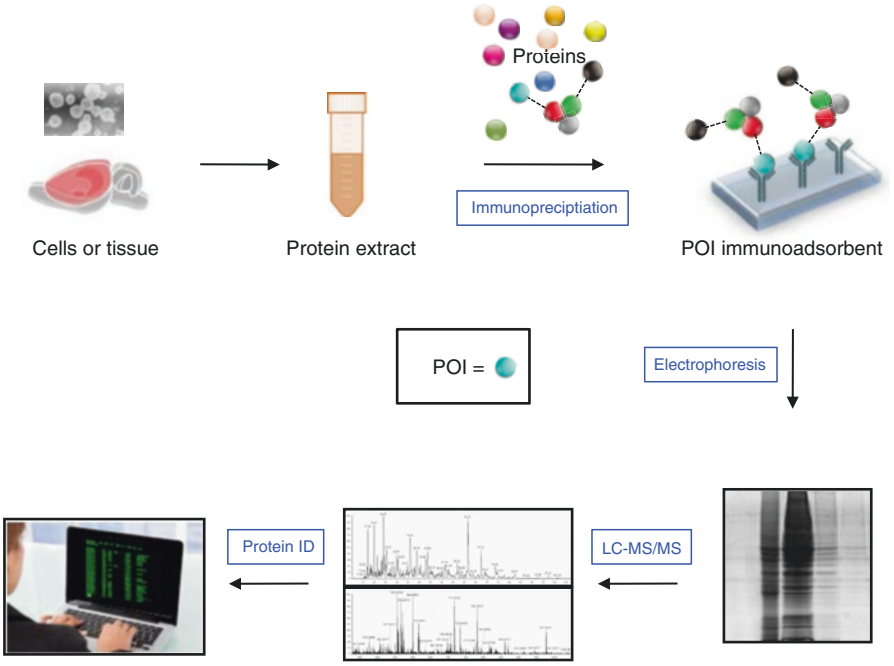

Fig. 19.1 Steps involved in the characterization of a protein of interest (*POI*) interactome

3. Sonicator
4. Cell manual grinding/maceration kit
5. Handheld cell homogenizer
6. Cell culture T75 flasks
7. Pierce spin columns with filtration frit, screw cap, and bottom plug

19.2.2 Solvents, Buffers, and Media

1. Dulbecco's Modified Eagle's Medium (DMEM): 4.5 g/L D-glucose, L-glutamine (+), sodium pyruvate (−), supplemented with bovine serum albumin (BSA) (*see Note 1*)
2. 20× Modified Dulbecco's PBS: 160 mM sodium phosphate, 40 mM potassium phosphate, 2.8 M NaCl, 200 mM KCl (pH 7)
3. Cells of interest (or tissue sample)
4. Affinity-purified antibody
5. 20× Pierce Biotechnology (Waltham, MA, USA)coupling buffer: 0.2 M sodium phosphate, 3 M NaCl (*see Note 2*)

6. 5 M sodium cyanoborohydride solution
7. Pierce Biotechnology quenching buffer: 1 M Tris•HCl (*see Note 2*)
8. Sodium azide (*see Note 3*)
9. Ultrapure water
10. Pierce Biotechnology elution buffer (pH 2.8) (*see Notes 2 and 4*)
11. Pierce Biotechnology AminoLink Plus Coupling Resin: 50% slurry in storage buffer (*see Note 2*)
12. Pierce Biotechnology Control Agarose Resin: cross-linked 4% beaded agarose as a 50% slurry in storage buffer (*see Notes 2 and 5*)

19.3 Methods (See Note 6)

19.3.1 Cell and Column Preparation

1. Proliferate cells in T75 flasks at 37 °C under 5% CO_2 (*see Note 1*) for 72 h (*see Note 7*) or expand until 10^7 cells are obtained.
2. Remove the cell layer with a cell scraper and by washing with 2 mL modified PBS (*see Note 8*).
3. Rinse and scrape with another 1 mL PBS to obtain maximum cell yield.
4. Centrifuge the cell suspension for 5 min.
5. Discard the supernatant and store pellets at −80 °C.
6. Prepare spin columns by adding 50 µL resin slurry, and remove the suspension buffer (*see Note 9*).
7. Wash the resin twice with 200 µL 1× coupling buffer, centrifuging between washes.
8. Add 10 µL 20× coupling buffer, antibody (*see Note 10*), and water to total 200 µL.
9. In a fume hood, add 3 µL sodium cyanoborohydride solution to the resin-antibody mixture (*see Note 11*).
10. Incubate for 120 min on a shaker at room temperature.
11. Centrifuge out the solution into a fresh tube and save the flow-through for resin-antibody coupling verification (*see Note 12*).
12. Perform the wash steps given in Table 19.1 and centrifuge after each addition for removal of the solution.
13. Add 200 µL coupling buffer for storage, cap and plug the column, and store at 4 °C (*see Note 13*).
14. To prepare the lysate, suspend the frozen cell pellets in 200 µL modified PBS.
15. Lyse the cells (*see Note 14*) by either sonication (*see Note 15*), manual grinding (*see Note 16*), or using a cell homogenizer (*see Note 17*).
16. Centrifuge the cells at 14,000–18,000 × *g* for 30 min.
17. Collect the supernatant and measure the protein concentration.

Table 19.1 Antibody cross-linking wash steps

Step	Solution(s)	Quantity (μL)	Number
1	Coupling buffer	200	2
2	Quenching buffer	200	1
3	Quenching buffer Sodium cyanoborohydride	200 3	1 [a]
4	Coupling buffer	200	2
5	Wash solution	150	6 [b]
6	Coupling buffer	200	2

[a]Incubate at the same time for 15 min before centrifugation
[b]Centrifugation for 30 s is sufficient

19.3.2 Preclearing and Co-immunoprecipitation

1. For every 1 mg of protein, place 80 μL of the control resin slurry into a spin column and centrifuge out the storage buffer.
2. Add the protein-containing supernatant, cap and plug the column, and incubate for 45 min on a slow shaker at 4 °C (*see Note 18*).
3. Centrifuge into a clean tube (*see Note 19*).
4. Add 10 μL elution buffer and centrifuge into a clean tube.
5. Add 50 μL elution buffer to the resin and incubate for 5 min at room temperature.
6. Centrifuge into the same tube as above (*see Note 20*).
7. Label and store the elution tube at −80 °C.
8. Combine the precleared lysate with the cross-linked antibody resin for 105 min (*see Note 21*).
9. Centrifuge into a clean tube and save the flow-through, label and store at −80 °C (*see Note 22*).
10. Wash three times with 200 μL 1× PBS.
11. Add 10 μL elution buffer to the column and centrifuge into a collection tube.
12. Add 50 μL more elution buffer, incubate for 5 min at room temperature, and centrifuge into the same tube.
13. Repeat steps 10 and 11 two more times to obtain three elutions (*see Note 23*).
14. Store elutions at −80 °C until ready for mass spectrometry (*see Note 24*).
15. To prepare the resin for later use, wash twice with 100 μL coupling buffer, centrifuge between washes, and discard the flow-through.
16. Plug the bottom of the column, add 200 μL coupling buffer, and store at 4 °C (*see Note 13*).

19.4 Notes

1. In our experiments related to schizophrenia, we used a human oligodendroglia cell line (MO3.13; Cedarlane Labs; Ontario, Canada). This medium was used due to previous successful experiments with this cell line. Different cell types may necessitate different media and incubation conditions.

2. Component of the Pierce Biotechnology Co-Immunoprecipitation Kit (Waltham, MA, USA).
3. Used as a preservative for long-term storage.
4. This contains primary amines to displace bound proteins from the resin.
5. This is used as a control to account for nonspecific binding to the resin.
6. Unless otherwise specified, centrifugation steps were performed at 1000 × *g* for 60 s.
7. It is recommended to change the buffer after 24 h to remove cell debris and provide more nutrients for the growing cells.
8. If possible, use a light microscope to ensure that the majority of cells have been scraped off the surface of the flask.
9. Use an agitator right before removing the resin to get as close to a homogeneous mixture as possible. The resin is not likely to fit through the opening of a standard pipette tip, so use clean scissors to snip off the end of the tip.
10. If the antibody is measured in terms of concentration, use 10 μg as per the kit protocol but up to 75 μg if there is difficulty binding all of the POI. In our case, the antibody was provided in terms of activity instead of pure concentration. We used the provided documentation to determine the amount of antibody to use. To reduce unintentional interactions, a monoclonal antibody is suggested instead of a polyclonal antibody.
11. This step activates the resin for coupling.
12. This can be done using a standard protein assay, ensuring the assay has the capability of detecting low protein concentrations. It is suggested to do this before continuing the procedure, especially if this is the first time the antibody is being used.
13. The columns can be stored at 4 °C for up to 2 weeks with no additional treatment. In our case, they were prepared during the cell proliferation stage. If longer storage is needed, add sodium azide to a concentration of 0.02% and wrap the column in laboratory film to prevent the resin from drying.
14. The lysis buffer must be carefully selected. Even sensitive detergents can sometimes denature proteins or disrupt protein-protein interactions. It was for this reason that the provided PBS was used and various methods were tested in an attempt to determine the best and cleanest lysis method. If none of these methods work, look into gentle, non-denaturing detergents, keeping in mind the potential for downstream complications (e.g., detergents may require removal prior to mass spectrometry analysis).
15. In our case, MO3.13 cells were suspended in 500 μL lysis buffer and sonicated at 30% duty cycle, output 1.3, for 20 s with the pulsar on. Always keep cells on ice.
16. To use the manual grinder, resuspend the cells in 250 μL lysis buffer and grind by hand for 5 min on ice.
17. For the handheld cell homogenizer, resuspend the cells in 450 μL lysis buffer and run the homogenizer for 25 s at full speed on ice.
18. The speed should be fast enough to keep the resin moving but gentle enough to not agitate the resin too much and lose more loosely-bound proteins.

19. This is the precleared lysate.
20. This will be used as a control to identify the proteins that bind nonspecifically to the resin. In this way, if the control resin was unable to remove all the nonspecific binding proteins, they can then be later removed from the list.
21. Too short and the yield will be too low. Too long and unintended protein interactions and/or degradation may occur. If you find your protein is not binding, you may need to either increase the binding time or increase the amount of antibody linked to the resin.
22. This can be used later to ensure that the POI has bound sufficiently to the antibody and resin. If too much of the POI flows through, there may be a problem with the antibody binding or there is not enough antibody binding capacity for the amount of POI loaded.
23. These three elutions will be used to ensure that the highest amount of protein possible leaves the resin without unnecessarily diluting the sample. Perform protein concentration tests to determine in which eluate the majority of the protein has elected. For example, if all protein eluted in the first fraction, discard the other two. Otherwise, combine and concentrate these eluates.
24. It is recommended to run a SDS-PAGE analysis to sort bands by apparent molecular weight before carrying out an analysis (e.g., LC-MS/MS). If the SDS-PAGE step is omitted, a desalting process will be required.

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Chapter 20
Sequential Co-immunoprecipitation and Immunoblot Approach to Determine Oligomerisation of G-Protein-Coupled Receptors

Paul C. Guest

20.1 Introduction

Most drugs used in the treatment of psychiatric disorders are targeted towards G-protein-coupled receptors (GPCRs) [1, 2]. These receptors are activated by different neurotransmitters, leading to changes in synaptic transmission in multiple brain functions, such as mood, behaviour and cognition. However, a number of studies over the past 15 years or so have suggested that these cell surface receptors may form oligomers with other GPCRs, which complicates the interpretation of their function and may alter their pharmacological profiles as well as function responses [3]. This could be an important determinant in the design of new drugs targeting key symptoms of mental disorder. Therefore, there is a need to develop new interdisciplinary approaches to investigate the oligomer forming potential of these receptors.

This chapter describes a combined co-immunoprecipitation and immunoblot protocol for determining whether or not the 5-hydroxy tryptamine (5-HT) 1A receptor (5-HT_{1A}) is capable of forming dimers with other 5-HT_{1A} receptors as homodimers as well as with other members of the 5-HT receptor family to create heterodimers. For this, HEK-293 cells co-expressing the c-myc-tagged $5HT_{1A}$ receptor with FLAG-tagged versions of the $5HT_{1A}$, $5HT_{1B}$ and 5HT1D receptor were immunoprecipitated using a c-myc immunoadsorbent and the precipitates subjected to immunoblot analysis using FLAG and c-Myc antibodies as described by Salim et al. [4] (Fig. 20.1). Thus, the c-Myc immunoprecipitation would pull down c-myc-tagged 5-HT_{1A} receptor and anything else that it is bound to such as the

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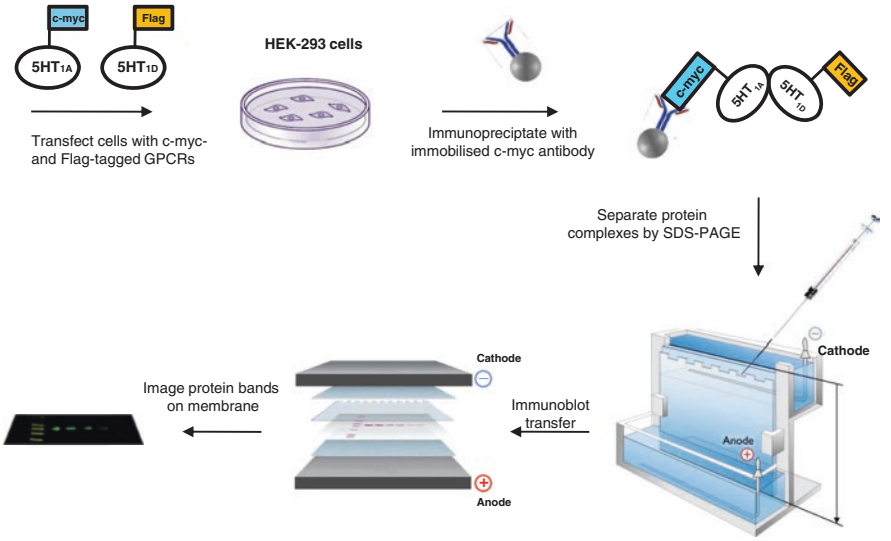

Fig. 20.1 Flow diagram showing experimental protocol

Flag-tagged 5-HT_{1A}, 5-HT_{1B} and 5-HT_{1D} receptors targeted in this study. Since the latter contain a Flag tag, their presence can be detected using a Flag-directed antibody by immunoblot analysis. Likewise Flag immunoprecipitation would pull down all Flag-containing constructs as well as the c-myc-tagged 5-HT_{1A} receptor, if such oligomers exist. If this is the case, the presence of the 5-HT_{1A} receptor can be detected by immunoblot analysis using the c-myc antibody.

20.2 Materials

20.2.1 Transfection

1. pCDNA3.1$^{(+)}$ expression vector (Invitrogen; Paisley, UK)
2. Restriction endonucleases
3. C-myc epitope (peptide, MEQKLISEEDL; nucleotide, ATG GAA CAA AAA CTT ATT TCT GAA GAA GAT CTG)
4. Flag epitope (peptide, DYKDDDDK; nucleotide, GAT TAC AAG GAT GAC GAT GAC AAG)
5. Dulbecco's Modified Eagle's medium
6. Foetal calf serum (FCS)
7. 250 mM $CaCl_2$
8. HEK-293 cells
9. RoboCycler with hot-top assembly (Stratagene; Amsterdam, Holland)

20.2.2 Immunoprecipitation

1. Anti-c-myc affinity agarose (Sigma-Aldrich; Poole, UK)
2. Anti-Flag M2 affinity agarose (Sigma-Aldrich)
3. Phosphate-buffered saline (PBS), pH 7.4
4. Lysis buffer (Sigma-Aldrich)
5. Wash buffer: 50 mM Tris (pH 7.5), 0.1% sodium dodecyl sulphate (SDS), 150 mM NaCl
6. SDS sample buffer (Sigma-Aldrich)

20.2.3 Western Blot Analysis

1. 180 × 160 × 1.5 mm slab gels polymerised from 10% acrylamide and 0.1% N,N′-methylenebisacrylamide in Tris/glycine/SDS buffer (Novex; San Diego, CA, USA)
2. 0.45 mm thickness Immobilon$^{(R)}$ P membranes (Merck Millipore; Watford, UK)
3. Transfer buffer: 25 mM Tris/190 mM glycine (pH 8.3), 20% methanol
4. Blocking buffer: 20 mM Tris (pH 7.4), 150 mM NaCl, 5% skimmed milk powder
5. Antibody incubation buffer: 20 mM Tris (pH 7.4), 150 mM NaCl, 0.1% Tween-20
6. Wash buffer: 20 mM Tris (pH 7.4), 150 mM NaCl
7. Mouse c-myc antibody (Sigma-Aldrich)
8. Mouse Flag antibody (Sigma-Aldrich)
9. Peroxidase-conjugated sheep anti-mouse serum
10. Enhanced chemiluminescence (ECL) detection reagents 1 and 2 (GE Healthcare; Little Chalfont, UK)

20.3 Methods

20.3.1 Transfection with Plasmid DNAs

1. Insert c-myc epitope into 5HT1A receptor cDNA by polymerase chain reaction mutagenesis as described [5] (*see Note 1*).
2. Insert Flag epitope into 5-HT_{1A}, 5-HT_{1B} and 5-HT_{1D} receptor cDNAs as above.
3. Clone PCR products into the pCDNA3$^{(+)}$ expression vector using standard techniques [6] (*see Note 1*).
4. Inoculate 1 × 10^6 cells/10 cm plate in 10 mL DMEM, 10% FCS, supplemented with L-glutamine, penicillin and streptomycin.
5. Incubate at 37 °C under 5% CO_2/95% air.

6. After 24 h, transfect cells using 1 mL 250 mM $CaCl_2$ containing 10 μg of each plasmid by slow drop-wise addition while gently swirling the plate (*see Note 2*).
7. Incubate at 37 °C under 5% CO_2/95% air.
8. After 16 hours, gently aspirate the medium and add 10 mL pre-warmed fresh medium to the plates (*see Note 3*).
9. Incubate at 37 °C under 5% CO_2/95% air.

20.3.2 Immunoprecipitation of GPCR Complexes

1. Harvest the cells 24 h later by first removing the media.
2. Scrape the cells off the plate using ice-cold PBS into a 15 mL Falcon tube.
3. Collect the cell pellet by centrifugation at 1000 × g for 5 min (*see Note 4*).
4. Aspirate the PBS and add 1 mL of cell lysis buffer (*see Note 5*).
5. Homogenise the cells by passing up and down through a 20 gauge syringe needle.
6. Centrifuge at 13,000 × g for 20 min at 4 °C.
7. Add the supernatant separately to 12.5 μL packed gel of c-myc or Flag immunoadsorbents.
8. Incubate overnight at 4 °C with gentle rocking.
9. Centrifuge at 700 × g for 5 min to recover the immunoadsorbents.
10. Resuspend in 1 mL of cell lysis buffer, centrifuge at 700 × g for 5 min and discard the supernatant.
11. Repeat step 10 two more times.
12. Resuspend the immunoadsorbents, centrifuge as above and discard the supernatants.
13. Repeat step 12.
14. Elute the bound proteins in 60 μL loading buffer.

20.3.3 Immunoblot Analysis

1. Heat eluted samples in loading buffer for 3 min at 100 °C and centrifuge at 700 × g for 10 sec (*see Note 6*).
2. Subject samples to SDS polyacrylamide electrophoresis using the discontinuous buffer system of Laemmli [7] (*see Note 7*).
3. Subject the electrophoresed proteins to semi-dry electrophoretic transfer onto Immobilon-P membranes (*see Note 8*).
4. Immerse membranes in blocking buffer and mix gently on a rocker for 2 h (*see Note 9*).
5. Remove the blocking solution and rinse twice in antibody incubation buffer.
6. Incubate membranes with either c-myc (1:1000) or FLAG (1:1000) antibodies in antibody incubation buffer overnight at 4 °C (*see Note 10*).

7. Remove the solutions and wash three times for 5 min in antibody incubation buffer.
8. Add peroxidase-conjugated anti-mouse serum (1:1000) to both membranes and incubate for 2 h at room temperature.
9. Rinse three times for 5 min in wash buffer and then twice in water.
10. Drain excess water from the membranes and place protein side up on smoothed cling film.
11. Mix an equal volume of ECL detection solution 1 with detection solution 2 and add this to both membranes such that the entire surface is covered and incubate for 1 min at room temperature.
12. Remove excess detection reagent by holding an edge with forceps and touching a corner to a tissue.
13. Place the membrane protein side down onto a fresh piece of cling film and wrap each one so that the cover is smooth with no wrinkles or air bubbles.
14. Insert membrane protein side up in a suitably sized film cassette.
15. Place a sheet of ECL Hyperfilm on top, seal the cassette and expose as required for detection of the immunoreactive bands (Fig. 20.2) (*see Notes 11 and 12*).

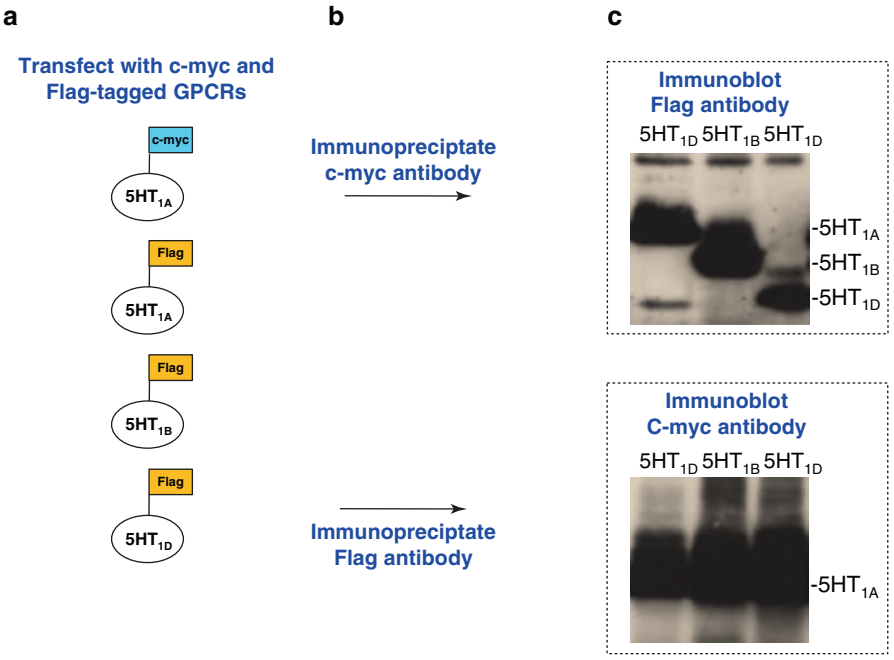

Fig. 20.2 (**a**) Cells co-expressing the c-myc-tagged 5HT1A receptor and FLAG-tagged versions of the $5HT_{1A}$, $5HT_{1B}$ and $5HT_{1D}$ receptors were obtained by co-transfection. (**b**) Cell lysates were immunoprecipitated using either anti-c-myc (*top*) or anti-FLAG (*bottom*) immunoadsorbents. (**c**) Immunoprecipitates were subjected to immunoblot analysis using FLAG (*top*) or c-myc (*bottom*) antibodies and subjected to ECL detection and imaging

20.4 Notes

1. Production of the tagged receptors and corresponding DNA plasmids was carried out as described by Salim and co-workers [4]. Users should adapt their procedures according to the epitope tags and target sequences of choice.
2. Cells should be 30–40% confluent for optimum transfection efficiency.
3. Be careful not to disturb the DNA precipitates on the bottom of the plate.
4. In most situations, centrifugation of cells should be carried out a low g force so that cell membranes are not disrupted. In this particular situation, the targets are integral membrane proteins, so this is not likely to be a factor.
5. This buffer consists of a mild detergent, bicine and 150 mM NaCl. This should cause minimal interference with protein interactions such as antibody binding.
6. Heating denatures the proteins for a more efficient separation on electrophoresis and the centrifugation helps to collect the heated loading solution at the bottom of the tube to aid recovery of the original volume.
7. In this experiment, the gels were chosen with acrylamide concentrations to resolve protein bands in the region of 20–120 kDa in order to detect GPCR monomers and dimers. For resolving higher molecular weight proteins, lower acrylamide concentrations should be used.
8. The transfer buffer contained 20% methanol to facilitate transfer of transmembrane proteins.
9. This helps to block nonspecific sites on the membrane for improved signal-to-noise ratio.
10. This step can also be carried out at room temperature for approximately 2 h.
11. Times will vary depending on the affinity and specificity of the antibodies and the abundance of the target proteins. Thus, it may be necessary to determine the optimum exposure times for each new experiment.
12. It is important to carry out controls to ensure oligomerisation is not the result of a co-transfection artefact. To rule out the possibility that oligomerisation is caused by nonspecific aggregation of receptors, extracts from cells separately expressing the c-myc- and Flag-tagged can be mixed and immunoprecipitated with anti-c-myc agarose and subjected to immunoblot analysis with Flag antibodies. In this case, the Flag-tagged receptor should not be detected in the precipitated material. A major prerequisite for the physiological oligomerisation of GPCRs is co-expression in the same cells. This should be demonstrated using detailed immunological or specific binding experiments that localise the studied receptors to the same cells combined with co-precipitation studies using receptor-specific antibodies or ligands. The $5HT_{1A}$, $5HT_{1B}$ and $5HT_{1D}$ receptors all show pre- and postsynaptic localisations and can be found together in some of the same brain regions [8–10]. Furthermore, studies using antibodies to untagged versions of the targeted GPCRs should be carried out to confirm their existence in endogenous tissues [11, 12].

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Chapter 21
A Clinical Study Protocol to Identify Serum Biomarkers Predictive of Response to Antipsychotics in Schizophrenia Patients

Johann Steiner and Paul C. Guest

21.1 Introduction

Approximately half of schizophrenia patients fail to respond favourably to an initial treatment with antipsychotic medications [1, 2]. Also, traditional treatment for schizophrenia sometimes involves the administration and switching of drugs multiple times until an adequate response is achieved [3]. Moreover, a high rate of non-compliance and relapse is a common occurrence [4, 5]. These issues are likely to be due to the fact that there is still insufficient understanding of the molecular pathways affected in this disease to guide treatment. Thus, the availability of objective biomarker tests to inform treatment selection is urgently needed, and a small number of studies have now been carried out in this area [6–9]. This is in line with the objectives of the Food and Drug Administration and other regulatory agencies to set out personalized medicine approaches for improved disease management [10]. However, studies to identify such biomarkers should be set up using a standardized design to minimize the effects of potential confounding factors on study outcome.

Here, we describe a clinical protocol involving 77 newly diagnosed schizophrenia patients to identify a baseline biomarker signature that could be used to predict response over a 6-week treatment period with the antipsychotics olanzapine, quetiapine, risperidone or others [6]. The main outcome measure was the Positive and Negative Syndrome Scale (PANSS) [11], and serum proteins were measured by using a multiplex immunoassay at Myriad RBM (Austin, TX, USA)

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as described previously [6, 7, 9]. Emphasis was placed on minimizing statistical effects of gender, age, body mass index (BMI), smoking and cannabis use on experimental outcomes.

21.2 Materials

1. Approval obtained from the institutional ethical committee for the study protocols (*see Note 1*)
2. Written consent obtained for all participants
3. A study plan developed according to the Declaration of Helsinki [12]
4. Patients diagnosed with paranoid schizophrenia according to the Diagnostic and Statistical Manual (DSM)-IV (Table 21.1) (*see Note 2*), with exclusion of psychosis resulting from other medical conditions or substance-induced psychosis by physical examination, routine blood analysis, screening for illegal drugs and magnetic resonance imaging of the brain
5. S-Monovette 7.5 mL serum tubes (Sarstedt; Numbrecht, Germany)
6. Low protein-binding Eppendorf tubes (Hamburg, Germany)
7. Human DiscoveryMAP$^{(R)}$ multiplex immunoassay platform service (Myriad RBM; Austin, TX, USA) (*see Note 3*)
8. Microsoft Office Excel software (Redmond, WA, USA) or equivalent
9. Principal component analysis (SIMCA-P+ vs12.0, Umetrics, Umea, Sweden)
10. Statistical software package R (http://www.r-project.org)

Table 21.1 Demographics of schizophrenia patients at baseline and after treatment for 6 weeks with antipsychotics [6]

	Baseline	6 weeks' treatment
Number	77	77
Sex (male/female)	50/27	50/27
Age (years)	35.6 ± 11.1	–
BMI (kg/m^2)	25.4 ± 4.6	26.1 ± 4.5
PANSS positive scores	23.0 ± 7.2	12.7 ± 5.3
PANSS negative scores	19.3 ± 8.7	14.4 ± 6.8
PANSS general scores	43.0 ± 10.3	28.7 ± 9.1
Smoking (cigarettes/day)	9.5 ± 9.1	9.6 ± 9.5
Marijuana (yes/no)	16 / 61	3 / 74
Drug (olanz/quet/ris/others)	–	18 / 14 / 24 / 21
Cumulative chlorpromazine units (mg)	–	17,123 ± 12,657

Abbreviations: *olanz* olanzapine, *PANSS* positive and negative syndrome scale, *quet* quetiapine, *ris* risperidone. Values are shown as mean ± sd

21.3 Methods

1. Record all indicated parameters (and others as necessary) using those given in Table 21.1 as a guide.
2. Carry out tests to determine whether or not statistical differences exist across the groups as appropriate, and exclude patients lying outside two standard deviations (*see Note 4*).
3. From the selected patient group, exclude those individuals with other medical conditions (*see Note 5*).
4. Treat participants as inpatients over the 6-week study period (*see Note 6*).
5. Collect blood from all subjects by venipuncture into serum tubes at the start of the study (T0) and after the 6-week treatment period (T6) (*see Note 7*).
6. Prepare serum by placing samples at room temperature for 90 min to allow blood coagulation, followed by centrifugation at 4,000 × g for 5 min to recover the supernatants.
7. Store serum at 80 °C in low protein-binding Eppendorf tubes prior to analysis.
8. Randomize and blind samples to analysts using code numbers until all biochemical assays are complete.
9. Analyse samples by multiplex immunoassay (*see note 8*).
10. Record all values such as molecular levels of each analyte on an Excel spread sheet.
11. Carry out data analyses using the statistical software package R (http://www.r--project.org).
12. Preprocess the data by filtering out analytes which contain measurement values outside the linear assay range in more than 30% of samples (*see Note 9*).
13. Assess data quality using principal component analysis (*see Note 10*).
14. Determine significant associations using non-parametric Spearman's correlation tests and adjust for false discovery rate [13] (*see Note 11*).
15. To identify a molecular fingerprint for prediction patient responses, apply a Random Forests analysis [14] (Table 21.2) (*see Note 12*).

Table 21.2 Significant associations between molecular levels at T0 and improved symptom scores [6] (*see Note 13*)

	Change in PANSS negative scores at week 6	
Protein	*P*-value	Correlation coefficient
Insulin	0.005	−0.037

21.4 Notes

1. In this study, approval was obtained from the University of Magdeburg.
2. The focus on paranoid schizophrenia, which comprises the most prevalent subtype of the illness, was intended to minimize variability. In this study, 36 patients were in the first stage of illness and had not taken antipsychotics at the start of the study, and 41 patients had not taken antipsychotics for at least 6 weeks before the start of the study.
3. This consists of approximately 200 immunoassays in multiplexed formats based on the Luminex technology (https://rbm.myriad.com).
4. In this study, we also attempted to control for smoking and cannabis use, considering the increased consumption of these drugs by schizophrenia patients and known links to psychiatric illnesses.
5. Clinicians had access to detailed clinical files of all patients including medical histories and referral letters from the general practitioners. Any patients with other conditions such as type 2 diabetes, hypertension, cardiovascular or autoimmune diseases were excluded to minimize potential confounding factors during data analysis.
6. Participants were treated as inpatients as they were acutely ill. This also had the added benefit of monitoring patients more closely to better control the study.
7. In addition to providing the material for biomarker analysis, a serum was used for therapeutic drug monitoring to assess compliance.
8. In this study, all samples were shipped to Myriad RBM (Austin, TX, USA) for multiplex immunoassay using the HumanMAP® panel comprised of approximately 200 analytes. The screening was carried out in a Clinical Laboratory Improvement Amendments (CLIA)-certified laboratory as described previously [6, 7]. Assays were calibrated using standards, raw intensity measurements converted to absolute protein concentrations by comparison to the standards and performance verified using quality controls.
9. Out of 191 analytes, 168 remained in the data set after this procedure, and only 6.8% of the values were missing.
10. This was used to detect any strong effects on the overall data variance. In this case no effects were identified across the first ten principal components. Outliers in each analyte as well as response variables were excluded from analysis if these differed by more than two standard deviations from the mean. This resulted in removal of less than three subjects per analyte.
11. Comparisons between the groups are based on non-parametric Wilcoxon rank-sum tests or paired Wilcoxon rank-sum tests for within-subject comparisons. Analysis of covariance (ANCOVA) was used to account for ‘regression to the mean’ effects between baseline and follow-up measurements [15]. This is a statistical phenomenon that can make natural variation in repeated data look like real change and can occur when large or small measurements tend to be followed by measurements that are closer to the mean. In addition, ANCOVA was used to estimate effects of BMI on analyte differences between the groups.

12. This method builds multiple decision trees and uses a majority decision of classification outputs across trees to assign patients to one out of two groups based on the levels of the serum molecules. Random Forests was also used to select the most important molecules for prediction by permuting the measured values of each molecule sequentially and determining the importance based on the impact of this randomization on the output (results not presented here).
13. No proteins were identified at baseline (T0) associated with improvements in PANSS positive or general scores. However, one protein (insulin) was associated with improvements in PANSS negative symptom scores. The negative correlation means that lower levels of insulin at T0 were associated with greater improvements [6].

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Chapter 22
A Protocol for Producing the Maternal Low-Protein Rat Model: A Tool for Preclinical Proteomic Studies

Dan Ma, Susan E. Ozanne, and Paul C. Guest

22.1 Introduction

One hypothesis on the pathophysiology of schizophrenia suggests that this disease can result from disruption of brain development during foetal and neonatal time periods, leading to disturbances in neuronal function in later life [1, 2]. A number of different environmental factors that occur during pregnancy have been linked to increased risk of schizophrenia in the offspring, including maternal infections, maternal physical or emotional stress, maternal malnutrition and foetal hypoxia [1]. One of the most studied cases of malnutrition in humans was that resulting from the blockade of occupied Holland in 1944 and 1945 that led to severe food shortages, particularly in the supply of protein [3]. The short period of famine meant that it was possible to study individuals who were in utero during the famine and compare them to those born the year before or the year after. Such studies demonstrated that those who were in utero during the famine have had increased incidences of a number of diseases including metabolic and mental disorders such as schizophrenia. The highest incidence of schizophrenia was observed for those who were conceived

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during the peak months of the famine and thus exposed to it during early development [3].

This effect has been modelled using early-life protein restriction in rodents which leads to changes in molecular pathways such as the insulin signalling cascade, similar to those observed in low-birth-weight humans [4, 5] and in schizophrenia patients at first presentation [6–9]. Thus, the low-protein rat model has been used extensively as a tool to study the mechanisms by which suboptimal nutrition in utero leads to increased risk of a number of diseases involving disturbed metabolic function [10], including schizophrenia [11, 12]. Maternal protein deprivation in rats causes behavioural effects in the offspring such as age- and sex-dependent decreases in sensorimotor gating and the initial startle response, both typical features of schizophrenia [11]. In addition a proteomic study found alterations in serum levels of insulin, adiponectin, leptin and a number of inflammation-related factors in low-protein rat offspring; similar changes have been reported in schizophrenia patients at first onset [12]. The same study also found schizophrenia-like changes in glutamatergic pathways in the frontal cortex as well as alterations in proteins involved in cellular signalling, cytoskeletal structure, hormonal secretion and synaptic remodelling in the hypothalamus [12].

This chapter presents a protocol for preparation of a 3-month-old offspring of rat dams fed a low-protein diet through pregnancy and lactation. This model can be used to study several diseases and conditions although the focus here was on the preparation of biologically relevant materials for proteomic investigations of schizophrenia.

22.2 Materials

1. Female Wistar rats weighing 240–260 grams housed individually at 22 °C on a 12 h light-dark cycle (*see Note 1*)
2. A control diet containing 20% protein
3. A low protein containing 8% protein (*see Note 2*)
4. S-Monovette 7.5 mL serum tubes (Sarstedt; Numbrecht, Germany)
5. QProteome Cell Compartment Kit (Qiagen; Crawley, UK)
6. EDTA-free protease inhibitors (Calbiochem; San Diego, CA, USA)
7. ProteoExtract Protein Precipitation Kit (Merck; Hull, UK)
8. DC Protein Assay Kit (Bio-Rad; Hercules, CA, USA)

22.3 Methods

1. Allow rats to mate (*see Note 3*).
2. On identification of a vaginal plug, feed dams ad libitum on either the control or low-protein diet throughout gestation (21 days) and lactation (21 days) (Fig. 22.1).
3. Reduce litters to eight pups/dams after birth (*see Note 4*).
4. Wean all pups onto a standard laboratory chow fed ad libitum.

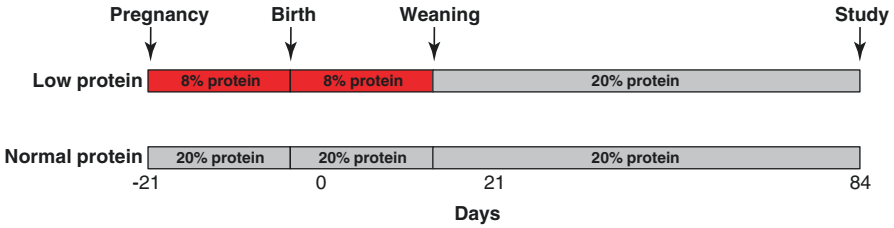

Fig. 22.1 Flow diagram showing generation of the maternal LP rat model

5. Remove access to chow the night before tissue collection (*see Note 5*).
6. Measure and record body and organ weights (*see Note 6*).
7. Collect trunk blood into serum tubes and allow to clot at room temperature (*see Note 7*).
8. Centrifuge at 1,000 × g for 15 min to remove any debris.
9. Transfer the supernatant to fresh tubes and store at −80 °C prior to analysis.
10. Dissect brain and store at −80 °C prior to analysis (*see Note 8*).
11. Mince 30–50 mg brain tissue using a metal blade to generate <0.5 mm^3 cubic pieces on a prechilled glass Petri dish.
12. Incubate in 1 mL ice-cold extraction buffer 1 from Q Proteome Cell Compartment Kit containing protease inhibitors on a rotary mixer for 20 min at 4 °C.
13. Collect supernatants following centrifugation at 1,000 × g for 20 min at 4 °C.
14. Suspend pellets in 0.5 mL of the same buffer, incubate and centrifuge as above.
15. Pool the supernatants from steps 13 and 14 on a sample-by-sample basis to obtain fraction 1 (*see Note 9*).
16. Resuspend the pellets in 1 mL ice-cold buffer 2 from QProteome Cell Compartment Kit containing protease inhibitors.
17. Incubate on rotary mixer for 20 min at 4 °C.
18. Centrifuge at 6,000 × g for 10 min at 4 °C and retain the supernatant.
19. Repeat the extraction in 0.5 mL ice-cold buffer 2 containing protease inhibitors and centrifuge as above.
20. Pool the supernatants from steps 18 and 19 to obtain fraction 2 (membrane protein associated) (*see Note 10*).
21. Determine protein concentrations of all samples using the DC protein assay (*see Note 11*).
22. If required, precipitate proteins in each fraction using the ProteoExtract Protein Precipitation Kit or similar to concentrate the proteins and remove buffer contaminants (*see Note 12*).

22.4 Notes

1. It should be ensured that all regulatory approvals are in place prior to carrying out any in vivo studies.

2. A number of low-protein diets are commercially available and used by various research groups. It is important that the low-protein diet is matched as closely as possible to the control diet used (e.g. caloric content) so that most components of the diets are identical.
3. When considering numbers of animals to mate, it should be noted that when studying the effects of maternal diet, the statistical unit is the mother. Therefore, when carrying out power calculations, the number required should reflect the number of dams included in the study.
4. Standardization of litter size is performed to ensure similar nourishment of pups in different litters during lactation. Ideally the same numbers of males and females should be included in the standardized litter.
5. Offspring can be studied at any age. However, for young adult offspring, we normally study animals at 12 weeks of age.
6. Low-protein rat offspring should have lower body weights compared to control offspring.
7. Ensuring clotting time is standardized helps to avoid potential confounding effects that differences in clotting time may cause.
8. The method used for dissection of the brain will depend on the brain region of interest. Possibilities are crude dissection on ice, micro-punching or laser capture microdissection. The amount of tissue generated will differ depending on methodology used.
9. Using this kit, fraction 1 should be enriched in cytosolic proteins. If other fractionation is desired, many other kits or protocols can be used.
10. As above, other fractionation procedures can be applied as required by the user.
11. Other assay kits can be used, but the user should ensure compatibility with the reagents used in the extraction buffer.
12. Removal of detergents in buffers is typically required prior to mass spectrometry-based experiments.

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Chapter 23
Generation of the Acute Phencyclidine Rat Model for Proteomic Studies of Schizophrenia

Dan Ma and Paul C. Guest

23.1 Introduction

Animal models are used routinely in different areas of medical research to gain a better understanding of the underlying disease mechanisms. The main objective is to mimic the human disease conditions as closely as possible. One of the most prominent animal models used for psychiatric diseases such as schizophrenia is the acute phencyclidine (PCP) rat model. PCP is an N-methyl-D-aspartate (NMDA) receptor antagonist which elicits behavioural alterations in rodents and "normal" humans that resemble some of the symptoms of first-onset schizophrenia [1–7]. In addition, these effects can be ameliorated or reversed by antipsychotic medications [6].

Although preclinical research for schizophrenia and other neuropsychiatric disorders has focused mainly on analysis of drug effects on behavioural readouts, the translation of rodent phenotypical changes to behavioural abnormalities in humans has not been successful and can also lead to false conclusions. This is due to the fact that animal models can only reflect certain behavioural traits of complex mental disorders, and even then it is impossible to determine mood states with any degree of certainty. For this reason proteomic studies have attempted to correlate behavioural readouts with molecular changes in analyses of disease-associated brain

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regions [8–10] and the peripheral blood [11–14] in clinical studies of schizophrenia. It is hoped that biomarker candidates emerging from these studies can be used for diagnostic purposes and provide new insights into specific molecular and cellular abnormalities. In this way, identification of translatable biomarkers in validated preclinical models would support their use in drug discovery and development. In addition, translational changes in blood serum proteins could be used to monitor disease progression and treatment response.

Here we describe a protocol for production of the acute PCP of schizophrenia. We also include a protocol for testing locomotion and stereotypical behaviours to assess the face validity [11] of the model as well as a standard operating procedure for preparation of brain tissue and blood serum to provide well-characterized biomaterials to support proteomic investigations.

23.2 Materials

1. Male, adult Sprague–Dawley rats (Charles River, Margate, UK), weighing 280–300 g housed in groups of four per box at 21 °C (±1 °C) on a 12 h/12 h light/dark cycle (lights on at 08:00) (*see Note 1*)
2. IR Actimeter System (Panlab; Barcelona, Spain) (Fig. 23.1) (*see Note 2*)
3. Vehicle: 0.9% sodium chloride in water
4. PCP: phencyclidine hydrochloride (Sigma–Aldrich; Poole, UK) in vehicle
5. 7.5 mL S-Monovette serum tubes (Sarstedt; Numbrecht, Germany)
6. Low-binding Eppendorf tubes (Hamburg, Germany)
7. Tissue fractionation buffer for total protein extraction: 7 M urea, 2 M thiourea, 4% (3-((3-cholamidopropyl) dimethylammonio)-1-propanesulfonate) (CHAPS), 2% ASB14 and 70 mM dithiothreitol
8. EDTA-free protease inhibitors (Calbiochem; San Diego, CA, USA)
9. Sonication device with microprobe
10. Bradford assay (Bio-Rad; Hemel Hempstead, UK) (*see Note 3*)

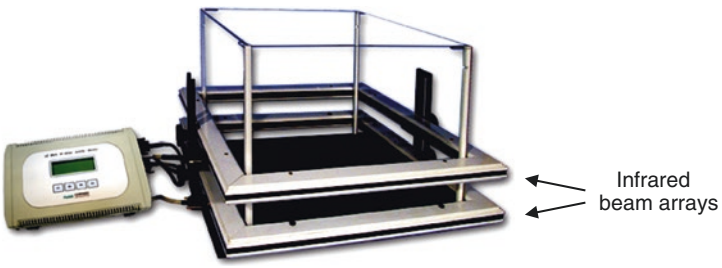

Fig. 23.1 Infrared locomotion chamber

23.3 Methods

1. Handle rats for 1 week prior to the experimental day to facilitate adjustment to the environment and procedures (*see Note 4*).
2. Two days prior to experiments, habituate rats individually for 30 min per day in the locomotion box (*see Note 4*).
3. Fast rats overnight on the day before the experiment (*see Note 5*).
4. After a further 30 min of habituation on the experimental day, inject rats subcutaneously with either vehicle or 5 mg/kg PCP and place back into the box (*see Note 6*).
5. Record locomotion for 90 min in 10 min intervals (*see Note 7*).
6. Record stereotypical behaviour simultaneously with step 4 in 10 min intervals by an observer blinded to treatment group (*see Note 8*).
7. In a parallel group of animals, cull and collect trunk blood 30 min after injection with PCP or vehicle into serum tubes (*see Note 9*).
8. Allow blood to clot for 90 min at room temperature (*see Note 10*).
9. Centrifuge at 3000 × g for 15 min at 4 °C and store the resulting supernatants in low-binding Eppendorf tubes at −80 °C (*see Note 11*).
10. Analyse by a proteomic technique such as multiplex immunoassay (Table 23.1).
11. Simultaneous with step 6, dissect brain tissue on ice and store at −80 °C (*see Note 12*).
12. Add tissue samples to fractionation buffer containing protease inhibitors.
13. Sonicate and vortex for 30 min to disrupt the tissue to homogeneity.
14. Centrifuge at 17,000 × g for 3 min at 4 °C and retain the supernatant for proteomic studies (*see Note 12*).
15. Determine protein concentrations of the lysates using the Bradford assay.
16. Precipitate protein as required using acetone (*see Note 13*).

Table 23.1 Multiplex immunoassay analysis of serum showing changes in hormones and inflammation factors in PCP-treated rats compared to those injected with vehicle. For each protein, the change is given as a PCP/vehicle ratio. Only those proteins with greater than 20% change in levels are shown [11]

Protein	PCP/vehicle	*P*-value
Aspartate aminotransferase	1.60	0.028
Progesterone	1.38	0.001
Adrenocorticotropic hormone	1.32	0.006
Insulin	0.76	0.018
Macrophage inflammatory protein 1 beta	0.75	0.006
Tumour necrosis factor alpha	0.72	0.005
Testosterone	0.54	0.050
Glutathione S-transferase alpha	0.22	0.002
Prolactin	0.12	0.001

23.4 Notes

1. Food and water should be available ad libitum. Experiments should be conducted during the light cycle and carried out in full compliance with the Home Office Guidance (UK Animals Scientific Procedures Act 1986) and the ethical policies of the Home Office (or similar).
2. The IR Actimeter System consists of a 45 × 45 × 35 cm plastic box with two infrared beam frames. The frames generate a matrix of 16 × 16 infrared beams for analysing horizontal and vertical motion.
3. Other assays can be used, but the user should ensure that no buffer incompatibility issues exist.
4. We found that this period of adjustment helps to ensure more consistent behavioural responses across the different animals.
5. Fasting all animals overnight prior to proteomic studies is important to help normalize the levels of proteins and small molecules involved in glucose homeostasis.
6. We used eight animals per group to help achieve statistical robustness.
7. Each beam break is recorded as one count. Typically, significant hyperlocomotion of the PCP rats occurs from 20 to 90 min after injection [11].
8. This can be achieved using a variety of rating systems, such as that devised by Sturgeon et al. [2]. This ranges from 0 (inactivity) to 5 (dyskinetic extension and flexion of the limbs, head and neck, gagging and weaving). Typically PCP-injected rats produce ratings of 2–4, whereas vehicle-treated rats have scores of 0–1 [11].
9. A parallel group of non-tested animals is used for the proteomic studies to eliminate any potential effects of the behavioural assessments.
10. It is important that the clotting time is kept consistent to minimize proteomic differences due to differences in coagulation.
11. Serum is ideal for analysis by proteomic methods such as multiplex immunoassay, label-free liquid chromatography mass spectrometry (LC-MS) and selective reaction monitoring (SRM)-MS.
12. Select brain regions as required in accordance with study objectives.
13. This is a whole cell extract protocol. Other procedures including subcellular fraction and soluble and insoluble procedures could also be used according to the experimental objectives and the proteomic procedures chosen [15]. The method described here is ideal for two-dimensional gel electrophoresis, LC-MS and SRM-MS.
14. This could be used as a concentration step or to remove buffer constituents which could affect subsequent proteomic steps.

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Chapter 24
A Protocol for Generation of a Corticosterone Model of Psychiatric Disorders

Paul C. Guest

24.1 Introduction

Psychiatric disorders affect a staggering one in four adults and one in ten children in any single year [1]. The precise mechanisms underlying these disorders have not been determined although considerable evidence suggests that events in early life such as stress or trauma may predispose susceptible individuals to these conditions in later life [2–5]. In humans, the primary response to stress is the increased release of the corticosteroid hormone cortisol which is regulated by the hypothalamic–pituitary–adrenal (HPA) axis [6, 7]. In addition, most patients with hypercortisolism resulting from adrenal or pituitary adenoma/ectopic tumours have been diagnosed with a psychiatric disease [8] with approximately half showing signs of major depression [9]. This most likely results from a multistep process in which the HPA axis imbalance leads to alterations in neurotransmitter, hormone and cellular signalling in the central nervous system (CNS) which may result in structural changes in the brain.

The corticosteroid hormones mediate their effects through a high-affinity mineralocorticoid receptor and a low-affinity glucocorticoid receptor [10]. The problem occurs when increased levels of corticosteroids saturate the mineralocorticoid receptor and cause activation of the glucocorticoid receptor, triggering negative feedback effects on the HPA axis. Both glucocorticoid and mineralocorticoid steroid receptors are present in high concentrations in the hippocampus, and prolonged hypersecretion of corticosteroid hormones can lead to dysfunctional neurons with decreased synaptogenesis and neurogenesis, along with increased neuronal loss in this brain region [11–13]. In fact, studies have shown that depressed individuals

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with the highest degree of hippocampal atrophy are linked to hypersecretion of corticosteroids [14, 15].

The chapter describes the generation and characterization of a chronic hypercorticosteroid mouse model for use in proteomic studies such as two-dimensional gel electrophoresis followed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry [16, 17].

24.2 Materials

24.2.1 Immunohistochemistry

1. Mice ($n = 16$) (*see Note 1*)
2. 5 mg (21-day release) corticosterone pellets (Sigma-Aldrich; Poole, UK)
3. Placebo pellets (Sigma-Aldrich) (*see Note 2*)
4. 5′bromodeoxyuridine (BrdU) (Sigma-Aldrich)
5. Phosphate-buffered saline (pH 7.4) (PBS)
6. Immersion fix: 4% paraformaldehyde in PBS
7. 30% sucrose, 4% paraformaldehyde
8. Ethylene glycol antifreeze solution (Sigma-Aldrich)
9. 50% formamide, 300 mM sodium chloride, 30 mM trisodium citrate (pH 7.0) (2× SSC)
10. 50 mg/mL sodium tetraborate (Borax) solution (Sigma-Aldrich)
11. 2 M HCl
12. 3% hydrogen peroxide
13. Blocking solution: 5% normal rabbit serum, PBS, 3% Triton X-100 (*see Note 3*)
14. Rat BrdU antibody (Abcam; Cambridge, UK)
15. Biotinylated anti-rat secondary antibody (Vector Laboratories; Burlingame, CA, USA)
16. Avidin–biotin complex (Vector Laboratories)
17. Diaminobenzidine (DAB)
18. Light microscope capable of 40× magnification

24.2.2 Plasma Analysis

1. Vacutainer-EDTA-coated tubes (BD Biosciences; Oxford, UK)
2. Coat-a-count rat corticosterone radioimmunoassay kit (Euro DPC Limited; Gwynedd, UK) (*see Note 4*)

24.2.3 Preparation of Samples for Proteomic Analysis (See Note 5)

1. Soluble protein extraction buffer: 30 mM Tris (pH 8), complete EDTA-free protease inhibitors (Roche Diagnostics, Mannheim, Germany)
2. Insoluble protein extraction buffer: 30 mM Tris (pH 8), 7 M urea, 2 M thiourea, 4% (w/v) dimethyl[3-(propyl]azaniumyl]propane-1-sulfonate (CHAPS)
3. Polytron® homogenizer (Kinematica; Lucerne, Switzerland)

24.3 Methods

24.3.1 BrdU Immunohistochemistry

1. Administer four corticosterone or placebo pellets per mouse by implantation under the skin at the back of the neck.
2. Dose (i.p.) with 50 mg/kg/day BrdU for days 10–14 of implantation.
3. On day 15, anaesthetize with 10 mg/kg euthatal.
4. Collect venous blood into EDTA tubes and prepare plasma.
5. Perfuse mice transcardially with PBS (*see Note 6*).
6. Remove brains, dissect into hemispheres along the midline and place into immersion fix for 7 days (*see Note 7*).
7. Transfer to sucrose/paraformaldehyde solution.
8. Cut 40 µm sections and store free-floating in antifreeze at –20 °C.
9. Place sections in formaldehyde/2× SSC solution at 65 °C for 2 h (*see Note 8*).
10. Wash in PBS.
11. Place sections in 2 M HCl for 30 min.
12. Place in Borax solution for 10 min followed by three washes in PBS.
13. Incubate for 30 min in 3% hydrogen peroxide (*see Note 9*) followed by three washes in PBS.
14. Incubate 1 h in blocking solution.
15. Incubate overnight at 4 °C with 1:200 rat BrdU antibody.

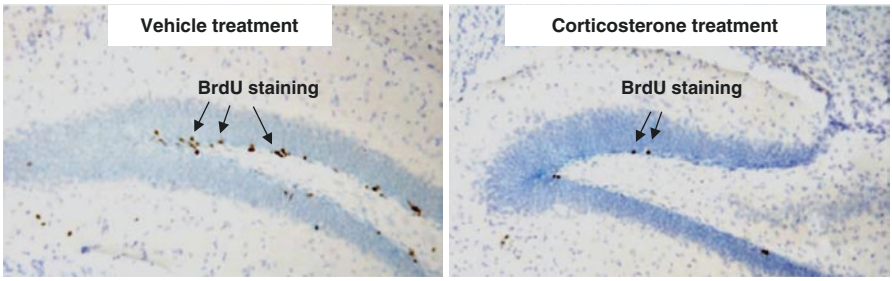

Fig. 24.1 Sections of mouse hippocampal dentate gyrus showing decreased numbers of BrdU-positive cells following chronic corticosterone treatment

Table 24.1 Effects of corticosterone treatment on BrdU incorporation, plasma corticosterone levels and hippocampus weight

	Placebo	Corticosterone
BrdU-positive cells (number)[a]	3642 ± 279	1808 ± 88
Plasma corticosterone levels (ng/mL)[b]	50.2 ± 7.2	113.1 ± 27.3
Hippocampus weight (mg)[c]	25.6 ± 7.5	18.7 ± 5.6

[a]Results of 6 placebo- and 5 corticosterone-treated mice
[b]Results of 8 placebo- and 8 corticosterone-treated mice
[c]Results of 7 placebo- and 8 corticosterone-treated mice

16. Wash sections three times in PBS and then incubate for 1 h with 1:200 biotinylated rat antibody.
17. Wash sections three times in PBS followed by amplification with avidin–biotin complex.
18. Visualize with DAB staining.
19. Identify BrdU-positive nuclei using light microscopy (Fig. 24.1) (*see Note 10*).

24.3.2 Plasma Corticosterone Measurement

1. After application of the anaesthesia, collect venous blood into EDTA tubes and prepare plasma according to standard protocols.
2. Centrifuge at 4 °C for 15 min at 3000 × g.
3. Remove upper plasma layer and store at –80 °C prior to analysis.
4. Determine plasma corticosterone levels as per the manufacturer's instructions (Table 24.1).

24.3.3 Sample Preparation for Proteomic Analysis

1. Dissect hippocampal tissue as completely as possible, blinded to treatment status of mice, and weigh the tissue (Table 24.1) (*see Note 11*).
2. Subject brain tissues to a differential solubility extraction procedure as described previously [17] (*see Note 12*).
3. Homogenize approximately 100 mg (wet wt) of tissue at 4 °C in 1 mL soluble protein extraction buffer using the Polytron homogenizer.
4. Centrifuge homogenates at 100,000 × g for 30 min at 4 °C in a TL30 rotor (Beckman; Palo Alto, CA, USA).
5. Collect supernatants and store at –80 °C (soluble protein extracts).
6. Homogenize pellets in 0.4 mL insoluble protein extraction buffer.
7. Repeat the centrifugation as above.
8. Collect and store the final supernatants (insoluble proteins) at –80 °C.

24.4 Notes

1. This can be almost any strain. The mice used in this study were obtained from Charles River (Margate, UK). Ensure that all procedures are conducted in accordance with the Animals Scientific Procedures Act of 1986 and its associated guidelines.
2. This control is important as the operational procedure itself could cause stress in animals.
3. This is an important control to block non-specific sites in the sections.
4. There are non-radioactive kits available such as the chemiluminescent kit offered by Arbor Assays (Ann Arbor, MI, USA).
5. Depending on the protein targets of interest, it may be important to fractionate the extracts as we have done here to increase proteome coverage.
6. This is done to remove excessive blood from the brain prior to proteomic analysis. Otherwise, a high proportion of known blood proteins may be detected, potentially obscuring detection of some of the low abundance indigenous brain proteins.
7. Note that this could be subject to some error and some hippocampal pieces could be missed and non-hippocampal tissue inadvertently collected. For ideal results, use the *Chemoarchitectonic Atlas of the Mouse Brain* as a guide when removing specific brain regions [18].
8. This step is carried out to denature the DNA.
9. This step should help to eliminate endogenous peroxidases, which could interfere with immunoreactive BrdU detection.
10. The microscope should be capable of magnifications up to 40x.
11. In studies where tissue weight is being measured, it is essential that the tissue in question is dissected in manner blinded to the experimental groups. This is to avoid any bias introduced by the experimenter.
12. The current protocol is ideal for a subsequent two-dimensional gel electrophoresis analysis. For measurements using other proteomic platforms, such as those based on mass spectrometry, it is likely that buffer exchange will be required so that particular constituents such as detergent do not interfere with the analysis.

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Chapter 25
MK-801-Treated Oligodendrocytes as a Cellular Model to Study Schizophrenia

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25.1 Introduction

The brain is the main organ of the central nervous system (CNS) and is composed of a heterogeneous group of cells, comprised of neurons and glia. Glial cells consist of astrocytes, oligodendrocytes, and microglia. For over a century, neurons have been perceived as being the most important cell type for brain function, and glia were seen simply as tissue glue. However, recent studies have clearly indicated the important role of glia in brain function. Glial cells are responsible for many functions, such as power support, neuronal control, maintenance, and plasticity of synaptic contacts [1]. Many studies have shown that neurons and glial cells communicate bidirectionally at both structural and functional levels. Thus, glial cells may act as partners of neurons in the formation of information processing mediated by glutamate, the most abundant excitatory neurotransmitter in the brain [1–3].

Glutamatergic transmission occurs throughout the CNS and is responsible for numerous essential brain functions such as cognition, learning, and memory. The inhibition of glutamatergic transmission can lead to disabling of the entire nervous system. Each glutamatergic synaptic event is initiated by an action potential that leads to Ca2+ influx in the presynaptic terminal, resulting in the release of the neurotransmitter into the synaptic cleft. After being released, glutamate rapidly crosses the synaptic cleft and activates specific receptors in the postsynaptic membrane [3]. The interaction of glutamate with glial cells is basically mediated by four types of

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receptors: G protein-coupled metabotropic receptors and three families of ionotropic glutamate receptors, comprised of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), kainate, and N-methyl-D-aspartate (NMDA) receptors [1, 4, 5]. Following the emergence of phencyclidine (PCP) around 1950, NMDA receptors have been implicated in the pathophysiology of neuropsychiatric disorders [6].

The NMDA receptor is a glutamate-activated cation channel, encoded by seven genes. It has four sites of pharmacological relevance: the glutamate recognition site, the glycine modulating site, the binding site for PCP and its analogues, and a cation-binding site within the channel, where magnesium acts [3, 7]. In Alzheimer's disease, impaired memory and learning has been linked to perturbations in NMDA receptor function [8]. Studies using PCP have also led to the glutamatergic hypothesis of schizophrenia due to similarities regarding symptomatic manifestations of PCP-induced psychosis and symptoms of this psychiatric illness [2, 7]. However, it has also been shown that the use of NMDA antagonists in patients with chronic pain reduced the need of opioids and these compounds also exhibit anticonvulsant properties in epilepsy patients [9].

In addition to PCP, other pharmacological agents have been used to study the pathophysiology of schizophrenia through the modulation of neurotransmitter systems. One of these is dizocilpine (also known as MK-801), which is the most powerful antagonist of the NMDA receptor [10]. Studies have revealed that neurons, astrocytes, and oligodendrocytes are affected by MK-801 treatment, although in a differential manner. For example, glycolysis appears to be more affected in MK-801-treated oligodendrocytes compared to the other cell types [11]. Furthermore, recent proteomic studies found that many proteins associated with energy metabolism were upregulated in the MK-801-treated oligodendrocyte cell line, MO3.13 [12].

The interest in understanding the role of oligodendrocytes in human disease led to development of oligodendroglial models for testing hypotheses under a controlled environment. The MO3.13 cell line was developed via fusion of a 6-thioguanine-resistant mutant of the human rhabdomyosarcoma RD with adult human oligodendrocytes cultured from a surgical specimen [13, 14]. Thus, proteomic profiling of these cells could help in understanding the mechanisms of action of pharmacological agents in studies of psychiatric disorders such as schizophrenia. Here, we present a protocol for culturing the MO3.13 oligodendrocyte cell line, treatment with MK-801, and mass spectrometry-based proteomic analysis.

25.2 Materials (See Note 1)

25.2.1 MO3.13 Cell Culture

1. Human oligodendroglia cell line MO3.13 (Cedarlane; Burlington, NC, USA)
2. Culture medium: DMEM, high glucose supplemented with 10% fetal bovine serum (FBS), and 1% 5000 U/mL penicillin-streptomycin (ThermoFisher Scientific; Waltham, MA, USA)

3. Flask Nunclon Delta-treated Vent/Close 75 (Sigma-Aldrich; São Paulo, SP, Brazil)
4. MAXYMum Recovery™ PCR Tubes (Axygen Scientific; Radnor, PA, USA)
5. Pipette tips (Axygen Scientific)
6. Serological pipette 25 mL (Sigma-Aldrich)
7. 15 mL conical centrifuge tubes (ThermoFisher Scientific)

25.2.2 Cell Collection

1. Phosphate-buffered saline (PBS)
2. Sarstedt cell scraper (Sigma-Aldrich)
3. Freezing medium: 60% DMEM, 30% FBS, 10% DMSO

25.2.3 Cell Treatment

1. MK-801 hydrogen maleate (Sigma-Aldrich)

25.2.4 Lysis, Reduction, Alkylation, and Digestion

1. Ultrasonic homogenizer (Cole Parmer Instrument Co.)
2. Lysis and reduction buffer: 6 M urea, 2 M thiourea, 10 mM dithiothreitol (DTT), 0.1 mM sodium pervanadate
3. Hydrogen peroxide solution
4. Protease and phosphatase inhibitors (cOmplete ULTRA) (Sigma-Aldrich)
5. Alkylation buffer: 20 mM iodoacetamide in 200 mM triethylammonium bicarbonate buffer (TEAB)
6. Digestion buffer: 2% sequencing grade modified trypsin (Promega; Madison, WI, USA)
7. Stop digestion buffer: 100% formic acid (FA)

25.2.5 Desalting and Concentration of Peptides

1. Oasis® HLB Short Cartridge (Waters Corporation; Milford, MA, USA)
2. Qubit® assay tubes (Thermo Fisher)
3. Qubit® protein assay kit (Thermo Fisher)
4. Concentrator plus (Eppendorf; Westbury, NY, USA)
5. Activation solution 1: 100% methanol (HPLC grade)
6. Activation solution 2: 100% LC-MS grade acetonitrile (ACN)

7. Reverse phase (RP) loading solution: 0.1% trifluoroacetic acid (TFA)
8. RP elution solution: 70% ACN, 01% TFA

25.2.6 NanoLC-MS/MS Analyses

1. Solvent A: 0.1% FA in water
2. Solvent B: 0.1% FA in ACN
3. Lock Spray solution: 100 fmol/μL [Glu1]-fibrinopeptide B standard (Waters Corporation) in methanol/water/FA (50:50:0.1%)
4. Ammonium hydroxide
5. ACQUITY UPLC M-Class system with 2D Technology: binary solvent manager; auxiliary solvent manager; sample manager (Waters Corporation)
6. First-dimension analytical column: M-Class peptide 130 Å, 5 μm, 300 μm × 50 mm BEH C18 trap column
7. Second dimension analytical columns: M-Class Symmetry 100 Å, 5 μm, 180 μm × 20 mm C18 Trap Column and 1.8 μm, 75 μm × 150 mm V/M and M-Class HSS T3 Column (Waters Corporation)
8. Autosampler vials: total recovery glass vials (Waters Corporation)
9. Instrument control software for UPLC: MassLynx (version 4.1; Waters Corporation)
10. SYNAPT G2-Si high-definition mass spectrometer (Waters Corporation)
11. NanoLock Spray dual electrospray ion source (Waters Corporation)
12. Pre-Cut Picotip emitter (Waters Corporation)
13. MS instrument control software: MassLynx (version 4.1; Waters Corporation)

25.2.7 Data Processing

1. Software for raw data processing, database searching, and label-free quantification: Progenesis QI for Proteomics version 3.0 (Nonlinear Dynamics; Waters Corporation)

25.3 Methods

25.3.1 Cell Culture (See Note 2)

1. Take off the cryogenic vial from liquid nitrogen and unfreeze quickly in a 37 °C water bath (*see Note 3*).
2. Promptly transfer the content to a centrifuge tube containing 10 mL of DMEM and centrifuge for 5 min at 1200 × g (*see Note 4*).

3. Discard the supernatant and distribute the cell pellet in a T75 cell culture flask with 25 mL of culture medium (*see Note 5*).
4. Grow MO3.13 cells at 37 °C in 5% CO_2 atmosphere.
5. Change the culture medium every 2–3 days, depending on rate of growth (*see Note 6*).
6. Remove cells from the flask using approximately 5 mL 0.25% trypsin-EDTA solution and leave for 3 min at 37 °C.
7. Transfer the cells to a centrifuge tube containing 10 mL DMEM.
8. Centrifuge for 5 min at 1,200 × g and discard the supernatant.
9. Divide the cell pellet into four to five new T75 flasks to continue cell culture growth or freeze the cells.

25.3.2 Freezing (See Note 7)

1. Prepare the number of cryogenic vials according to the amount of cells to be frozen.
2. Take the flask containing the cells and discard the medium.
3. Remove the cells from the flask by leaving in 5 mL 0.25% trypsin-EDTA for 3 min at 37 °C and then transfer the cells into a centrifuge tube containing 10 mL DMEM.
4. Centrifuge for 5 min at 1,200 × g and discard the supernatant.
5. Resuspend the cells with the target concentration of 10^5/mL of freezing medium.
6. Freeze the vials first by placing in a −20 °C freezer for 2–3 h and then into a −80 °C freezer.
7. Transfer the vials the next day into a liquid nitrogen tank (*see Note 8*).

25.3.3 Acute 8 h Treatment with MK-801 (See Note 9)

1. Dilute MK-801 in HCL solution.
2. Dilute the MK-801 solution with 15 mL DMEM to a final concentration of 50 μM.
3. Discard the medium and transfer 5 mL new medium containing 50 μM MK-801 in each flask.
4. After 8 h, collect the cells.

25.3.4 Collecting the Cells

1. Discard the culture medium.
2. Add 600 μL PBS to the flask and harvest cells by scraping them off the flask.

3. Wash the cells with 600 μL PBS 1x containing protease and phosphatase inhibitors (optional).
4. Collect cells into 15 mL falcon tube and pellet them by 5 min centrifugation (1,200 x g).
5. Remove supernatant and snap-froze the cell pellet containing lysis buffer in liquid nitrogen until further sample preparation.

25.3.5 Lysis, Reduction, Alkylation, and Digestion (See Note 10)

1. Lyse the cells by adding 100 μL of lysis buffer containing protease and phosphatase inhibitors to the MO3.13 pellet (*see Note 11*).
2. Stir well and incubate for 2 h at 37 °C (*see Note 12*).
3. After incubation, dilute the sample 10x with 20 mM TEAB pH 7.5 and sonicate on ice (*see Note 13*).
4. Add 100 μL 200 mM iodoacetamide in 20 mM triethylammonium bicarbonate to achieve final concentration of 20 mM iodoacetamide, and incubate the sample for 20 min in the dark at room temperature.
5. After incubation, digest the sample using 5.5 μL of 2% trypsin at an enzyme/substrate ratio of 1:50 overnight (12–16 h) at 37 °C.
6. To stop the reaction, add 100% FA to a final concentration of 5% and leave for 5 min at room temperature.
7. Centrifuge for 45 min at 14,000 × g at 4 °C to remove pellet lipids and other vestiges.
8. Transfer the supernatant to another tube (*see Note 14*).

25.3.6 Desalting and Concentration of Peptides

1. Use 0.1% TFA to dilute the peptide sample achieving a final volume of 1 mL and adjust the pH to 2.0.
2. Wash the cartridge with 1 mL activation solution 1 followed by 1 mL of activation solution 2 (*see Note 15*).
3. Balance the cartridge twice using 2 mL of 0.1% TFA.
4. Load the sample onto the cartridge slowly and collect the flow through (FT).
5. Apply the FT again slowly to the same cartridge.
6. Wash the cartridge twice with 1 mL 0.1% TFA.
7. Elute the peptides into a new microtube with 1 mL RP elution solution.
8. Dry the sample in a concentrator or lyophilizer.
9. Reconstitute the sample in 20 mM ammonium formate (pH 10).

25.3.7 NanoLC-MS/MS Analysis (See Note 16)

1. Create the LC-MS/MS method.
2. Load samples containing 500 ng protein into a M-Class HSS T3 column.
3. Set peptide elutions using ACN gradient from 7% to 40% for 90 min at a flow rate of 0.4 μL/min directly into a Synapt G2-Si HDMS.
4. Use 100 fmol/μL [Glu1]-fibrinopeptide B as a lock mass compound, and use the auxiliary pump to deliver it to the reference sprayer of the NanoLock Spray source at 0.5 μL/min.
5. Turn MS acquisition on in the LC software and align the gradient with the beginning of MS acquisition.
6. Perform MS analysis in DIA mode using ion mobility separation and CID fragmentation.
7. Ramp the transfer cell collision energy from 25 to 55 eV in the elevated energy scan.
8. Perform triplicate LC-MS analysis of each sample (*see Note 17*).

25.3.8 Data Processing

1. Perform initial signal processing of continuum LC-IMS-MS^E data using Progenesis QI for Proteomics, and create a new project for your experiment.
2. Add the acquired raw data files to the project.
3. Provide lock mass m/z as 785.8426 to perform the calibration.
4. Set up processing parameters: MSe experiment, 150 counts for the low energy threshold, 50.0 counts for the elevated energy threshold, and 750 counts for the intensity threshold.
5. Import the data.
6. Start automatic processing, selecting for automatic alignment of the runs (*see Note 18*).
7. Proceed with automatic peak picking using 8 as the maximum ion charge, and adjust the sensitivity method of the automatic peak picking algorithm to 4.
8. Define your experiment design (optional).
9. Set the parameters for automatic peptide identification: choose your target-decoy database for peptide and protein identification; trypsin should be selected as the digestion enzyme; and one missed cleavage can be allowed.
10. Set carbamidomethyl of cysteine and oxidation of methionine as fixed and variable modifications, respectively.
11. Choose relative quantitation using Hi-N and three peptides to measure per protein.
12. Use protein grouping.

25.4 Notes

1. Make sure that all materials are ready for use. For example, the medium should already be at the right temperature (37 °C).
2. All the solutions and buffers should be prepared with Milli-Q water (UHQ), analytical grade reagents, and highest purity chemicals. Organic solutions should be prepared fresh.
3. During unfreezing, it is indicated to move the cryovial circularly inside the bath.
4. For centrifuge cells, it is better to use mobile centrifuge rotor angle.
5. Depending on the number of cells, it is possible to divide the pellet in more than one T75 cell culture flask.
6. Cultures should be split at ~90% confluency.
7. It is recommended to freeze a few aliquots of the cells promptly after the initial growth/split to help maintain a stock of the cell line.
8. It is recommended to test the cells for regrowth after freezing to be sure that the freezing procedure was performed correctly.
9. It is recommended to grow MO3.13 cells until a confluency of 90% is reached in T25 cell culture flasks to initiate the treatment. It is also suggested to treat the cells in triplicate and ensure that everything is clean.
10. Topics 3.5 and 3.6 are optimized versions of Melo-Braga et al. [15].
11. The lysis buffer volume used depends on the pellet size. Here we used 10^7 cells.
12. Mix by vortexing and pipetting the cell pellet up and down. The cell lysate will form a viscous solution due to the presence of DNA.
13. Probes/tips must be submerged properly into the solution for efficient sonication. If the tip is not submerged enough, the sample will foam or bubble. If the tip is too deep, it will not circulate the sample effectively. Foaming can also be caused when the amplitude setting on the sonication device is too high. In addition, the tip must not touch the sides of the tube to avoid releasing plastic into the sample.
14. It is better use microtubes with low protein retention (i.e., LoBind from Eppendorf or Maxymum Recovery from Axygen).
15. The option of cartridges depends on the quantity of material. For peptide samples with quantity higher or equal to 500 µg, the Oasis HLB cartridges are usually a good choice.
16. Before starting a gradient for peptide separation, make sure that your LC system is set up properly and that you use freshly prepared and degassed solvents. Set and keep the temperature of the sample manager at a constant temperature of 6 °C, while samples are stored therein.
17. This is carried out to increase accuracy and reproducibility.
18. Use this to assess all runs in the experiment for suitability.

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Chapter 26
Combining Patient-Reprogrammed Neural Cells and Proteomics as a Model to Study Psychiatric Disorders

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26.1 Introduction

The molecular mechanisms underlying the pathophysiology of psychiatric disorders such as schizophrenia and major depressive disorders still remain a conundrum. Consequently, over the last years, there have not been improvements regarding treatment effectiveness [1]. One reason for this is that most of the studies on psychiatric disorders have been conducted on post-mortem tissues, hindering observations of the disorder in living cells [2] and making it impossible to investigate brain tissue in the different developmental stages of disease [3].

Ten years ago, Kazutoshi Takahashi and Shynia Yamanaka developed a technique for the generation of pluripotent cells by genetically reprogramming somatic cells from both mice [4] and humans [5]. Reprogramming occurs due to transduction of four transcription factors (oct-4, sox-2, Klf-4, and c-Myc). These cells have been

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named induced pluripotent stem cells (iPSCs), which are capable of generating cells from all three embryonic layers once they enter this pluripotent state [6] and have thus been used as models of multiple human diseases [7, 8]. For the study of brain diseases such as psychiatric disorders, the iPSC approach also helps to overcome the impractical and poor accessibility of neural cell types within the human brain [3]. Another great improvement provided by iPSCs is the possibility of studying developing human neural cells before they reach a mature stage. This characteristic is particularly important for studying diseases such as schizophrenia, which are believed to have a neurodevelopmental component. For example, neural progenitor cells (NPCs) can give rise to radial glia and neuronal populations, prior to formation of glial and neuronal subtypes [9]. The study of iPSCs also makes it possible to study the relationship between genotype and phenotype, taking into consideration the physiological status of each patient at the time of sampling [9, 10]. The capability of investigating disease-specific mechanisms prior to and during onset allows the detection of molecular signatures of the disease in living cells. This allows the potential for identifying molecular targets for development of novel biomarkers and pharmacological treatments [11].

The combination of iPSC technology with proteomics is recent and makes it possible to study proteins in a given cellular stage and also at a subcellular level. Different studies have shown that alterations in mitochondrial physiology contribute to the onset, progression, and treatment response of patients with psychiatric disorders, making proteomic studies of such organelles particularly useful. These mitochondrial changes include morphological deformation, reduction in number and density, dysfunction of the oxidative phosphorylation system, and altered mitochondrial-related gene expression [12]. Thus, the possibility of studying the proteome of patient-derived cells at the organelle level could bring insights into psychiatric disease mechanisms and lead to new perspectives regarding potential biomarkers and drug response.

Here, we present a cell culture protocol for differentiation of human iPSCs or embryonic stem (ES) cells into NPCs followed by an approach for subcellular proteomics.

26.2 Materials (See Note 1)

1. Microtubes 1.5 mL maximum recovery (Axygen Scientific; Radnor, PA, USA)
2. 5–25 mL serological pipettes
3. 15 and 50 mL conical centrifuge tubes
4. Sterile transfer pipette

26.2.1 PSC Differentiation to NPCs

1. Human ES or iPS cells
2. Culture medium: Essential medium 8 (Thermo Fisher Scientific; Waltham, MA, USA), mTeSR™1 feeder-free cell culture medium (StemCell Technologies; Vancouver, Canada), or similar (*see Note 2*)

3. 75 cm^2 cell culture-treated flasks
4. 60 and 100 mm cell culture-treated dishes
5. Cell lifter and scraper
6. Embryoid body (EB) formation medium: Dulbecco's Modified Eagle Medium (DMEM)/Nutrient Mixture F-12, 20% KnockOut Serum Replacement (KSR) (*see Note 3*), 1% L-glutamine, 1% penicillin/streptomycin, 1% non-essential amino acids (NEAA), 100 μM β-mercaptoethanol (Thermo Fisher Scientific)
7. Neural progenitor cell medium: DMEM/F12, with 0.5x N-2 supplement (*see Note 4*), 0.5× B-27 supplement (*see Note 5*), 20 ng/mL fibroblast growth factor (FGF), and 20 ng/mL epidermal growth factor (EGF) (Thermo Fisher Scientific)
8. Phosphate-buffered saline (PBS) without calcium and magnesium
9. Accutase (Merck-Millipore; São Paulo, SP, Brazil)
10. Poly-ornithine (Sigma-Aldrich; São Paulo, SP, Brazil)
11. Laminin (Thermo Fisher Scientific)
12. Dorsomorphin (Sigma-Aldrich)
13. Y-27632 Rho-associated kinase (ROCK) inhibitor (Merck-Millipore) (*see Note 6*)

26.2.2 Subcellular Fractionation and Protein Extraction

1. Teflon-glass Dounce homogenizer
2. Fixed-angle centrifuge capable of 7000 × g
3. Ultrasonic homogenizer
4. Vivaspin 6 centrifugal concentrators (Sartorius AG; Goettingen, Germany)
5. 2.5× homogenization buffer: 525 mM mannitol, 175 mM sucrose, 12.5 mM Tris-HCl, 2.5 mM EDTA (pH 7.5) (*see Note 7*)
6. Hypotonic buffer: 10 mM NaCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl (pH 7.5)
7. Protein extraction buffer: 7 M urea, 2 M thiourea, 4% Chaps, 70 mM dithiothreitol (DTT)
8. 2% cOmplete ULTRA Tablets protease inhibitor cocktail (Sigma-Aldrich)

26.2.3 Tryptic Digestion Procedure and Sample Preparation for LC-MS^E

1. Qubit® assay tubes (Thermo Fisher Scientific)
2. Qubit® protein assay kit (Thermo Fisher Scientific)
3. 0.2% RapiGest SF solution (Waters Corporation; Milford, MA, USA) (*see Note 8*)
4. 50 mM ammonium bicarbonate
5. 100 mM DTT(*see Note 9*)
6. 300 mM iodoacetamide (IAA)

7. Sequencing grade modified porcine trypsin (Promega; Madison, WI, USA)
8. 1 N ammonium formate
9. 5% trifluoroacetic acid

26.3 Methods

26.3.1 iPSC Differentiation to NPCs (See Note 10)

1. Change medium 1 day before iPSCs are ready for passage with EB medium.
2. On the day of embryoid body formation, start by washing the plate once with 5 mL PBS and incubate for 5 min at 37°C.
3. Aspirate PBS, add 1 or 2 mL Accutase, and incubate at 37°C (*see Note 11*).
4. To harvest cells, aspirate Accutase and add fresh EB medium containing 2 μM dorsomorphin (*see Note 12*) and 10 μM ROCK inhibitor.
5. Lift cells using a cell lifter.
6. To form self-aggregate EBs, aspirate cell clumps off the plate, using a 5 mL serological pipette.
7. Transfer clumps to a low attachment petri dish and incubate at 37 °C (*see Note 13*).
8. Change media the next day removing dead cells (*see Note 14*).
9. Change medium every day for 6 days to formation of EBs (Fig. 26.1) and proceed to plating.
10. On day 7, plate EBs on matrigel by first transferring them to a 15 mL conical tube and letting them decant (*see Note 15*).
11. Remove the old medium and add 1 mL neural progenitor cell medium containing EGF and FGF (*see Note 16*).
12. Dissociate the EBs, pipetting up and down using a 1 mL tip.
13. Transfer the dissociated EBs to a 100 mm dish containing matrigel and 6 mL of neural progenitor cell medium.
14. Wash the tube with 1 mL medium and transfer to the dish.
15. On the next day, change the medium and remove dead cells (*see Note 17*).
16. Change medium every 2 days (*see Note 18*).
17. Following formation of rosettes (Fig. 26.1), wash the plate with 5 mL PBS, add 2 mL Accutase, and incubate 3 min at 37 °C.
18. Harvest cells with PBS and centrifuge at 200 × g for 5 min.
19. Add fresh NPC medium and transfer the dissociated rosettes to a new dish coated with 100 μg/mL poly-ornithine and 1 μg/mL of laminin.
20. Complete the volume with 8 mL fresh medium per dish (*see Note 19*).
21. Change NPC medium every 2–3 days.
22. Grow to confluence up to 1 week.
23. Passage NPCs using 2 mL Accutase (100 mm dish) for 2–5 min at 37 °C (*see Note 20*).

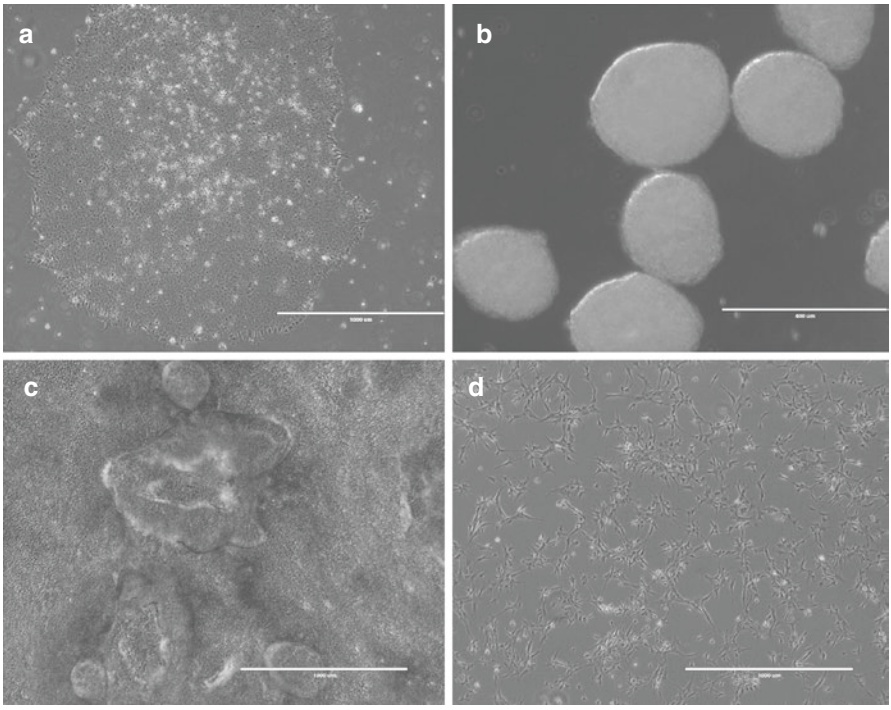

Fig. 26.1 Different stages of iPSCs culturing toward the generation of neural progenitor cells. (**a**) A colony of iPSCs. (**b**) Embryoid bodies. (**c**) Rosette formation. (**d**) Neural progenitor cells

24. Remove cells with PBS and centrifuge at 200 × g for 5 min.
25. Split NPCs to new dishes containing poly-ornithine and laminin at a 1:3 or 1:4 ratio, using NPC medium (*see Note 21*).
26. For harvesting cells, discard the medium, add PBS, and scrape cells off the plate using a scraper (*see Note 22*).
27. Collect the cells into a 15 mL tube and pellet them by 5 min centrifugation at 200 × g.
28. Discard the supernatant and retain the pellet.
29. Proceed to immediately subcellular fractionation or store the pellet at −80 °C for further use.

26.3.2 Subcellular Fractionation and Protein Extraction

1. Add 5 mL ice-cold hypotonic buffer to the pellet and allow the cells to swell for 5–10 min.
2. Place the swollen cells into a pre-chilled Dounce glass tube.

3. Break open the swollen cells using a tight-fitting Teflon pestle by slowly stroking the pestle up and down (*see Note 23*).
4. Transfer the homogenate to a centrifuge tube, rinse the homogenizer with 3.6 mL 2.5× homogenizer buffer, and add it to the homogenate.
5. Rinse the homogenizer again with a small amount of 1× homogenizer buffer and add it to the homogenate.
6. Place the tube in a precooled rotor and centrifuge at 2,100 × g for 5 min.
7. Decant and save the supernatant in a clean centrifuge tube for isolation of mitochondria and cytosol.
8. Save the pellet on ice (*see Note 24*).
9. For the isolation of mitochondria, centrifuge the tube at 7,000 × g for 15 min.
10. Save the pellet as the mitochondrial fraction and the supernatant is the cytosol.
11. For a cleaner isolation of mitochondria, wash this fraction by resuspending the pellet in 1× homogenizer buffer, and repeat the 7,000 × g sedimentation.
12. Save the pellet as the final mitochondria fraction.
13. Use a Vivaspin 6 centrifugal concentrator to reduce the volume of the cytosol fraction until approximately 10% of the initial volume is attained (*see Note 25*).
14. Add 50 µL protein extraction buffer to the subcellular fractions and sonicate on ice.
15. Centrifuge for 10 min at 20,800 × g at 4 °C and collect the supernatant (*see Note 26*).

26.3.3 Tryptic Digestion and Sample Preparation for LC-MS^E Analysis

1. Perform protein quantification and place 50 µL of 1 µg/µL sample in a capped microcentrifuge tube (*see Note 27*).
2. Add 10 µL 50 mM ammonium bicarbonate.
3. Add 25 µL 0.2% solution RapiGest SF and vortex.
4. Place tube in a block heater set at 80 °C for 15 min.
5. Centrifuge briefly to collect all fluid at the bottom of the tube.
6. Add 2.5 µL 100 mM DTT and vortex.
7. Place tube in a block heater set at 60 °C for 30 min.
8. Allow cooling to room temperature and centrifuge as above.
9. Add 2.5 µL 300 mM IAA and vortex.
10. Place sample in dark at room temperature and allow 30 min reaction time.
11. Add 5 µL sequencing grade trypsin solution and vortex.
12. Allow to digest at 37 °C overnight.
13. Following the digestion, hydrolyze the RapiGest by adding 10 µL 5% TFA and vortex.
14. Incubate samples at 37 °C for 90 min.

15. Centrifuge the samples at 14,000 × g, 6 °C for 30 min.
16. Transfer the supernatant to a Waters Corporation Total Recovery vial.
17. After transferring the supernatant, adjust the pH by adding 5 µL 1 N ammonium hydroxide for effective trapping on the first dimension column.
18. Adjust the final volume if necessary with 20 mM ammonium formate (pH 10).
19. Carry out nanoLC-MS/MS analysis (*see Note 28*).

26.4 Notes

1. All solutions and buffers should be prepared with Milli-Q water (UHQ), analytical grade reagents, and the highest purity chemicals. All plastic materials should be of the highest quality, and organic solutions should be prepared freshly prior to use.
2. We use the mTeSR™1 medium and the presented protocol is optimized for this. However, it is possible to use other such media, but some optimization may be required.
3. KnockOut Serum Replacement is a more defined, fetal bovine serum (FBS)-free medium supplement for growth of PSCs cultured on fibroblast feeder cells. It is designed to replace FBS in existing protocols.
4. N-2 supplement is typically used for growth of neuroblastomas and postmitotic neurons in primary culture, as well as in studies of NPCs.
5. B-27 has been optimized as a serum-free supplement to support growth and viability of CNS neurons.
6. In human embryonic stem cells, Y-27632 Rho-associated kinase (ROCK) inhibitor treatment helps to decrease dissociation-induced apoptosis, increases cloning efficiency, and facilitates subcloning after gene transfer as well as cell survival and differentiation.
7. The buffer should be ice cold before use.
8. RapiGest surfactant (SF) is used to enhance enzymatic digestion of proteins by making them more susceptible to cleavage without inhibition of enzyme activity.
9. DTT is used in tryptic digestion experiments for the reduction of intra- and intermolecular disulfide bonds in proteins. Reduction is typically followed by alkylation of the free sulfhydryl residues used as reagent such as IAA.
10. Human pluripotent stem cells (ES or iPSCs), cultivated on matrigel or feeder layer, should be 70–80% confluent, showing dense colonies with defined borders (Fig. 26.1a).
11. This is for a 60 mm and 100 mm dish, respectively. The cells should separate easily from each other within the colonies.
12. Dorsomorphin inhibits potentially interfering AMP-activated protein kinases.
13. Overnight, the clumps should aggregate and form EBs.

14. Tilt the plate and allow all formed EBs to gather together at a corner of the dish. Gently remove most of the old medium with a pipette, and replace the medium with EB medium containing 1 μM dorsomorphin and 1 μM ROCK inhibitor.
15. Let the EBs decant but not for too long because the cell debris will also start to precipitate.
16. The growth factors should be added immediately prior to use.
17. A flat dissociation will generate better rosettes, and the EB cells should migrate out of clumps.
18. The NPCs will migrate for 3–5 days.
19. This stage represents the first NPC passage.
20. The cells should completely detach.
21. This is a procedure for preparation of precursor cells only. For differentiation in the various neuronal cells, it typically requires removal of growth factors from the culture medium for several days, in addition to various other conditions depending on neuronal cell type [14, 15].
22. For the subcellular fractionation protocol, a minimum of 5×10^6 cells is necessary.
23. The degree of homogenization can be monitored with a phase-contrast microscope. Naked nuclei, smaller organelles, and a small number of unbroken cells (large spheres with a granular appearance) should be present if cell lysis is successful.
24. The pellet contains dense organelles such as nuclei.
25. For extraction of proteins present in the cytosol fraction, it is necessary to reduce the volume of this fraction.
26. Be careful not to collect the lipid layer located on top of the supernatant.
27. We use the Qubit assay system for protein quantitation, but other methods can be used depending on reagent compatibility.
28. This analysis can be carried out in many ways, including as described previously [13].

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Chapter 27
SILAC Mass Spectrometry Profiling: A Psychiatric Disorder Perspective

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27.1 Introduction

Despite decades of research, the disease mechanisms underlying mental illness are not completely understood. The main hypotheses have focussed on perturbations in neurotransmitter systems that regulate mood, behaviour and cognition, and currently prescribed psychiatric medications mostly target these systems [1]. However, diseases such as schizophrenia [2, 3], major depressive disorder [4], bipolar disorder [5] and autism spectrum conditions [6, 7] have also been associated with peripheral deficits in metabolism including dyslipidaemia, hyperinsulinaemia and type 2 diabetes. For example, perturbations of the cholesterol system and lipid transport proteins have been described in most psychiatric disorders [8]. Although such effects can result from currently available medications, they have also been observed in some patients at first onset before any medications have been administered [9, 10]. Thus peripheral cells involved in regulation of metabolism as well those from central neuronal systems are now under intensive investigation as a means of identifying novel biomarkers and drug targets for improved diagnosis and treatment of individuals affected by these disorders [11]. It is also important to know the proteomic responses of such cells to current or novel medications [12].

Stable isotope labelling by amino acids in cell culture (SILAC) is a mass spectrometry (MS)-based proteomic profiling method that has been used to identify proteomic changes in various experimental models [13]. It is a quantitative method based on labelling of cellular proteomes using two sets of amino acids that have been tagged with different molecular isotopes (e.g. ^{13}C and ^{14}C) [14]. This generates a mass difference between two proteomes, creating light and heavy versions of each peptide that can be distinguished and quantitated by mass spectrometry (MS) analysis [14, 15]. The classical version of SILAC involves growing cells in media with natural "light" or "heavy" amino acids for several days to allow virtually complete incorporation of the mass-tagged amino acids into the respective cellular proteomes. Pulsed SILAC (pSILAC) is a variation of this method that can be used to investigate biosynthetic regulation of proteins [16, 17]. This approach is based on growing two distinct cell cultures in the presence of normal amino acids and prior to challenging the system with a specific compound, changing media of both cell cultures with media containing medium-heavy and heavy amino acids, respectively. Both cell cultures are then cultivated for a short period of time after the challenge to investigate the compound effects on protein translation. Both methodologies, SILAC and pSILAC, allow monitoring the possible differences in de novo protein production after the respective treatment.

Here, we present a detailed protocol for identification and quantification of protein translational changes between two cultured cell populations. These could be, for example, cultured primary cells from animal models of a psychiatric disorder or cell lines cultured in the presence or absence of a specific pharmacological agent. Examples of cell lines that could be utilized include those modelling pituitary cells

(e.g. AtT-20) [18], adipocytes (e.g. 3T3-L1) [19] or pancreatic beta cell lines (e.g. INS1) [20]. In a typical experiment, cells are grown in two different culture dishes containing standard medium, which contains normal light amino acid (Lys0). After an appropriate incubation period and before treatment with the pharmacological agent, the Lys0 standard medium is replaced with one containing either an intermediate (Lys4) or heavy (Lys8) amino acid in the cultures. Following the treatment with the agent, protein extracts from both cell populations are combined and subjected to sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). After this, the protein bands are excised and enzymatically digested with an enzyme such as trypsin, and the resulting peptides are analysed by liquid chromatography tandem MS (LC-MS/MS). The ratio of heavy and medium-heavy peptides reflects differences in translation of the corresponding proteins as a consequence of the two different culture conditions (e.g. ± pharmacological agent) (Fig. 27.1).

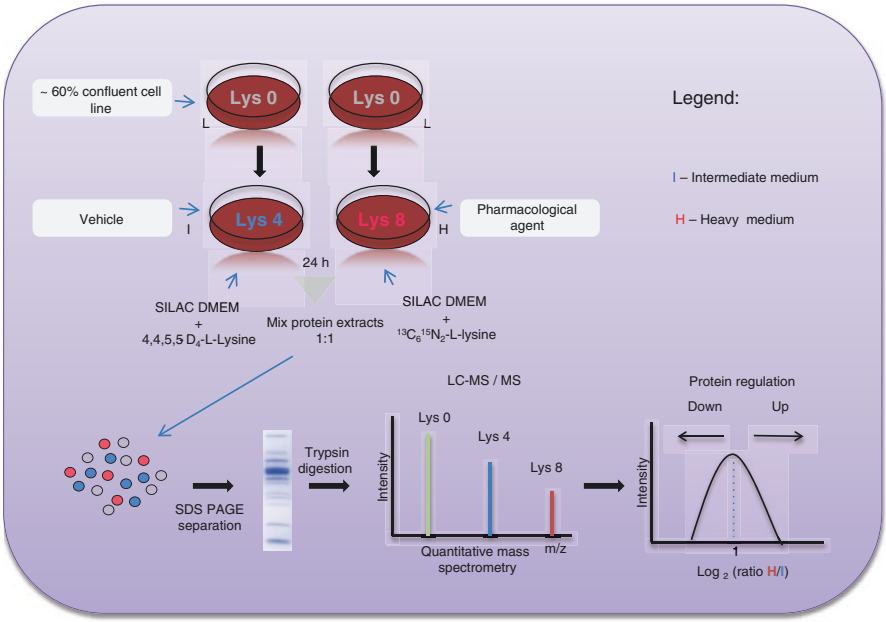

Fig. 27.1 pSILAC experimental workflow. Cells are cultured in standard media containing lysine [Lys 0; *green font*] in two cell culture dishes. Next, the standard media from both cell populations are subsequently replaced with SILAC medium containing either intermediate lysine [4,4,5,5-D_4-L-lysine (Lys4); *blue font*] or heavy lysine [$^{13}C_6{}^{15}N_2$ -L-lysine (Lys8); *red font*] and treated with the vehicle and the pharmacological reagent (compound x), respectively. After a 24 h incubation, proteins from both cell populations are extracted, combined and subjected to a SDS-PAGE separation, trypsin digestion and the resulting peptides analysed by LC-MS/MS. The ratio of the heavy and intermediate peptides reflects differences in newly translated corresponding proteins as a result of the pharmacological agent. The light peptides are ignored as these arise from the total cellular protein population

27.2 Materials (See Note 1)

27.2.1 Reagents, Buffers and Media

1. L-arginine (Arg0) in phosphate buffered saline, pH 7.4 (PBS)
2. 4,4,5,5-D_4 L-lysine-2 HCl (Lys4) in PBS (*see Note 2*)
3. $^{13}C_6{}^{15}N_2$ L-lysine-2 HCl (Lys8) in PBS
4. Dulbecco's Modified Eagle's Medium (DMEM), 10% antibiotic-free calf serum (heat-inactivated)
5. SILAC-DMEM minus Lys and Arg
6. Dialysed foetal bovine serum (FBS) (*see Note 3*)
7. Lys4 SILAC-DMEM: 0.08 mg/mL L-Arg, 0.15 mg/mL Lys4, 10% FBS(10%) in 500 mL 0.22 μm filter-sterilized SILAC-DMEM (*see Note 4*)
8. Lys8 SILAC-DMEM: L-Arg (0.08 mg/mL), 0.15 mg/mL Lys8, 10% FBS in 500 mL 0.22 μm filter-sterilized SILAC-DMEM

27.2.2 Cells and Culture Materials

1. Cell line or cultured primary cells of choice (*see Note 5*)
2. Sterile 10 cm cell culture dishes (*see Note 6*)
3. 15 mL Falcon tubes (*see Note 6*)
4. 1.5 mL microcentrifuge tubes (*see Note 6*)
5. Trypsin (cell culture quality for cell dissociation)
6. Pharmacological agent and vehicle control as appropriate (*see Note 7*)
7. RIPA buffer: 25 mM Tris-HCl (pH 7.6), 150 mM NaCl, 1% NP-40, 0.1% sodium 3α, 12α-dihydroxy-5β-cholan-24-oic acid (SDS)
8. 100× stock Halt™ protease and phosphatase inhibitor (Thermo Fisher Scientific; Waltham, MA, USA)
9. BCA protein assay kit or similar
10. 0.125–1.0 mg/mL bovine serum albumin (BSA) protein standards

27.2.3 SDS-PAGE Reagents

1. Sample loading buffer
2. SDS-PAGE gel and solutions (*see Note 8*)
3. Stain: 1 mg/mL filter-sterilised Coomassie Blue R250, 40% methanol (CH_3OH) and 10% acetic acid (CH_3CO_2H)
4. Destain: filter-sterilized 20% CH_3OH and 10% CH_3CO_2H

27.2.4 In-gel Digestion Solutions

1. 100 mM ammonium bicarbonate (NH_4HCO_3)
2. 100 mM NH_4HCO_3, 50% acetonitrile (CH_3CN)
3. Reduction agent: 100 mM NH_4HCO_3, 10 mM dithiothreitol (DTT) (*see Note 9*)
4. Alkylation agent: 100 mM NH_4HCO_3, 20 mM iodoacetamide (IAA) (*see Note 9*)
5. Trypsin: 12.5 µg/mL MS grade porcine trypsin in 20 mM NH_4HCO_3 (*see Note 10*)
6. 1% formic acid (HCO_2H)

27.2.5 HPLC Solvents, MS Analysis Equipment and Data Processing

1. MS solvent: 0.1% trifluoroacetic acid ($C_2HF_3O_2$), 3% CH_3CN
2. Buffer A: 0.1% HCO_2H
3. Buffer B:100% CH_3CN
4. 100 Å, 5 µm, 180 µm × 20 mm C18 Symmetry Trap and 130 Å, 1.7 µm, 75 µm × 250 mm C18 BEH analytical columns (Waters Corporation; Milford, MA, USA)
5. NanoACQUITY UPLC (Waters Corporation)
6. High-resolution MS instrument (*see Note 11*)
7. MaxQuant (v1.5.3.30) (www.maxquant.org)

27.2.6 Data Analysis

1. Microsoft Office Excel (https://www.office.com/), R (https://www.r-project.org) and in conjunction with Bioconductor (http://www.bioconductor.org)

27.3 Methods

27.3.1 Cell Culture and Amino Acid Incorporation

1. Seed cells in two dishes in a 10 mL DMEM medium (or other appropriate medium) and incubate overnight at 37°C under 5% CO_2/95% air (*see Note 12*).
2. Remove the medium from one dish and replace with a 10 mL pre-warmed (37°C) Lys8 SILAC medium.

3. Add pharmacological agent to this dish and incubate for 24 h at 37°C under 5% CO_2/95% air.
4. Remove the medium from the other dish and replace this with a 10 mL pre-warmed (37°C) Lys4 SILAC medium.
5. Add vehicle to this dish and incubate 24 h at 37°C under CO_2/95% air (*see Note 13*).

27.3.2 Protein Extraction

1. Add trypsin solution to the dishes to remove the cells.
2. Wash the recovered cells by centrifugation in ice-cold PBS at 750 × g for 5 min at 4°C in a 15 mL tube.
3. Gently suspend pellets in PBS and repeat the centrifugation as above.
4. Resuspend cells in 10 mL PBS (ice-cold) and transfer 5 mL aliquots to 15 mL tubes on ice.
5. Pellet cells by centrifugation as above.
6. Add a 250 µL ice-cold RIPA buffer containing protease and phosphatase inhibitors to one cell aliquot.
7. Sonicate samples on ice with a probe sonicator (10 × 3 sec pulses, 50% power).
8. Leave on ice 5 min, and centrifuge at 13,000 × g to remove cell debris.
9. Transfer the supernatants to fresh tubes (*see Note 14*).
10. Quantify the amount of protein using a BCA assay kit.
11. Combine 50 µg protein from pharmacological agent-treated cells with 50 µg of protein from vehicle-treated cells.

27.3.3 SDS-PAGE and Preparation of Gel Pieces for Trypsinization

1. Add a 4× sample buffer to the extracted protein mixture to make a 1× final concentration.
2. Subject the samples to electrophoresis.
3. Stain the gel in Coomassie Blue reagent for approximate 2 h.
4. Destain the gel overnight on a rocking table and take a photo when bands are visualised.
5. Slice the gel lanes into horizontal pieces and cut these further to produce 1 mm^3-sized pieces using a scalpel blade (*see Note 15*).
6. Wash the gel pieces in 1 mL water for 15 min.
7. Centrifuge at 750 × g for 5 min and discard the supernatant.
8. Add 300 µL of CH_3CN and leave for 15 min.
9. Centrifuge as above and discard the supernatant.
10. Wash the pieces with 300 µL 100 mM NH_4HCO_3 for 15 min.
11. Centrifuge as above and discard the supernatant.
12. Wash the pieces with 300 µL 100 mM NH_4HCO_3 containing 50% CH_3CN for 15 min.

13. Centrifuge as above and discard the supernatant (*see Note 16*).
14. If the gel is still blue, repeat steps 10–13.
15. Once the gel pieces are clear, wash as above with 100 µL CH_3CN for 5 min (*see Note 17*).
16. Centrifuge as above and discard the supernatant.
17. Dry the pieces in a sterile laminar flow hood for 15 min.
18. Add 50 µL reducing solution and incubate 1 h at 56°C.
19. Centrifuge as above and discard the supernatant.
20. Add a 50 µL alkylation solution and incubate 30 min at room temperature.
21. Centrifuge as above and discard the supernatant.
22. Wash the pieces twice with 300 µL 100 mM NH_4HCO_3 for 15 min.
23. Centrifuge as above and discard the supernatant.
24. Wash the pieces with 300 µL 100 mM NH_4HCO_3 containing 50% CH_3CN for 15 min.
25. Centrifuge as above and discard the supernatant.
26. Add 100 µL CH_3CN and incubate for 5 min.
27. Centrifuge as above and discard the supernatant.
28. Dry the gel pieces as above for 15 min.

27.3.4 Trypsin Digestion

1. Add a 30 µL digestion solution, and leave for 30 min at room temperature (*see Note 18*).
2. Add 20 mM NH_4HCO_3 so that it just covers the gel pieces.
3. Incubate at 30°C for at least 16 h (*see Note 19*).
4. Add an equal volume of CH_3CN and incubate at 30°C for 30 min.
5. Centrifuge and transfer the supernatant containing the digests to a fresh tube.
6. Add 50 µL 1% HCO_2H and incubate for 20 min at room temperature.
7. Centrifuge as above and add the supernatant to the same tube as above.
8. Repeat steps 4–7.
9. Add 50 µL CH_3CN and incubate for 10 min (*see Note 20*).
10. Centrifuge and add the supernatant to the same tube as above giving a final volume of 200–400 µL.
11. Dry samples under vacuum at 60°C (*see Note 21*).
12. Suspend the peptide pellets in 50 µL 1% formic acid.
13. Mix gently at room temperature for 30 min and store at -80°C.

27.3.5 LC-MS/MS and Data Processing (See Note 22)

1. Thaw samples and centrifuge 10 min at 13,000 × g.
2. Gently transfer aliquots of the supernatant to an MS tube.
3. Dry the tryptic peptides to virtual completion in a vacuum concentrator.
4. Suspend the semi-dry pellets in a 10 µL MS solvent.

5. Analyse using buffer A and B over the desired linear gradient range by LC-MS/MS.
6. Process raw MS files in parallel using MaxQuant.
7. Search the data against the International Protein Index database for the relevant species using Mascot Daemon and/or Andromeda software.

27.4 Notes

1. All solutions should be made using ultra-pure water. It is important to work under sterile conditions, and all solutions should be made fresh.
2. These stock solutions can be stored at −20°C. A number of companies such as Cambridge Isotope Laboratories, Thermo Fisher and Life Technologies provide SILAC kits, which usually contain only one heavy amino acid. Therefore, most of these lack a second heavy amino acid, which is essential for pSILAC experiments. We therefore purchase the Lys4 and Lys8 reagents separately. There is a 4 Da mass difference between the resulting intermediate and heavy peptides, enabling identification and quantification via MS analysis.
3. Dialysed foetal bovine serum should be used in pSILAC experiments. It should be noted that some cell lines show altered growth patterns in SILAC media because of the lack of growth factors in dialysed serum. Therefore, it is recommended to determine whether or not growth of the selected cells is affected by the medium during optimisation experiments.
4. The amount of arginine and lysine added to SILAC medium was established based on their respective concentrations in the standard medium we use. These may need optimization for different media.
5. Cell lines should be chosen according to experimental aim.
6. Dish size should be chosen according to the cell line because it is important to extract at least 100 μg protein from each sample (50 μg for LC-MS and 50 μg for validation assays).
7. It is important to optimise the correct dose and incubation time for the pharmacological agent and to use the appropriate vehicle control.
8. Precast gels are the best option, but it is possible to use home-made gels if necessary. When making homemade gels, prepare fresh stocks of all solutions.
9. Prepare this solution fresh on the day of use. Approximately 200 μL will be needed per gel slice.
10. Prepare 40 μL of this solution for each gel slice fresh on the day of use.
11. We use the LTQ Orbitrap XL (Thermo Fisher Scientific), but other similar models would be suitable.
12. Optimization of cell number for the cell type being used will be required. For the 3T3L1 adipocyte cell line, we seed 0.4×10^6 cells in a 10 mL dish. One dish should be designated for each condition being studied. Cells should reach approximately 60% confluence for the intermediate and heavy SILAC media incubation step. Thus, the starting amount of cells should be defined in order to

attain this. The period of culture in the presence of the pharmacological agent should also be optimised.
13. We run at least 4 independent biological replicates.
14. For each sample, it is important to save at least 50 µg protein extract (prior to trypsin digestion) for validation studies (e.g. by Western blot or immunoassay analyses).
15. Use a new scalpel blade for each original slice to avoid contamination.
16. You should notice that the gel pieces shrink during this step.
17. You should notice that the gel pieces shrink and turn white during this step.
18. The gel pieces should be restored to their original sizes in this step.
19. After 30 min, check to see if there is enough solution covering the gel pieces. If not, more should be added. It is important to make a note of how much buffer was added in total to each set of gel pieces.
20. The gel pieces should shrink and turn white again.
21. This could take 2–3 h.
22. There are many protocols for this which will vary depending on the experimental aims [21–24].

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Chapter 28
Preparation of Peripheral Blood Mononuclear Cells (PBMCs) as a Model for Proteomic Studies of Psychiatric Disorders

Hassan Rahmoune and Paul C. Guest

28.1 Introduction

Schizophrenia has now been studied using proteomic approaches in more than 250 studies (October 2016 PubMed search using the terms SCHIZOPHRENIA and PROTEOMICS). Early studies using these technologies were focused mainly on post-mortem brain tissues, which resulted in identification of altered protein profiles associated mainly with synaptic function and energy/metabolism pathways [1–3]. However, at least some of these effects may be confounded by factors such as anti-psychotic drug treatment, poor diet and unhealthy life style that are associated with chronic stages of the disease [4, 5]. Thus, studies of living patients could be more informative, although this is made difficult by the fact that obtaining brain tissues for such investigations is not possible.

One option that has been explored in an increasing number of publications is the study of peripheral tissues and body fluids from living patients. Although schizophrenia is mainly considered as a brain disease, it is now clear that this disorder can also be manifested at the proteome level in the periphery. This can be explained by the fact that instead of working alone, the brain works in harmony with the rest of the body to carry out most of its functions [6]. Looking at peripheral tissues also has the added benefit of enabling the availability of relevant biosamples for use in clinical and drug discovery studies [7, 8].

A number of investigations have now been carried out using peripheral blood mononuclear cells (PBMCs) taken from patients and controls as a potential cellular

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model of psychiatric diseases. These cells are also likely to be useful models for high-throughput drug screening approaches, considering that they contain many of the same receptors and corresponding signalling pathways that are found in brain tissues [9, 10]. Herberth and co-workers carried out liquid chromatography tandem mass spectrometry (LC-MS^E) analysis of PBMCs from first onset schizophrenia patients who had never been treated with antipsychotics at the time of sample collection [11]. Of the 18 proteins that they found to be differentially expressed, 8 were part of the glycolytic pathway, recapitulating some of the effects on this pathway seen in studies of post-mortem brain tissues from patients with psychiatric disorders, as described above. A similar study acquired PBMCs from bipolar disorder patients and carried out LC-MS^E analysis to identify altered protein networks [12]. This analysis found changes mostly associated with cytoskeletal and stress response proteins.

Here we present a protocol for preparation of PBMCs from whole blood along with culture of these cells to provide the conditioned media and cellular extracts for use in proteomic profiling studies.

28.2 Materials

1. Blood samples collected into 9 mL EDTA S-Monovette tubes (Sarstedt: Leicester, UK)
2. Dulbecco's phosphate-buffered saline (DPBS) (Invitrogen; Paisley, UK)
3. Ficoll-Paque Plus (density 1.077 g/mL) (GE Healthcare; Little Chalfont, UK)
4. Sterile 15 and 50 mL Falcon tubes
5. Centrifuge with swinging bucket rotor
6. Cell storage medium: 10% DMSO/90% fetal bovine serum (FBS)
7. Thawing medium: RPMI 1640 (Sigma-Aldrich; Poole, UK), supplemented with 10% fetal calf serum, 1% glutamine, penicillin, streptavidin and DNase
8. Stimulation medium: thawing medium without DNase containing 1 mg/mL staphylococcal enterotoxin B (SEB; Sigma-Aldrich) and 1 mg/mL CD28 (BD Bioscience; Oxford, UK)

28.3 Methods

1. Warm the Ficoll-Paque to room temperature and ensure that it is mixed to homogeneity by repeated inversion (*see Note 1*).
2. Add 15 mL Ficoll-Paque to a 50 mL tube (*see Note 2*).
3. Dilute 8 mL blood with an 8 mL DPBS (*see Note 3*).
4. Gently layer the above mixture on the top of the Ficoll solution (*see Note 4*).
5. Centrifuge the tube at 750 × g for 30 min at room temperature (*see Note 5*).
6. Remove as much of the upper plasma layer as possible using a clean pipette (*see Note 6*).

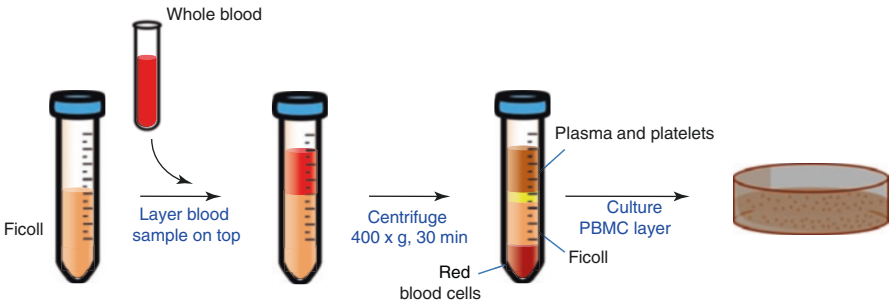

Fig. 28.1 Schematic showing experimental protocol for isolation of PBMCs from whole blood

7. Collect PBMCs from the plasma/Ficoll interface with a sterile pipette (Fig. 28.1) *(see Note 7)*.
8. Transfer the cells into a new sterile 50 mL Falcon tube.
9. Add 30 mL DPBS and centrifuge and centrifuge at 350 × g for 5 min (*see Note 8*).
10. Suspend the cell pellet gently in 10 mL DPBS and add this to a sterile 15 mL Falcon tube.
11. Centrifuge at 350 × g for 5 min.
12. Remove the supernatant and suspend the cell pellet gently in 10 mL DPBS.
13. Repeat steps 9–12 and freeze cells under liquid nitrogen in a cell storage medium (*see Note 9*).
14. Suspend cells in a thawing medium and culture overnight at 37°C in 5% CO_2 (*see Note 10*).
15. The next morning suspend 7×10^6 cells in either a thawing (without DNase) or stimulation media (*see Note 11*).
16. Culture at 37°C for 72 h in 5% CO_2.
17. Collect cell media and pellets by centrifugation at 1,000 × g for 4 min at 4°C.
18. Centrifuge the media at 13,000 × g for 4 min.
19. Remove the media and store at −80°C (*see Note 12*).
20. Washed the pellets from step 17 twice with ice-cold DPBS by centrifugation at 1,000 × g.
21. Remove the media and store at −80°C before use (*see Note 13*).

28.4 Notes

1. The Ficoll solution may settle into heterogeneous density layers in cold storage.
2. The amount of Ficoll depends on the volume of blood to be added.
3. Similar media can be used here such as a culture medium containing FBS.
4. Care should be taken to minimize mixing of blood with the Ficoll-Paque layer.

5. Centrifugation should be carried out with the brake in the off position to decrease chances of unwanted layer mixing during deceleration.
6. Again, care should be taken so that the layers below are not disturbed.
7. For a 50 mL tube, the volume of collected cells should be 6–8 mL. PBMCs are found at the interface between the plasma and the Ficoll-Paque layer as these have intermediate density between the plasma/DPBS and the Ficoll-Paque and therefore form a tight cushion between these two layers (Fig. 28.1). The bottom layer contains red blood cells, which have a higher density.
8. This is a washing step.
9. The user can proceed immediately to an experimental stage as we do here. Immediate use of PBMCs may be a better option than freezing to ensure maximum cell survival in subsequent steps.
10. Culturing overnight allows cell recovery after the isolation and/or freeze/thawing procedure.
11. The aim of this basic experiment was to determine the effects of stimulation on protein levels.
12. The media will contain molecules such as cytokines which have been differentially released from the cells under stimulatory and non-stimulatory conditions. This can be analysed by a method such as multiplex immunoassay [11, 13], although a concentration step may first be required to improve sensitivity of detection.
13. A number of proteomic analyses can be carried out here to determine effects of stimulation on cellular proteins, such as LC-MS^{E} and two-dimensional gel electrophoresis (2DE). Previous studies have shown that several components of the glycolytic pathway are differentially regulated by stimulation in PBMCs from schizophrenia patients and controls [11].

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Chapter 29
Proteomic Profiling of Skin Fibroblasts as a Model of Schizophrenia

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29.1 Introduction

Schizophrenia affects approximately 1% of the world's population. The disease is characterized by psychosis, hallucinations, disordered thoughts, negative feelings and cognitive deficits [1]. Accordingly, most of the research into this disease has focused on identifying abnormalities within the central nervous system. However, several groups have also detected differences in peripheral systems in schizophrenia patients including immunological abnormalities [2–5], decreased levels of circulating transport proteins [5, 6], altered hypothalamic-pituitary-adrenal (HPA) axis function [7–10], perturbations in growth factors [5, 11], increased coagulation capacity [12, 13] and increased levels of insulin-related peptides [14–16]. Such findings support the hypothesis that there is a systemic biochemical defect in schizophrenia that affects the whole body and not just the brain [17, 18].

Many proteomic studies have been carried out using post-mortem brain tissue, with the aim of gaining insights into the pathophysiological pathways affected in schizophrenia. However, these have several limitations including potentially poor protein integrity after lengthy post-mortem intervals and confounding factors linked with the chronic nature of the disorder, such as unhealthy lifestyles of the patients and their long-term treatment with antipsychotic medications [19, 20]. Since many molecular pathways in neuronal cells are also present in other cells of the body, the study of

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easily accessible peripheral cells can circumvent the problems associated with post-mortem tissue and provide a useful model for investigating the disorder. Furthermore, such cells can be obtained from living patients at different stages of the disease.

With this in mind, studies of fibroblast cultures have identified changes in cell growth and morphology [21], as well as decreased cellular adhesion [22] and altered apoptosis, or programmed cell death [23]. Many of these effects may be analogous to the growth and movement of neurons required in synaptic growth and plasticity. Here, we present a standard protocol for preparation and cell culture of fibroblasts, along with preparation of cellular protein extracts which can be used for proteomic profiling studies. We also present a cell proliferation assay and Western blot method for validation of the model.

29.2 Materials

29.2.1 Cell Culture

1. Skin biopsies (*see Note 1*)
2. Culture medium: RPMI 1640 (Sigma; Poole, Dorset, UK), containing 10% fetal calf serum (FCS) and 1% L-glutamine/penicillin/streptomycin solution
3. Step-down medium: RPMI 1640 containing 1% FCS
4. 75 cm^2 culture flasks
5. Detachment media: 0.25% Trypsin, 0.02% ethylenediaminetetraacetic acid (EDTA)
6. MycoFluor™ Mycoplasma Detection Kit (Thermo Fisher Scientific; Loughborough, UK)

29.2.2 Cell Proliferation Assay

1. Phosphate buffered saline (PBS)
2. ^{3}H-thymidine (GE Healthcare; Little Chalfont, UK) (*see Note 2*)
3. 5% trichloroacetic acid (TCA)
4. 5% TCA/95% ethanol
5. Scintillation liquid and scintillation counter

29.2.3 Western Blot Analysis

1. Total cell extraction buffer: sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer

2. Sonifier and microprobe
3. SDS-PAGE running equipment (*see Note 3*)
4. Semi-dry Western blotting apparatus and polyvinylidene fluoride (PVDF) membranes (*see Note 4*)
5. Blocking solution: PBS, 0.1% Tween 20, 5% skimmed milk powder
6. Wash solution: PBS, 0.1% Tween 20
7. Primary antibody: rabbit anti-calpain 1(Abcam; Cambridge, UK) (*see Note 5*)
8. Species-specific secondary antibodies: horseradish peroxidase-conjugated anti-rabbit antibody
9. ECL reagents (GE Healthcare)
10. ECL Hyperfilm (GE Healthcare)

29.2.4 Mass Spectrometry Profiling

1. ProteoExtract Subcellular Proteome Extraction Kit (S-PEK) (Merck-Millipore; Watford, UK)
2. Detergent compatible DC protein assay (Bio-Rad; Hercules, CA, USA)
3. Exchange buffer: 50 mM ammonium bicarbonate (pH 8)
4. Spin columns for buffer exchange
5. Split-less nano-Ultra Performance Liquid Chromatography system (10 kpsi nanoAcquity; Waters-Corporation; Milford, MA, USA)
6. Quadrupole time-of-flight mass spectrometer (Q-Tof Premier; Waters-Corporation)
7. Glu-Fibrinopeptide B reference peptide
8. ProteinLynx Global SERVER (PLGS; Waters-Corporation)
9. Statistical analysis: R software package (www.r-project.org)

29.3 Methods

29.3.1 Fibroblast Culture

1. Grow tissue in culture medium in 75 cm^2 flasks for 2 weeks at 37°C under 5% CO_2/95% air, changing the medium every 3 days (*see Note 6*).
2. Add detachment medium to remove cells.
3. Cultivate further using a starting density of 5×10^5 cells in 75 cm^2 flasks.
4. Add detachment medium when cells reached 80–90% confluence and continue culturing by changing the media every 3 days.
5. Harvest cells on the final day after reaching 80–90% confluence.
6. Test the cultures periodically for infection using the Mycoplasma Detection Kit.

29.3.2 Proliferation Assay

1. Plate 2.5×10^4 fibroblasts in 12-well plates in culture medium and culture for 24 h at 37°C in 5% CO_2/95% air.
2. Wash twice with 0.5 mL PBS and then incubate in step-down medium for 10 h at 37°C in 5% CO_2/95% air.
3. Pulse label cells with 0.037 MBq ^{3}H–thymidine for 24 h (*see Note 7*).
4. Wash the cells and culture for a further 7 days in medium without ^{3}H–thymidine.
5. Wash cells twice in PBS and then add 1 mL of 5% TCA and leave the cells at 4°C for 2 h (*see Note 8*).
6. Wash cells twice with 5% TCA/95% ethanol and leave to dry for 15 min at 37°C.
7. Add 0.5 mL 0.2 M NaOH for a 3 h incubation at 37°C.
8. Add the mixtures to 5 mL vials containing 3.5 mL scintillation liquid and leave overnight.
9. Determine incorporation of ^{3}H-thymidine into the DNA using a liquid scintillation counter (Fig. 29.1) (*see Note 7*).

29.3.3 Western Blot Analysis

1. Extract proteins from harvested cells by brief sonication in SDS-PAGE loading buffer and heating at 95°C for 5 min.
2. Separate 20 µg protein on SDS-PAGE gels (*see Note 3).*
3. Transfer proteins from the gel onto a PVDF membrane by semi-dry electrophoresis (*see Note 4*).
4. Incubate the membrane for 1 h in a blocking solution.
5. Rinse the membranes three times for 5 min in wash buffer.
6. Incubate with 1:1,000 calpain 1 antibody overnight at 4°C.
7. Rinse the membrane three times for 5 min in wash buffer.

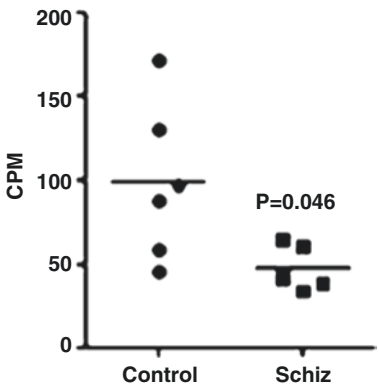

Fig. 29.1 Scatter plot showing ^{3}H–thymidine incorporation into fibroblasts from control (n=6) and schizophrenia (schiz; n=5) subjects after a 1-day pulse and 7-day chase incubation. The P-value was calculated using a two-tailed t test

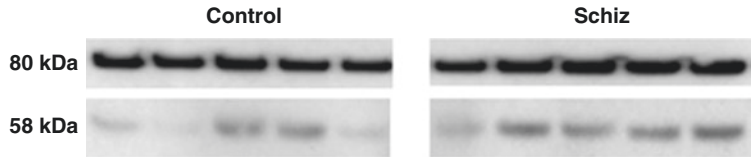

Fig. 29.2 Western blot analysis showing the levels of immunoreactive calpain 1 in fibroblasts from control ($n = 5$) and schiz ($n = 5$) subjects

8. Incubate the membrane with 1:1,000 HRP-conjugated mouse antibody in PBS for 90 min at room temperature.
9. Visualize the immune complexes using the ECL kit and by subsequent exposure to Hyperfilm (Fig. 29.2) (*see Note 9*).

29.3.4 LC-MS^E Profiling (See Note 10)

1. Extract proteins using the ProteoExtract kit to generate a soluble protein fraction (*see Note 11*).
2. Determine protein concentrations using the DC protein assay.
3. Remove contaminating salts and mild non-ionic detergents by buffer exchange into 50 mM ammonium bicarbonate using spin columns.
4. Carry out trypsin digestion as described previously [24].
5. Carry out one-dimensional separation of peptides using the10kpsi nanoAcquity chromatography system coupled online to the Q-Tof Premier for MS^E analysis as described previously [24].
6. Infuse the Glu-Fibrinopeptide B reference compound using the LockSpray and scan every 30 s to maintain mass accuracy.
7. Process raw data using PLGS (*see Note 12*).
8. Set the following parameters for protein detection:
   (i) Ions should be detected in at least 2 out of 3 injections of each sample.
   (ii) Ions should be present in at least 60% of the samples.
   (iii) Protein identification should be based on at least two peptides.
   (iv) Set the maximum random false identification rate to 4%.

29.4 Notes

1. Ensure that all documents from the appropriate institutional ethical review board are in place for the study of human tissues and for data protection. Also, ensure that all participants provide written agreement to participate in the study.
2. The ^{3}H–thymidine is incorporated into nascent DNA strands in dividing cells as an indication of the proliferation rate.

3. For a good range of high to low molecular weight separation, we suggest the use of gradient gels such as 4–12% NuPAGE Novex Bis-Tris gels (Invitrogen; Paisley, UK)
4. We suggest the use of PVDF membranes for the electrotransfer step for efficient transfer and to maximize retention of proteins.
5. Calpain 1 is involved in cell growth and is therefore a biomarker of proliferating cells.
6. These conditions favour growth of fibroblasts.
7. Previous studies have shown that incorporation of ^{3}H-thymidine is lower in samples from schizophrenia patients compared to controls [24].
8. This leads to precipitation of the DNA.
9. The figure shows increased levels of the smaller chain (58 kDa) of calpain 1 in fibroblasts from schizophrenia patients compared to controls [24].
10. A variety of MS-based methods could be used here. We present the basic set up using the LC-MS^{E} approach as described previously [24].
11. This kit separates the total lysate into four cellular fractions: cytosolic, membrane, nuclear and cytoskeletal. Only the cytosolic fraction was analysed in this study because this contains the highest number of abundant soluble proteins detectable by mass spectrometry.
12. PLGS produces quantitative and qualitative information automatically according to user-defined thresholds [25].

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Chapter 30
Proteomic Profiling of the Pituitary Gland in Studies of Psychiatric Disorders

Divya Krishnamurthy, Hassan Rahmoune, and Paul C. Guest

30.1 Introduction

In humans, the pituitary is located at the base of the brain and consists of two regions known as the anterior and posterior lobes [1]. The anterior lobe contains distinct cells which produce vital hormones such as prolactin, proopiomelanocortin (POMC) and growth hormone along with several others. The posterior lobe consists mostly of neuronal projections from the hypothalamus region of the brain and produces the hormones oxytocin and arginine vasopressin. The secretion of these hormones into the bloodstream is regulated by distinct stimulatory and inhibitory systems in the process of maintaining bodily homeostasis [2].

Pituitary abnormalities have been implicated in various pathological conditions, including psychiatric disorders [3–5]. Higher levels of hormones such as corticotropin-releasing hormone, arginine vasopressin, the POMC-derived peptide adrenocorticotropic hormone (ACTH) and cortisol have been reported in cases of schizophrenia and other psychiatric diseases [6–9]. Functional magnetic resonance imaging (MRI) studies have also found larger pituitary volumes in first-episode patients with psychosis or schizophrenia spectrum disorders [3, 10].

In addition to the hormones, the different cellular systems of the pituitary contain distinct combinations of enzymes and other accessory proteins involved in prohormone trafficking, processing and packaging into nascent secretory vesicles. These include the endoproteases prohormone convertase 1 (PCSK1) [11] and PCSK2 [12],

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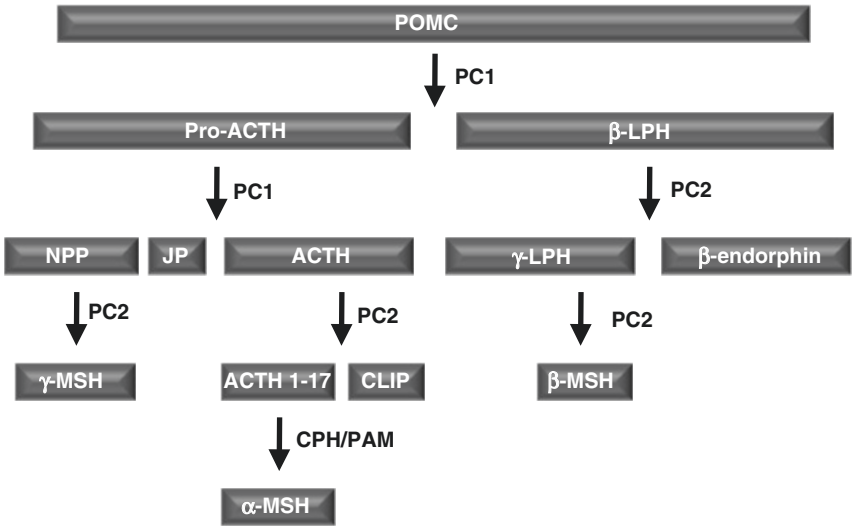

Fig. 30.1 Diagram showing the proteolytic maturation of proopiomelanocortin to multiple bioactive peptides. *ACTH* adrenocorticotrophic peptide, *CLIP* corticotropin-like intermediate peptide, *JP* joining peptide, *LPH* lipotropin, *MSH* melanocyte-stimulating hormone, *NPP* N-terminal peptide of proopiomelanocortin

the exopeptidase carboxypeptidase H (CPE) [13] and the carboxy-terminal amidating enzyme peptidyl-glycine alpha-amidating monooxygenase (PAM) (example for POMC processing shown in Fig. 30.1) [14].

Given these effects, further characterization of the pituitary proteome may provide additional insights into the underlying causes and effects of these diseases. Furthermore, the fact that the hormones and many of the accessory proteins are co-released into the bloodstream under stimulated physiological conditions means that they may also serve as blood-based biomarkers in clinical studies [15]. Here, we present a protocol for preparation and extraction of proteins from this tissue to support shotgun liquid chromatography-tandem mass spectrometry (LC-MS^E) approaches for proteomic characterization. Given the reasons described above regarding co-secretion, it was of particular interest to confirm identification of the major pituitary hormones and accessory proteins, such as the prohormone-converting enzymes.

30.2 Materials

1. Post-mortem pituitary glands frozen in liquid nitrogen (*see Note 1*)
2. Alumina mortar and pestle (Sigma-Aldrich; Poole, UK)
3. Homogenization buffer 1: 50 mM Tris-HCl (pH 8.0) containing EDTA-free protease inhibitor cocktail (Merck Calbiochem; Nottingham, UK) (*see Note 2*)

4. Homogenization buffer 2: 8 M urea, 50 mM $NaHCO_3$ (pH 8.0) (*see Note 3*)
5. Sonifier and microprobe (*see Note 4)*
6. Pierce BCA assay kit (Thermo Fisher Scientific; Loughborough, UK) (*see Note 5*)
7. 50 mM $NaHCO_3$ (pH 8.0)
8. Reduction buffer: 5 mM dithiothreitol (DTT)
9. Alkylation buffer: 10 mM iodoacetamide (IAA)
10. Trypsin-digested 25 fmol/ µL yeast enolase (Waters Corporation; Milford, MA, USA) (*see Note 6*)
11. Porcine trypsin (Promega; Madison, WI, USA)
12. Nano-Ultra Performance 10 kpsi nanoAcquity liquid chromatography system (Waters Corporation)
13. NanoESI emitter (7 cm length, 10-mm tip) (New Objective; Woburn, MA, USA)
14. Quadrupole time-of-flight (QTOF) Premier mass spectrometer (Waters Corporation) (*see Note 7*)
15. Glu-Fibrinopeptide B (Sigma-Aldrich)
16. ProteinLynx Global Server (PLGS) v.2.4 (Waters Corporation)
17. Rosetta Biosoftware's Elucidator version 3.3 (Seattle, WA, USA) (*see Note 8*)
18. UniProt database

30.3 Methods

1. Grind whole frozen pituitary to powder using the mortar and pestle on dry ice (*see Note 9*).
2. Aliquot and store powdered pituitary at –80 °C.
3. Sonicate frozen tissue aliquots in homogenization buffer 1 at a 5:1 (µL:mg) buffer/tissue ratio.
4. Centrifuge at 13,000 × g for 20 min at 4 °C and collect the supernatant (soluble fraction).
5. Suspend pellet in homogenization buffer 2 and repeat sonication.
6. Centrifuge at 13,000 × g for 20 min at room temperature and collect the supernatant (insoluble fraction) (*see Note 10*).
7. Determine protein concentrations of both fractions.
8. Make up sample aliquots to 100 µg protein/100µL in 50 mM $NaHCO_3$ (pH 8.0).
9. Incubate samples in reduction buffer for 30 min at 60 °C (*see Note 11*).
10. Incubate samples in alkylation buffer for 30 min at 37 °C in the dark (*see Note 12*).
11. Add porcine trypsin at a 1:50 (trypsin/protein) ratio and incubate for 16 h at 37 °C.
12. The next day, add a further trypsin aliquot as above and continue incubation for 2.5 h (*see Note 13*).
13. Stop reactions by addition of 1.67 µL 10 M HCl.
14. Spike samples with trypsin-digested yeast enolase.

Table 30.1 Parameter settings for UniProt database searching

Fixed modifications
Carbamidomethylation of cysteines
Variable modifications
Oxidation of methionine
Phosphorylation of serine, threonine or tyrosine
Criteria for peptide identification
≥ Fragment ions/peptide
≥ Fragment ions/protein
≥ Peptides/protein
Detection in 2/3 replicates
Detection in 60% of the samples

15. Desalt samples in 97% buffer A (3% buffer B) using the trapping column on the nanoAcquity system.
16. Carry out peptide separation using the BEH nanocolumn at a flow rate of 0.3 μL/min coupled online to the mass spectrometer via the nanoESI emitter.
17. Initially use 3% B and then a gradient of 3–30% B over 90 min, 30–90% B over 25 min, 90–95%.
18. Infuse Glu-Fibrinopeptide B using a LockSpray, and scan every 30 s for external calibration.
19. Acquire data in MS^E mode (*see Note 14*).
20. Process data using PLGS and the Elucidator software for time and m/z alignment of raw MS1 data.
21. Extract aligned peaks and obtain abundance measurements by integration of time, m/z and intensity volumes (*see Note 15*).
22. Use data to search the UniProt database using the PLGS server and parameters set as shown in Table 30.1 and employing the ion accounting algorithm described previously [16] (*see Note 16*).
23. Compile a list of all identified prohormones and prohormone-converting enzymes using the above parameters as quality control step of both the biological material and the method (Table 30.2) (*see Note 17*).

30.4 Notes

1. Ensure that all documents from the appropriate institutional ethical review board are in place regarding study of human tissues and data protection.
2. This was aimed at producing a soluble protein fraction.
3. This buffer should solubilize the less soluble material such as membranous and high-molecular-weight polymerized proteins.
4. We used a Branson Sonifier 150 (Thistle Scientific; Glasgow, UK).
5. Other kits can be used, but the investigator should ensure that buffer reagents are compatible with those used in the kit.

Table 30.2 List of prohormones and converting enzymes identified

Gene	Protein name	Soluble (% coverage)	Insoluble (% coverage)
Prohormones			
7B2	Neuroendocrine protein 7B2	36	9
GHGA	Chromogranin A	80	56
COLI	Proopiomelanocortin	76	83
CSH	Chorionic somatomammotropin	64	51
FSHB	Follitropin β-chain	17	0
GALA	Galanin	43	12
GLHA	Glycoprotein hormones α-chain	32	22
LSHB	Lutropin β-chain	70	79
PCSK1N	ProSAAS	57	76
PRL	Prolactin	74	79
NEU1	Oxytocin-neurophysin 1	70	70
NEU2	Vasopressin-neurophysin 2-copeptin	70	69
SCG1	Secretogranin-1	73	71
SCG2	Secretogranin-2	80	76
SCG3	Secretogranin-3	19	4
SCGN	Secretagogin	17	28
SOM2	Growth hormone variant	71	66
SOMA	Somatotropin (growth hormone)	89	76
TSHB	Thyrotropin β-chain	63	98
VGF	Neurosecretory protein VGF	11	30
Prohormone-converting enzymes			
CPE	Carboxypeptidase H	69	69
PCSK1	Prohormone convertase 1	9	16
TPP1	Tripeptidyl-peptidase	13	0

Note: PCSK2 (prohormone convertase 2) was not identified by mass spectrometry. However, Western blot analysis showed that this enzyme was present (data not shown)

6. This was used to monitor chromatography.
7. Many other instruments can be used here. The user should ensure that the setup accounts for similar principles to those described here, regarding operating conditions and data analysis.
8. This software was used for alignment of peptide precursor ions in time and mass/charge (m/z). The use of this software increased percentage coverage of protein identifications, as described previously [17].
9. This was carried out as a means of overcoming tissue heterogeneity issues, since the pituitary is comprised of multiple secretory cell types (e.g. somatotrophes, lactotrophes, corticotrophes). The option of dissecting the tissue would lead to unwanted (in this case) enrichment of particular cell types.
10. Do not freeze or refrigerate as urea-containing solutions can precipitate under these conditions, making resuspension of the proteins difficult after thawing.

11. This reduces any disulphide bonds in proteins and converts them to free sulphhydryl groups.
12. This alkylates the free sulphhydryl groups to help minimize modification of proteins during subsequent mass spectrometry steps such as reformation of disulphide bonds.
13. This should help to more completely digest all proteins and thereby increase sequence coverage.
14. In MS^E mode, the quadrupole transfers ions to the collision cell, where the energy is cycled rapidly from low to high. Intact peptide ions are measured during low-energy scans, and peptide fragment ions are measured in the high-energy scans. The fragment ions are matched to the corresponding peptide precursor ions based on retention time, mass accuracy and other physiochemical properties [16]. In this study, the collision energy was 5 eV for low-energy scans and ramped from 17–40 eV in high-energy scans with a cycle time of 1.3 s.
15. This is achieved by normalization to the total ion current.
16. The data were also searched against a randomized database, and the maximum false identification rate was set to 4%.
17. This revealed identification of all major pituitary prohormones, given their high abundance in this tissue (this can be seen by the number of peptides identified for each in the two fractions). However, two of the major targeted enzymes, PC2 and PAM, were not identified. This was most likely due to the lower abundance of these proteins. Therefore, analysis of these enzymes may require enrichment approaches or application of other more sensitive methods such as Western blot or immunoassay analyses.

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Chapter 31
Development of an Assay for Measuring Proprotein-Conversion Activity on a Multiplex Magnetic Bead-Based Array Platform

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31.1 Introduction

Most proteomic platforms such as those based on multiplex immunoassay [1, 2] can be used for the simultaneous measurement of the levels of multiple proteins in body fluids and tissues down to low limits of detection. However, these methods do not necessarily generate information on the functional implications of a particular proteomic profile change. Increased levels of a particular protein could be associated with either increased or decreased activity of a biological pathway, depending on whether this protein is an activator or an inhibitor. In addition, it would be difficult to ascertain whether the change in the levels of a given protein is a cause or consequence of the physiological change under study. As an example, measuring the levels of an enzyme would not necessarily provide information about its activity, especially when the levels of its substrates and products are not detectable in the same system.

Several methods have now been described for detecting and measuring proteolytic activity of prohormone- and proneuropeptide-converting enzymes. For example, mass spectrometry (MS)-based methods have now been used to quantify the intact and cleaved forms of specific peptides, thereby measuring cleavage activity [3]. Furthermore, targeted MS techniques have been used for quantitation of the intact and cleaved forms of synthetic peptides after incubation with sample extracts [4]. Fluorescence-based methods have also been used for detecting the cleavage of

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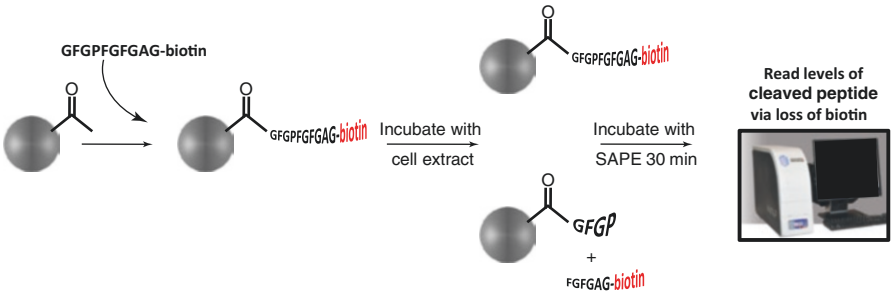

Fig. 31.1 Experimental flow diagram showing coupling of biotinylated peptide to magnetic beads, incubation with sample containing POP activity, incubation with streptavidin phycoerythrin (SAPE) and reading the sample for loss of biotinylated C-terminal fragment (cleavage after proline [P])

labelled peptide substrates [5], although such methods are not readily adaptable to a multiplex format.

This chapter describes a protocol for detection of proprotein-converting activities on a multiplex platform. As an example, a method is given for quantifying the activity of prolyl oligopeptidase (POP). This enzyme removes amino-terminal peptides from small proteins through cleavage on the carboxy-terminal side of proline residues [6]. POP is found at high levels in the brain, consistent with its role in processing of neuropeptides. In addition, circulating POP activity can be measured and has been found to be increased in psychiatric diseases such as bipolar disorder and schizophrenia [7, 8]. The POP activity assay described here is constructed using a biotin-labelled peptide substrate, covalently linked to dye-loaded magnetic microspheres (Fig. 31.1). Cleavage of the peptide is detected by measuring the release of the label in a flow cytometry-based reader [9]. This instrument allows multiplexing since it can simultaneously determine bead identity and label quantity using laser detection.

31.2 Materials

31.2.1 Tissue Extraction

1. Post-mortem pituitaries from control subjects and schizophrenia patients (*see Note 1*)
2. Alumina mortar and pestle (Sigma-Aldrich; Poole, UK)
3. Homogenization buffer: 50 mM Tris HCl (pH 8.0) containing EDTA-free protease inhibitor cocktail (100 mM AEBSF, 80 mM aprotinin, 5 mM bestatin, 1.5 mM E-64, 2 mM leupeptin, 1 mM pepstatin A) (Merck Calbiochem: Nottingham, UK) (*see Note 2*)
4. Sonifier with microprobe

31.2.2 Assay Construction

1. Polystyrene beads encapsulated with magnetite (Luminex; Austin, TX, USA) (*see Note 3*)
2. Phosphate-buffered saline (PBS)
3. Activation buffer: 50 mg/mL sulfo-N-hydroxysulfosuccinimide
4. Coupling buffer: 2-(N-morpholino)ethanesulfonic acid (MES) (pH 5.0) (*see Note 4*)
5. Biotinylated peptide: GFGPFGFGAG-biotin (*see Note 5*)
6. Blocking buffer: PBS containing 1% glycine (*see Note 6*)

31.2.3 POP Measurements

1. Assay buffer: 50 mM HEPES (pH 7.5), 250 mM NaCl, 1 mM EDTA and 1 mM dithiothreitol (DTT) (*see Note 7*)
2. Detection buffer: 4 ng/mL streptavidin phycoerythrin (SAPE) (Invitrogen; Carlsbad, CA, USA) in PBS
3. MAPIX reader and xPONENT 3.1 acquisition software (Luminex)

31.3 Methods

31.3.1 Preparation of Pituitary Extract

1. Grind frozen pituitaries using a mortar and pestle and sonicate in homogenization buffer.
2. Centrifuge at 13,000 × g for 20 min at 4 °C and retain the supernatants (*see Note 8*).

31.3.2 POP Assay

1. Wash beads in 100 μL deionized water followed by washing in 100 μL PBS.
2. Incubate beads in 100 μL activation buffer for 20 min at room temperature in the dark (*see Note 9*).
3. Collect beads using the magnetic separator and remove the supernatant (*see Note 10*).
4. Wash the beads three times in coupling buffer using the magnetic separator.
5. Incubate the beads with 500 μL biotinylated peptide in the same buffer for 2 h at room temperature in the dark (*see Note 11*).

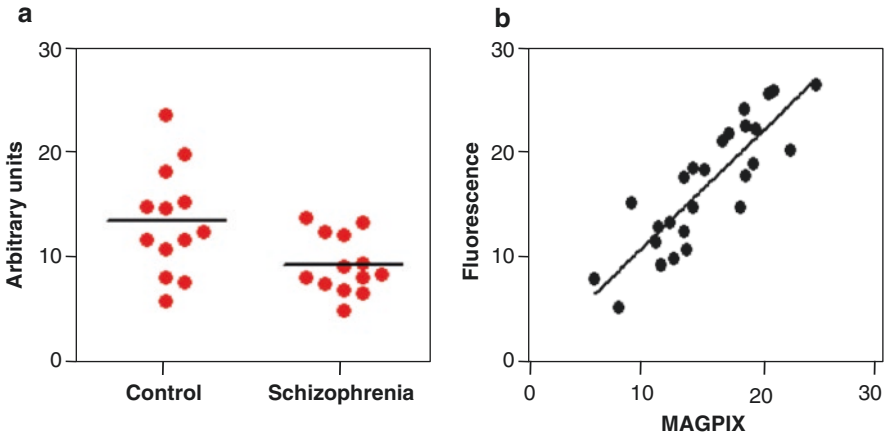

Fig. 31.2 (**a**) POP activity levels in pituitary extracts from control subjects and schizophrenia patients as determined using the new assay (approximately 30% lower activity is detected in schizophrenia samples). (**b**) Correlation ($R = 0.86$, $P < 0.001$) of POP activity as determined using the new MAGPIX assay compared to those found using a fluorescence-based assay [5, 14]

6. Isolate the beads and remove the supernatant.
7. Wash the beads two times in blocking buffer.
8. Resuspend in the same buffer and store at 4 °C prior to use.

31.3.3 Determination of POP Activity in Pituitary Extracts

1. Add 3,000 beads to each well of a 96-well microtitre plate.
2. Wash two times in PBS.
3. Add 100 mL of 1:20 diluted pituitary extract in assay buffer (*see Note 12*).
4. Incubate for 2 h at 37 °C to allow peptide cleavage to occur.
5. Wash two times in PBS.
6. Incubate with 50 µL detection buffer for 30 min at room temperature.
7. Analyse samples in the MAGPIX reader and xPONENT software to quantify cleavage of the biotinylated peptide (Fig. 31.2) (*see Note 13*).

31.4 Notes

1. Ensure that all documents are up-to-date from the appropriate institutional ethical review board regarding study of human tissues and make certain that data protection procedures are in place. Here, post-mortem pituitaries from 13 schizophrenia patients and 13 control subjects were studied. The tissues did not

differ between the two experimental groups according to age at death, pH or post-mortem interval [10].

2. None of the substances in the inhibiter cocktail have been found to inhibit POP activity in a previous study [11]. When designing other assays, the user should ensure that particular buffer components do not inhibit their target enzyme(s). For example, many prohormone-converting enzymes are Ca^{2+} dependent [12]. Therefore, in these cases, the chelation agent EDTA should not be included in the buffer.
3. Here the MAGPIX® system is used. However, the standard Luminex® system can also be used as both allow for multiplexing of assays [13].
4. MES buffer does not contain primary amines or carboxyl groups and so should not interfere with the coupling reaction.
5. This is the single amino acid code for NH_2-glycine-phenylalanine-glycine-proline-phenylalanine-glycine-phenylalanine-glycine-alanine-glycine-biotin. Biotinylated peptides can be synthesized by several different companies or in-house given a peptide synthesis facility.
6. Glycine blocks the remaining carboxyl groups on the beads.
7. The addition of EDTA and DTT has been found to increase POP activity [11].
8. This produces a soluble extract given that most prohormone-processing enzymes are not membrane associated [12].
9. This step activates carboxyl groups on the beads for coupling to molecules containing primary amines (such as peptides).
10. When using the standard Luminex system, use gentle centrifugation at this step and remove the buffer above the pellet.
11. This step involves covalent binding of the peptide primary amine to the activated carboxyl group on the bead.
12. This dilution must be worked out empirically for each specific case such that control activity falls within the midrange of a titration curve.
13. Low POP activity would be indicated by detection of a high proportion of intact peptide-biotin substrate. High POP activity would be reflected by lower intact substrate levels, due to increased cleavage and consequent loss of the biotin signal. In the example shown, POP activity was approximately 30% lower in pituitary extracts from schizophrenia patients compared to those from controls [14] (Fig. 31.2).

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Chapter 32
Phenotyping Multiple Subsets of Immune Cells In Situ in Formalin-Fixed, Paraffin-Embedded Tissue Sections

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32.1 Introduction

Mental illnesses such as schizophrenia, major depression and bipolar disorder can affect up to 25% of people worldwide at some point in their lifetime [1]. Currently, diagnosis of these diseases is based on the presence of mood, behavioural and cognitive symptoms, and this relies on a subjective clinical interview. On some occasions, physicians are guided in this process by using the Diagnostic and Statistical Manual of Mental Disorders 5 (DSM-5) [2] or the International Statistical Classification of Diseases and Related Health Problems tenth Revision [3]. However, these volumes merely qualify the symptoms of mental disorders and do not address any physical or molecular correlates of the disease, nor do they describe the underlying molecular pathways which are disturbed. To make matters worse, diagnosis can be confounded by the fact that many psychiatric disorders have similar or overlapping symptoms [4]. Furthermore, there are also a number of somatic diseases which can be misdiagnosed as a psychiatric disorder. For example, episodic hypoglycaemia induced by an insulinoma can mimic a neurological or psychiatric presentation [5–8]. In a similar manner, excessive growth hormone release or hypercortisolaemia resulting from pituitary adenomas can present with psychiatric or neurological symptoms [9–11].

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Given these issues, the availability of molecular biomarker tests that can reveal the underlying pathophysiology of psychiatric illnesses would aid diagnosis. This could also lead to a more holistic view of the underlying cause and manifestation of these diseases, which have now been established to affect multiple organ systems of the body and not just the brain [4]. Evidence for this has been seen through the identification of distinct molecular profiles in body fluids including blood serum and plasma [12–14] and in tissues such as peripheral blood cells [15–17] and skin fibroblasts [18, 19], as well as in whole endocrine tissues like pituitary glands [20, 21]. One of the most frequent findings has been fluctuations in circulating proteins which are indicative of a dysfunctional inflammatory system, as seen by elevated levels of cytokines, chemokines and acute-phase response proteins [22–24].

Given the role of the immune system in peripheral tumours, as well as neurological and psychiatric disorders, it is important to be able to understand the specific role that each immune cell type plays in situ. There has now been decades of research in this area, and thousands of immune cell types have been described [25]. Flow cytometry has been used widely in this field as one of the primary tools for differentiating the various types of immune cells [26]. This approach relies on multiplex fluorescence labelling of cellular protein markers followed by passing these one at a time through a flow chamber so that the fluorescent signal of each marker can be measured [27, 28]. The phenotype of each cell is identified through separation of the various cell types based on the fluorescence intensity of each marker combination. However, flow cytometric methods cannot be used to derive information about the distribution of the various cells in solid tissues such as tumours and endocrine glands, since it relies on analysis of single cells. In cancer immunology and immunotherapy research, such an approach is likely to be insufficient since the phenotypes in question may require studying the expression of many proteins simultaneously in multiple cell types. A good example of this is the flow cytometric definition of the markers that constitute a regulatory T cell (CD3+, CD4+, CD25 high and FOXP3+) [29]. It may also be critical to visualize the phenotypic distributions of the various immune and other cells in order to investigate how the inter-distributions of these cells relate to the functionality of a tissue or of a tumour and its microenvironment. This requires obtaining phenotypic information of cells in situ.

This chapter will describe a multispectral imaging protocol as a means of deciphering the array of molecular pathways affected in complex diseases such as cancer, neurological disorders and psychiatric conditions. Specifically, this will describe simultaneous multispectral analysis of formalin-fixed, paraffin-embedded (FFPE) tissues as a model system and will cover the staining, imaging and image analysis stages of this procedure. There are many means of obtaining a sample multiplexed with four markers. The fluorescent tyramide signal amplification (TSA) protocol described here is one of these. However, it should be noted that there are many others such as multiple quantum dots [30, 31], bright-field chromogenic immunohistochemistry (IHC) [32] and multiple species primary-secondary methods [33].

32.2 Materials

32.2.1 Required Reagents (See Note 1)

1. Primary and secondary antibodies of interest.
2. 10% neutral buffered formalin (NBF).
3. Wash solution: 0.1 M TRIS-HCl (pH 7.5), 0.15 M NaCl and 0.05% Tween®20.
4. Ultrapure, peroxidase-free water (*see Note 2*).
5. DAKO cytomation fluorescent mounting medium (*see Note 3*).
6. 4′,6-Diamidino-2-phenylindole (DAPI) counterstain (*see Note 4*).
7. Diluent/blocking reagent (PerkinElmer; Waltham, MA, USA).
8. Opal Polymer HRP Ms Plus Rb (PerkinElmer).

32.2.2 Required Materials (See Note 1)

1. Microwave oven with a carousel and ten power settings, rated at 1000–1200 W (*see Note 5*)
2. Hellendahl-type staining jars (Fisher Scientific; Pittsburgh, PA, USA) (*see Note 6*)
3. Baths and solvents for deparaffinization and rehydration of FFPE tissue (*see Note 7*)
4. Slide incubation/humidity tray
5. Hydrophobic barrier pen
6. Glass coverslips
7. Control tissues
8. Fluorophores for use in Opal/TSA multiplexed IHC labelling (*see Note 8*) (Table 32.1)
9. PerkinElmer multispectral microscope (*see Note 9*)
10. inForm® V2.0.2 image analysis software

32.3 Methods (See Note 10)

32.3.1 Controls and Development

1. Determine antibody and Opal fluorophore dilutions experimentally by titration using monoplex slides, DAPI and a single optical fluorophore (*see Note 11*).
2. Once the optimal antibody and Opal fluorophore dilutions are determined, prepare single-colour slides that do not contain DAPI (*see Note 12*).

Table 32.1 Available fluorophores for use in Opal/TSA multiplexed IHC labelling

PerkinElmer Opal fluorophores	Thermo Fisher (Life Technologies/Invitrogen) TSA fluorophores
Opal 520	TSA Alexa Fluor 350
Opal 540	TSA Alexa Fluor 488
Opal 570	TSA Alexa Fluor 546
Opal 620	TSA Alexa Fluor 555
Opal 650	TSA Alexa Fluor 568
Opal 670	TSA Alexa Fluor 594
Opal 690	TSA Alexa Fluor 647
TSA fluorescein	TSA biotin
TSA cyanine 3	
TSA tetramethylrhodamine	
TSA cyanine 5	
TSA biotin	
TSA coumarin	

32.3.2 Slide Preparation/Dewaxing

1. Place slides in 60 °C oven for at least 1 h prior to dewaxing (*see Note 13*).
2. Dewax three times with xylene for 10 min.
3. Rehydrate with ethanol through a series of ethanol solutions:
   - 100% ethanol two times for 3 min
   - 95% ethanol two times for 3 min
   - 70% ethanol one time for 3 min
4. Rinse slides with distilled water briefly twice.
5. Fix tissue in 10% neutral buffered formalin for 10 min (*see Note 14*).
6. Rinse slides in distilled water (*see Note 15*).
7. Rinse slides in wash solution for 2 min.

32.3.3 Antigen Retrieval via Microwave (See Note 16)

1. Fill the slide jars with the AR buffer and place the slides in the jar (*see Notes 17 and 18*).
2. Carry out controlled microwaving at 95 °C for 15 min, placing one slide jar at the edge of the microwave platform at a time to ensure even distribution of the microwave power using the settings below ensuring that the slides do not dry out (*see Note 19*).
3. After microwaving, let the slides cool to room temperature before proceeding (*see Note 20*).
4. Wash slides in distilled water or TBS for 2 min.
5. Wash slides in TBST for 2 min.

32.3.4 Blocking

1. Use PAP or another hydrophobic pen to completely surround the tissue section on the slide (*see Note 21*).
2. Add the antibody diluent/blocking solution to the slides for 10 mins at room temperature in a humidified chamber.

32.3.5 Primary Antibody Incubation (See Note 22)

1. Balance the overall intensity for each biomarker so that all can be spectrally unmixed using Table 32.2 as a guide (*see Note 23*).
2. Once an optimal primary concentration is determined, empirically determine dilutions of the TSA/Opal fluorophore (*see Note 24*).
3. Prepare a negative control (no primary) to determine if background levels are being affected.
4. Remove the blocking solution via pipette or aspirator and apply the primary antibody diluted in the same blocking solution used in the previous step.
5. Use enough volume (normally 100–400 mL) to cover the tissue.
6. Incubate the primary antibody according to the manufacturer's guidance (*see Note 25*).
7. Wash the slide three times for 2–5 min in TBST at room temperature with gentle agitation.

32.3.6 Secondary Antibody Incubation

1. Incubate slides in Opal Polymer HRP Ms Plus Rb for 10 min at room temperature (*see Note 26*).
2. Wash the slide three times for 2–5 min in TBST at room temperature with gentle agitation.

Table 32.2 Excitation filter and Opal/TSA reagent matching

Excitation filter	Opal/TSA reagent
DAPI	DAPI; TSA coumarin
FITC	Opal 520; Opal 540; TSA fluorescein
Cy3	Opal 570; Opal 620; TSA Cy3; TSA tetramethylrhodamine
Texas Red	Opal 570; Opal 620; TSA Cy3; TSA tetramethylrhodamine
Cy5	Opal 650; Opal 670; Opal 690; Opal Cy5

32.3.7 Opal/TSA Signal Generation

1. Remove blocking solution via pipette or aspirator.
2. Apply 100–400 μL Opal/TSA fluorophore working solution onto each slide, and incubate at room temperature for 10 min (*see Note 27*).
3. Discard any unused solution.
4. Wash the slide three times for 2–5 min in TBST at room temperature with gentle agitation.

32.3.8 Microwave Treatment

1. Repeat the microwave treatment as above taking care not to let the slides dry out.
2. After the microwave cycle, let the slides cool to room temperature (15–30 min) before proceeding.
3. Wash slides in distilled water or TBS for 2 min.
4. Wash slides in TBST 2 min.

32.3.9 Counterstaining

1. If using the Opal Kit, apply the DAPI working solution for 5–10 min (*see Note 28*).

32.3.10 Mounting

1. Wash the slide three times for 5 min each with TBST.
2. Wash the slide for 5 min in TBS.
3. Add the minimal mounting medium to cover the coverslip area (*see Note 29*).
4. Place the slide positioned at the edge of the bench.
5. Place the sample covering slip into the drop of mounting medium.
6. Turn a second coverslip 45° where the point supports the sample coverslip.
7. Slowly lower the second coverslip until it touches the microscope slide.
8. If bubbles appear slowly raise the second coverslip to help this escape.
9. When all bubbles are removed, remove the second coverslip and discard (*see Note 30*).

32.3.11 Imaging

1. Perform imaging according using a fluorescence-capable system according to the manufacturer's recommendations (*see Notes 9 and 31*).

2. Optimize the system to minimize crosstalk (*see Note 32*).
3. Minimize autofluorescence (*see Note 33*).

32.3.12 Image Analysis

1. Acquire and analyse images according to the manufacturer's instructions (*see Notes 1, 34 and 35*).

32.4 Notes

1. For further information, see the PerkinElmer Phenoptics Opal Assay Guide (http://scimed.nz/wp-content/uploads/2016/01/012447_01-GDE-Assay-Development-Guide.pdf).
2. Autoclaved Milli-Q® waterworks for this purpose. Other options should be validated.
3. Other options are available but should be validated independently. These include VECTASHIELD® HardSet Antifade Mounting Medium (Vector Laboratories; Burlingame, CA, USA) and ProLong® Diamond (Thermo Fisher Scientific; Waltham, MA, USA).
4. We find that DAPI works best undiluted. Users should validate independently.
5. Most conventional home microwaves work well in the Opal process. However, Panasonic® products with Inverter® technology have more precise power control and are recommended. Users should validate microwave performance independently.
6. These jars or something similar are important as they hold enough buffer to ensure that slides do not dry out.
7. We recommend the use of xylene for deparaffinization, and histological grade ethanol is required for rehydration.
8. TSA is created and patented by PerkinElmer and licensed to Life Technologies-Invitrogen. Opal is a rebranding of TSA.
9. Other capable PerkinElmer microscopes are required such as the Mantra Quantitative Pathology Workstation and Nuance MultiSpectral Imaging System for use with the PerkinElmer kits for five-, six- or seven-colour labelling.
10. This is based on original work by Toth and Mezey [34] and further developments by PerkinElmer [35]. Cycle through the steps until all of the biomarkers have been labelled using a different Opal/TSA fluorophore for each biomarker:

    (i) Slide preparation
    (ii) Epitope retrieval
    (iii) Blocking
    (iv) Primary antibody incubation
    (v) Introduction of horseradish peroxidase (HRP)

(vi) Signal amplification
(vii) Antibody removal via microwave
(viii) Counterstaining
(ix) Mounting

The current practical limitation is six biomarkers plus the nuclear counterstain (DAPI). There are other protocols available which use commercially available autostainers. This can be helpful as TSA multiplexing methods can take several days to complete and are labour intensive if done manually as described in this protocol. One has been published under peer review. In addition, Ventana Medical Systems, Inc. (Tucson, AZ, USA) offers multiplex fluorescent TSA staining methods that can be fully automated [36].

11. Library slide controls are required for the best spectral unmixing.
12. These slides must receive the correct number of sequential microwave cycles, so if an antibody will be used in the sixth cycle in the multiplex, it must receive six rounds of microwave in this control. Antibody sequence does not usually matter, although anecdotal evidence supports using cell surface antibodies in earlier cycles (CD4 specifically seems to be susceptible and should be used first). Use multiplex slides for data collection once all conditions have been worked out and unstained slides to account for autofluorescence and as an area size control.
13. Ensure even heating. Do not place in slide holder.
14. Alternatively, tissue can be fixed with 100% methanol plus a 10% solution made from 30% hydrogen peroxide for 20 min.
15. Alternatively, Tris-buffered saline (TBS) or phosphate-buffered saline (PBS) can be used twice for 2 min each time. Some users find improvements when all water washes are replaced with TBS or PBS washes for retention of tissues on the slides.
16. The microwave is used in this protocol to perform antigen retrieval, for quenching of endogenous peroxidase activity and for removal of antibodies after the antibody target has been labelled by the TSA probe. This step is one of the most critical processes and is made much simpler through the use of a temperature-controlled microwave such as the EZ-Retriever® from BioGenex Laboratories (San Ramon, CA, USA). This controlled microwave maintains temperature within 3° and does not start critical retrieval time until the temperature is met. It is our experience that the controlled microwave substantially improves the retrieval process and retains tissue on the slide better than a noncontrolled microwave.
17. Experimentally, determine which antigen retrieval (AR) buffer works best for your tissue samples (e.g. AR6 [pH 6] or AR9 [pH 9]). These buffers are available from PerkinElmer and DAKO, and both work well.
18. If a controlled microwave is not used, the user must determine and monitor this step closely, and the timing for each step in the procedure must be determined empirically depending on the microwave you are using.

19. Use a power setting of 100% until boiling point is reached (typically 45–90 s), and then lower power to ~20% for 15 min. Ensure that boiling or evaporation of the antigen retrieval buffer does not occur.
20. Cooling takes 15–30 min.
21. Not all PAP pens are equal. In many cases, the markings undergo autofluorescence and some wash or wear off during the multiple rounds of microwaving required in this protocol. Electron Microscopy Sciences Aqua-Hold PAP pen (Cat# 71311) is the best for this protocol, but may need to be reapplied after three or more rounds of microwaving.
22. Each colour in the multiplex IHC protocol must be optimized separately to obtain the correct concentrations for antibodies and times for incubations.
23. There are two primary controls of intensity for each biomarker, the primary antibody concentration and the dilution of the Opal/TSA fluorophore. In short, each excitation filter on the microscope will capture data for at least two biomarkers as detected by the TSA/Opal fluorophore. If you have prior IHC knowledge of the antibody clone you are using, test either the same concentration or at least 3–4 serial dilutions of this known concentration, and then prepare a negative control (no primary) to determine if background levels are being affected. The optimal concentration of any antibody or TSA/Opal fluorophore is the concentration that yields the highest signal relative to the lowest background with complete staining patterns.
24. Typically, this dilution ranges from 1:50 to 1:250 in 1× amplification diluent to create the Opal/TSA fluorophore working solution. For this, dilute the TSA/Opal fluorophore working reagent from 1:50 to 1:250 on the two most optimal primary antibody concentrations to determine if improvements in staining are recognized.
25. If not possible, try a minimum of 60 min at room temperature in a humidified chamber.
26. Recommended for experiments with human tissues and mouse or rabbit antibodies. Use enough volume to cover the tissue, typically 100–400 μL. The Thermo Fisher SuperPicture Polymer Detection Kit HRP is an alternative to this reagent.
27. As mentioned above, the fluorophore can be diluted in a range from 1:50 to 1:250 in amplification diluent to create the working solution (1:150 is typically optimal).
28. The DAPI working solution is created by adding two drops of DAPI solution into 1 mL TBST. If using your own DAPI solution, resuspend in dimethylformamide at a concentration of 5 mg/mL, dilute this solution 1:2000 in TBS and use as described above.
29. Some researchers report that 2–3× greater signal is found with Opal 650 and Opal 690, when ProLong Diamond Antifade Mounting Medium is used. Quality mounting has also been achieved using DAKO aqueous mounting medium.
30. The procedure using the second coverslip will result in a bubble-free mounting. We suggest that the user practises the skill prior to the experiment.

31. The choice of the number of high-powered fields (HPF) to analyse per slide and how many slides to analyse per biopsy is a difficult problem. This depends on the heterogeneity of a sample on a slide or across a biopsy. Furthermore, as many different filter cubes will be needed for the imagery, the system should preferably be automated. It should also be capable of scanning or imaging large areas of the sample or taking sufficient images of important regions of the slide to obtain a good representation of the sample. A high-quality, high numerical aperture objective (20–40x) is also a requirement for this kind of work. Some groups use a laser-scanning confocal imaging system to acquire the multispectral data for these kinds of multiplex IHC samples, as these may be available at most academic centres. However, these can be difficult to optimize and are used rarely for automated image collection from multiple fields from a single section. In addition, they typically do not come with autoloaders for multiple slides so they could be cumbersome to use. For further review on the subject, see [37, 38].
32. With five or more colours (i.e. with a four- or five-plex assay, plus a counterstain), it is difficult to image without crosstalk. This means that fluorescence from one filter cube will bleed into another image, causing spurious signals and incorrect results. Crosstalk can be minimized through careful selection of excitation and emission filters. However, at some point (typically around five colours), crosstalk is inevitable. This can only be corrected using an unmixing scheme [39].
33. All tissue samples, especially FFPE sections, are autofluorescent and this can sometimes mask signals. The TSA method used in this protocol is an amplification strategy, which helps to increase signals over autofluorescence. However, an unmixing scheme may also be required as above.
34. There are many generalized software packages that can analyse multiplexed IHC imagery, including Definiens™, Indica Labs™ and Visiopharm™. This protocol utilizes PerkinElmer's inForm software, which was designed specifically for phenotyping cells from multiplexed immunohistochemistry imagery. Analyses should be done according to the manufacturer's recommendations [40].
35. There are a few things to consider if using a software other than inForm to analyse multiplex immunohistochemistry imagery. The goal of analysing these kinds of data is to obtain something analogous to flow cytometry data: phenotyped and counted immune and other cells. It is easy to assume that a flow cytometry-like gating scheme would be the most appropriate way to analyse cells in a multiplexed imaging method such as this. However, since the cells in a tissue section are in physical contact and often spatially overlapping, it is generally a poor assumption to use flow-like gating, which assumes that each cell is measured independently. In addition, cells in situ need to be segmented accurately and their locations relative to other cells and to the compartmental boundaries (tumour-stroma in particular) determined. Each software package has its pros and cons relative to these needs and should be assessed individually.

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Chapter 33
Lab-on-a-Chip Proteomic Assays for Psychiatric Disorders

Harald Peter, Julia Wienke, Paul C. Guest, Nikitas Bistolas, and Frank F. Bier

33.1 Introduction

Psychiatric disorders such as schizophrenia and major depression are serious diseases which can affect individuals at different ages and seriously impair health, quality of life, social well-being and productivity. In addition, these disorders can have a significant negative impact on society and the economy, and projections show that they are likely to be the second leading cause of disability worldwide by the year 2020 [1]. This has created an urgent need in this field as fast and accurate diagnoses need to be made for a more effective therapy. For example, diagnosis of major depression in primary care settings by general practitioners has a low success rate and around 70% of these patients are undertreated [2, 3].

Currently, diagnosis of psychiatric disorders is performed by clinicians and psychiatrists through evaluation of symptoms, and this process relies on a clinical interview. Obviously the outcome of this subjective approach can vary depending on the clinician's experience, training and adherence to guidelines in the *Diagnostic and Statistical Manual of Mental Disorders 5th Edition* (DSM-5) [4] or the International Statistical Classification of Diseases and Related Health Problems 10th Revision (ICD-10) [5]. There is now an urgent need for new technologies using biomarker-based approaches that are fast, cost-effective and simple to use for the operators to help improve this process. This will also reduce the duration of illness and improve compliance of patients by helping to place them on the right treatments as early as possible.

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A number of research groups have now identified perturbations in circulating inflammation-related molecules in psychiatric disorder patients at their first clinical presentation [6, 7]. Although it has not been established whether these changes are a cause or effect of the disease, two recent studies have shown that they can occur months to years before full symptom manifestation in schizophrenia patients [8, 9]. Thus, the measurement of inflammation-related biomarkers may provide a useful aid to diagnosis in many areas of psychiatry. In line with this objective, multiplex immunoassay tests have been described for diagnosis of schizophrenia [10, 11] and major depressive disorder [12]. In addition, multiplex tests have been developed which can be used to identify individuals who are at risk of developing these disorders [8, 9, 13, 14] as well as those that show the best chances of responding positively to psychiatric medications [15, 16]. Several products have been commercialized such as VeriPsych for diagnosis of schizophrenia [18] and MDDScore for diagnosis of major depression [17] using this platform. However, these assays typically take several days for sampling, testing and readout, and can cost more than $800 for analysis of a single sample [18–20]. There are other disadvantages regarding current multiplex immunoassay protocols that hinder the broad use of this approach in routine analysis. These include relatively long experimental times and the necessity of complex laboratory-based procedures as well as the need for skilled operational personnel.

The use of lab-on-a-chip-based systems may help to overcome these limitations since they offer a rapid and automated solution, which integrates many of the lab-based procedures. Here we describe a protocol which makes use of the Fraunhofer integrated lab-on-a-chip in vitro diagnostic (ivD) platform. The platform can be used to run single or multiplex immunoassays to identify a biomarker "score" in blood samples [21]. The combination of antibody microarrays with microfluidics makes an efficient combination to automate and increase the speed of multiplex proteomic assays. The ivD platform consists of a microfluidic cartridge and a base unit. The credit card-sized cartridge contains all relevant elements necessary for attachment of antibodies targeting the protein(s) of interest. This includes reservoirs for all of the reagents, integrated pumping systems, the specific microarray and an optical transducer to allow integrated sensing (Fig. 33.1) [21–23]. The base unit contains all necessary electronics to control the cartridge, an optical readout system to analyze the results directly after binding, and a touch screen to control the assay and monitor the results. With this setup, results can be achieved in less than 15 min, yielding equivalent results as found using the corresponding single assays.

In this chapter, we present a microarray-based sandwich immunoassay and its automation in a lab-on-a-chip system, the Fraunhofer ivD platform, for the detection of C-reactive protein (CRP) due to its role in psychiatric disorders and the clinically relevant detection limits of the assay. In the first step, a polyclonal antibody against CRP is immobilized in a 10 × 10 layout on acyclic olefin polymer slide using a noncontact piezo spotter (Fig. 33.2). Further antibodies can be immobilized individually here to expand the detection parameters. Nonspecific antibodies can also be spotted to serve as negative controls. Next, the sample (containing CRP) is incubated on the slide and the sensor field is flushed with a washing buffer to remove

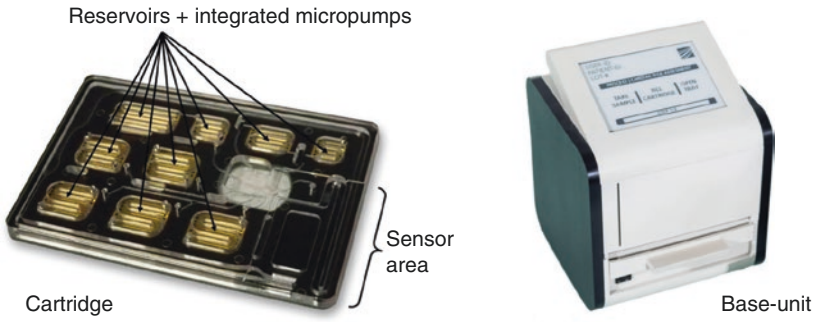


Fig. 33.1 Fraunhofer ivD platform, allowing a multiplex immunoassay analysis and readout from a single drop of blood within 12 min. (*Left*) Lab-on-a-chip cartridge (size: 60 × 40 mm) with nine reservoirs and integrated micropumps, microfluidic channels, thermal control elements, electronics, and a sensor area for microarrays with up to 400 spots. (*Right*) Base unit (size, 14 × 14 × 14 cm) for control, readout, and data analysis of the lab-on-a-chip cartridge. Results can either be transferred to a computer or analyzed directly and presented on the display

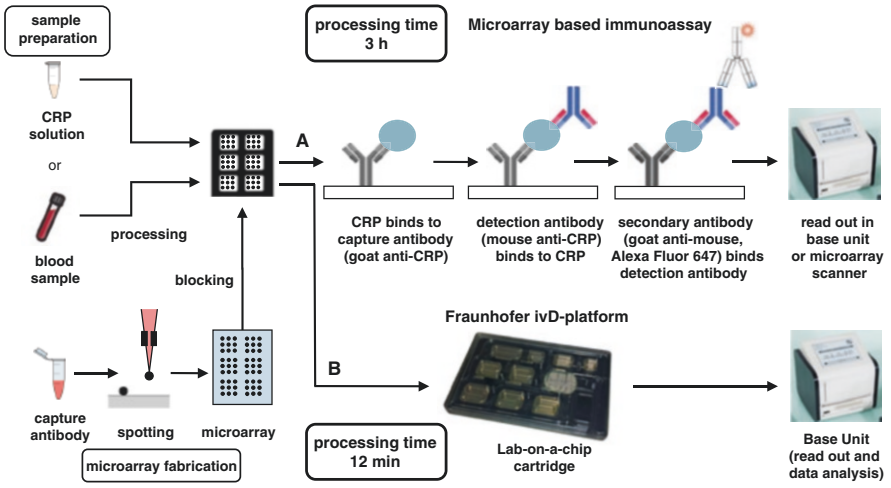


Fig. 33.2 Schematic of a microarray-based immunoassay for the detection of CRP, including sample preparation and microarray fabrication. (**a**) Laboratory-based microarray protocol, using glass slides as support for the microarray, followed by several washing steps and a readout on a microarray scanner or the Fraunhofer ivD platform base unit (3 h processing time). (**b**) Fully automated lab-on-a-chip-based immunoassay allowing a detection of CRP in blood directly after 12 min. All antibodies and washing solutions are pumped automatically over the microarray within the cartridge followed by a readout and data analysis within the base unit

nonspecifically bound molecules. A detection antibody is then washed over the microarray, which specifically binds to the analyte, followed by another washing step. Finally, a fluorescently labeled secondary antibody is pumped over the sensor field so that the concentration of the bound CRP protein can be quantified using the bound fluorescence on the optical readout unit.

33.2 Materials

33.2.1 Microarray Fabrication

1. QuadriPERM square tissue culture dish (Sarstedt, Nümbrecht, Germany)
2. Microarray spotter: sciFLEXARRAYER S11 (Scienion AG, Berlin, Germany) (*see Note 1*)
3. Epoxysilane slides: 3D epoxy polymer slides (PolyAn, Berlin, Germany)
4. Phosphate-buffered saline (PBS): 1.5 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl, and 8 mM Na_2HPO_4 (pH 7.4)
5. PBS-IT: PBS with 0.004% IGEPAL and 1% trehalose
6. Capture antibody: goat antibody to human C-reactive protein (CRP), Cat. No. K62350G (Meridian Life Science, Memphis, TN, USA)
7. Internal spotting control antibody: goat anti-rabbit IgG (H + L), Alexa Fluor 647, Cat. No. A-21245 (Life Technologies, Darmstadt, Germany) (*see Note 2*)
8. Internal control antibody: rabbit anti-goat IgG (H&L) (Min X Human serum proteins), Cat. No. 605–4113 (Rockland Immunochemicals, Limerick, PA, USA)
9. Blocking solution: 1% bovine serum albumin (BSA), 0.05% Tween 20, and 0.02% sodium azide (*see Note 3*)
10. PBS-T: PBS with 0.05% Tween

33.2.2 Immunoassay

1. QuadriPERM square tissue culture dish (Sarstedt)
2. ProPlate multi-array system (Grace Bio-Labs, Bend, OR, USA)
3. ProPlate adhesive seal strips (Grace Bio-Labs)
4. Antigen: human CRP, Cat. No. A6939,0001 (AppliChem, Darmstadt, Germany)
5. Detection antibody: monoclonal antibody to human CRP, Cat. No. M86842 M (Meridian Life Science)
6. Secondary antibody: goat anti-mouse IgG (H + L), Alexa Fluor 647, Cat. No. A-21236 (Life Technologies)
7. CRP-free human serum (Fitzgerald Industries International, North Acton, MA, USA)
8. Tris-buffered saline (TBS): 150 mM NaCl and 20 mMTris base (pH 7.4)
9. TBS-T: TBS with 0.05% Tween 20

33.2.3 Microarray Scanning

1. Laser scanner (*see Note 4*)
2. Tecan Array-Pro Analyzer (Tecan Group) or alternative quantification software

33.2.4 Automated Hybridization, Washing, and Readout (Fraunhofer ivD Platform)

1. Fraunhofer ivD cartridge (Fraunhofer IZI-BB, Potsdam; BiFlow Systems, Chemnitz, Germany)
2. Fraunhofer ivD platform (base unit)

33.2.5 Microarray Data Analysis

1. Spreadsheet software such as Microsoft Excel (Microsoft, Redmond, WA, USA)

33.3 Methods

33.3.1 Microarray Fabrication

1. Dilute the capture antibody to 0.1 mg/mL in PBS-IT.
2. Dilute the internal spotting control and the internal control to 0.075 mg/mL in PBS-IT.
3. Pipette 30 µL of spotting solutions into designated wells of a 384-well microtiter plate.
4. Use a noncontact spotter to print the probes onto epoxy-coated slides, setting the number of dots to three, the volume per dot to approximately 0.5 nL, and the grid to 500 µm distance between the spots (*see Note 5*).
5. Shortly after the spotting process, the slides must be kept in a humid chamber with saturated NaCl at room temperature and protection from light (*see Note 6*).
6. To inactivate free epoxy groups, the slides need to be blocked directly before performing the immunoassay as indicated in the next seven steps (*see Note 7*).
7. Let the slide dry for about 30–60 min at room temperature, protected from light and dust.
8. Immerse the slide in blocking solution for 30 s with fast up and down movements (*see Note 8*).
9. Incubate the slide in blocking solution for about 1 h at room temperature while shaking, protected from light.
10. Wash the slides for 10 s in TBS-T with fast up and down movements.
11. Afterward wash the slide in TBS-T, TBS-T, and TBS each for 10 min at room temperature while shaking, protected from light.
12. Wash the slide 5 s in H_2O with fast up and down movements.
13. Remove liquids carefully from the slide with a flow of nitrogen (*see Note 9*).

33.3.2 Immunoassay (See Note 10)

1. Prepare serial dilutions from the CRP antigen ranging from 100 µg/mL to 1 ng/mL in a 1:2 series either in human blood serum or blocking solution (*see Note 11*).
2. The following steps can be carried out to either measure an unknown blood sample or the samples from the calibration curve.
3. Assemble the ProPlate multiarray system on the slides.
4. Transfer 70 µL from each CRP dilution or blood sample to be analyzed into the wells ensuring that no air bubbles form.
5. Seal the chamber with adhesive seal strips, and incubate for 1 h at room temperature while shaking slightly (*see Note 12*).
6. Prepare 10 µg/mL detection antibody (mouse anti-CRP) and 10 µg/mL secondary antibody (goat anti-mouse, Alexa Fluor 647) in blocking solution shortly before use.
7. Perform the following washing procedures between the incubation periods by removing the previous solution each time with a multichannel aspirator.
8. Wash the arrays three times for 30 s with 250 µL TBS-T while shaking slightly.
9. Add 70 µL detection antibody into each well ensuring that no air bubbles form.
10. Seal the chamber with adhesive seal strips, and incubate for 1 h at room temperature while shaking slightly.
11. Wash the arrays three times for 30 s with 250 µL TBS-T while shaking slightly.
12. Add 70 µL secondary antibody into each well ensuring that no air bubbles form.
13. Seal the chamber with adhesive seal strips, and incubate for 1 h at room temperature while shaking slightly.
14. Wash the arrays three times for 30 s with 250 µL TBS-T while shaking slightly.
15. Remove the ProPlate multiarray system from the slides, and wash directly for 10 s in TBS and for 5 s in H_2O with fast up and down movements.
16. Remove liquids carefully from the slide with a flow of nitrogen without allowing droplets to dry on the surface.
17. For fluorescence image acquisition, image the slides using a laser scanner at 633 nm with the corresponding filter and an appropriate PMT/gain setting depending on the signal-to-noise ratio.
18. Quantify the fluorescent signals with the software provided from the scanner manufacturer.

33.3.3 Automated Immunoassay Procedure with the Fraunhofer ivD Platform

1. Prepare the CRP solution or blood sample to be analyzed as described above and transfer it to the corresponding sample reservoir of the ivD cartridge (*see Note 13*).

2. Insert the cartridge into the ivD platform base unit and start the immunoassay program.
3. The fluorescence image data can either be analyzed automatically within the base unit or exported for external analysis (*see Note 14*).

33.3.4 Data Analysis

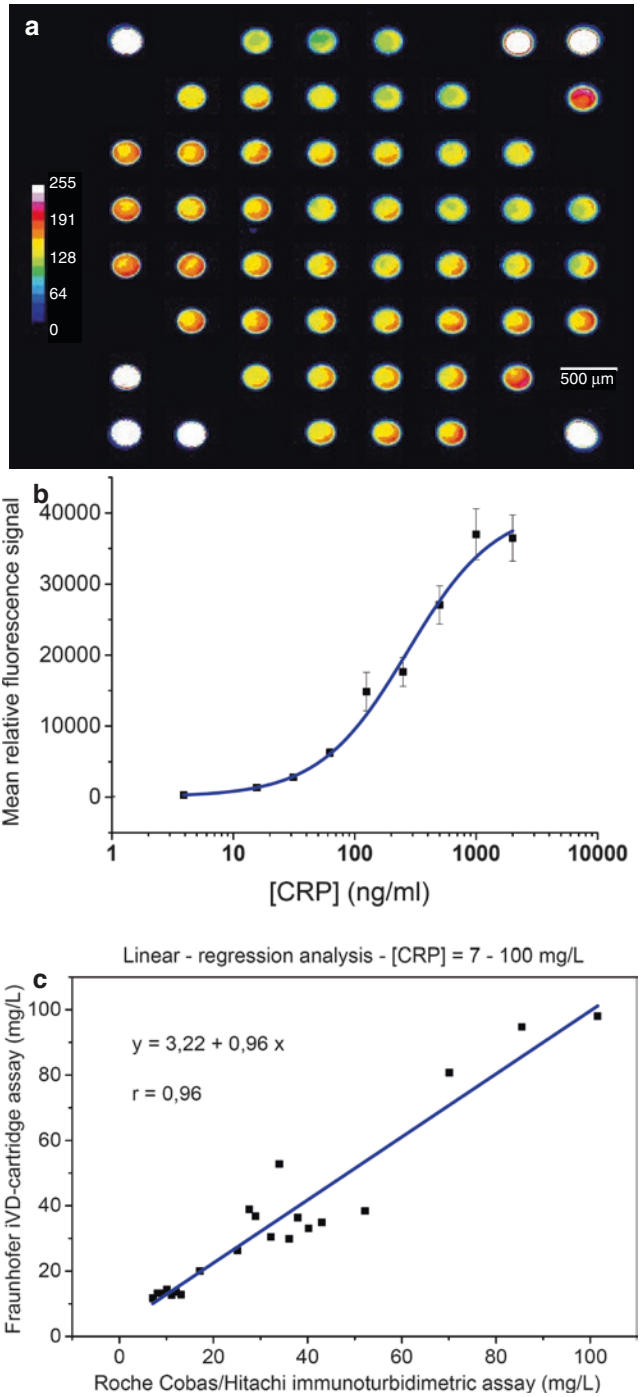
1. After image acquisition and fluorescent signal quantification, subtract the local background of each spot from the raw spot intensity value, and calculate the mean net signal intensity (NI) and standard deviation (SD) of the replicates.
2. Afterward analyze the data depending on the individual scientific/clinical question (*see Note 15*).

33.4 Notes

1. Other spotters can be utilized, but compatibility with the used materials and reagents is essential.
2. An alternative product for the internal spotting control could be goat anti-mouse IgG (H + L), Alexa Fluor 647, Cat. No. A-21235 (Life Technologies).
3. As an alternative, 2.5% casein hydrolysate, 50 mMTris base, 0.05% Tween 20 and 0.02% sodium azide can be used.
4. We used the Tecan LS Reloaded (Tecan Group, Männedorf, Switzerland).
5. We use the sciFLEXARRAYER S11 for spotting and PolyAn polymer slides as the array surface. Using the indicated settings, the spots will have a diameter of about 150 µm.
6. At this stage, the slides should be processed within several days.
7. If not stated otherwise, use the quadriPERM dish for incubation.
8. We recommend using a Wafer Tweezer (Bernstein-Werkzeugfabrik Steinrücke GmbH, Remscheid, Germany).
9. Proteins can easily be removed or blown away if too much nitrogen pressure is applied without allowing droplets to dry on the surface.
10. If not stated otherwise, all solutions containing proteins should be kept on ice while working. In addition, to obtain reliable results when performing an immunoassay with an unknown target antigen concentration, a freshly created calibration curve should be prepared to serve as a reference.
11. The concentrations of CRP can be customized by using a higher initial concentration and/or more or fewer dilution steps. By using human blood serum, the resulting calibration curve is closer to reality (see Fig. 33.3b). In contrast, using the blocking solution gives less bias concerning the standard deviation. It is not necessary to perform the measurements in both solutions every time.

12. Make sure the slides are protected from light during all incubation periods as the fluorophores are light sensitive.
13. All other reservoirs are already prefilled with the necessary buffers and solutions. If an empty cartridge is being used, each reservoir can also be filled with custom buffers and reagents individually.
14. As an example, a fluorescent image from a CRP microarray within the flow channel of the ivD platform is shown in Fig. 33.3a.
15. As an example, Fig. 33.3c shows the data analysis of 20 clinical samples in comparison to the Roche/Hitachi cobas immunoturbidimetric assay laboratory-based reference system.

Fig. 33.3 (**a**) Fluorescent image of a small (64 spot) immunoassay microarray in the flow channel of the ivD platform, containing 46 CRP detection spots as well as positive-, negative-, and spotting-control spots. (**b**) Calibration curve of CRP samples in serum, measured with the ivD platform lab-on-a-chip system, allowing a quantification of CRP in the range of 3.5–1000 µg/L. (c) Concordance analysis, showing the results of measurements with the ivD platform in comparison to the Roche/Hitachi cobas immunoturbidimetric assay system, analyzing 20 clinical samples (patient sera with CRP in the range of 7–100 mg/L). For the analysis with the ivD platform, the samples were diluted by factor 300 to fall into the sensitive detection range of the lab-on-a-chip system


a
255
191
128
64
0
500 µm
b
Mean relative fluorescence signal
40000
30000
20000
10000
0
1
10
100
1000
10000
[CRP] (ng/ml)
Linear - regression analysis - [CRP] = 7 - 100 mg/L
c
y = 3,22 + 0,96 x
r = 0,96
Fraunhofer iVD-cartridge assay (mg/L)
100
80
60
40
20
0
0
20
40
60
80
100
Roche Cobas/Hitachi immunoturbidimetric assay (mg/L)

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Chapter 34
Development of a User-Friendly App for Testing Blood Coagulation Status in Schizophrenia Patients

Johannes Vegt and Paul C. Guest

34.1 Introduction

Schizophrenia is characterized by a range of symptoms such as hallucinations, delusions, social isolation, low motivation, and cognitive impairment [1]. The fact that current antipsychotic medications are only partly effective highlights the need for more effective therapies. Proteomic studies which aim to explain the underlying molecular dysfunction in this disorder could lead to identification of new therapeutic targets for better prognostic outcomes. A recent study found that schizophrenia patients who were on chronic warfarin therapy for deep-vein thrombosis had a reduction in their psychotic symptoms, which suggests that defects in the blood coagulation pathway might be involved in schizophrenia pathogenesis [2]. This is consistent with the findings of a mass spectrometry profiling study of sera from schizophrenia patients and controls, which showed prominent phosphorylation differences of multiple proteins in the coagulation pathway [3]. Another study showed that patients with other psychiatric disorders may experience worse anticoagulation control, compared with normal control subjects [4]. Thus, regular monitoring of the clotting activity in schizophrenia patients might provide real-time biomarker readouts. In addition, drugs which reduce clotting potential, such as warfarin, might offer an alternative or adjunct treatment strategy for this psychiatric disorder.

Extrinsic blood coagulation involves a series of reactions in which enzyme precursors are successively cleaved, which activates each of these to catalyze the next

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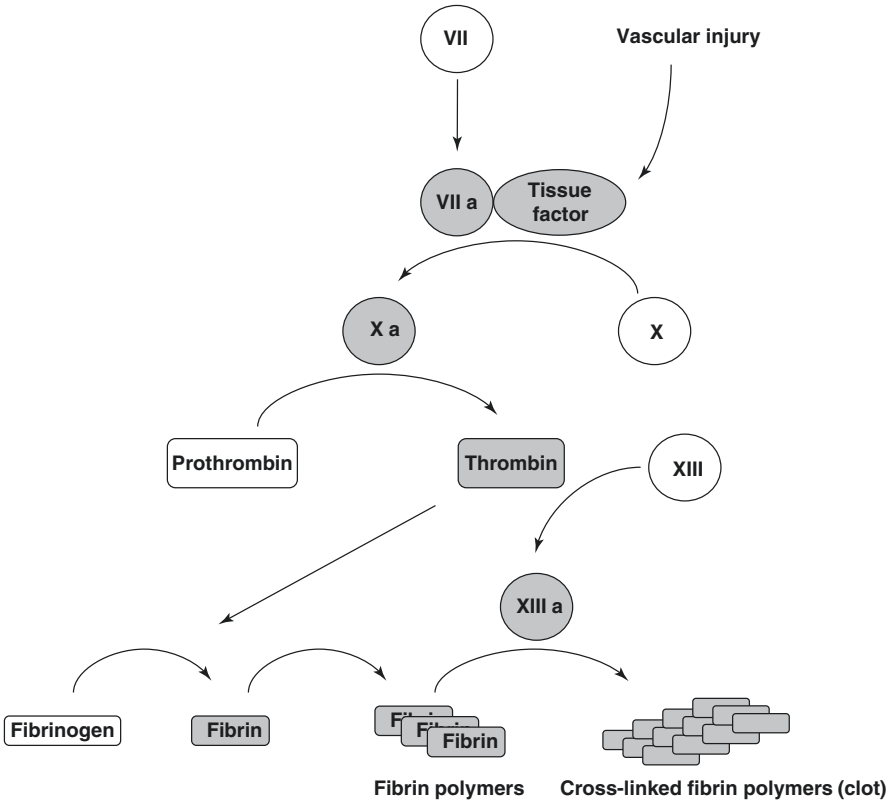

Fig. 34.1 Diagram showing clot formation via the intrinsic pathway

step in the reaction in a cascade manner, ultimately resulting in cross-linking of fibrin chains and formation of a clot (Fig. 34.1) [5]. The cascade proceeds in the following sequence: (1) tissue damage, (2) activation of tissue factor, (3) activation of factor VII, (4) activation of factor X, (5) cleavage of prothrombin to generate thrombin, (6) cleavage of fibrinogen to produce fibrin, and (7) polymerization of fibrin. Many scenarios can lead to excessive blood clotting, which can cause limitations in blood flow. This can result in severe medical problems such as heart attack, stroke, organ damage, or even death. Anticoagulants are often used in medicine to help prevent blood clots from forming and to reduce the risk of blockages in arteries and veins [6].

When anticoagulants are prescribed for a patient with a heart condition, it is essential to carry out periodic monitoring blood clotting times using the international normalized ratio (INR) in order to adjust the anticoagulant dose as necessary [7] and patients are advised to perform these checks using coagulation test strips

and a suitable reader [8]. Based on tested INR values, the appropriate dosage of anticoagulant should be administered to achieve the target range. Management of this process is facilitated by the use of Coagu app, developed by Appamedix in Berlin, Germany [9]. This app works as a functional readout of the activity of the coagulation cascade. It was recognized for its usability by the International Design Centre Berlin in 2013 and as an example of a particularly user-friendly product on the International Funkausstellung (IFA) [10]. It works on a universal design and this confers high usability by people of all ages. The Coagu app is now used by patients in over 70 countries, and the results show that greater use of self-monitoring offers clinical and patient benefits that are likely to result in reductions in blood clot-related heart attacks and strokes [11].

34.2 Materials

1. Smartphone, tablet or similar device (*see Note 1*)
2. Coagu app
3. Clotting time test strips and meter (*see Note 2*)

34.3 Methods

34.3.1 Preparation: Determination of INR (Fig. 34.2)

1. Insert test strip into coagulation meter.
2. Pierce the tip of a finger using a sterile designated lance.
3. Apply the blood drop immediately to the strip.
4. Read the measured value which appears on the meter display in 60 s.

34.3.2 App Usage: Determination of Anticoagulant Dosage (Fig. 34.3) (See Note 3)

1. Open the Coagu app on the smartphone or other devices.
2. For the first use, each patient configures the app according to their specific needs including target INR range, drug names and dose (*see Note 4*).
3. Enter the measured value which is stored and displayed in the calendar and histogram of the app (Figs. 34.4 and 34.6) (*see Note 5*).

Fig. 34.2 (*A*) The readings of the last international normalized ratio (INR) measurement gradually fade over a 7-day period as a reminder for the patient to take the next measurement. (*B*, *C*) The tip of a finger is pierced lightly to draw a small drop of blood. (*D*) Immediately apply the blood drop to the test strip housed in the measurement device (*see Note 2*). (*E*) After 60 s, the measured value appears on the meter display. (*F*) The patient enters the INR value, using a number picker, and this is stored. (*G*) The measured and saved value appears with the actual date on the start page of the Coagu app

4. The patient should next determine which medication and what dosage they should take based on the INR reading (Figs. 34.2, 34.5 and 34.6).
5. The values are entered in the measurement history and this can be visualized as a trend over a 6-month period in histogram format (Fig. 34.6) (*see Note 6*).
6. The patient is reminded up to twice a day via a notification which occurs as an audible and visual signal with advice to set the time for taking their medication or some other action (Fig. 34.3) (*see Note 7*).
7. The patient may add a comment on the entry each day individually (Fig. 34.3) (*see Note 8*).

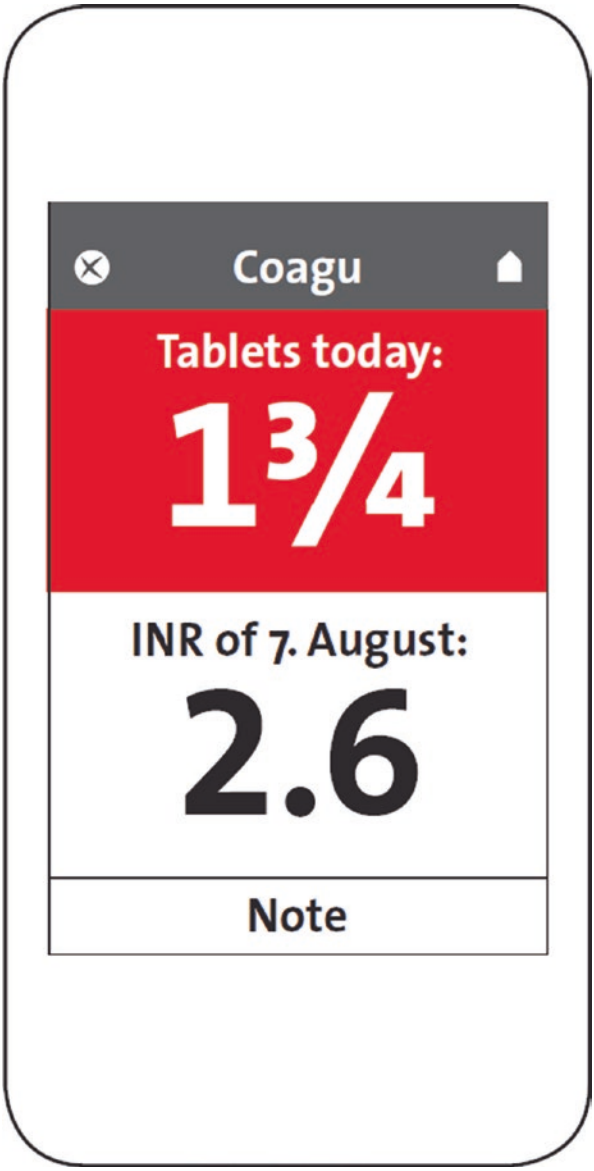

Fig. 34.3 Patients on anticoagulants should take their medication every day. First, the app reminds them of the dose to be taken that day. Second, the patient confirms that they have taken the tablets with a tap on the display. Last, this is registered on the histogram and calendar

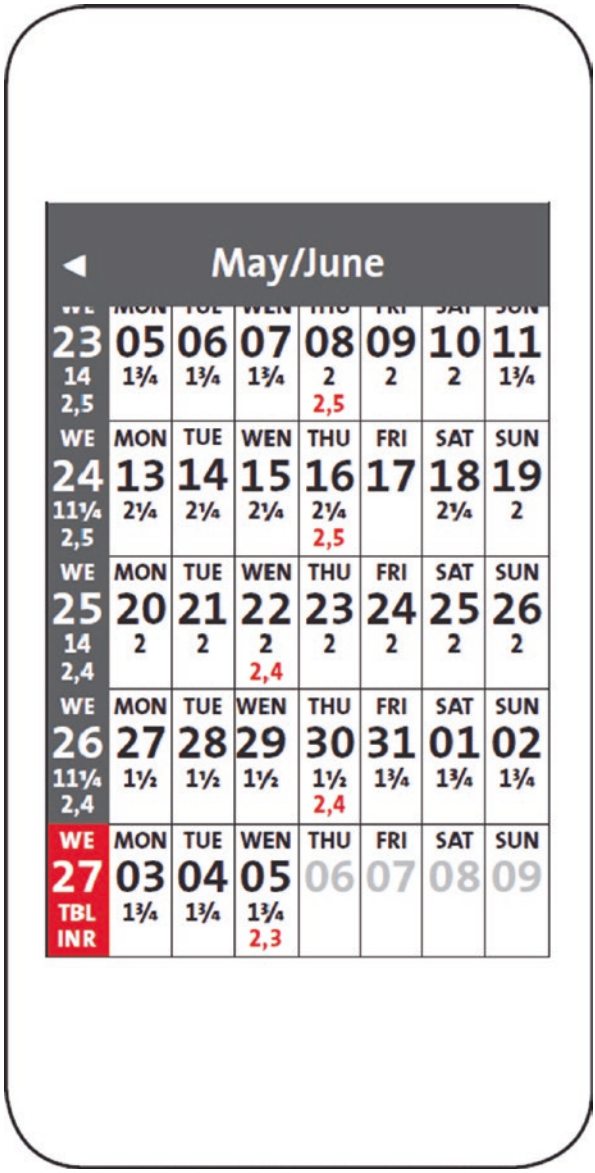

Fig. 34.4 Calendar showing the daily INR values which have been entered by the patient (*red* values indicate a high reading)

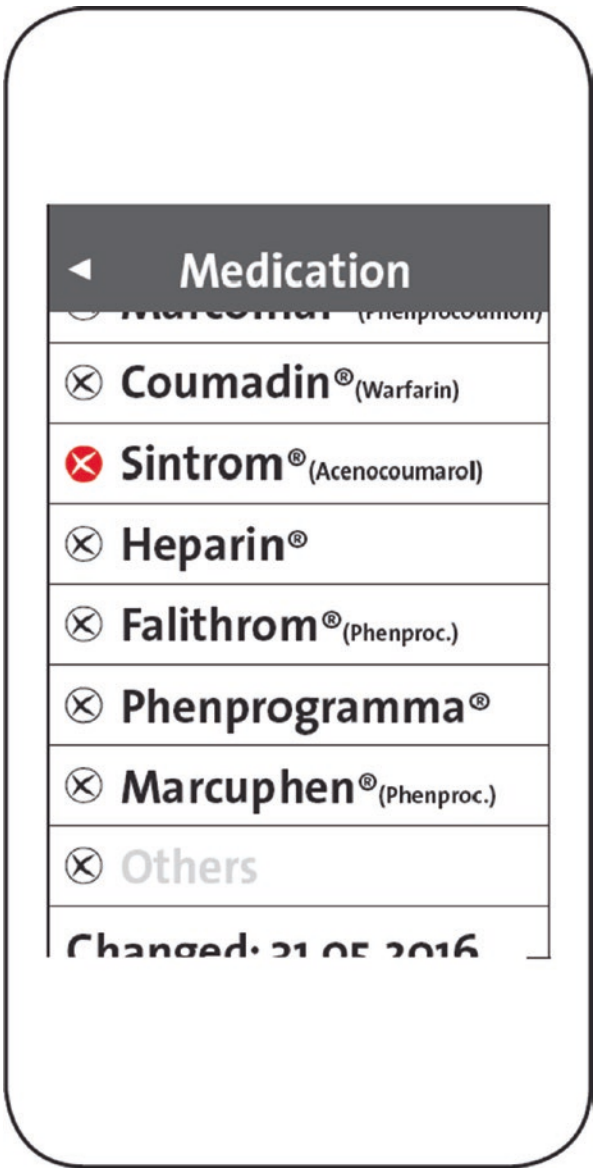

Fig. 34.5 The patient chooses a prescribed medication from a list, and a confirmation that the medication has been taken is registered automatically in the calendar. With a tap on the calendar, a daily summary appears (not shown here). The entered notes can now be read by the patient or new notes can be entered

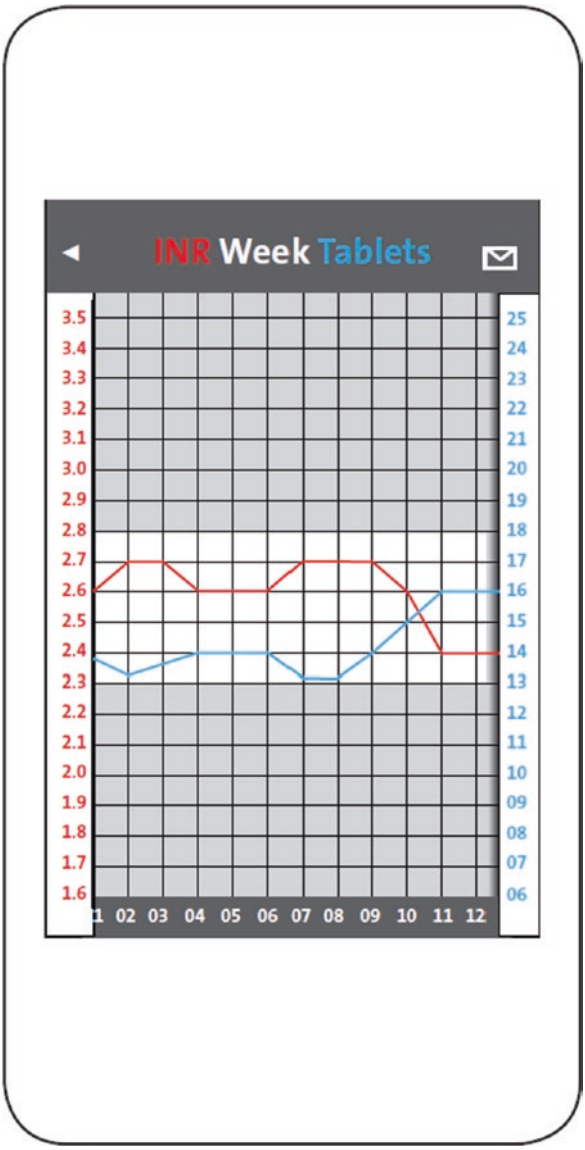

Fig. 34.6 The histogram shows the measured INR values and medication use over time. Tendencies can be seen over a period of 6 months

34.4 Notes

1. Many devices can be used such as Apple iOS and Google Play android smartphones and tablets. Note that there is no connection between Johannes Vegt (developer of the Coagu app) and the producer of the CoaguChek measurement device (Hoffmann-La Roche AG).
2. A lancing implement comes with the CoaguChek instrument, although other similar devices can be used.
3. Data protection is of paramount importance in the functioning of the app. Data on the smartphone or related device should be managed by the patient alone and not stored on an external server or as cloud data. It is important to note that the Coagu app is not a medical product in its present form. Since the app is sold worldwide, this would require testing and approval by the regulatory authorities in the host countries. Elevation of the app to a medicinal product would be possible if the relevant health insurance company accepts liability.
4. The target range is the maximum and minimum INR values determined by each patient's doctor.
5. Currently, the average age of the app user is about 60 years. Accordingly, relatively large touch-sensitive surfaces were created to compensate for possible motor inaccuracies. In addition, larger font sizes are used at crucial points to compensate for potential visual impairments. If the device is also put into use for other disorders such as schizophrenia, the average user age will decrease.
6. With this, the user can see the correlation between drug dose and INR reading.
7. For one example, any interruption of the medication can be listed in the app.
8. Comments are stored in the calendar and available for 6 months. Through the app, the patient can also contact their physician by phone, SMS or email. Technically, it would be possible for the patient to share their information with their doctor so that their history could be updated, as required. This is only possible in cooperation with the health insurance agencies and higher health authorities.

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Part III
Future Perspectives

Chapter 35
Proteomic Approaches to Enable Point-of-Care Testing and Personalized Medicine for Psychiatric Disorders

Francesca L. Guest and Paul C. Guest

35.1 Introduction

Psychiatric disorders such as schizophrenia, major depression and bipolar disorder are debilitating mental health diseases which affect around one-third of the people in the world at some point in their lifetime. These diseases can impair the quality of life, well-being and productivity, along with significant secondary effects on society, the workforce, healthcare services and the wider economy [1]. According to World Health Organization (WHO) findings, psychiatric disorders will become the second leading cause of disability in the world by the year 2020 and the leading cause by 2030 [2]. Considering major depression separately, this disease affects approximately one-fifth of the people in Europe, with socio-economic costs approximating 1% of the gross domestic product [3, 4]. In addition, the Global Burden of Disease study has reported that psychiatric diseases account for more than 12% of the years lived with disability (YLD) out of the total disease that affects the world. This is compounded by the fact that current medications for psychiatric diseases are around only effective at relieving symptoms around 50% of the time and they often have serious side effects [5, 6].

As a potential impediment to improvements in this area, diagnosis of psychiatric disorders has remained essentially unchanged over decades and is still based on the

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evaluation of symptoms in clinical interviews. This method can be a problem since diagnoses may vary significantly dependent on the training and adherence of the relevant medical practitioners to the criteria outlined in the currently used diagnostic manuals [7, 8]. Diagnosis can also be hampered by the overlap of presenting symptoms displayed in different psychiatric disorders. This symptom overlap also complicates diagnosis by the existence of different subtypes within a so-called single disease [9]. Disease heterogeneity may explain why some patients respond well to specific treatments and others fail to respond or even worsen in their conditions [10]. These difficulties in diagnosis create a major challenge towards therapeutic intervention and effective disease management. Such factors have also likely contributed to the high attrition rate in the development of new drugs to treat psychiatric disorders and the resulting near abandonment of efforts in this area by the major pharmaceutical companies [11]. These companies have recently reduced research in this area due to the lack of understanding of the underlying molecular pathways affected in psychiatric conditions and the low availability of surrogate biomarkers to support continued drug discovery efforts. This creates a vicious cycle that reduces their ability to develop new medicines.

35.2 Clinical Needs

Despite the urgent need for improved diagnosis and better pharmaceutical treatments in this field, no clinically validated molecular biomarker tests exist for any psychiatric disorder. In the near future, it is likely that increased biomarker testing by clinicians will lead to “bio-”signatures in individuals that reflect the molecular changes that occur in healthy or mental illness states. The ideal diagnostic test should have a high accuracy for identifying patients suffering from a particular psychiatric illness as well as those who have another illness or who are “psychiatrically normal”. Currently, tests for schizophrenia and depression have been developed on multiplex immunoassay formats, although these still require validation testing and translation to clinically useful and inexpensive platforms for point-of-care use [12, 13].

There is now an increasing interest for new technologies and biomarker-based approaches that are fast, cost-effective and simple to use for all operators. The use of accurate in vitro diagnostic devices that can accurately classify patients according to the type or subtype of a psychiatric disorder will help to reduce the duration of untreated illness and improve compliance by placing patients on the treatment that is right for them as early as possible. This will help to change the prevailing strategy in psychiatry from a reactive medical care to a more personalized medicine approach. The existing methods leave considerable room for improvement and are financially costly due to typically required large-scale instrumentation and associated long processing times, as well as the usual delay in final generation of the diagnostic test results.

35.3 New Miniaturized Technologies

Several means of overcoming such issues have been emerging over the last decade. These include portable mass spectrometry (MS) devices [14], lab-on-a-chip (LOC) [15] and smartphone apps [16]. Thus far, none of these devices have been used for measurement of biomarkers in psychiatric disorders although this is likely to change soon considering their success in other areas of medicine and the critical need for such point-of-care devices in psychiatric medicine.

35.3.1 Portable Mass Spectrometers

Traditionally, mass spectrometry instruments consist of multiple components with a large footprint and require considerable technical expertise for operation. Portable gas chromatography (GC)-MS systems have now been developed in order to overcome these issues and even allow their use in a portable manner [14, 17–19]. These portable systems allow their use in environments such as airports for detection of hazardous substances in real time. For use in airports, this usually involves an attendant swiping a traveller's luggage with gauze and then applying the gauze to an instrument for a readout comparison with known hazardous substances (Fig. 35.1). Thus, many other types of users have the potential to benefit from this technology. These potential users include emergency responders, the military, law-enforcement organizations, the health agencies and forensic and clinical scientists. The use of matrix-assisted ionization (MAI) portable MS instruments has also been applied for rapid on-site analysis of small molecules, drugs, lipids and peptides, which makes this a highly suitable platform for biomarker testing [19]. For the most direct applications in psychiatry, more work is required testing such devices in the analysis of blood biomarkers.

Fig. 35.1 Emerging technologies for point-of-care psychiatry

35.3.2 Lab-on-a-Chip Devices

LOC in vitro diagnostic devices provide several advantages over existing larger-scale methods, such as the consumption of lower biosample volumes, less waste, lower fabrication and reagent costs, improved faster system response times and compactness due to a high degree of integration and parallelization of functions [20, 21]. Many tests can be constructed on these credit card-sized devices, such as DNA hybridization and multiplex immunoassays [15]. Most importantly for psychiatric patients, this translates to a reduced waiting time for results, and tests can be performed at the point of care during a consultation with a doctor. Recently, a company called ClonDiag developed a LOC device for HIV detection that employs static image analysis and counting of CD4-positive cells in a compact and transportable platform [22]. This device requires only a single drop of blood and can deliver results within a 20 min time frame (Fig. 35.1). A similar LOC instrument has been developed consisting of a printed microchip that can be used to detect other viruses through an electrical sensing approach [23]. Another company has developed a tuberculosis diagnostic immunofluorescence-based LOC device that is 96% accurate for detection of tuberculosis in saliva within 30 min [24].

Most existing biomarker tests for psychiatric disorders have used multiplex immunoassay platforms for the analysis of serum or plasma [11–13]. It is potentially of great interest in this case that multiplex immunoassays have also been developed on credit card-sized LOC devices. One such card can be used to detect prostate cancer with high accuracy using either surface-enhanced photon scattering or voltage-based readouts [25, 26]. The procedure involves application of a drop of blood to a small sample well, followed by insertion of the card into a portable analyser which then generates a diagnostic score in less than 15 min. One anticipated major benefit of all of these LOC tests is that a rapid diagnosis will help to cut down on waiting times for results of laboratory tests which often take several days or even weeks, using existing methods. In addition, these devices contain a universal serial bus (USB), enabling transmission of the data to other devices such as computers, tablets and smartphones.

35.3.3 Smartphone Apps

Point-of-care testing also consists of disposable strips within small cassettes such as in widely available home pregnancy tests [27]. The cassette structure provides a space for sample addition, conduction of the test and generation of a result that is usually interpreted visually or by using a low-cost reader. Strip tests are already in use for the detection of diabetes, some infectious diseases and even for measuring the levels of substances such as alcohol and illicit drugs. Coupling the readouts of

such devices to smartphone apps would be a major step forward in patient self-monitoring (Fig. 35.1). This is also in line with global companies such as Apple and Google which have displayed an increasing interest in the market through connecting diagnostic test scores with app readouts via smart software. Therefore, an important requirement of new point-of-care devices involves incorporation of mobile communication and internet capabilities so that the associated data can be formatted for ease of presentation and interpretation by the users on a portable device. In parallel, there has also been an increased move to incorporate networked computing to aid in disease prediction, diagnosis, prognosis and monitoring of medication compliance. Eventually, this may lead to the development of "bioprofiles" for each individual, consisting of genomic, proteomic, transcriptomic, metabolomic and/or imaging data, along with physiometric readings and medical histories.

In the twenty-first century, mobile phones have become ubiquitous, with an estimated 6 billion product users at the end of 2011 [28]. This is a staggering figure considering that the estimated world population as of September 2016 was approximately 7.45 billion persons [29]. To add to this, there has now been a convergence in the technology base of the mobile phone which has resulted in the emergence and evolution of the smartphone and other smart devices. Thus, in addition to the general voice and text messaging capabilities, smartphones incorporate functions to support various apps and sensors and also allow wireless Internet accessibility and connectivity with other smart devices. This combination of features makes the smartphone a versatile and user-friendly technology for health and disease management [30, 31]. Indeed, the testing of smartphone apps in the medical area has already shown promise with improved outcomes in a variety of health conditions, diseases and control of certain habits. For example, clinical trials which incorporated healthcare interventions assisted through smartphone apps resulted in improvements in more than 60% of the outcomes [32]. This included parameters such as better attendance of patients at appointments, faster diagnosis and treatment and improved communication, as well as clinical factors like improved blood sugar control and decreased asthma symptoms. Other benefits included behavioural effects such as smoking cessation, better medication compliance and reduced stress levels.

35.3.4 Hybrid Technologies

In the field of biomarker tests, there have now been developments of multiplex biomarker assays on smartphone-based devices, using a three-dimensional printed optomechanical interface for imaging reaction wells on a plate [33]. The data can be transmitted to a database for analysis and the results returned to the user in under a minute. This mobile platform has already proven successful in clinical settings through rapid analysis of multiplex assays for mumps, measles and herpes simplex I and II virus immunoglobulins. In each case, these use the smartphone camera optics function for accumulation and transmission of the readouts. Other

optical-based smartphone display systems include flu virus subtyping by immunoassay [34], haemoglobin and HIV levels by immunoassay [35] and prostate-specific antigen using enzymatic amplification [36]. It is likely that other LOC and smartphone-based systems will be developed for other disease areas such as neurological and psychiatric disorders.

35.4 Conclusions

This chapter has described the recent movement towards improving care of psychiatric patients through the initial study of psychiatric diseases using multiplex biomarker approaches and then deploying these technologies as handheld microfluidic devices linked with smartphone apps for ease of use in the clinical setting. This is currently our best approach of achieving a paradigm shift in line with personalized medicine objectives. This will allow persons to be treated based on their individual biomarker profiles rather than as one of many with a particular disease using a standard blockbuster drug. In addition, the use of multiplex biomarker tests on credit card-sized devices and/or handheld instruments which are capable of distinguishing disease subtypes may be useful for rapid identification of patients who are most likely to respond to specific medications, either alone or in combination with other drugs. This approach has already been used successfully in the field of cancer treatment. For example, measurement of human epidermal growth factor receptor 2 can be used to identify those breast cancer patients who are most likely to benefit from treatment with the recombinant antibody therapeutic Herceptin [37]. Similar approaches in psychiatry could result in more effective treatment of patients with fewer side effects and, thus, better overall treatment outcomes.

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