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Harald Seitz
Sarah Schumacher *Editors*

Molecular Diagnostics

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This book series reviews current trends in modern biotechnology and biochemical engineering. Its aim is to cover all aspects of these interdisciplinary disciplines, where knowledge, methods and expertise are required from chemistry, biochemistry, microbiology, molecular biology, chemical engineering and computer science.

Volumes are organized topically and provide a comprehensive discussion of developments in the field over the past 3–5 years. The series also discusses new discoveries and applications. Special volumes are dedicated to selected topics which focus on new biotechnological products and new processes for their synthesis and purification.

In general, volumes are edited by well-known guest editors. The series editor and publisher will, however, always be pleased to receive suggestions and supplementary information. Manuscripts are accepted in English.

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Editors

Molecular Diagnostics

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Preface

Nearly 20 years ago the concept of molecular diagnostics moved into the focus of academic and industrial research. This shift was mainly driven by new techniques like DNA Microarrays which often result in miniaturization of assays and the automatization of processes. Since, then the field of molecular diagnostics has been expanded and clinical diagnostic laboratories function as a playing field for this expansion. Vast and dynamic changes in the test menus, instrumentation and clinical applications have been some of the impacts molecular diagnostics has had. Nevertheless, so far only a few promising techniques have become standard routine.

Human beings differ in their level of health. Even individuals with the same disease can have specific differences in their clinical picture. Therefore it is important to adjust therapy and medicaments individually. Today this approach is known as "personalized medicine". Molecular Diagnostics covers current molecular biological techniques used not only to identify the underlying molecular defects in inherited disease, but also to monitor therapies. Multiple studies in modern science have shown that changes on a molecular level are often directly linked to the origin of diseases. Consequently, new targets have been recognized and often used as potential biomarkers for intervention. These targets are able to act on different levels, like proteomics, genomics, or metabolomics.

The aim is to develop disease-specific biomarkers, which can be applied *in vivo* in the patient or *in vitro* by analyzing human samples like blood, tissues, or urine. Ideally, these markers allow not only a distinction between healthy and diseased people, but also, e.g., the classification of different types of cancer. Moreover, multiplexing multiparameter analyses often have a higher sensitivity, wide dynamic range, and need shorter incubation time. So the quite new field of "Molecular Diagnostics" is able to open new ways for detecting diseases, even at an early stage. This prevents severe harm for a patient, reduces costs and the effort of medical examinations, gives access to new methods in modern medicine, and is well-suited for point-of-care diagnostics.

Newly developed assays, devices for *in vitro* diagnostics and the corresponding imaging technologies should not only be available in specialized institutes or laboratories. Even common hospitals and surgeries, as well as general medicine, should gain access to these techniques.

The development of molecular diagnostic tools is an interdisciplinary task of clinicians, experimental and theoretical groups in universities, research institutes and industrial facilities. This multidisciplinarity gives the opportunity to use knowledge and resources of diverse institutes and researchers in an ideal way. Contributors of the chapters are well-known experts in their field, and come from a variety of disciplines, to ensure breadth and depth of coverage.

Therefore this volume contains contributions from scientists working in different fields and institutes.

We want to thank Springer-Verlag for giving us the opportunity to edit this volume. Furthermore for the constant help and support during the preparation of this volume, especially Ms. Karin Bartsch, Project Coordinator, and Ms. Elizabeth Hawkins, Chemistry Editor.

We also want to acknowledge the authors for distributing their chapters and spending their time preparing interesting articles.

Finally, we want to thank all colleagues who made this volume possible.

In conclusion, we hope that in this volume you will find inspiring literature and useful information about molecular diagnostics.

Sarah Schumacher
Harald Seitz

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Integration in Bioanalysis: Technologies for Point-of-Care Testing

Frank F. Bier and Soeren Schumacher

Abstract Biosensors, Lab-on-Chip technologies, and sensor-actor molecules are steps towards the integration of bioanalysis into small devices that will help in providing analysis where it is needed: the point-of-care. This article gives a brief overview of recent achievements and future prospects.

Contents

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1 Introduction

1.1 The Concept of Molecular Biomarkers

The last two decades have seen extensive effort put into genome and proteome research which has led to a deeper understanding of the molecular basis of diseases, their occurrence, development, and cure. As a consequence of this knowledge, more suitable therapies are on the horizon and are discussed widely as "personalized medicine". Molecular diagnostics will be an integrated part of this concept, since medication, success of treatment but also early occurrence of specific biomarkers for early detection of disease or even presymptomatic diagnosis will become the focus of medical treatments. Also genetic markers for risk screening and all aspects of companion diagnostics that define medication by the genetic constitution of a patient will help provide improved therapy.

Therefore, the molecular in vitro diagnostics market has good forecasts and is regarded to be a worldwide increasing market. Especially combined with point-of-care testing (sometimes better described as point-of-need) in vitro diagnostics might significantly improve the benefit obtained from molecular knowledge. Biochip- and Lab-on-Chip technologies designed for routine application open up the opportunity of performing complex analysis and multiparameter analysis on a small scale. Lab-on-Chip systems have the potential to transfer molecular diagnostics to the point-of-need.

A key component of the future development of diagnostics is the concept of biomarkers. In general biomarkers are all kinds of parameters that may be obtained from a patient and that are quantitative and correlate to a particular disease. Usually a biomarker qualifies to be called a surrogate marker when evidence has been gained from clinical studies that the biomarker represents a certain disease, a disease stage, or the patient's reaction towards a particular treatment or medication. While the general concept of a biomarker includes all kinds of physiological data such as heart beat and lung volume, molecular biomarkers concentrate on biochemical or genetic parameters and patterns thereof, sometimes named signatures.

In addition, diagnostics becomes more complex when a deeper look at these various biomarkers is necessary (Fig. 1). Within the Human Genome Project about 25,000 genes were identified. Considering the dogma of molecular biology these genes are transcribed to various forms of RNA, then translated into proteins and post-translationally modified. Going one step further, also variation in metabolism may be linked to diseases as well. In this regard, genomic, proteomic, glycomic, and metabolomic research has led to the need for detection and quantification of completely different types of analytes ranging from genes and proteins to small molecules and combinations thereof.

A multitude of detection methods are needed to match the requirements of each analyte. However, in many cases these methods are too sophisticated for routine diagnostics and in most cases too expensive as well. Hence, there is a need for new, more user-friendly technologies. This will lead to benefits for many patients and additionally may help to reduce costs caused by false and delayed treatments.

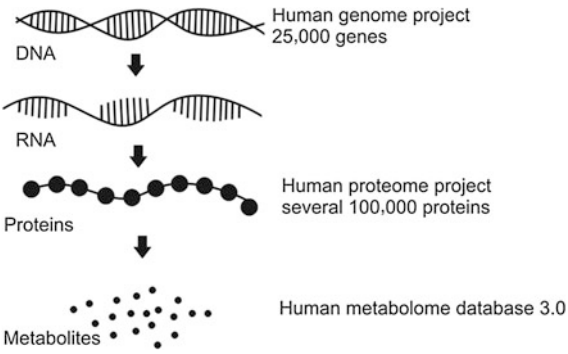

Fig. 1 Differentiation of biomarkers and number of possible diagnostic targets (data retrieved from corresponding homepages [30])

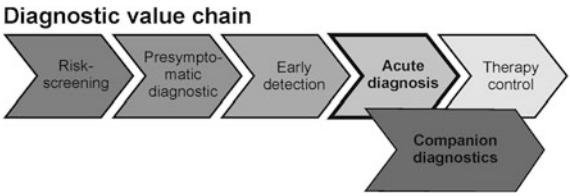

Fig. 2 Biomarkers will help to provide diagnostics at various stages of a disease even before a patient shows symptoms. Also during or after medical intervention biomarker based diagnostics will help to follow the success

1.2 Societal Needs

Having established this type of biomarker-related diagnosis, the accumulation of relevant data over a time period by electronic means will lead to increasing insight into long-term effects and later presymptomatic or even prognostic diagnosis may be achieved (Fig. 2).

Taking advantage of these new biomedical findings will provide an opportunity for improved patient-centered care. Hence, biomarker-based diagnostics will not only be used for curative purposes but also for prevention of diseases, enhancement of therapy success, and in general for increasing the quality of life.

From a more societal point of view diagnostics can effectively reduce costs within health-care systems. In terms of personalized medicine, collecting data over time will allow more rational access to the best therapy, and for large collectives of patients gathering data will lead to valuable information for a health-care system. Biomarker-based diagnostics will thus help to reduce health-care costs by reducing the number of second line therapies, reducing treatment costs, reducing the number of follow-up therapies, reducing nursing, reducing consequential costs, and reducing the period of sickness absence. In this regard, there are only very few economic studies about the impact of point-of-care testing. One example has been undertaken for emergency departments in the U.S. It was shown that fast measurement of the diagnostic marker Troponin directly at the patient can reduce costs

by a factor of four, by enabling faster patient management time and an overall better outcome for the patient [11].

From a more holistic viewpoint and considering the aging and increasing population, but also the ramifications of globalization, more versatile diagnostic technologies will be necessary. Moreover, diagnostics will have to be more frequently available and hence technologies have to be found that enable patient-near testing with the same quality as from the laboratory.

The scenario of bedside analysis is the first field of application of point-of-care testing (POCT). A short return time from sample to the location of the decision-maker, the so called "turn-around time" (TAT), is of great interest to the physician during his visit in the clinic. Also in the doctor's office it often might be of great help, if the doctor had access to the blood parameters or other results from the laboratory while the patient is still in the office. The decision for therapy could be better targeted and the patient would be pleased to be well informed about the physician's decision.

The role of biomarkers is to support the decision, which therapy might be most promising. A well-established biomarker might also be a guide for medication and for the appropriate dose for the individual patient. This scenario is called "companion diagnostics" and refers to the need of most medications to be appropriately adjusted to the physiological and genetic constitution of each patient individually to be most effective or sometimes even effective at all. Many drugs are known to be metabolized more or less effectively by different patients but up to now this information is only seldom available and useable by a doctor in his office. This information would be of help only if it were available immediately.

The utility and usefulness of biomarkers will increase, if samples other than blood, like saliva, urine, or other easily accessible body fluids are tested, which may help to make diagnosis less invasive.

Individual consultation and personalized therapy are the major trends of modern health care and both require diagnostics at the point-of-need and are what economists call the "market pull" for the development of Lab-on-Chip technologies for POCT [4].

1.3 Integration as a Key Parameter

Technologies that can be used for point-of-care testing have to match various requirements—especially because of the circumstances point-of-care testing is used in. Here, samples are not taken in a lab environment which enables users (medical personnel or physicians) to perform steps of sample preparation and its purification until results are obtained. More precisely, the technology has to provide user-friendly devices, that perform automatic processing of any sample of a body fluid and gives an interpretation of the measured results on a display. With this scenario in mind the following key features for technologies may be defined:

- User-friendliness: The devices have to be as easy as possible to use. This includes not only sample preparation, but also handling of the device and the small sample volumes required for testing.
- Miniaturization: In most application scenarios the device and also a possible base unit have to be as small as possible. By thinking about the assay itself, miniaturization of the assay will decrease the amount of sample needed for a particular analysis and will be of benefit in terms of faster reaction times.
- Parallelization: Because of the increasing knowledge in biomedicine in many cases a parallel analysis of different biomarkers can be beneficial. Therefore, technologies have to deliver not only a single parameter. Moreover, the possibility to determine a multiple of different parameters to make a diagnosis not only on the basis of one parameter can lead to faster therapeutic action and hence better patient outcome.
- Speed: Speed of analysis can be seen as crucial since nowadays applications in point-of-care testing are described as being linked to direct therapeutic action. For example, point-of-care testing for diabetes is directly linked to the injection of insulin, or a test for the determination of a cardiac infarction is directly linked to therapeutic action. These are two examples where patients directly benefit from a fast diagnosis and where speed especially in the second example is of great importance.
- Interdisciplinary: The key to obtaining such devices and fulfilling the above-mentioned criteria is the convergence of different technologies. Hence, an interdisciplinary approach has to be chosen which combines not only biochemistry, but also electroengineering, microfabrication, material sciences, and knowledge about production which all have to work together.

Taking these five key features into account, it is necessary to start as early as possible within the design process to think about a holistic system solution. In this process the concept of integration is essential since integration of steps, materials and processes may lead to the desired device features. In this regard, the following sections describe different degrees of integration and try to outline necessary design rules for implementation of interdisciplinary technologies for realizing systems for point-of-care testing.

2 Integration Steps

Point-of-care testing has to integrate laboratory-like procedures and guarantee laboratory standards. Moreover, POCT has to be connected to the data management system of the clinic or of the physician who is in charge of the patient. Figure 3 shows how integration of bioanalysis proceeds and which steps have to be taken during further development. It can be regarded as a road map for integration in POCT for the upcoming years.

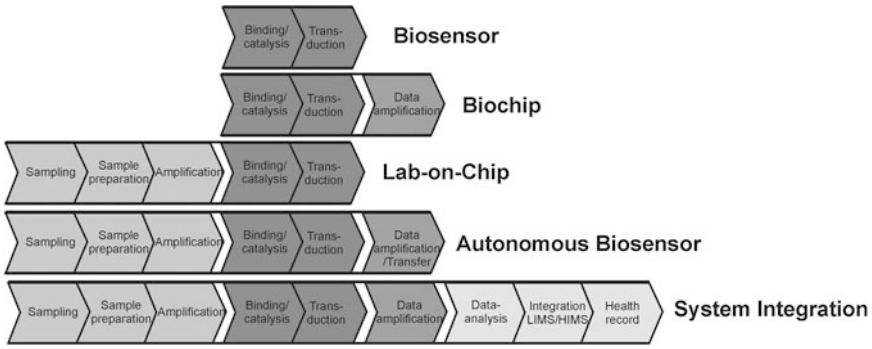

Fig. 3 Different steps of technological integration

2.1 Biosensors and Biochips

The concept of biosensors has a long history; usually Clark's glucose electrode proposed in 1962 is named as the birth of the technology [6]. Biosensors were defined by IUPAC in 1992; a biosensor is "a device that uses specific biochemical reactions mediated by isolated enzymes, immune systems, tissues, organelles or whole cells to detect chemical compounds usually by electrical, thermal or optical signals" [12]. By this definition classical biosensors are made from two components, the biological receptor molecule and the transducer, which are responsible for linking a biochemical reaction to a readout that may be quantified such as an optical or electronic signal.

Having a look at the technological side, in the early days of biosensors, there was a clear separation between the receptor molecules and the transducer which was made by physical entrapment of the receptor molecule within a membrane. The membrane itself was also used as a separation tool which only allows the analyte of interest to pass through. The next step in the process of integration was the generation of biosensors in which the membrane, the receptor molecule, and the transducer were all combined in one compartment [19]. Hence, the process of separation, binding, and transduction were located next to each other enabling faster electron transfer, better biosensor response, and higher sensitivities. These so-called membrane sensors where then replaced by second-generation biosensors in which the membrane was no longer necessary. This could be accomplished by new and more specific recognition elements which made the first separation step redundant. As fabrication technologies in microelectronics and microsystems progressed in the later 1980s smaller and affordable production of microelectronic and mechanical systems (MEMS) was achieved and thus the integration of receptor molecules, transducer, and the electronics necessary for data generation could be combined. The convergence of now three components led to the production of third-generation biosensors which were sometimes also termed "biochips" [19].

The literature contains a vast variety of attempts and concepts for biosensors and the number of publications is still increasing. Because of the impact of various technologies the improvements can be seen in all of these components with special emphasis on their interfaces. For example, the communication between enzymes and an electrode in an electrochemical sensor is of huge importance for its performance. In an amperometric detection mode electrons are measured which corresponds to the conversion of a substrate. To enhance the amount of electrons traveling from the enzyme to the electrode, two different methods can be chosen. One method is the possibility of using a sophisticated connecting layer in which the enzyme can be embedded. By adding a redox-mediator to this layer there is the possibility for an indirect electron transfer from the enzyme over the redox-mediator to the electrode. In a recent example, Nagel et al. showed the synthesis and application of a redox-polymer based on poly(*N*-isopropylacrylamide) (PNIPAM) with incorporated ferrocene moieties for an indirect electron transfer using NAD-dependent glucose dehydrogenase (NAD-GDH) or pyrroloquinoline quinone-dependent GDH (PQQ-GDH) and glucose as the analyte) [15]. The authors detected a heterogeneous electron transfer rate of 80 s^{-1}. This is twice as high compared to a normal self-assembled monolayer of a ferrocenepentanoate. Hence, by using hydrogels with incorporated mediators such as ferrocene a more effective electron transfer may be achieved. The other method is to modify the recognition element itself. To describe one example here, Demin and Hall modified a glucose oxidase (GOx) [10]. By different methods such as NMR spectroscopy and in silico calculations two considerations could be revealed: (i) oligosaccharide structures on the surface of the GOx are responsible for a larger space between the enzyme and the electrode; (ii) the path of the electron through the GOx could be shown hence the hemisphere of the enzyme could be determined through which the electron can pass to the electrode. From that, a genetically modified GOx was derived and produced bearing no oligosaccharide structures and a certain surface modification to facilitate direct immobilization. Hence, a better and direct electron transfer from the enzyme to the electrode could be accomplished.

This is a nice example of how the modification of biological recognition elements can lead to improved biosensor performance for applications such as glucose detection. Nevertheless, there is a trend to overcome the limitations of biological recognition elements such as stability problems under harsh conditions or batch-variations and to replace them with artificial receptor molecules. To obtain artificial receptors besides their chemical synthesis which is in most cases tedious and time-consuming, two approaches have been established in the last few decades. The first is the use of artificial DNA- or RNA-molecules called aptamers which may act as an antibody-like recognition element. For their synthesis a process called systematic evolution of ligands by exponential enrichment (SELEX), invented simultaneously by Gold and Szostak [9, 25], is used in which the tightest binding DNA- (or RNA-) strands are selected via a selection process. Through a generic approach aptamers against different molecules can be generated and used in biosensor applications [13]. Since the binding event is not directly linked to a signal generation most applications using aptamers are combined with an optical

transducer. One example is the detection of TNT by an aptamer within a fiber-optic biosensor. Because of the selectivity of the aptamer it was possible to discriminate TNT from other explosives [7].

The second is the concept of molecularly imprinted polymers (MIP). Here, a polymerization is carried out in the presence of the analyte which is also the template during the imprinting process. Within the polymerization mixture monomers, so-called functional monomers, are also used which can specifically interact with the template molecule by covalent or noncovalent means. After polymerization the template is extracted leaving an artificial binding site in which the analyte may (re-)bind. First adaptations of the MIP concept to biosensors can be traced back to the work of Mosbach [14]. This concept is also a generic approach and may be used for a great variety of different analytes. Using the noncovalent approach it was, for example, possible to obtain a binding polymer against nitrofurantion, an antibiotic frequently used in farming in former times, however nowadays prohibited due to toxic side effects. With these polymers it was possible to detect nitrofurantion directly from bird seed avoiding tedious mass analytical measurements [2]. One prominent example of a covalently imprinted polymer is the use of boronic acids as functional monomers for the detection of saccharides such as glucose, fructose, or saccharide derivatives such as fructosyl-valine [18, 20]. Since the binding event is also not linked to a direct detection in many cases the transducer chosen for molecularly imprinted polymers is either based on a mass change measured by quartz crystal microbalance (QCM) or cantilevers or based on the measurement of the latent heat of the binding using calorimetry. For a fructosyl-valine imprinted polymer it could be shown that the thermometric response of the binding event is about forty times higher compared to a control polymer without imprinted cavities [18].

Not only improvements on the recognition site are responsible for better biosensors. As already mentioned also improvements in the design and production of transducers may lead to great advancements in how biosensors will perform in various applications. Miniaturization of transducers is beneficial for cost reduction as well as user-friendliness. Because of the still ongoing race in miniaturizing electronics, especially electrochemical- and MEMS-based sensors may be miniaturized. A limitation of this trend will be, when problems arise from the small surface area with small possible surface loadings of enzymes leading to small signal amplitudes. In this regard different amplification methods have to be applied to gain signals with a high signal-to-noise ratio. Besides microelectronic devices also the manufacturing of micromechanical devices finds its way into the research field of biosensors. To give an example, the fabrication of a microcantilever enables the measurement of mass changes or changes in viscosity. In a biosensor for glucose detection Birkholz et al. used resonating microcantilevers to measure the change in viscosity of a hydrogel in which glucose was bound [5].

To summarize, improvements in recognition elements as well as in transducers are responsible for miniaturization and integration of biosensors and biochips. With smaller devices and more specific and direct biochemical reactions there is the potential for many more applications. Because of the specificity of the

biocomponent (or biomimetic component) biosensors are principally capable of working in crude samples without prior purification, like the measurements made by glucose sensor measures in undiluted blood samples. However, many biochemical assays, especially those related to the determination of nucleic acids need sample preparation. Also the introduction of labels for improvement of sensitivity requires preanalytical sample treatment. For this reason, further integration steps have to be performed to fulfill the needs of more complex analyses.

2.2 Lab-on-Chip Systems

While in biosensors and biochips data generation and amplification are included, preceding steps such as sample preparation, purification and washing steps for performing assays are not. For point-of-care testing, nevertheless, these steps should be included, for example when cells have to be destroyed to analyze interior compounds. Especially for the analysis of nucleic acids, usually lyses and amplification (with polymerase chain reaction, PCR) are necessary steps before determination of origin or more specific sequence details. Since user-friendliness was defined as being one of the key challenges all these steps have to be automated and integrated into the analytical device.

One concept to do so is a Lab-on-Chip system; the term derived from the notion of a "laboratory on a (microelectronic) chip" program. All the above-mentioned preanalytical steps have to be performed within the Lab-on-Chip device at laboratory quality and it has to be designed in such a way that an unprocessed sample is applied and finally measured by the biosensor which is part of the Lab-on-Chip. Work on Lab-on-Chip systems in general has been reported for many years. However, most approaches lack the possibility of serial production which hinders the development that finally might lead to commercialization. Also, in many studies just single steps of the analytical process were displayed and designed in a chip-like format but the whole process has not been covered yet.

Recently, the current authors published work on a Lab-on-Chip system called the "Fraunhofer ivD-platform" that was designed with the potential for serial production and offers a huge degree of modularity [21]. The system which will be described in greater detail in a later chapter of this book consists of a credit-card-sized cartridge (used as a consumable) and a read-out unit. Considering the concept of integration within the cartridge, which is the actual Lab-on-Chip system, all steps from sampling to transduction are integrated. Processes such as data amplification and displaying of the results are integrated into the read-out and processing unit. In this way, the combination of the cartridge and the read-out unit cover the whole process chain necessary for point-of-care testing.

To perform a test, blood from the finger pad is used and applied onto the cartridge. Besides a reservoir for the sample, the cartridge holds the reagents necessary for the particular test (assay), the sensor and actuators for displacing reagents and sample towards the sensor. Hence, after adding a drop of blood the

cartridge is inserted into the read-out unit and processes such as washing, labeling, incubation, and read-out are automatically performed within 15 min.

The platform has been designed to be modular; it therefore allows a choice between an optical and an electrochemical sensor. Both sensors can measure different analytes in parallel depending on the used capture molecules which are immobilized on the surface of the sensor. While the electrochemical sensor is able to measure 16 different analytes at once, the optical sensor offers the possibility to measure as many biomarkers as necessary for the particular application (up to 500). To obtain such high numbers of parameters, a microarray is used [3]. In a microarray different capture molecules are deposited on a surface in small spots having diameters of around 50 μm and volumes in the nL-range.

For the detection of small molecules, proteins or antibodies either antibodies as capture molecules or antigens can be spotted. After addition of the sample from which the analytes can bind to the capture molecule different washing and labeling steps are performed to measure the amount of bound analyte by means of a fluorescence signal. This signal is measured and quantified within the read-out and processing unit in this case with a CCD-camera and software which quantifies the intensity of each spot.

For the detection of DNA the steps to be performed include sample preparation, sample amplification, labeling, and detection steps which have to be integrated within the Lab-on-Chip system. Within such systems purification of DNA may be carried out in two ways: After disruption of the cells either nanobeads bearing DNA-strands for purification or silica matrices may be used. After both purification procedures the purified DNA has to be amplified. The most common method for amplification is the polymerase-chain reaction (PCR), in which cooling and heating cycles are used for annealing, elongation, and denaturation of new DNA-strands with a polymerase. In the case of the ivD-platform an external peltier element is used for cooling and heating since the high rates cannot be accomplished within the cartridge itself. For detection, also a microarray now bearing DNA-probes is used.

Although in the case of the Fraunhofer ivD-platform the degree of integration can be further improved the system was chosen to be as simple as possible. This guarantees an easy transfer of already established assays.

2.3 Integration on the Chip: "Active Arrays"

An even more elegant way by means of biochemical integration especially for DNA-detection is given by the concept of "active arrays" (von Nickisch-Rosenegk et al. [29]. Here, the reverse primers are immobilized on a substrate as "capture molecules." During the annealing step the templates from the sample bind to the primer and are elongated. After denaturation of the elongated DNA-strand a new primer anneals and the strand will be elongated again. Since the first primer is immobilized on the sensor the final PCR product can directly be measured

spatially resolved within the microarray spot. In this case amplification and detection are combined enabling the possibility for real-time detection. However, heating and cooling cycles are still necessary for the amplification.

Moving one step further towards simplification, a procedure called helicase-dependent amplification has been adapted as an active array. In an example to measure three sexually transmitted diseases *Neisseria gonorrhoeae* (NG), *Chlamydia trachomatis* (CT), and *Herpes simplex virus* (HSV) with OnChip-HDA at once this isothermal amplification method was used [1]. In this reaction a polymerase is responsible for the elongation of the immobilized primer. In contrast to a normal PCR, the denaturation of the double strand is performed via a helicase followed by binding of a 5′-primer to the single strand for reverse elongation [28]. The main advantage of these types of amplification is that just a single temperature has to be applied making these concepts suitable for point-of-care applications. Even so, an easy adaption of already established assays is not possible and the primer design particularly for multiplexing is challenging.

Although the concept of active arrays increases the degree of integration, data acquisition and analysis are still performed within the read-out unit: Integration has to continue.

2.4 Autonomous Biosensors

As integration proceeds the next technological design has to integrate process steps from sampling to data acquisition and wireless transmission of the data to the physician or a data hub. This type of sensor may be implanted or operate in remote situations such as the home. Hence, this integration is particularly based on the miniaturization of electronic parts such as the transducer itself or application-specific integrated circuits (ASICS) for its processing or data acquisition. However, the technological development is not driven by biochemical or bioanalytical and diagnostic needs. The production of electronic parts and especially the reduction of their power consumption have to be named as key drivers of the development.

In general, all components ranging from sample taking to data acquisition are integrated in such kinds of autonomous sensors. Recent projects have aimed at the development of sensors that are able to measure small analytes, like ions or metabolites. These projects are oriented towards the example of capsule endoscopy which comprises a "pill"-like camera system sending pictures from the gastrointestinal tract through the body [8]. This concept has been broadened towards sensors by first reports from Philips adding pH-measurement to the camera system [17]. The concept has consequently been worked out by a recent consortium working on implantable sensor concepts with the aim of implantable sensors for intensive care supervision [23]. Application areas for such autonomous biosensors can be the time-limited transplantation or digestion of such devices. With more than three autonomous biosensors, networks can be established. For example, in

biotechnological processes such as fermentation or on fields for agriculture they can provide added value in terms of distribution of nutrients. Therefore, there is a lot of work left for IT-engineers to develop software which allows for communication between sensors and a base unit including expert systems to qualify data for decision support.

2.5 Sensor-Actor Molecules: Molecular Integration

In contrast to technological integration which is highly developed in autonomous biosensors also biochemical integration will contribute to finding new ways for point-of-care testing. The first steps in this direction have been accomplished in the context of the above-mentioned "active arrays" where enzymes act on samples to derive analytical information. More artificial are newly developed molecules that generate signals upon binding like the molecular beacons invented for nucleic acid characterization [26]. The potential of multiplexing of the approach has led to a variety of applications especially in the detection and characterization of infectious diseases [27]. The concept of molecular beacons has been adapted to aptamers [16] as well as to peptide-based systems [24]. Both of the latter, however, are less universal than the original molecular beacon concept and much more detailed work on the recognition site has to be performed to make aptamer- or peptide-based molecular beacons work. More general approaches may be undertaken to link binding to signaling on the molecular level. All these newly developed molecules may be integrated in future easy-to-use analytical or diagnostic systems. With this new method of biochemical integration the processes of recognition and signal generation are fully integrated. Application areas are various; the concept of "a-lab-in-a-tissue" which named the project [22] makes the need for such a testing possibility quite clear. Because of globalization and the growing world population fast, qualitative tests for viruses such as influenza are desired for improved pandemic control and governance.

3 The Goal: Systems Integration

The possibilities given by different technologies for in vitro diagnostic testing at the patients' bed-side show a high degree of integration. From a technological point of view integration of the various features of a POCT device may be carried out with regard to different scales or levels such as the molecular level, electronic integration, optimization of production steps, and packaging. By integrating process steps for detection a faster and more user-friendly diagnostic method will be available in the near future. In addition, integration is again a key factor for their implementation into clinical workflows. In this regard it is not sufficient to supply technologies for a faster analysis. The supply chain has to be covered fully

meaning that also integration of data into clinical information systems and finally into electronic health records are essential. In this way diagnostics is not only a service but becomes a directly available product in terms of values gained from an analysis. Hence, in vitro diagnostics and point-of-care testing has to be connected with information and communication technologies to give added value to patients, physicians as well as to society.

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Future of Medicine: Models in Predictive Diagnostics and Personalized Medicine

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Abstract Molecular medicine is undergoing fundamental changes driving the whole area towards a revolution in modern medicine. The breakthrough was generated the fast-developing technologies in molecular biology since the first draft sequence of the human genome was published. The technological advances enabled the analysis of biological samples from cells and organs to whole organisms in a depth that was not possible before. These technologies are increasingly implemented in the medical and health care system to study diseases and refine diagnostics. As a consequence, the understanding of diseases and the health status of an individual patient is now based on an enormous amount of data that can only be interpreted in the context of the body as a whole. Systems biology as a new field in the life sciences develops new approaches for data integration and interpretation. Systems medicine as a specialized aspect of systems biology combines in an interdisciplinary approach all expertise necessary to decipher the human body in all its complexity. This created new challenges in the area of information and communication technologies to provide the infrastructure and technology needed to cope with the data flood that will accompany the next generation of medicine. The new initiative 'IT Future of Medicine' aims at driving this development even further and integrates not only molecular data (especially genomic information), but also anatomical, physiological, environmental, and lifestyle data in a predictive model approach—the 'virtual patient'—that will allow the clinician or the general practitioner to predict and anticipate the optimal treatment for the individual patient. The application of the virtual patient model will allow truly personalized medicine.

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Keywords Distributed computing · Health care revolution · High-throughput data analysis · Nonlinear information and communication technology · Personalized medicine · Predictive model · Systems medicine · Virtual human

Contents

1 Introduction

The emergence of modern technologies in molecular biology has triggered a paradigm shift in the life sciences. The major breakthrough in this field has been the sequencing of the human genome. In a concerted worldwide effort the first draft of the human genome was published in 2001 [1, 2]. The development process of the sequencing technology and gathering of genomic data lasted over 10 years at a cost of 3 billion USD. Since then genome research has been in a phase of rapid development. The modern high-throughput technologies in the 'omics' field for high-throughput analyses of genomes, transcriptomes, proteomes, or other aspects of biological information are now more powerful and efficient and are used to unravel the inherent complexity of living systems. These technologies allow an analytical depth that was not predictable 10 years ago. With this huge spectrum of analytical tools and the increasing throughput, the amount of data produced from biological samples increased tremendously with the effect that new ways had to be implemented to handle, analyse, evaluate, and interpret molecular biology data. Bioinformatics as a new scientific discipline has been established to facilitate data handling and support with tools and methods. As the amount, complexity, and heterogeneity of the data increase it has become a new challenge to integrate the information and generate an understanding of the whole biological system. Systems biology as the next wave in molecular biology addresses these issues and generates computational and mathematical tools to elucidate the functional and regulatory networks [3] of complex biological systems. Today, modeling

approaches are employed to understand fundamental principles in biology and allow predictions about the functioning of, for example, the human body. These novel approaches in biology create a demand for software solutions and computational power that is unprecedented and will become a new driver for innovation in the information and communication technologies [4].

The transfer of these innovative technologies from molecular biology to the medical field caused a paradigm shift and opened the route for a new medicine. With the first draft of the human genome and the subsequent technological development the tools are now available to create a fundamental understanding of diseases in light of this molecular information. Also the entire field of human diagnostics is changing according to the tools that are available today [5]. This newly available spectrum of diagnostic tools exceeds by far what was possible only a few years ago. Recent advances in the 'omics' technologies drive the development even further towards whole genome, whole proteome, or whole metabolome analysis of a given cell, tissue, organ, or even the whole body [6]. Cutting-edge technology drives the development of the instruments towards an increase in speed and precision at a higher throughput of samples that can be analysed at the same time. To bring these cutting edge technologies to the bedside and into clinical practice still needs further development and investment for the analytical machinery to implement easy-to-use molecular diagnostics at a reasonable price per patient sample. The sequencing technologies again take the lead in this; very recently two market leaders in sequencing technology announced the launching of a new product that is able to analyse a human genome within 1 day at a price of 1,000 USD. To produce the sequence of an individual human for 1,000 USD was the dream of the scientific community, and today, only 10 years later, we are for the first time close to the point where it will be possible to generate the genomic sequence of every patient. The availability of this technique will finally allow us to establish the basis for truly personalized medicine [7].

Personalized medicine can be provided as novel tools for molecular diagnostics become increasingly available at an affordable price. With appropriate diagnostics the clinician or general practitioner can tailor a medical treatment or a prevention strategy to an individual patient according to the responding markers on the gene, protein, or metabolite level. Personalized medicine can be considered as a paradigm shift transforming treatment-centered medicine to patient-centered disease management [8]. The 'omics' technologies, especially the sequencing of the individual human genome, will be of primary importance as they offer a comprehensive set of data about each single patient to the clinician. Stratified medicine will be replaced by decisions on therapy and medical treatment on the basis of the individual molecular make-up of patients [9].

From pharmacogenomic studies it became obvious that the common drug blockbuster strategy ('one drug fits all') must be revised according to individual responses. Observations with treated cancer patients indicate that only 25 % of the patients respond positively to the applied drugs; for most other cancer patients a treatment will only generate severe side-effects without leverage for the disease itself [10].

The new model-based approaches in human life science emerging out of the systems biology field are so promising that they are expected to induce the next paradigm shift in modern medicine towards individual-centered health care. Based on molecular methods, systems medicine will break boundaries deciphering the complex mechanisms of diseases, accelerate the discovery of new treatments, and support the evaluation of clinical trials. But systems medicine can also be used to design new molecular diagnostic tools [11–13].

2 Molecular Diagnostics as a Driver for Systems Medicine

As life can be regarded similar to a computational process, a biological system computes the phenotype out of the genomic information given a specific environment [14]. Following this paradigm the analysis of the molecular basis is one of the major cornerstones in modern medicine.

The joint effort of sequencing the first human genome had a huge impact on molecular biology and transformed the whole field. Following the central dogma of biology, the biological information stored in the DNA is translated via the RNA into the building of proteins [15, 16]; the metabolites that are found in a given organ, tissue, or cell are determined by the presence and activity of proteins in the given context. Technology development for deciphering biological information has now reached a level that enables the access and measurement of components on all levels and even beyond striving to analyse the complete set of molecules in organelles, cells, whole pathways, or even organs to get a comprehensive view of a biological system. The 'omics' technologies also comprise methods to detect protein–protein interaction, protein glycosylation, or the phosphorylation status of a given protein [17–20]. The development of the 'omics' technologies aims at precision, throughput, parallelization of processes, speed, miniaturization, as well as reducing the amount of sample needed for the analysis, but also reduction in costs for the machines and the price per sample [21].

After a decade of research following the publication of the first draft of the human genome the next elementary milestone in DNA sequencing was reached in early 2012 when technology developers published that the magical threshold had been reached to allow the sequencing of a human genome within one day at a price of 1,000 USD. This 'third generation' sequencing technology opens the gate for implementation of whole genome sequencing for routine molecular diagnostics in clinical application [22]. Further development of this breakthrough technology will allow, for example, the detection of single molecules omitting the amplification step for the libraries; real-time detection will speed up the analytical process beyond the recent timeframe giving rise to possibilities such as online sequencing during surgery, detection of structural variation, mutations and genetic variability, modifications, epigenetics, and transcription factor analysis. Transcriptomics reflects the current status in a given tissue or organ of a patient, and it employs the same technological basis and is additionally striving for sample reduction towards

single-cell and single-molecule analysis with possible transcriptional modifications. Of great importance will be the instant comparison of tissues or organs also in a time-dependent manner. Therefore, simultaneous transcriptome analysis of different samples in real-time will be a future challenge. Real-time PCR as an example has been a powerful technology to meet these demands, but has not yet been introduced in routine clinical bedside diagnostics. The limiting factor for the roll-out of third generation sequencing is the bioinformatics, that is, the mathematical analysis and computational basis behind it. Computer hardware and software solutions are not yet available to enable genome sequencing, for example, instantly at a patient's bedside.

Also the sector of protein analysis is developing fast towards whole proteome detection in cell tissues and organs, as well as the subsequent modifications and interactions. The use of mass spectrometry and NMR for protein analysis have been a major breakthrough, and mass spectrometry has developed now to a leading technology in the analysis of proteins and other biological molecules. These technologies are advancing fast, and it will soon become routine in clinical applications to employ mass spectrometry more frequently for diagnostic purposes. Similar to nucleic acid analysis, further technological breakthroughs are needed in the analysis of whole proteomes in complex samples, or single cells, simultaneously detecting modifications and interactions, and also taking time dependency into consideration. Also methods to analyse protein kinetics in a high-throughput approach are still needed for in-depth understanding of the complex system of cells. Awareness of metabolites as indicators for metabolic activity and energy status in a cell, tissue, or organ has increased over the last decade, and mass spectrometry was again a major technology allowing a much more comprehensive analysis than it was possible before [23]. Although the dream to get a full metabolome analysed in one analytical step cannot be fulfilled with today's methods, but precision and throughput are advancing tremendously, and have now reached a point where transfer of this technology to the bedside as one important molecular diagnostic tool seems to be approaching fast. Efforts to bring protein and metabolite analysis into clinical practice are increasing because these types of biological molecules will reflect the physiological status of a patient in contrast to the genomic information that represents just the 'blue print' of a body.

Microarray technology followed sequencing technology and is a powerful tool for a highly parallelized analysis of a broad spectrum of biological molecules, from nucleic acids via proteins down to metabolites. The most recent advances have been achieved in the employment of array technology for glycomics, the detection of carbohydrates and oligosaccharides in a given tissue or cell as important biomarkers. In the future, array technology will also be one of the major platforms that allow the transfer of molecular assays to clinical practice as 'lab-on-a-chip'. Small and integrative handheld devices such as the ivD platform [24] are an interesting target for engineering to provide cheap and fast diagnostics close to the patient and nearly real-time with a minimum of sample needed for each analysis.

As the future of medicine is shifting from a reactive to a more pro-active and therefore preventive medicine, information about lifestyle and environment will

become increasingly important. In this respect imaging and sensor technology develop into very important life science technologies as they allow monitoring of patients over time. A new spectrum of non-invasive diagnostic tools will emerge from this fast-expanding field. These new approaches advance analytics to the whole-body level. Through new and fast computing systems, the imaging and sensing techniques enable an immediate interpretation of the body and its parts, as well as its biological functions. The combination of molecular data with the imaging and sensing data generate an interior vision with a resolution of the information in space and time. At the moment these techniques are advancing rapidly in speed, precision, and application range, but it is still an engineering, information and communication technologies (ICT) challenge to develop and adapt these techniques for real-time data collection, transfer, and processing [25–27]. In addition, appropriate interfaces among the patient, data, and health care professionals are needed to produce the appropriate information output. These methods will give rise to the hope that in the near future more sophisticated non-invasive diagnostic tools will be available, with the possibility of integration of monitoring the health status and environmental aspects. As technologies advance, other sample sources in addition to blood and biopsy become accessible to provide the necessary information via non-invasive diagnostic tools. In the future, urine, blood, saliva, perspiration, and breath can also be analysed via portable devices that do not require high-tech machinery such as fluorescence microscopy, surface plasmon resonance, or colorimetry. The new devices will enable the monitoring of body fluids or breath at the bedside and as point-of-care (PoC) tools in the general practitioner's office or even at home. Connection of the devices to personal health records is a necessary requirement to integrate the recordings directly and in real-time with existing information and allow the general practitioner a faster evaluation of the results.

The combined use of monitoring systems and enhanced data analysis will be necessary to further advance in vitro diagnostics systems to provide precise and complex analysis and follow-up. The in vivo systems address specific needs of continuous monitoring *via* sensors; they help patients with chronic diseases or senior citizens to live an autonomous life. This type of technology supporting the use of remote systems and mobile computing technology also opens the perspective for better health care in remote areas, for poorer regions, or developing countries where citizens have only limited access to health care. The basis for the implementation of these technologies of ambient assisted living require a central data repository including a personal health record and advancements in hardware and software. With the approach of these technological opportunities legal and ethical frameworks have to be discussed concerning the accessibility and use of the data for the practitioner or other representatives of the health care system.

Immunohistochemistry and fluorescence in situ hybridization are techniques with many variants that have already arrived in clinical practice to localize proteins and nucleic acids in cells and tissues and monitor their distribution. Remaining challenges are the automation of sample preparation, resolution beyond subcellular levels, and the automation of image analysis with high precision and

fast data output. Technologies to analyse features such as detection of mutant genes and transcripts, interacting sets of molecules including complexes of nucleic acids and proteins, as well as variants introduced by chemical modifications of specific nucleic acids or proteins also remain to be developed. Today positron-emission tomography (PET) or PET-MR images already provide the possibility for a quantitative analysis of the localisation of certain active substances in the body. The new integrated PET-MR scanners allow the integration of complex, multi-parametrical, multi-resolution data for 3D reconstructions. The new developments in detection techniques and algorithms will improve both resolution and sensitivity by an order of magnitude or more. X-ray tomography provides volumetric information about the body or a particular organ. When used in conjunction with the injection of a contrast agent and with an appropriate data analysis, it enables, for example, efficient cardiovascular, pulmonary, and tumor imaging [28–30].

An issue that is gaining increasing importance is the multitude of micro-organisms interacting with the human body. New publications [31, 32] show that the metabiome—the microbes living in our intestines—has much more influence on human health than expected. In a few years time we will have access to knowledge regarding how the microbiome interacts with the human body and triggers or influences health status or certain diseases. The study of the microbiome (the identification of the specific micro-organisms and their composition in the intestines) was very demanding in the past because these microbes prefer anaerobic conditions, and it was very difficult if not impossible to cultivate the specific intestinal microbes in the lab for characterization. The fast and cheap next generation sequencing techniques now open up the possibility to study these microbiomes via metagenomics approaches to characterize the composition of the population in a given environment and with an appropriate time resolution. To transfer metagenomic approaches into clinical practice new approaches in automated data assembly and annotation are needed.

One essential prerequisite to implement personalized medicine *via* a patient model is the use of electronic health records (EHR) of patients. Today, in most European countries we face the situation that the patient data are distributed and fragmented, and most of this information is only available on paper. Finland, Sweden, and Scotland as well as regions such as northern Italy have already implemented EHR for their citizens, and more countries will follow. The EHR will be the information core where all the data from a patient is collected and will constitute the interface between patient and health professionals. In particular, the monitoring of risk patients or patients with chronic diseases via sensors and imaging needs a central repository connected to an alert system and to to clinicians. With higher mobility of citizens, EHRs are necessary to ensure patient data can be accessed and updated independently from various locations and times. Given this perspective of future personalized medicine it is important that platforms implemented in different countries work according to standards and allow interoperability between systems. The consent of the citizens in a society on legal and ethical issues connected to an EHR are of crucial importance for the set-up of EHRs, and one of the main questions will be related to the safe storage of the data

and who has access to those data. The legal and ethical framework might differ substantially between countries, therefore initiatives such as the European Union–United States eHealth initiative are very important to start a discourse about the set-up of new systems between countries at the earliest possible stage to facilitate the mobility of citizens (http://ec.europa.eu/digital-agenda/en/about-health).

All these technologies contribute to the generation of highly detailed information about an individual's genetic make-up and physiological status and allow unprecedented insight into the functioning of an individual as a whole [33].

For all these tools enabling molecular diagnostics it will be highly important to define generally accepted standards and protocols to eliminate errors and false responses of the systems. The standardization of all upstream and downstream processes in molecular diagnostics gain highest priority when the data are integrated in the EHR and the virtual patient model to support decision making in the clinical routine. Sample preparation, measurements, data generation, data handling and processing, and mathematical analysis must follow the standards strictly to avoid false responses or predictions based on the model [34–36].

3 Systems Medicine: A Paradigm Shift in Personalized Medicine

Systems biology is a novel approach to decipher biological complexity on a systems level. In systems biology, methodologies and tools for mathematical analysis, integration, and interpretation of biological data as well as simulation and visualization methods are employed to build mathematical models of biological processes such as cellular interaction networks [37–40]. The present analytical tools need enlargement to address more complex questions and the integration of aspects not yet considered to be relevant, including environmental conditions and lifestyle data [41].

Mathematical models support the integration and interpretation of increasingly large datasets and combine these with existing knowledge to allow the interpretation of the data. Model approaches have been used widely for pattern recognition, data mining, and network analysis of complex and heterogenous molecular data. The novel approach of systems medicine using a virtual patient model will drive this approach further to reach the level of simulation and subsequent prediction of possible scenarios that cannot be measured directly.

Systems biology represents a complement to the current 'reductionistic' way that science is performed and how the results are transferred to medicine [41]. The systems approach includes the dynamics and nonlinear behavior of networks in biological systems. One prerequisite is knowledge about all the components that are determining and influencing the behavior of a biological system, how they interact, and their resolution and behavior in space and time. Biological systems use mechanisms to achieve robustness and stability while being flexible and

dynamic: These are feedback control, structural stability, redundancy, modularity, and adaptation [42–44]. Because biological systems are multiparametric, complex, and dynamic it can easily be recognized that complex diseases such as cancer or asthma are not monocausal. Diagnosis as well as treatment and prevention decisions must be based on this nature of biological systems and the individuality of patients, demanding tools to support health professionals for optimal use of the knowledge [41].

Modeling approaches help us understand the function and behavior of these highly complex biological systems, and also support predictions for parameters or situations that are not possible to measure and analyse with current methods. Therefore, modeling approaches are also recognized as powerful tools in future medicine to integrate the available data of a patient and support health professionals in their decision making. The translation of these novel approaches into clinical application will allow a general practitioner or a clinician to identify the optimal medical therapy or prevention treatment for each person based on the individual data that are available.

4 The 'IT Future of Medicine' Approach

The international research consortium 'Information and Communication Technology for the Future of Medicine' (ITFoM) anticipates the medicine of the future and is based on molecular, physiological, and anatomical data from all individual patients. The aim of the ITFoM project is the creation of the 'virtual patient' model integrating all different layers of genomic, physiological, and other relevant information to support health professionals in their decision-making process for therapy and prevention strategies for each individual patient.

ITFoM (www.itfom.eu) is a flagship pilot project developing a research programme to achieve truly individualized medicine in 2025. Initiated by a call from the European Future and Emerging Technologies programme, the ITFoM pilot connects the experts in Europe and worldwide to address this challenge and enable data-driven personalized medicine in the future [4, 9, 45].

Systems biology offers the methodologies and tools to analyse, integrate, and interpret biological data, providing mathematical concepts, and models, of biological processes which are then subjected to computational simulations. The ITFoM project will establish the 'virtual patient' system allowing for integration of all relevant data not only to improve and facilitate personalized medicine, but also enabling the prediction of the consequences of lifestyle choices and medical interventions on a tailored case-by-case basis.

To implement this vision and see the use of 'virtual patients' become part of standard clinical practice, substantial advances must be made in underpinning hardware and software infrastructures, computational paradigms, and human–computer interfaces, as well as in the instrumentation and automation of techniques required to gather all relevant information useful for the model approach.

ITFoM is a consortium of more than 160 academic institutions and industrial partners with unparalleled expertise in ICT, life sciences, public health, and medicine. This consortium will address for the first time the ICT implications of worldwide individualized patient care in combination with genomics and medical requirements on a large scale. This joint effort will contribute to a revolution in the health care system with enormous benefits for prevention, diagnosis, and therapy. It is expected that this model-based approach will also lead to increased efficiency in patient treatment by individualizing combinations of a limited number of drugs and reducing side effects of treatments. On a long-term perspective, the model-based approach of integrating the patient's genome data and other information on diseases, lifestyle, and environment could develop into an efficient system for prevention, and avoiding treatment, thereby playing an important role in concepts to reduce health care costs.

The resulting innovations emerging from the joint effort in ITFoM will answer to the European Digital Agenda and major health and societal challenges by building consistent value chains such as drug development, diagnostics and prevention, health devices, and PoC diagnostics; intelligent hardware and software solutions together with robust and secure data pipelines support the flow of the circulating medical data.

ITFoM aims at creating the new ICT that is necessary to enable models of human biochemical pathways, cells, tissues, diseases, and ultimately of the human as a whole.

5 Modeling Approaches for the Virtual Patient

Modeling approaches have been developed for all areas in the life sciences; we find anatomical, physiological, and molecular models. For an appropriate 'virtual patient' the integration of all information and models is a central challenge.

5.1 Anatomical Models

Imaging methods are the core to build anatomical models. Imaging technology and computer hardware and software recently developed very quickly in this field; for example, magnetic resonance imaging (MRI) and positron-emission tomography (PET) offer a whole spectrum of technologies increased by the possibility of combined approaches [46]. Other innovative approaches such as ultrasound and electromagnetic imaging or (nano)sensors increase the number of opportunities for high-resolution imaging and are efficient and inexpensive alternatives for existing technology. Anatomical models consider cells, tissues, organs, or even the whole human body. Some models are already used in clinical practice; they are used as support in surgery for careful planning and execution as in cancer or heart surgery

[47]. An example recently published is the PREDIBIRTH model software that uses MRI image analysis to predict if a mother might face difficulties during the birth process. The MRI images of the mother's pelvis and the fetus are integrated and analysed in a 3D model providing different scenarios of the expected birth process. These predictions or scenarios will help the clinician to prepare optimally for the birth process and support decisions in critical situations [48].

5.2 Physiological Models

Physiological models study the parts and functional behavior of the human body using physical and biological knowledge of the underlying processes. Physiological models are based on metabolic information and integrate population data that have clinical relevance [49]. Physiological models study the dynamic processes and the functional behavior of the physiological state of a tissue, an organ, or even an organism. Information about the structure and composition of the object of interest is relevant for the physiological models, but also the nature and functioning of the relevant elements, as well as growth processes. Knowledge of the physical parameters of the system including forces and electrical charges and how they are transmitted are of crucial importance for the models. Also thermal energy and its influence on the system can be studied with physiological models. A prominent example is the Virtual Liver Network, a scientific programme across Germany that is financed by the Federal German Ministry of Education and Research. A predecessor programme—HepatoSys—started in 2004 to quantitatively study the liver as one of the major organs in the human body and elucidate the cellular processes underlying detoxification, endocytosis, iron regulation, and regeneration in mammalian hepatocytes (www.hepatosys.de). The Virtual Liver Network (www.virtual-liver.de) followed this approach as a multidisciplinary research programme aiming at the development of a whole-organ model of the human liver and describing the physiological functions under normal and pathological conditions [49, 50].

The Virtual Physiological Human is a European scale initiative developing patient-specific computer models for personalized and predictive health care. Computational tools are the core that enable modeling and simulation of human physiology in healthy and disease-related physiological processes (www.vph-noe.eu). Projects under the VHP umbrella address diseases of the heart, brain, and intestines, but also develop infrastructure for data generation and ICT, and work on the enabling of data integration and modeling. An issue to further advance physiological models is information about how the different parts interact, such as cell–tissue interaction or interaction of different tissues in the human body. The integration of molecular data into these models will allow comprehensive understanding of the mechanisms and the pathophysiology of diseases.

5.3 Molecular Models

Many diseases have molecular causes. Therefore knowledge about the genetic make-up of the human body, the molecular components on all levels along the line of the flow of biological information, as well as their behavior and interrelations, need to be integrated in consistent approaches to understand the underlying mechanisms of diseases. As only a limited number of diseases are monocausal, the challenge now is the understanding of the high number of multifactorial diseases.

Molecular models address individual pathways including information from different layers of biological information such as metabolic and signal transduction pathways and cell-cycle regulation among others. With the new technologies already available for research purposes it is expected that in the near future these technologies will also enter clinical practice and will be increasingly used for patient health care. The genome is the basis, but depth and complexity will increase tremendously with integration of new data from transcriptomics, proteomics, metabolomics, and the other 'omics' analyses. Still there is a lack of knowledge about how these components in the system interact and depend on each other and how control and regulation mechanisms achieve stability and robustness of the system. This still existing lack of knowledge is a limiting factor for full implementation of molecular models in the medical field. But world-wide research efforts in this area continuously complement existing knowledge. The integration of Monte Carlo approaches in molecular models is currently established to fill the existing gaps in knowledge and use simulations and predictions to account for missing experimental data [51].

PyBios is a currently implemented model approach using an object-oriented modeling environment (pybios.molgen.mpg.de). It is based on interacting molecular models representing different cells or tissues integrating the patient's personal genome and other data. Individual models describe different objects representing the biological modules within the complex biological network acting in the cell in interacting compartments. These objects might represent different characteristics; for example, one object could describe the Ras protein representing one set of functions, whereas an object representing the mutant Ras protein shows altered functions resulting from the mutation. The PyBios system uses a Monte Carlo approach to simulate and predict the elements that cannot be measured yet. In a current pilot project (2010–2013) on melanoma patients in Germany (TREAT20) the PyBios modeling approach is used for the first time in a clinical setting [52]. The cancer model used in TREAT20 is based on the publications by Hanahan and Weinberg 2001 (with an update in 2011) [53, 54] representing the signalling pathways relevant in cancer. Tumor tissue collected from each patient is sequenced for the genome and transcriptome, respectively, exome data. These data are the basis for establishing an individual patient model that is later combined with a drug database to predict a possible drug response (Fig. 1). In addition, with this approach drugs that are unusual in clinical practice or even new drugs can be tested for their possible efficiency with these 'virtual patients'. The results from the

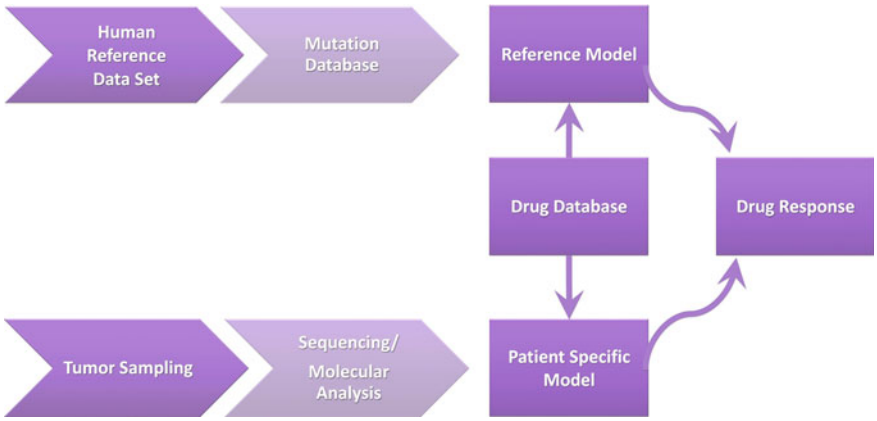

Fig. 1 The individualization of the virtual patient model and subsequent modeling for the prediction of possible drug responses in the individual patient

modeling approach and the predicted drug response support the clinician in the decision on drug treatment strategy.

With the new analytical technologies and the different model approaches that are already available, the next challenge is the issue of integration. Integration must be achieved in many different aspects including integration of data, technology, and models, but also existing knowledge on the functioning of the human body as well as on biomarkers or diseases. Only an intelligent strategy for achieving integration in an efficient and reliable way allows the transfer of this knowledge into clinical application via the ‘‘virtual patient’.

The reliability of the information output using the ‘virtual patient’ model also depends strongly on the quality of the existing data and information. The construction of the model, therefore, needs a very careful proof and quality check of the data before their integration into the system. New reference datasets need to be generated to feed the model with highly precise and reliable information. For the whole workflow a consensus for standards from the clinic via the analytical technologies down to data processing and integration has to be developed and agreed upon by all participating entities across Europe and even worldwide. Many steps within the workflow are still handled and controlled manually, but a system that will process and maintain the data of millions of patients needs the development of automated processes such as sample preparation, data processing, and analysis.

To achieve real-time health care in the future, these developments must be supported by an adequate infrastructure not only in biomedicine, but also in the ICT field. Data must be made available from the individual, and also a broad range of sources such as population-based data, environment, and lifestyle must be integrated in the system if sufficient support of health professionals is the aim.

To make full use of these novel modeling concepts in clinical practice, we have to solve the problem of demand for computing power that exceeds by far the

capacities of existing systems. Therefore, one of the most important challenges for the computer hardware and software fields is the development of solutions for the data-rich next generation medicine. The aim must be to speed up the computational processes and reduce power consumption dramatically before this virtual patient model can be used in clinical applications.

Only a concerted effort involving all necessary scientific disciplines from biomedicine to engineering and hardware and software system specialists can achieve this aim.

6 Information and Communication Technologies Are the Drivers for Systems Medicine

The ITFoM initiative aims at developing the data-driven 'virtual patient' model not only for European countries, but with the long-term perspective to be available to all patients worldwide. To achieve this aim it clearly needs enormous advancements in the ICT field to handle, store, and process all the data of the patients in the system. Already today we face bottlenecks in many areas that will be addressed in the ITFoM initiative.

To demonstrate the needs we face in the near future, a calculation example might elucidate the situation: The European health system includes around 500 million citizens. For each person the genomic information has to be generated, and transcriptional information at least needs to be collected continuously from each individual. If other information on the proteomics and metabolomics levels also needs to be included, the amount of data per person increases tremendously. To run an appropriate model, at least 1,000 cell types under 1,000 conditions per person have to be considered. If the model needs to be used at least twice per year (when the patient is consulting the clinician) the computing time is in need of around 10^{14} core years. Google uses 900,000 servers with estimated 100 cores per server; this infrastructure consumes approximately 0.01 % of worldwide electricity. Using the 'virtual patient' model for every European citizen, 100 times the worldwide electricity production would be needed to run the simulations (personal information H. Lehrach).

To allow 'virtual patients' to become part of standard clinical practice, substantial and intelligent advances must be made in hardware and software infrastructures, computational paradigms, and human–computer interfaces, as well as in the instrumentation and automation of techniques and processes required to gather all relevant information [4, 9].

When forming foundations for large-scale ICT, computing science was driven by 'large' physics and commercial applications, and medicine played only a minor role. ICT requirements of the new, data-rich, individualized medicine will soon surpass the demands of all other (ICT) fields. As data-intensive analysis and computer-intensive modeling technologies become common clinical practice,

medical ICT implementation will require far more resources. The enormous demands of computing power to perform the necessary Monte Carlo simulations or other mathematical approaches to describe biochemical systems and allow predictions and simulations of the whole human being pose unprecedented challenges to ICT, that is, in hardware and software constructions for storage, retrieval, and management of data, but also for communication, visualization, governance, and policy issues.

7 Implementation in the Health Care System

The future health care system—the patient in the first line—will increasingly benefit from these novel approaches, but our attitudes and daily routines will also change dramatically with respect to health. Despite the rapid development of modern information and communication technologies, an eHealth system to collect patient information in a central repository has not yet been implemented in most countries of Europe and worldwide. An eHealth system provides the huge advantage that general practitioners and clinicians do not have to rely on a patient's personal memory to report earlier diseases or which drugs are prescribed; an eHealth record provides all important information about a patient instantly to a GP or clinician which can be especially important and highly advantageous when an emergency case occurs. The implementation of an eHealth system certainly has to follow a strict legal and ethical framework, and it needs the consent of the citizens in a country to accept such a system. An eHealth system also provides the chance to allow elderly patients or patients with chronic diseases a more independent life. Concepts for Ambient Assisted Living are rapidly emerging along with a huge spectrum of technologies that provide independence for the patient [55]. In diagnostics the devices have increasingly implemented the possibility for a wireless connection to central data repositories where the data are processed and linked to patient data [24]. More and more web services also provide platforms where a person can collect and monitor his or her body functions such as blood pressure, pulse, and body weight. If we expect that in the future the lifestyle and environmental data will be an important element integrated in the 'virtual patient', then these data become increasingly important as background information for decisions regarding therapy and prevention [56].

Pilot projects to integrate molecular information in these patient records have been started recently in some countries in Europe, respecting all the legal and ethical implications connected to these projects. As molecular information becomes available for a reasonable price *via* advanced analytical technologies, it can be anticipated that soon it might become routine for patients to agree to include their personal genome information in their health records. Initiatives such as '23 and me' (www.23andme.com) offer a sequencing service and an analysis for disease risk factors and predicted drug response for the customer. Since services like this emerged, there has been an observable trend of increasing numbers of

customers, indicating the citizens' interest in this type of personal information, thus raising the hope that in the near future it will be possible to integrate molecular information on a broader—e.g. genomic—scale if the legal and ethical framework is carefully respected.

With the availability of modern communication technologies we are able to distribute and share information in a way that was unthinkable only a few years ago. As a consequence we face a situation where the general prescription or clinician is no longer the only group having proprietary information about health, diseases, or drugs. The average patient has become more autonomous and now demands participation in the decision-making process about therapy approaches and drug prescription. For the provision of specific treatment on an personalized basis, co-decision and empowerment are key terms [57]. As a consequence citizens assume increasing responsibility for their personal health with all the advantages, but also the disadvantages of this new situation.

The research in pharmacogenomics has shown the diversity of responses to drugs [10], and with increasing knowledge in this area it becomes obvious that the stratified medicine approach is no longer sufficient. The 'virtual patient' approach with the integration of molecular information and computational evaluation *via* the model will decrease the number of non-responders and make medical treatment more safe and efficient [58]. If a consequent information policy is followed by all stakeholders and parties involved, patients will have the freedom to participate actively in the decision process about their medical treatments and, consequently, also about their individual (health) behavior. To realise this, opt-in mechanisms on different levels and with the possibility to individually access the information must be developed, accompanied by an adequate informed consent. This framework will allow the individual to have decision power over the knowledge that can be potentially obtained.

The new concepts and developments in medicine empowering the virtual patient model open the perspective for truly personalized, efficient, safe, and less cost-intensive health care in the future. On the technological basis all necessary disciplines such as life sciences, engineering, ICT, and the like combine their expertise and efforts to achieve this ambitious goal to enable the patient model as a powerful tool in medicine. Cooperation with and integration of all stakeholder groups is also of crucial importance to allow implementation of the patient model in the health care systems on a sound ethical and legal basis for the benefit of the citizens. These new routes to personalized and efficient medicine will transform existing health care systems and change business models and markets in the near future.

This innovative approach will benefit not only European and first-world countries, and it will be the duty of the scientific community to transfer and actively support the access and use of knowledge for less-favoured countries worldwide to address global challenges in health and leverage the powerful new tools to eliminate inequalities [3].

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A Highly Versatile Microscope Imaging Technology Platform for the Multiplex Real-Time Detection of Biomolecules and Autoimmune Antibodies

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Abstract The analysis of different biomolecules is of prime importance for life science research and medical diagnostics. Due to the discovery of new molecules and new emerging bioanalytical problems, there is an ongoing demand for a technology platform that provides a broad range of assays with a user-friendly flexibility and rapid adaptability to new applications. Here we describe a highly versatile microscopy platform, VideoScan, for the rapid and simultaneous analysis of various assay formats based on fluorescence microscopic detection. The technological design is equally suitable for assays in solution, microbead-based assays and cell pattern recognition. The multiplex real-time capability for tracking of changes under dynamic heating conditions makes it a useful tool for PCR applications and nucleic acid hybridization, enabling kinetic data acquisition impossible to obtain by other technologies using endpoint detection. The paper discusses the technological principle of the platform regarding data acquisition and processing. Microbead-based and solution applications for the detection of diverse biomolecules, including antigens, antibodies, peptides, oligonucleotides and amplicons in

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small reaction volumes, are presented together with a high-content detection of autoimmune antibodies using a HEp-2 cell assay. Its adaptiveness and versatility gives VideoScan a competitive edge over other bioanalytical technologies.

Keywords Autoimmune diagnostic · Hybridization kinetics · Immunofluorescence patterns · Microbeads · Microscope imaging technology · Multiplex · Real-time

Contents

1 Introduction

The stunning achievements in the life sciences are based mainly on the development of innovative bioanalytical technologies. Many of these technological platforms are of high technical complexity but are suitable for just one application. Most of them are capable of detecting either proteins/peptides or nucleic acids at different multiplex levels ranging from low-density (<100 features) to high-density (>1,000 features) assays. Although highly sophisticated, many technologies like the planar microarray are unable to measure desirable kinetic data but provide endpoint measurements instead.

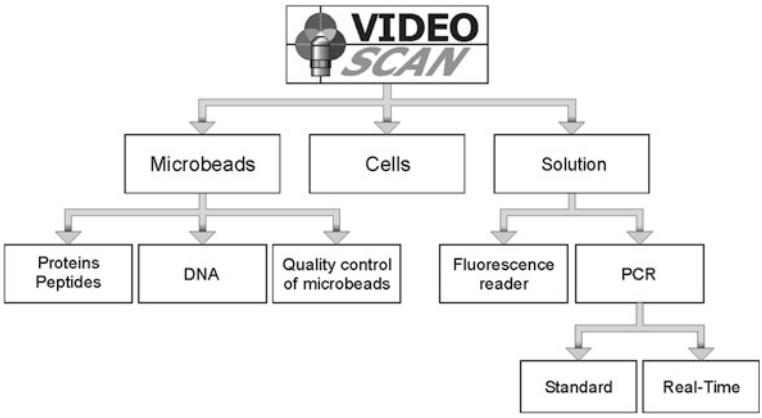

Fig. 1 Applications of the VideoScan platform. The highly versatile VideoScan platform covers microbead assays, cell pattern analysis, and assays in solution, with varying degree of features per application. Assays in solution include standard fluorescence read-out or (real-time) PCR. Cell pattern recognition is used for high-content screening in autoimmune diagnostics but also bacteria detection and enumeration. The main applications of the VideoScan technology are microbead assays for diverse analytes (DNA, peptides, proteins) both as endpoint analysis and in real time

In general these platforms only allow minor variations in the experimental setup because of pre-manufactured reagents optimized for a limited number of applications. Usually these technologies are highly priced and the necessary consumables are often quite expensive. On the other hand bioanalytical demands vary quite frequently depending on the subject studied. Within a project it might be necessary to perform measurements ranging from enzymatic assays, nucleic acid amplification and hybridization to antigen/antibody detection. Providing the equipment for all these different tasks might be fairly expensive and will require a lot of space in the laboratory.

Here we describe a microscope-based technology, designated VideoScan, which provides the user with a high degree of freedom to adapt the system to the specific requirements of the experimental conditions, including kinetics (Fig. 1). It uses standard commercial hardware components together with a modular software package. It is possible to identify and quantify biomolecules in solution or attached to microbeads, and assay temperatures are variable and precisely controllable within one experiment using the heating/cooling unit. On the other hand, the image processing capabilities enlarge the spectrum of applications allowing high-content screening of autoimmune antibodies on immobilized cells.

We describe a combined approach which can be used for the analysis of cell patterns [1], microbead-based assays [2, 3] and in solution.

2 The VideoScan Platform

Flow cytometry technologies are common for measuring multiplex microbead assays [4–6]. The measuring period of microbeads in suspension is limited to the time it takes for a microbead to pass through the detector, i.e., a few microseconds, so a detector of very high sensitivity is required, e.g., a photomultiplier [7]. Using flow cytometry it is also extremely difficult to measure the same microbead several times to acquire reaction kinetics. To overcome these disadvantages we developed the patented VideoScan technology [8, 9]. VideoScan is based on automatic capture and analysis of two-dimensional fluorescence images utilizing algorithms of digital image processing. Similar approaches have been reported previously [10–13].

The VideoScan measures and stores the signal of individual microbeads as a single-event detection principle already described for flow cytometry or microbead-based arrays [4]. This technology utilizes miniaturized wells and can be used in combination with standard technologies like multiplex PCRs and direct hybridization assays. The arrays are characterized by randomly ordered microbeads which carry specific capture probes. Within a microbead mixture, each microbead is characterized by a specific dye ratio and a given size. Microbeads which match these parameters are grouped into one microbead population. The population is assigned to one analyte only, allowing an unambiguous identification of individual microbeads in multi-analyte assays. This is a very simple yet powerful encoding strategy [14]. Other microbead encodings were reported previously [2, 14, 15]. In contrast to other technologies, VideoScan decodes the randomly dispersed microbeads prior to every measurement of the ligand by their intrinsic color-code and microbead size. This is different to Illumina BeadArray technology which defines microbead spots prior to the assay by sequential hybridization [14, 16]. Real-time decoding has multiple benefits. The redundancy per microbead population is adjustable. There is no requirement for a defined spot size for one population nor for a defined spot position. Assays are customizable by adaptation of number and analyte of microbead populations for each experiment. The readout concept is adaptable to scenarios where endpoint or real-time measurement at defined but variable temperatures is desirable.

2.1 Hardware and Software Components

VideoScan consists of our software package *FastFluoScan* developed in-house and commercial available hardware components (inverse motorized fluorescence microscope with a minimum of three fluorescence channels, motorized scanning stage, digital camera with at least 1.3 megapixels, and optionally a heating/cooling-unit (HCU)) (Fig. 2a).

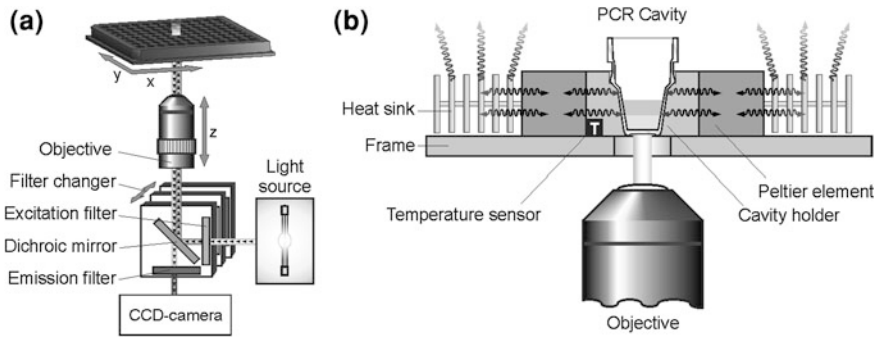

Fig. 2 Schematic drawing of the VideoScan hardware components and the heating/cooling-unit (HCU). **a** The main hardware components are a motorized fluorescence microscope, a stage, and a CCD camera controlled by a personal computer. **b** Scheme of the HCU for dynamic heating of a single well of standard PCR modules. Changes in fluorescence intensity can be monitored over a broad temperature range in up to six "ligand channels" for both solution and microbeads

FastFluoScan contains a hardware abstraction layer, so it can deal with hardware from different vendors. Currently we are using a Olympus IX81 microscope illuminated by xenon lamp or LED unit, and a Märzhäuser ScanStage IM 120 × 100. Our cameras (Kappa DX2H, DX2HC, DX4C–285, PS4C–285) are equipped with a Sony ICX285AL CCD-chip. Camera cooling is only required to detect extremely weak signals, e.g., luminescence applications. *FastFluoScan* has a modular design. It can plug in a variety of analysis modules for different measurement tasks. Currently there are modules for microbead measurement, fluorescence reading, and bacteria detection and counting. Any kind of sample carrier with a transparent, planar bottom can be used, e.g., 96-, 384-well-plates, microscope slides, or Advalytix® slides. In particular, Advalytix® slides are interesting because they provide 48 "spots" for small reaction volumes of less than 2 µL even under PCR conditions (see Sect. 5).

VideoScan can be operated with a HCU. This unit, using Peltier elements, fits into the scanning stage (Fig. 2b). Currently we are using a prototype made by Berthold Detection System (Germany) for a single module (NucleoLink™, Nunc) with heating and cooling rates of up to 15 and 10 K/s, respectively, in a temperature range between 15 and 105 °C. With a temperature resolution of less than 0.1 °C, high-resolution melting curve analysis is feasible (see Sect. 6).

2.2 Data Acquisition

Microbeads are located on the transparent bottom of a microplate well. Microbead immobilization is not mandatory. In each well, simultaneous measurement of several microbead populations (multi-parametric) is enabled. Microbead measurement is usually done with a 10 × /0.30 objective, which is able to image microbeads

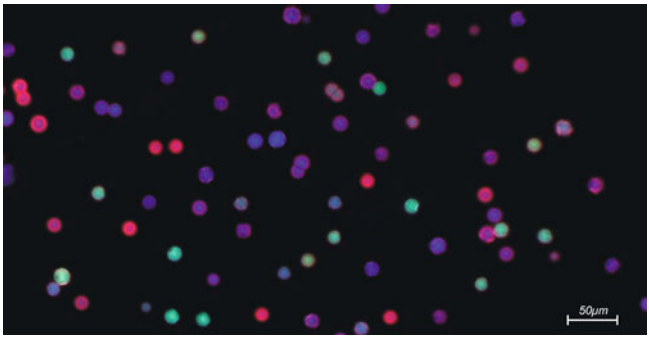

Fig. 3 Image of seven randomly arranged microbead populations. The seven microbead populations were used in a DNA hybridization assay at a packing density of about 700 microbeads per mm^2. Each microbead population can be identified by its PopID, and ligand fluorescence (red outer circle = positive) is quantified and reported as refMFI. Microbeads of one population are represented redundantly

with a diameter of 6–30 µm. A microbead of 6 µm creates an image with a diameter of nine pixels on the camera sensor (pixel size 6.5 × 6.5 µm), which is a sufficient resolution for image processing. The Nyquist–Shannon [15] sampling theorem is not violated at any wavelength used, so visual artifacts are avoided. Because depth of focus is only about 2.7 µm [16], auto-focusing is mandatory. This is automatically done by software algorithms. Each image captured with the above configuration covers an area of 670 × 900 µm. Such an image theoretically contains up to 500 single microbeads.

The intensity of fluorescence excitation is not constant, neither in time nor in the camera field of view. To solve this serious problem for measurement, VideoScan uses only ratios between different fluorescence channels, never absolute values. The ratio of these dyes is specific for every microbead population. Additionally the diameter can be used as a classification parameter. The surface of each microbead population is modified with specific probes to detect different analytes in a sample. The analyte is stained with a third fluorescence dye. The binding of an analyte leads to a fluorescent halo around the microbead (Figs. 3, 4 and 5). The intensity is determined by the image processing algorithm. This absolute value is referenced to one or both encoding dyes. The resulting referenced mean fluorescence intensity (refMFI) is independent of the device used and time point of measurement.

The measurement process is controlled by software without any user interaction necessary. After automatic focusing, an image is captured with a filter set matched to the first encoding dye (first encoding channel). This image is analyzed to identify single microbeads. Because fluorescence intensities between microbeads vary significantly due to the differences in the encoding dye concentration, an edge-based detection algorithm is applied [17]. Microbeads can be identified unambiguously by their circular shape, because microbeads are nearly perfect spheres. Non-circular shaped or overexposed objects are excluded from further processing, because they are considered to be artifacts, e.g., agglomerations, dust,

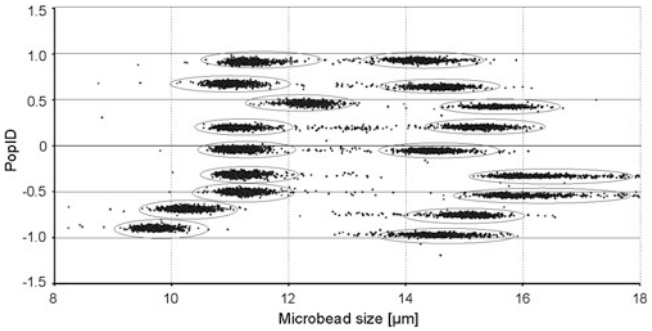

Fig. 4 Representative encoding of 18 microbead populations. Microbead populations are encoded by a combination of two fluorescent dyes at varying intensity levels which are processed as gray values. The decadal logarithm of the gray value ratio defines the PopID. Additionally, the microbead size can be used as an encoding parameter. Due to variations in impregnated dye quantity and microbead diameter, a negligible fraction is excluded from analysis (outer range of ellipsoids)

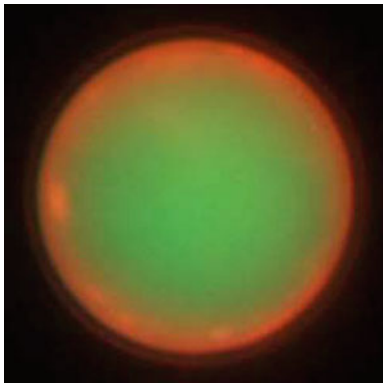

Fig. 5 Microbead with red halo of ligand dye (analyte). The intensity of the ligand dye is quantified on the edge of the microbeads

bubbles, etc.; centre coordinates and diameters of the remaining microbeads can be easily obtained from the circular area. Because of the edge-based detection method, the diameter calculation is independent from the intensity. Then a second image is taken at the same position with a filter set matched to the second encoding dye (second encoding channel). Gray values of the first and second encoding channel are determined in the centre of every microbead detected. This so-called PopID of every microbead is calculated by the decadic logarithm of the ratio of these two gray values (Eq. 1).

$$\mathrm{PopID} = \log_{10}\left(\frac{\mathrm{Grayvalue\,(encoding2)}}{\mathrm{Grayvalue\,(encoding1)}}\right) \tag{1}$$

If the two encoding dyes are graded in a ratio from 1:10 to 10:1 for every microbead, a PopID from −1 to +1 can be calculated. The diameter and PopID

ensure a unique assignment of every microbead to a specific population (Fig. 4). The probability of misclassification using this method is well below 1%.

Finally, the fluorescence intensity of the labeled analyte captured on the surface of the microbead is determined by taking an image of the same scene with a filter set corresponding to the wavelength of the "ligand channel". The initial integration time of routine images is typically 500 ms. In contrast to the encoding dyes, the ligand dye is located on the surface of microbeads only, so it creates a halo around the microbead. The intensity of this halo, resulting from binding analyte, is quantified on the edge of every microbead (Fig. 5).

To increase the dynamic range of a given assay, the intensity of every halo is checked. If its value exceeds a predefined limit, overexposure is assumed. If one or more microbead halos are overexposed, an additional image is captured with the integration time halved. This procedure is repeated until no halo is overexposed. Considering the integration time used, the absolute intensity value of the halo is recalculated and finally referenced to the intensity of one or both encoding channels. Referencing compensates completely for different intensities of fluorescence extinction and other long-term drifts of device-specific parameters. This allows comparison of numeric results measured with different devices at different time points or in different experiments. To get comparable results from different microbead populations, despite different concentrations of encoding dye, intensity differences in the encoding channel(s) are compensated by a population-specific correction factor (Eq. 2).

$$\mathrm{refMFI}_{\mathrm{Population\_i}} = \frac{\mathrm{Correction}_{\mathrm{Population\_i}}}{\mathrm{Beadcount}_{\mathrm{Population\_i}}} \cdot \sum_{\text{all beads population\_i}} \frac{\mathrm{Greyvalue(halo)}}{\mathrm{Greyvalue(encoding1)}} \tag{2}$$

The process is repeated at different positions in a well until a predefined minimum number of microbeads is identified. The measurement value for an analyte is calculated by the quantile-filtered mean value of all microbeads of the corresponding microbead population (refMFI). A user-defined quantile filter (default 3%) can be applied to handle outliers. Microbead measurement leads to a refMFI ranging from 0.003 to 300 (± 10%). In this dynamic range (100 dB), the measurement value is linear (deviation less than 3 dB).

3 Design Principles of VideoScan Microbead Assays

3.1 Immobilization of Microbeads

An optional step during VideoScan microbead array fabrication is microbead immobilization. The assay workflow of sequential handling steps is simplified considerably and refocusing is reduced to a minimum. Most importantly, reactions

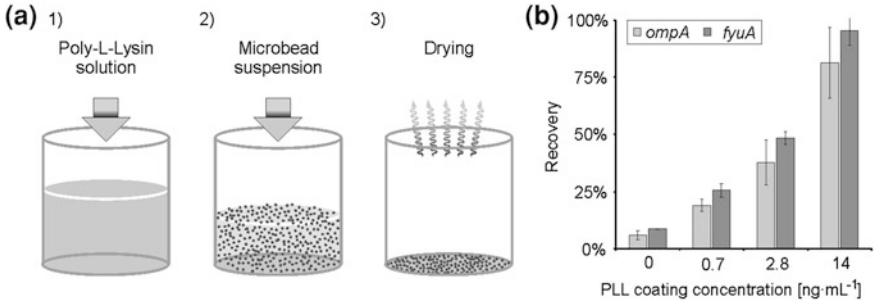

Fig. 6 Principle of microbead immobilization on PLL-coated wells. **a** (1) PLL is used to coat the bottom of the well. After 2 h, coating is finished and remaining PLL solution is removed. (2) A suspension of capture probe-loaded microbeads is added to the well. Gravitational force leads to a rapid sedimentation (~ 10 min) of microbeads which adhere to the PLL. (3) The evaporation of suspension supernatant leads to permanent immobilization of the microbeads. **b** Effect of PLL concentration on microbead recovery. Two different microbead populations were loaded with capture probes *fyuA* or *ompA and subsequently immobilized on PLL-coated NucleoLink*® stripes. Coating wells with 14 ng mL^{-1}PLL resulted in a recovery rate above 80% of microbeads applied and this concentration was used throughout further experiments

with dynamic temperature profiles at elevated temperatures suffer from intense microbead motion, due to convection and Brownian motion. We identified the polycationic polymer poly-L-lysine (PLL) as an excellent substrate for immobilization capture of molecule-loaded microbeads on standard plastic wells. PLL was previously reported for the immobilization of cells and different biomolecules on glass and plastic materials [18, 19]. This simple procedure fulfilled all requirements: (a) to keep various polydispersed microbead populations randomly mixable, (b) to introduce no adverse effect into the optical well properties, (c) no non-specific binding of reagents, (d) general applicability for various biomolecules, (e) no adverse effect on the ligand value and (f) equal distribution of single microbeads as a densely packed monolayer on the well bottom. It therefore made obsolete complicated methods developed for microbead immobilization, like the use of etched surface structures (e.g., Illumina BeadArray systems), mechanical retention or microbead embedding in hydrogel-coated slides [11, 13, 20–23]. To determine optimal conditions for the immobilization of microbeads in multiplex assays, we started with two microbead populations. Oligonucleotide capture probes encoding for *fyuA* or *ompA* (Table 3) were coupled onto the microbead surface (see Sect. 3.2). PLL hydrobromide (Sigma-Aldrich, MW 30,000–70,000) was resolved in phosphate-buffered saline (PBS) in concentrations ranging from 0 to 14 ng mL^{-1}. The NucleoLink® modules were incubated with 40 µL of the PLL solutions for 2 h at 40 °C (Fig. 6a(1)). The supernatant was removed and the PLL-coated modules were washed with 100 µL of 1:100 PBS for 5 min at room temperature. Afterwards, microbeads suspended in 1:100 PBS were added to the PLL-coated modules (Fig. 6a(2)). The microbead suspension was left to dry (Fig. 6a(3)). Residues of buffer substances were removed by washing in ddH_2O.

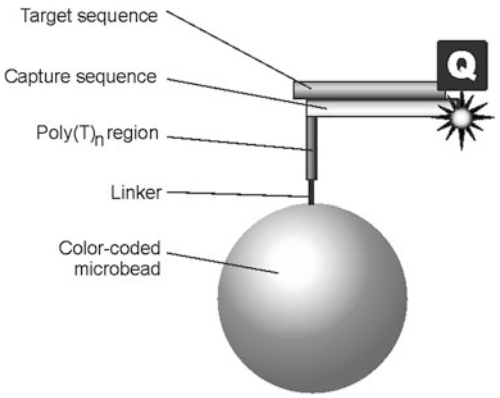

Fig. 7 Schematic representation of capture probes as used in a standard direct hybridization [25] fluorescence resonance energy transfer (FRET) assay. Capture probes are immobilized (5′ → 3′) via C6 carbon or hexaethylenglycol linker to the microbead surface by either covalent cross-linking via amide bonds or streptavidin–biotin binding, respectively. The capture probes contain a poly(dT)$_{10–20}$ region which is used as spacer, and for the relative quantification and characterization of non-labeled capture probes. The gene-specific region (15–35 nt) is complementary to the target sequence. For FRET assay, capture probes carry a reporter dye at the 3′ position

The recovery rate was determined as the ratio of immobilized microbeads to the number of microbeads after seeding (Fig. 6(b)). A coating concentration of 14 ng mL^{-1}PLL is sufficient to obtain a microbead recovery rate of at least 80%.

3.2 Immobilization of Biomolecules on the Surface of Microbeads

VideoScan predominantly utilizes thermo-tolerant color-coded carboxylated PMMA microbeads (PolyAn GmbH, Germany) with a diameter of 9–18 μm. This simple approach allows rapid developmental cycles with comparatively low technical demands. Biomolecules are immobilized by three different procedures: passive adsorption, cross-linking and immobilization of biotinylated biomolecules on streptavidin-coated microbeads. In the following sections we will refer to protocols for the covalent and highly thermostable immobilization of DNA capture probes via amide bonds according to Rödiger et al. [3] or Armstrong et al. [24] (Fig. 7). The immobilization of biotinylated oligonucleotides was done via biotin-streptavidin interaction according to Rödiger et al. [3].

A set of 18 microbead populations was used to evaluate the success of capture probe immobilization and target accessibility. We independently tested two fluorescence-labeled (Atto 647N) capture probes (*MLC-2v-cap*, *aCS-cap*, Table 8) containing a poly(dT)$_{20}$ and target-specific region (Fig. 7). Microbeads were loaded

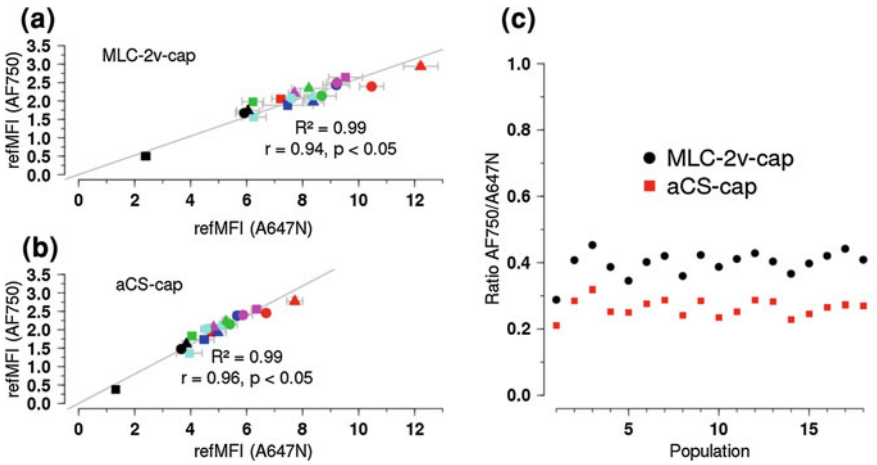

Fig. 8 Quality control of DNA capture probe loading and probe accessibility to hybridization. The quantities (refMFI) of two fluorescence-labeled (Atto 647N) DNA capture probes (**a**) *MLC-2v-cap* or (**b**) *aCS-cap*, Table 8) were determined simultaneously on 18 microbead populations (eight replicates). Each colored symbol for "●", "■", or "▲" represents the mean of one microbead population. *Error bars* are given as standard deviation. Microbeads with different quantities of capture probes were prepared as described in Rödiger et al. [3]. The poly(dT)$_{20}$ region (Fig. 7) common to both probes was interrogated by direct hybridization with fluorescent Alexa Fluor 750 (AF750)-labeled *poly(dA)$_{20}$* showing that capture probes on all microbead populations are highly accessible. Data were fitted using the *RKWard* integrated development environment (Rödiger et al. [27]) using the *lm()* function. Pearson's r correlation coefficient was calculated using the *cor()* function. (**c**) Ratios of ligand channels of AF750 and Atto 647N (A647N, ordinate) and microbead populations (abscissa) shows a high agreement throughout all microbead populations and capture probes (●*MLC-2v-cap*, ■*aCS-cap*). This suggests that both fluorescence channels can be used in bioassays

with different quantities of capture probes according to Rödiger et al. [3]. The fluorescence signal of Atto 647N (A647N) indicated a successful probe immobilization and allowed an inference regarding the quantity. The hybridization with Alexa Fluor 750 (AF750) labeled *poly* $(dA)_{20}$ was indicative of the quantity (compare Day et al. [26]) and functionality of the immobilized capture probe. We found a very strong correlation ($\geq$0.94, $p < 0.05$) and a linear relationship for immobilized probes interrogated with the AF750-labeled *poly*$(dA)_{20}$ (Fig. 8). Thus the use of labeled *poly*$(dA)_{20}$ is an elegant and cost-effective approach to characterizing the quantity and functionality of surface-bound capture probes. We also calculated the ratio of the ligand channel values of AF750 and Atto 647N and found a general agreement independent of the capture probe used in all microbead populations (Fig. 8c). The two different channels for ligand value measurement provide an important feature for assay development.

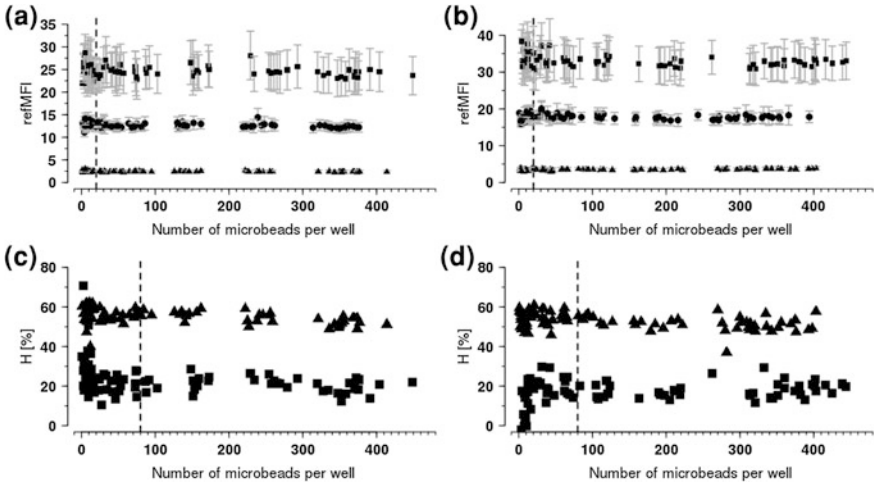

Fig. 9 Minimum number of microbeads required for a stable ligand value. Microbeads were either loaded with a capture probe for *MLC-2v-cap* or an artificial sequence designated *aCS-cap*. Microbeads with different capture probe densities were used (▲"low", ● "medium", and ■ "high" surface probe density). *Vertical dashed black line* is the cut-off for a stable ligand value. For (**a**) *MLC-2v-cap* and (**b**) *aCS-cap* the number of microbeads were plotted against their refMFI. *Error bars* represent the standard deviations. Quencher oligonucleotides for (**c**) *MLC-2v-cap* and (**d**) *aCS-cap* were given to the samples and the hybridization efficiency "H [%]" calculated as Eq. 3, where H_0 is the non-quenched and H_1 the quenched state in refMFI, respectively. Microbeads with a medium (not shown) and high (■) capture probe density reported higher variability at low (<80) microbead numbers which was not the case for microbeads with a low (▲) capture probe density

3.3 Determination of Minimum Number of Microbeads Per Assay

The universal advantage of microbeads is their *n*-fold redundancy at a desired number in a single reaction environment, e.g., a well of a microplate. In theory, statistical measures, including mean and median, can be provided at high data quality and reproducibility. In reality, however, there is a limitation for the minimum number of features, mainly attributed to the variance within one microbead population (see Rödiger et al. [3] for details). Therefore we determined the minimum required number of microbeads and the influence on the variance of the ligand value.

Three microbead populations were loaded with different quantities of Atto 647N-labeled capture probes *MLC-2v-cap* or an artificial sequence designated as *aCS-cap*, according to Rödiger et al. [3]. We found that the minimum number of microbeads required for a reliable ligand value is about 20 per population, independent of microbead population and capture probe. The reported signals were uniform above 20 microbeads per population (Fig. 9a, b). In this study we demonstrated that about 20 microbeads are statistically sufficient for reliable test results, a quantity not achievable by standard flow cytometry.

$$H\,[\%] = \left(1 - \frac{H_1}{H_0}\right) \cdot 100 \tag{3}$$

3.4 Determination of Functional Capture Probe Density

To perform assays with a good sensitivity and specificity it is important to determine the density of immobilized capture probes that is useful for VideoScan assays. It is well known that the surface capture probe density is negatively correlated with the hybridization efficiency ("H") of oligonucleotides probes. A high density may lead to steric hindrance, the so called "hook effect" [28].

To determine the range of functionally immobilized capture probe densities, a standard hybridization FRET assay was applied (Fig. 7). Again, three microbead populations were loaded with low, medium and high quantities of Atto 647N-labeled capture probes *MLC-2v-cap* or *aCS-cap*. The complementary target sequence for *MLC-2v-cap* or *aCS-cap*, respectively, was added at a final concentration of 150 nM. The samples were set to hybridize initially at 95 °C for 2.5 min and finally at 60 °C for 60 min. Under these conditions we found again that the minimum number of microbeads is 20 per population. Although showing an increased signal-to-noise ratio, microbeads with a medium or high capture probe density reported higher signal variability at low microbead numbers (<80), which was not the case for microbeads of lower capture probe density. Increasing the number of beads measured could easily compensate for this effect (Fig. 9c, d). We therefore conclude that VideoScan assays designed with microbeads of variable capture probe densities do not suffer from the adverse hook effect [28]. This broadens the range of suitable microbead populations and enables a multi-purpose assay design.

4 VideoScan Technology Applications

4.1 AKLIDES® Application for the Automated Detection of Autoantibodies by Cell-Based Assays

Based on VideoScan technology development, an automated indirect immunofluorescence (IIF) microscopy system for the assessment of autoantibodies (AAB) employing cell- and microbead-based assays was recently commercialized under the brand name AKLIDES® (Medipan GmbH, Dahlewitz/Berlin, Germany) [29]. Autoantibodies play a pivotal role in the serological diagnosis of systemic rheumatic and autoimmune liver diseases [30–32]. Autoantibody assessment is used to monitor disease activity, sub-classify, and predict autoimmunity [33, 34].

Modern laboratory standards require high reproducibility and standardization of IIF interpretation which is difficult to achieve due to subjective visual reading by

laboratory experts. The automation of AAB IIF reading, including pattern recognition, would therefore be effective in reducing intra- and inter-laboratory variability, and meeting the growing demand for cost-effective assessment of large numbers of samples (i.e., high throughput). This automated IIF interpretation system has previously been developed for ANA (anti-nuclear antibody) detection on HEp-2 cells, assessment of dsDNAab on *Crithidia luciliae* and of ANCA on human neutrophiles and found to be reliable for the positive/negative differentiation of IIF patterns as well as pattern recognition of ANA and ANCA findings [29, 30, 35]. The AKLIDES® system uses common standard protocols for IIF staining of immobilized cells on glass slides by the AAB to be detected. A secondary antibody labeled with FITC is employed to reveal the specific AAB binding. Additionally, 4′,6-diamidino-2-phenylindol (DAPI) staining is used for focusing procedures and nucleus detection. Image acquisition, evaluation of image quality, image segmentation for object detection, feature extraction for object description, and classification of objects have been automated to provide a standardized detection method for even subcellular structures. Images of IIF patterns are automatically taken and autofocusing is the most critical step in IIF reading since insufficient focusing in the range of a few microns may bring about false classification of staining patterns [36]. Typical IIF images obtained by AKLIDES® are shown in Fig. 10.

Currently, cell-based IIF is usually interpreted by an experienced immunologist in routine laboratories, assessing areas of the image with sufficient quality for their pattern-recognition decision. In terms of automated reading, certain image areas are not suitable for interpretation due to artifacts or staining failures. This creates the need to implement a preselection algorithm for suitable scenes for pattern analysis. Taking into account the structure and the staining of adherent cell monolayers, quality criteria, e.g., the maximum plausible cell size, were defined for proper image acquisition. For quality control of image reading, the quality of images is measured by descriptive parameters (e.g., sharpness, brightness, number of cells). Each image below a certain quality level is excluded from further pattern analysis.

This guarantees that only correctly acquired scenes are processed by the following more complex algorithms. To differentiate distinct patterns, IIF image signals were processed further in hierarchical processing steps: (i) positivity, (ii) localization (nucleus, cytoplasm, chromatin of mitotic cells), and (iii) main nuclear pattern. For the deep analysis of acquired image data, the following steps were processed sequentially: acquisition, quality control, segmenting, description of object and object classification. Segmented objects were defined finally by boundary, regional, topological, and texture/surface descriptors. Digital features were combined into rules, resembling the rules employed by experts for pattern recognition.

In summary, accumulating data have shown a good agreement between AKLIDES® and manual IIF interpretation for ANA, ANCA, and dsDNA antibody detection in patients with autoimmune diseases. We therefore conclude that

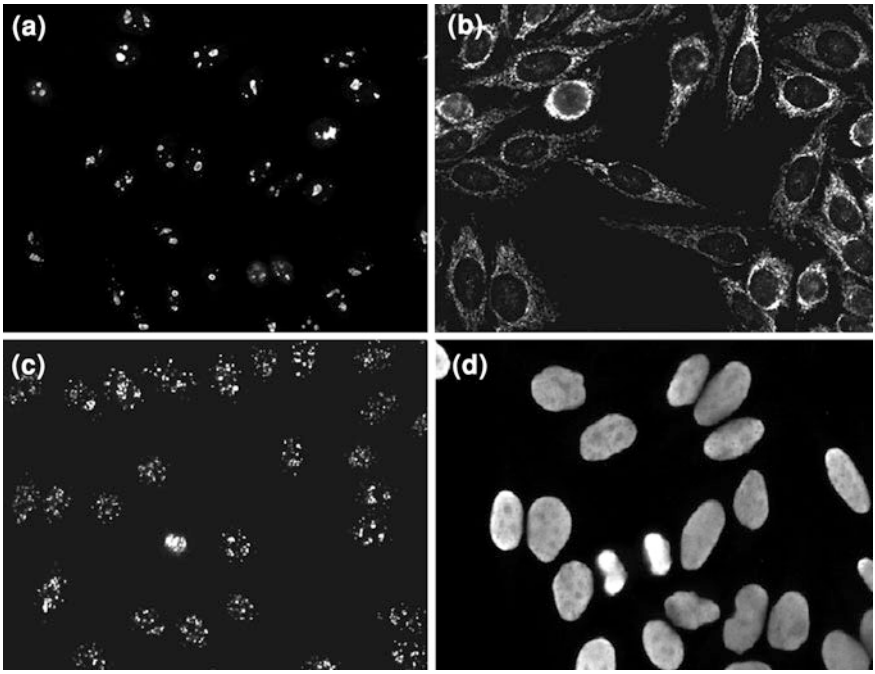

Fig. 10 Immunofluorescence patterns of sera assessed positive by AKLIDES® system. Immunofluorescence image data were evaluated according to the following hierarchy: (i) positivity, (ii) localization of staining (nuclear, cytoplasmic, chromatin of mitotic cells), and (iii) determination of nuclear patterns: homogeneous, speckled, nucleolar, centromere, multiple nuclear dots, as described elsewhere [29]. **a** Nucleolar pattern, **b** cytoplasmic pattern, **c** centromere pattern, **d** homogeneous pattern

AKLIDES® has the potential to become the new standard in automated autoantibody detection.

4.2 Semiquantitative Autoimmune Diagnostics of Antinuclear Antibodies

Pre-screening of patient sera for antinuclear antibodies using cell-based systems is only the first step in the diagnostic workflow, and positive results need an independent confirmation. Usually, autoimmune diagnostic multiplex test formats like strip tests or multi-well ELISA are widely used for this purpose although they possess specific disadvantages with respect to manufacturing costs or test processing [2, 8, 9]. Alternatively, planar biochips were found to barely fulfill diagnostic quality, since their manufacturing requires nanoliter liquid handling of protein solutions, a highly sophisticated, expensive, and unstable process that

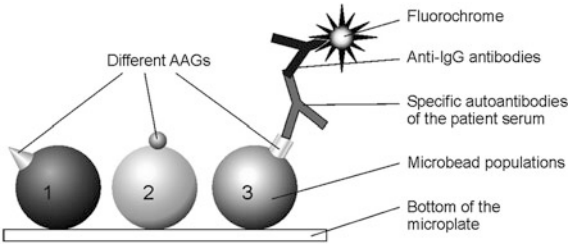

Fig. 11 Scheme of the antibody microbead assay. Three microbead populations (1, 2, 3) carrying different autoantigens (AAGs) were immobilized on a microplate. Specific autoantibodies (AABs) bind to their corresponding AAG (microbead 3) only. The reactive AAB are marked by secondary fluorophore-labeled anti-IgG antibodies generating a ligand fluorescence on the microbead surface

needs intensive quality assessment of each manufacturing step [37]. A suspension microbead array [25] that uses microbead populations encoded by fluorescence and/or size of their microbeads is a completely new approach. Each microbead population carries a specific autoantigen (AAG) and by suspending different microbead populations within one sample, a multiplex test can be performed. However, a flow cytometer is required [38]. The aim of this study was to characterize the analytic performance of the Attomol® ANA Microbead Assay (ANA-BA) for qualitative multiplex detection of autoantibodies (AAB) using a standard fluorescence microscope.

The antinuclear antibody microbead assay (ANA-BA) is provided as a ready-to-use kit that provides all reagents for detection (Attomol GmbH; Bronkow; Germany). It contains 11 different microbead populations, nine of which carry the AAGs dsDNA, Sm, RNP/Sm, Ro60, Ro52, La/SS-B, Scl-70, CENP-B, and Jo-1 respectively. In addition, a positive and a negative control population are included. Microbead populations are mixed and permanently immobilized with PLL in a standard 96-well microplate. Using four batches of the ANA-BA, two different serum panels were analyzed to detect AABs according to the manual (Fig. 11.) as follows: (a) incubation of patients' serum diluted 1:50 in sample buffer, 1 h, (b) wash buffer, three wash cycles each for 1 min, (c) fluorophore-labeled anti-human IgG conjugate, 1 h, (d) wash buffer, four wash cycles each for 1 min, (e) automated VideoScan measurement of relative fluorescence units.

All incubations were performed without agitation at ambient temperature. Serum panel A comprises 100 sera of blood donors and panel B comprises 750 sera of patients with suspected autoimmune diseases. Since Jo1-AABs are extremely rare, this parameter could not be used throughout the evaluation of test sensitivity/specificity in the study.

Sera of panel B, exhibiting significant immune reactivity to one of the antigens, were repeatedly analyzed to determine coefficient of variation (CV) [%] of intra- and inter-assay, and inter-batch precision. To proof test stability of all microbead

populations, eight sera were tested on ANA-BA stored at 4 or 40 °C, respectively, over 8 days.

The signal cut-off of each parameter was adjusted using panel A so that at least 99% of these control samples were negative. Using these settings, 750 sera of panel B were analyzed by ANA-BA. In addition, a subgroup of sera from this panel was tested with commercially available IVD-CE-marked reference ELISA. Data were processed to calculate kappa coefficient [39], relative sensitivity, and specificity. In the case of some antigens, fine-tuning of the cut-off was done using receiver operating characteristic analyses (data not shown) to exceed at least 95% specificity for each parameter.

Intra-assay precision was calculated from eight parallel determinations for some of the parameters which reacted with the sera used. The mean of the relative fluorescence units (rfu) of these different parameters ranged between 0.01 and 2.22, and the intra-assay precision varied between 1.8 and 6% (Table 1). Inter-assay precision was calculated from determinations of three different runs using microplate modules of the same batch. The mean of the rfu of these different parameters ranged between 0.01 and 1.70, and the intra-assay precision varied between 1.8 and 12% (Table 1).

Inter-batch precision was determined using microplate modules of three different batches in parallel. The mean of the different immune reactivities ranged between 0.01 and 2.23. The mean of all immune reactivities was 0.8. Inter-batch precision ranged between 1.2 and 19.9%.

To demonstrate ANA-BA stability, test modules were stored at 4 °C and a second set of modules at 40 °C for 1 week. Comparison of both differentially stored sets of modules showed that all the tested parameters did not lose their immune reactivity at higher temperatures (Fig. 12).

The analytical performance of ANA-BA was studied in comparison with commercial reference ELISA using 59–400 patient sera for each parameter (Table 2). The kappa coefficients are mostly above 0.8, which means “very good” accordance between ANA-BA and reference test results. The dsDNA and Scl-70 tests, with kappa of 0.678 and 0.668, respectively, are classified as “good”. The specificity of ANA-BA ranges between 96 and 100%. The sensitivity of all parameters with the exception of dsDNA and Scl-70 is above 90%. In the cases of RNP/Sm and Sm, sensitivity as well as specificity are both 100%.

The VideoScan application ANA-BA combines the advance of microplate technology with a high potential for automation of test manufacturing as well as test processing in routine laboratory workflows. In comparison with the 96-well ELISA format, each well of the ANA-BA comprises nine AAGs as diagnostic parameters and two integrated controls that enhance test reliability. Positive control indicates general test function as, e.g., altered anti-IgG conjugate, or tests inhibition if washing steps were performed inefficiently. Increased negative control values may indicate non-specific binding of “sticky” serum samples. Generally, the technology permits the determination of up to 18 AAB species so far, and processing of several hundred samples per day.

Table 1 Precision of ANA-BA

Precision	*n*		PosC	NegC	dsDNA	Ro60	Ro52	La/SSB	RNP/Sm	Sm	Scl-70	CENP-B	Jo-1
Intra-assay	8	mean	1.60	0.01	0.85	0.91	2.22	0.45	0.91	0.34	0.44	0.22	0.28
		CV%	2.4		2.5	6.7	4.9	1.8	6	3.8	3.6	4.9	2.6
Inter-assay	3	mean	1.70	0.01	0.75	1.20	2.27	0.32	1.22	0.38	0.46	0.28	0.38
		CV%	10.4		4.8	9.7	12	7.9	3.2	2.4	1.8	4.5	6.6
Inter-batch	3	mean	1.59	0.01	0.82	1.06	2.23	0.43	1.13	0.37	0.48	0.29	0.36
		CV%	1.2		6.2	14.1	2.8	5.1	16.4	8.6	8	19.5	19.9

All values determined for negative control (NegC) population were omitted from calculations of means. PosC, positive control

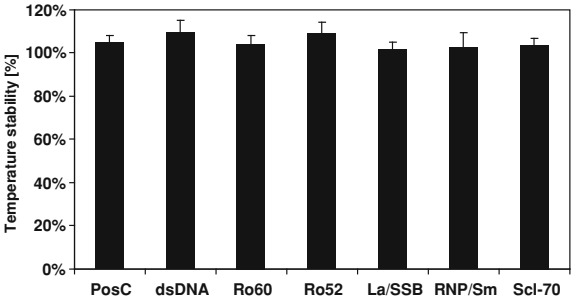

Fig. 12 ANA-BA stability at higher temperatures. Stability of ANA-BA at 40 °C was between 100 and 110% in comparison with ANA-BA stored at 4 °C

Table 2 Analysis of analytical performance of ANA-BA

	Ro60	Ro52	Scl-70	dsDNA	CENP-B	Sm	RNP/Sm	La/SS-B
Specificity (%)	96	96	96	96	100	100	100	97
Sensitivity (%)	94	91	63	74	90	100	100	95
Kappa	0.903	0.879	0.668	0.678	0.920	0.943	0.986	0.910

All values determined for negative control (NegC) population were omitted from calculations of means. PosC, positive control

ANA-BA shows a dynamic measurement range over at least three log10 steps. Intra-assay precision mostly does not exceed 5%, a value achieved by most common ELISA for antibody detection [40]. It seems that some antigens like Ro60 generally show a reduced precision, perhaps because it is more difficult to protect their immune reactive epitopes during the manufacturing process and storage. Inter-assay precision was found to be below 12%. This general finding for inter-assay precision is similar to other microbead-based assays [38, 41] or diagnostic biochips [37] and seems to be sufficient in semi quantitative testing. Kappa values of ANA-BA are comparable with standard ELISA and are in most cases "very good" (see below). However, inter-assay precision below 10% would be desired if quantitative antibody detection is the aim. Interestingly, the inter-assay CV of the anti-dsDNA antibody detection is 4.8%, the only parameter that requires quantification to increase the ANA-BA's clinical value [42]. In contrast, inter-batch precision with a mean over all parameters of 10.2% is, as expected, higher than intra- and inter-assay precision, since the batches applied in this study were manufactured during the not fully standardized final product development process.

Comparison of ANA-BA with reference ELISA provided relative sensitivities of $\geq$ 63%, and relative specificities of 100%, which is in good agreement with the findings of Wagner et al. [43]. Because considerable conflicts between ELISA and microbead assay results have been described [38, 44–46], it is important to mention that the degree of accordance between most of the reference ELISA and ANA-BA is kappa $\geq$ 0.8 and therefore "very good". The high specificity of ANA-BA is underlined by the findings that none of the blood donor sera showed any specific AAB activity, which is in accordance with the low AAB prevalence in the healthy population [42].

The data of this study demonstrate that ANA-BA is a test system with great potential for routine diagnostics of systemic rheumatic diseases. The analytic performance and the test handling are comparable with standard ELISA tests that were used as references for each analytic parameter. The test precision and stability of ANA-BA were also found to be in the range of diagnostic semiquantitative ELISA tests.

4.3 Immobilization and Detection of Biotinylated Antigens from Crude Bacterial Lysates

The development of multi-parameter microbead-based assays requires the immobilization not only of DNA capture probes; proteins or peptides are equally important to serve as capture probes for the detection of autoantibodies in patient sera or for epitope mapping. Different biomolecule immobilization strategies, including passive adsorption and covalent attachment, have been described previously [47, 48]. Binding of biotinylated capture probes to streptavidin-coated microbeads is also an elegant approach. The high affinity and specificity of the biotin–streptavidin interaction is widely used for biomolecule labeling or for purification purposes [49]. A major drawback of this approach is the tedious, time-consuming and expensive production of these essential biotinylated antigens. Instead of protein/peptides synthesis and chemical biotinylation, we aimed for a fast, easy and inexpensive alternative approach using a technology first described by Beckett et al. [50] and patented as AviTag™ technology (Avidity) (Fig. 13a). In this, peptides are expressed as fusion peptides with an AviTag™ (Fig. 13b). This tag is a 15-amino-acid peptide sequence with a lysine residue, which is biotinylated by a biotin ligase. The biotin ligase is overexpressed in *Escherichia coli* cells (Fig. 13c). Biotinylated peptides can be directly immobilized on streptavidin-coated microbeads merely by disrupting cells and incubation with the lysate. Unbound material is subsequently removed by washing. In a proof-of-principle approach, we used the well-characterized glutathione S-transferase (GST) as model protein.

In contrast to the notion that the expression of biotinylated proteins using the AviTag™ is not suitable [51] because the overexpression of a biotin ligase in *E. coli* causes cell damage and in efficient biotinylation, we observed a functionally sufficient biotinylation efficiency of different peptides (data not shown). Biotinylation takes place very specifically since only the AviTag™ is biotinylated but not the fusion protein itself. This should minimize the risk of severely affecting binding properties by peptide modifications. Since there is only one biotinylation site per tagged peptide, it is well orientated in regard to the microbead surface and it is easily accessible for potential binding partners compared to randomly biotinylated or covalently immobilized molecules, which are attached to microbeads via many different binding sites. The ease of this method, in addition to time and cost savings, makes it very attractive for use with VideoScan.

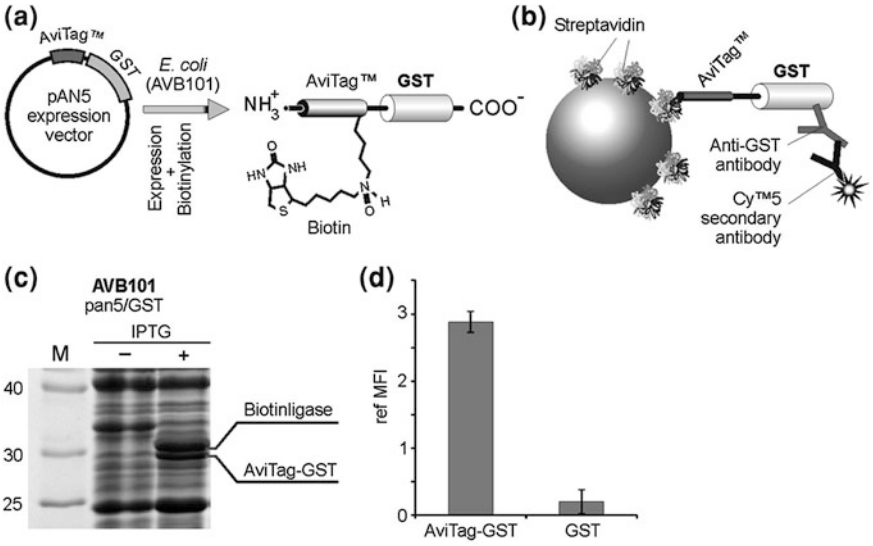

Fig. 13 Principle of the expression of in-vivo biotinylated GST in *E. coli* (AVB101): **a** GST-encoding cDNA was cloned into the expression vector pan5. Subsequent AVB101 cells were transformed to overexpress GST including AviTag™ and overexpress biotin ligase which specifically biotinylates a lysine residue. **b** Detection principle of biotinylated GST: AVB101 cells expressing either non-biotinylated GST or biotinylated GST (AviTag™-GST) were grown in 50 mL LB medium at 37 °C. At an OD of 0.7, protein expression was induced (1.5 mM IPTG) in the presence of 50 μM biotin. After 3 h cells were washed three times with 50 mL ice-cold water, pelleted and resuspended in 6 mL PBS (50 mM Na-phosphate, pH 7.4, 150 mM NaCl) including inhibitor cocktail plus (Roth) according to the manufacturer's instructions. Cells were lysed by French pressing. All cell debris was removed by centrifugation (20 min, 16,000g). The cleared lysates (200 μL) were incubated on ice for 15 min with about 200 streptavidin-coated microbeads, which were prepared as described in Rödiger et al. [3]. The microbeads were then washed three times with PBST (PBS, 0.1% Tween®20, pH 7.4) by resuspension of microbeads in 200 μL PBST, centrifugation (3 min, 2,250g) and removal of the supernatant. Microbeads were shaken for 1 h with 25 μL (1:100 in PBS) monoclonal rabbit-anti-GST-antibody (Sigma-Aldrich), then washed three times with PBST (see above). The microbeads were then incubated with 25 μL (1:100 in PBST) anti-rabbit-Cy5-labeled secondary antibody (Dianova) for 1h. Finally, microbeads were washed three times with PBST. **c** Western blot analysis of expressed GST: AVB101 was transformed with pan five containing cDNA coding for GST. Samples of 1 mL culture medium were taken before IPTG induction and after finishing the protein expression. Lysates of bacteria were analyzed by SDS-PAGE (M, marker in kDa). **d** VideoScan detection of biotinylated GST: Bound GST was quantified by Cy5 fluorescence using VideoScan

The VideoScan detection of biotinylated protein/peptide presented here is the first step in the development of a multiplex protein microbead assay. Building on this proof-of-principle, we are developing a medium-throughput system for VideoScan epitope mapping using biotinylated GST-peptide fusion antigens expressed in *E. coli* via the AviTag™-System.

5 VideoScan Assays for the Detection of Nucleic Acids

As described so far, applications are not limited to the detection of antigens, peptides and proteins but are also useable for nucleic acids. In the following sections we will focus on endpoint and real-time applications for the detection and analysis of nucleic acids. In respect to the analysis of this important biomolecule, planar surface technologies like microarrays are dominant for the simultaneous analysis of transcriptional changes, single-nucleotide polymorphisms, and identification of genes [52, 53]. Commonly the modus operandi is a direct hybridization of the labeled target to the complementary capture molecule on a defined spot (e.g., microarray) [54]. Though this approach results in a low signal bias during hybridization, it is well known that the planar paradigm also leads to a decreased sensitivity [55]. Moreover, preparation of microarrays requires tight control of the spotting. Spots may vary significantly and have only a little redundancy. With the introduction of microbeads, however, the aim was to overcome these limitations [4]. Capture-probe-loaded microbeads can be prepared in large bulks with optimized coupling chemistry, which leads to a reduced signal variation. They can be used at high redundancy. In contrast to planar spots, the spherical character of microbeads leads approximately to a hybridization behavior, as in aqueous solution [4]. Moreover small spot (microbead diameter) sizes are positively correlated with hybridization efficiency of target to capture probes due to transition from mass transfer to kinetically limiting factors [56, 57]. Microbeads also offer a larger surface area compared to planar spots of the same diameter. Conventional microbead-based assays are used in suspension but recently the paradigm changed to surface microbead arrays where microbeads reside on the well bottom [4, 14, 25, 58]. In contrast, VideoScan utilizes randomly ordered microbeads which are spatially distributed on the surface of a well [59]. A disadvantage of this approach is the reduced density of microbeads per area compared to spatial defined positions, for example on patterned surfaces, as found elsewhere [20, 52, 54, 60]. However, manufacturing of such test systems is more complex.

5.1 The VideoScan Multiplex Hybridization Assay

Escherichia coli (*E. coli*) is a common bacterium of the intestinal microflora of mammals and birds but also an important cause of bacterial infections. *E. coli* can be grouped into numerous pathovars and can cause intestinal as well as extraintestinal disease. Within the family of Enterobacteriaceae, *E. coli* shows the largest known variability of virulence as well as the highest known number of virulence-associated genes (VAGs). Typical VAGs in *E. coli* from diarrheic hosts (iVAGs) code for adhesins and toxins. Typical VAGs in extraintestinal pathogenic *E. coli* (eVAGs) code for adhesins, toxins, iron acquisition systems, lipopolysaccharides, capsules of polysaccharides and invasins. The primary method for testing

Table 3 Primers and capture probes for the detection of PCR products in *E. coli*

Name	Oligonucleotides and labeling (5′-3′)
vat	f: GTGTCAGAACGGAATTGTC#, r: GGGTATCTGTATCATGGCAAG
	p: $(dT)_{10}$CCGGGGTTGCTTTATTTGAGAAATTAATATTTCCC
tsh	f: TGGTGCAATGCCCATTTATGG, r: CTTCCGATGTTCTGAACGT
	p: $(dT)_{10}$TAATGAAGACAATGATGCCC
iucD	f: CCTGATCCAGATGATGCTC#, r: CTGGATGAGCAGAAAATGACA
	p: $(dT)_{10}$TGGTCAGTAAAGAATCGGCAGTGATGCC
cvi/cva	f: CGCAGCATAGTTCCATGCT#, r: GCAATTTGTTGCAGGAGGA
	p: $(dT)_{10}$TCATATATTGCACCTCCAGCCACACCCC
papC	f: GCTCCATGGTCATATAGTTTCG, r: TGATATCACGCAGTCAGTAGC
	p: $(dT)_{10}$ACGTTCTCTCTCCCTCAATACG
iss	f: GGACAAGAGAAAACTGTTGATGC, r: CAGCGGAGTATAGATGCCA
	p: $(dT)_{10}$CCAGTTATGCATCGTGCATATGG
EAST1	f: TGCCATCAACACAGTATATCC#, r: TAGGATCCTCAGGTCGCGAGTGACGGC
	p: $(dT)_{10}$CCAGTTATGCATCGTGCATATGG

The forward primer (f) was unlabeled, the reverse primer (r) contained a 5′-Cy5 label and the capture probe (p) was 5′ amino modified with an additional poly(dT)10 region. # oligonucleotide sequence according to Ewers et al. [62]

E. coli for VAGs is multiplex-PCRs (mPCR) with subsequent agarose gel analyses. This however is time-consuming, prone to errors and laborious. We therefore developed a VideoScan multiplex hybridization assay with direct fluorescent labeling of PCR products (see also [52, 61]).

The mPCR was performed according to the following protocol: a 20-μL reaction mixture was prepared including 2 μL 10× PCR buffer, 2 μL 50 mM $MgCl_2$, 2 U Taq DNA polymerase (Rapidozym, Germany), 0.5 μL of each 10 mM dNTP, 0.1 μL (100 pmol) primer pair (BioTez Berlin, Germany) (Table 3), and 50 ng template DNA, supplemented with appropriate volumes of water. mPCR was performed in a thermal cycler (Eppendorf, Germany): initial denaturation for 3 min at 94 °C, 40 cycles of 30 s at 94 °C, 30 s at 52 °C, 1 min at 72 °C, with a final cycle of 5 min at 72 °C. Hybridization of the fluorescence-labeled PCR fragments to the immobilized (Sect. 3.1) capture probe (Table 3) loaded microbeads (Sect. 3.2) was achieved by hybridization for 120 min at 50 °C.

The fluorescence intensity determined by VideoScan for each microbead population corresponded well with the amount of PCR product analyzed in parallel with agarose gel electrophoresis (Fig. 14). The analysis of three genes of *E. coli* (*iucD, cvi/cva, papC*) by the VideoScan hybridization assay, in comparison to standard agarose gel analysis, with a concentration gradation of template DNA ranging from 0 to 50 ng is shown in Fig. 14. The comparison of repetitive analysis of seven VAGs of *E. coli* is shown as an example for three runs with three different *E. coli* strains in Fig. 15.

We have shown a multiplex hybridization assay for the detection of virulence-associated genes (VAGs) as a sensitive diagnostic tool with a detection limit below that of agarose gel band analysis. Besides the high sensitivity, it allows for very specific mPCR analysis due to the exclusion of non-specific PCR products which

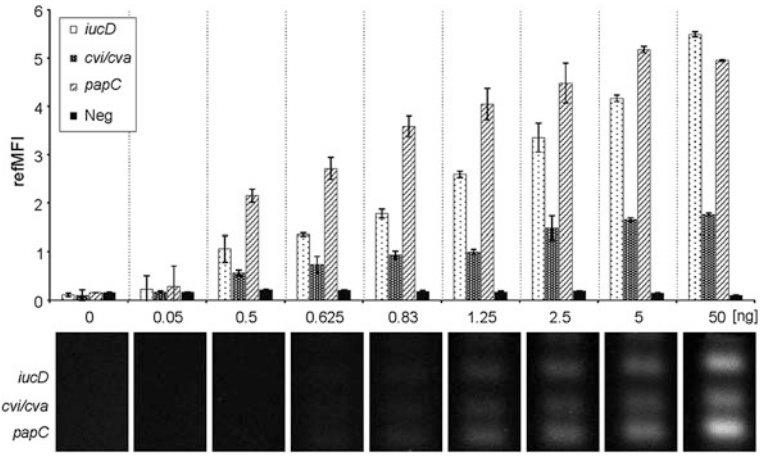

Fig. 14 Sensitivity/detection limit of the VideoScan hybridization assay: analysis of three VAGs from *E. coli* (*iucD, cvi/cva, papC*) by the VideoScan hybridization assay in comparison to standard agarose gel analysis. Comparison of refMFI and agarose gel analysis of a gradation of template DNA amounts used for PCR (0–50 ng). Increasing refMFIs correspond to increasing agarose gel band intensities. The signal pattern was independent of the PCR product quantity used. Down to 0.83 ng PCR product, VideoScan and the agarose gel reported identical results. However, below this, VideoScan was still able to detect PCR products. A gene non-specific sequence served as negative control (Neg)

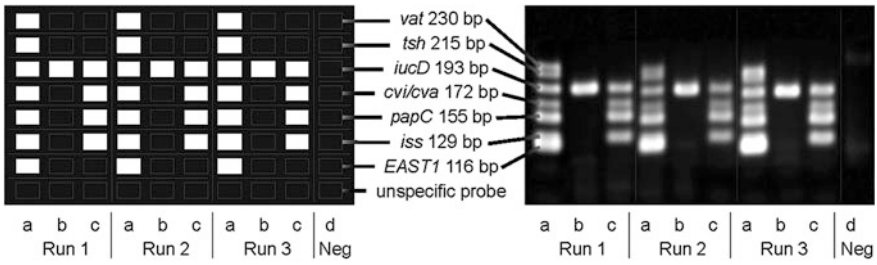

Fig. 15 Reproducibility of the VideoScan multiplex hybridization assay. Comparison of results of three runs of simultaneous amplification of seven virulence-associated genes (names and number of base pairs in the middle) of *E. coli* by VideoScan multiplex hybridization assay (*left*) and standard agarose gel (*right*). Additionally, a non-specific probe was coupled to microbeads to exclude non-specific hybridizations. Results for each run are similar between the VideoScan multiplex hybridization assay and standard agarose gel analysis which is shown as an example for the three *E. coli* strains IMT2470 (**a**), PS2506 (**b**), PS2507 (**c**). Positive values of refMFI which are represented as white boxes (*left*), and bands in the agarose gel (*right*) correspond to the detection of the gene of interest. **d** Negative control (Neg)

do not hybridize to the gene-specific capture probes. The VideoScan multiplex hybridization assay is fast, sensitive and highly reproducible. On the basis of these interesting features, we conclude that the VideoScan hybridization assay is a

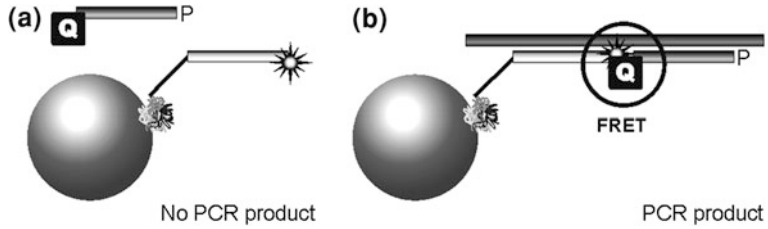

Fig. 16 Homogeneous microbead hybridization probe assay. **a** A single-stranded hybridization capture probe labeled at its 3′ end with Atto 647N is bound via biotin to streptavidin-coated microbeads. The "detector probe" is labeled with a black hole quencher "Q" (BHQ2) at its 5′ end. In the absence of PCR product hybridization, the capture probe and detector probe are kept in solution and FRET does not occur. **b** A FRET signal is only detectable when hybridization of all three molecules occurs due to an increase of the amount of PCR product

useful tool for semi-automated mPCR studies. We use this assay routinely in a 96-well format for medium-throughput gene analysis with high sensitivity and specificity. The analysis of more than 400 *E. coli* strains is an ongoing project.

5.2 *Homogeneous Microliter-Volume Hybridization Using Advalytix® Slides*

Rapid and sensitive detection of DNA targets is crucial in many disciplines of nucleic acid analysis (e.g., gene expression, single nucleotide polymorphism). For analytic platforms, the detection limit is found to be one of the most critical parameters. We applied a homogeneous multiplex oligonucleotide hybridization assay based on FRET to determine the lowest detectable oligonucleotide concentration. Since VideoScan can process multiple well geometries, we utilized the AmpliGrid system (AG480F, Advalytix®, Beckman Coulter) and performed a homogeneous multiplex direct hybridization assay (Fig. 7) and microbead hybridization probe assay (Fig. 16) in a volume as small as 1 μL.

Three microbead populations were loaded with capture probes *GAPDH*, *VIM* and *aCS-cap* (Table 8) as described in Sect. 3.2. *GAPDH* was included as negative control for non-specific quenching. The microbead populations were pooled and adjusted to 20–50 microbeads per μL per population. A droplet of 0.5 μL microbead suspension was transferred to a spot of an AmpliGrid slide. Further 0.5 μL aliquots of the sample were added with increasing concentrations, from 0 to 200 nM final concentration, of target molecules for both *VIM* and *aCS-cap*. All spots were sealed with 5 μL mineral oil. The hybridization (2.5 min at 95 °C, 30 min at 50 °C, hold at 20 °C) was performed in the AmpliSpeed ASN 400 thermal cycler.

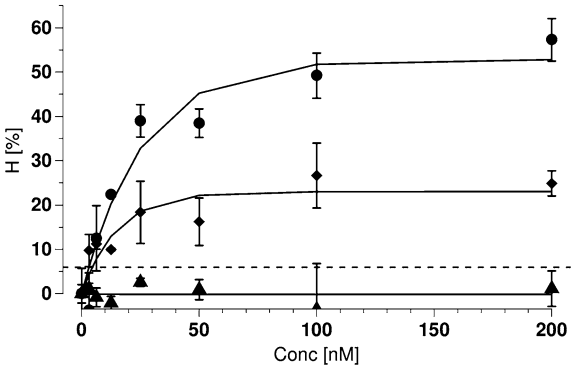

Fig. 17 Homogeneous microliter-volume VideoScan hybridization assay detection limit. Varying concentrations of *VIM-cap* (●) and *aCS-cap* (◆) were quantified in 1 μL on spots of Advalytix® slides. We used the direct hybridization assay (Fig. 7) for *aCS-cap* (◆) and the microbead hybridization probe assay (Fig. 16) for *VIM-cap* (●). *GAPDH-cap* (▲) was used as negative control for non-specific quenching and to provide a threshold (—) calculated by the three-sigma rule. Above the threshold, signals were considered significant. H, hybridization efficiency (Eq. 3)

It was possible to combine the VideoScan system with AmpliGrid slides for the quantitative detection of analytes in a sample volume of 1 μL (Fig. 17). *GAPDH-cap* was used as negative control for non-specific quenching and for estimating a threshold value by the three-sigma rule. Signals above threshold were considered significant for *VIM-cap* and *aCS-cap*. As determined by graphical analysis, the detection limit is about 9 nM of oligonucleotides in 1 μL reaction volume. Interestingly, this was similar for both the homogeneous multiplex direct hybridization assay (Fig. 7) and the microbead hybridization probe assay (Fig. 16).

The VideoScan system has been adopted for direct hybridization and FRET hybridization assays. It offers a platform for parallelized reactions for various nucleic acid applications. This design enables customized assays in small reaction volumes to save reagents, samples and processing times.

6 VideoScan PCR: Analysis and Application

To date, the simple hybridization of nucleic acids has been analyzed using VideoScan. The extension of the hardware with a heating/cooling unit (HCU) (Fig. 2b) dramatically increases the application range enabling dynamic heating, e.g., PCR conditions. Melting curves and hybridization temperature optimization are possible now—both are important to design and performance well performing PCR assays.

6.1 PCR in Solution

Quantitative real-time PCR (qPCR) is a well established technology for the simultaneous amplification and quantification of PCR product synthesis in a homogeneous format [63]. The scope of this technology includes gene expression analysis, pathogen detection and SNP analysis. Non-specific qPCR chemistries make use of DNA intercalators like EvaGreen® (Biotium) which reversibly bind to DNA without sequence selectivity [64]. This cheap and simple chemistry is optimal for single plex qPCRs. For multiplex qPCRs, specific chemistries which make use of probes are required. Hydrolysis (TaqMan®) probes play a dominant role and are regarded as one of the simplest and best chemistries available [65]. The introduction of multiplex PCRs has led to cost savings (hands-on time, reagents), savings of precious sample material and increased reliability since endogenous controls are included [63, 66–69].

We characterized the VideoScan HCU in solution by performing mPCRs, singleplex qPCRs and duplex qPCRs. In proof-of-principle experiments we used a set of human receptors and cardiac-specific genes as target. The VideoScan HCU performance regarding C_T values, amplification efficiency and feasibility of melting curve analysis was compared to the iQ5 (Bio-Rad Laboratories) real-time thermal cycler. We chose the iQ5 thermal cycler based on design similarities, i.e., the Peltier-based technology for temperature regulation [67].

The human heart sample was obtained from the explanted heart of a patient suffering from dilated cardiomyopathy [70] and transplanted in "Deutsches Herzzentrum Berlin" after written informed consent. Total RNA was isolated using peqGOLD TriFast™ (PEQLAB Biotechnologie GmbH) as described by the manufacturer. The cDNA was synthesized after DNase I (Invitrogen) digestion from 1 μg total RNA by using the SuperScript II synthesis kit (Invitrogen) with oligo$(dT)_{18}$ primer according to the manufacturer's instructions.

The iQ5 thermal cycler (Eppendorf) and the VideoScan HCU were run in parallel. PCR reactions were conducted in 20 μL Maxima PCR buffer (Fermentas) as follows: initial denaturation at 95 °C for 10 min, followed 50 PCR cycles of 40 s at 95 °C, 90 s at 58.5 °C, and 90 s at 68.5 °C. During mPCR (I)*GAPDH*, *VIM*, *cTnT*, *SERCA2* and *MLC-2v* or (II)*GAPDH*, $ADRB_{1/2}$, $ET_{A/B}$ (Table 4) were amplified. In qPCR experiments, fluorescence was measured during the annealing stage. EvaGreen® (Biotium) was used to monitor the amplification of *MLC-2v* from diluted cDNA in a singleplex qPCR. For duplex TaqMan® qPCR, *HPRT1* and *MLC-2v* were used as target.

Raw fluorescence VideoScan data were processed by background fluorescence subtraction utilizing a custom-made plugin for *RKWard* [27]. The amplification efficiencies and C_T values were determined using the dedicated functions from the qpcR package [71]. Amplicons were separated in 3% TBE agarose gel.

The multiplex amplification of (I)*GAPDH*, *VIM*, *cTnT*, *SERCA2*, *MLC-2v* or (II)*GAPDH*, $ADRB_{1/2}$, $ET_{A/B}$ from heart tissue cDNA performed with the VideoScan HCU showed an identical pattern compared to results obtained with the

Table 4 Primers for the amplification of PCR products from human heart tissue

Gene	Primer
GAPDH[§]	f: TGCACCACCAACTGCTTAGC[¥], r: GGCATGGACTGTGGTCATGAG[¥]
HPRT1[§]	f: GGCAGTATAATCCAAAGATGGTC, r: TTCAAATCCAACAAAGTCTGGC d: Atto465-AGGTCGCAAGCTTGCTGGTGAAAAGG-BHQ1
VIM	f: CCCTTGACATTGAGATTGCC, r: CCAGATTAGTTTCCCTCAGGT
cTnT[#]	f: AGTGGGAAGAGGCAGACTGA[#], r: CGAACTTCTCTGCCTCCAAG[#]
SERCA2[#]	f: GTCACTCCACTTCCTGATCC[#], r: CTCCAGTATTGCAGGTTCCA[#]
MLC-2v[#]	f: ACAGGGATGGCTTCATTGAC[#], r: ATGCGTTGAGAATGGTTTCC[#] d: Atto647N-CAGGGTCCGCTCCCTTAAGTTTCTCC-BHQ2
ADRB1[*]	f: AGGGATTTCTACCTCACACTG, r: ACAGAGTCACATGTCACAGAG
ADRB2[*]	f: GATTTCAGGATTGCCTTCCA, r: GTACTACAATTCCTCCCTTGTG
ET_A^*	f: AATTGTTTCCAGTCATGCCTC, r: AGTTCATGCTGTCCTTATGG
ET_B^*	f: TCTGTTGGTATTGGACTATATTGG, r: AGCATAAGCATGACTTAAAGCAG

Forward primer (f), reverse primer (r), TaqMan® hydrolysis probe (d) # [72], ¥ [73]. Primers for *GAPDH*, *HPRT1* and *VIM* were used at 100 nM, hydrolysis probes at 125 nM and the remaining primers at 400 nM final concentration. *: human receptor, #: cardiac specific gene, §: reference gene

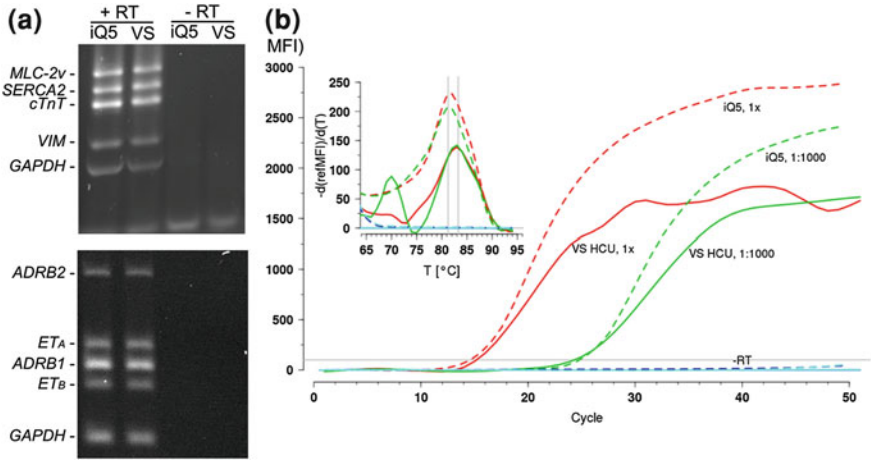

Fig. 18 Comparison of the PCR performance between the iQ5 thermal cycler (iQ5) and the VideoScan HCU. **a** Representative agarose gel: mPCR for human (*upper part*) *GAPDH*, *VIM*, *cTnT*, *SERCA2*, *MLC-2v* and (*lower part*) *GAPDH*, ET_B, *ADRB1*, ET_A, *ADRB2* was amplified with the iQ5 and VideoScan HCU. All targets were amplified similarly, indicating that the VideoScan HCU is comparable to the iQ5. **b** Amplification plot of qPCR with EvaGreen®: The amplification of *MLC-2v*. *Dashed lines* "iQ5" thermal cycler, solid lines VideoScan (VS HCU). Two cDNA concentrations (1×, 1:1,000) were used. The *inset* shows two melting peaks in reverse-transcribed samples. The non-reverse-transcribed samples remained negative

iQ5 thermal cycler (Fig. 18a). There was no amplification detected in non-reverse-transcribed samples. Thus the VideoScan HCU can be used for qualitative multiplex gene expression analysis of human receptors and cardiac-specific genes

Table 5 Results of the singleplex qPCR with EvaGreen® for *MLC-2v*

cDNA	C_T	Efficiency [%]	T_M[C]
1×			
VideoScan	16.77	77.2	~83
iQ5	16.54	73	~81
1:1,000			
VideoScan	27.12	69.8	~83
iQ5	27.23	69.9	~81

Table 6 Results of a duplex qPCR for the amplification data of genes *HPRT1* and *MLC-2v*

Target	C_T	Efficiency [%]
HPRT1		
VideoScan	23.18 ± 1.3	~77
iQ5	25.01 ± 0.7	~88
MLC-2v		
VideoScan	27.12 ± 0.9	~75
iQ5	27.03 ± 0.5	~68

(see [74–77] for details of the chosen targets). Next we performed a qPCR for the amplification of *MLC-2v*. The cDNA of heart tissue was used at two concentrations (1×, 1:1,000). Despite the different appearance of the amplification curves, mathematical analysis of the data revealed that the efficiency and $C_T$values (Table 5) were very similar between both systems independent of the dilution at given C_T values (Fig. 18b). Melting peak analysis confirmed the sole amplification of *MLC-2v*.

The duplex TaqMan® qPCR *HPRT1* and *MLC-2v* performed similarly. In both systems the simultaneous detection of *HPRT1* and *MLC-2v* was possible with comparable C_T values (Table 6). However, it appears that the amplification efficiency was about 10% lower in PCRs with HCU (Table 6). There was no amplification detected in non-transcribed samples (not shown).

In conclusion, we have shown that the VideoScan HCU can be used for a broad range of PCR applications including mPCR, single and duplex qPCR with a performance comparable to highly specialized technologies. The presence of all targets in the mPCR indicates that both the iQ5 thermal cycler and the VideoScan HCU have similar conditions in the reaction vessel. At least for the duplex TaqMan® qPCR, we observed slightly decreased amplifications efficiencies. This may in part be explained by minor differences in the temperature offset of 2 °C as described in Sect. 6.2. In its current state the VideoScan system offers only a single module HCU, which limits the throughput. However, the aim of the HCU is not to replace existing systems but to offer a platform for other, i.e., microbead-based, assay formats (see following sections). A multi-vessel version is currently under development.

Table 7 Synthetic DNA derived from sequences of Vimentin (*VIM*), *avim* (artificial derivate of VIM) or *MLC-2v*

Target	Size	Sequence
avim	53 bp	ACCTGCTCAATGTTAAGAT GGCCCTTGACATTGAGATTGCCGCGGCC GCAAAA
VIM 1	60 bp	CCAGCAGCTTCCTGTAGGTGGCAATCTCAATGTCAAGGGCCATCTTAA CATTGAGCAGGT
VIM 2	111 bp	CCCTTGACATTGAGATTGCCACCTACAGGAAGCTGCTGGAAGGCGAG GAGAGCAGGATTTCTCTGCCTCTTCCAAACTTTTCCTCCCTGAACC TG AGGGAAACTAATCTGG
MLC-2v	192 bp	ACAGGGATGGCTTCATTGACAAG AACGATCTGAGAGACACCTTTGCT GCCCTTGGGCGAGTGAAC GTGAAAAATGAAGAAATTGATGAAAT GATCAAGGAGGCTCCGGGTCCAATTAACTTTACTGTGTTCCTCAC AATG TTTGGGGA GAAACTTAAGGGAGCGGACCCTGAGGAAACC ATTCTCAACGCAT

Complementary strands not shown

6.2 Melting Curve Analysis in Solution

One essential requirement for real-time PCR analysis is the identification of the amplified DNA fragments using melting curve analysis. This procedure requires a precise temperature regulation in the reaction vessel. We therefore performed a proof-of-principle experiment to analyze the melting characteristics of DNA with VideoScan HCU and compared it with results from the real-time iQ5 (Bio-Rad Laboratories) thermal cycler. A set of four synthetic DNA sequences (Table 7) was purchased from IBA GmbH (Germany) and diluted to a final concentration of 2.5 μM in PCR buffer [3], including the HRM DNA-binding dye EvaGreen® (Biotium). The melting curve conditions were set to the same conditions (70–95 °C, 1 °C/step, dwell time 15 s).

The raw fluorescence data were processed utilizing a custom-made plugin for *RKWard* (Rödiger et al. [27]) and custom-made scripts. In brief, refMFI fluorescence values were normalized to zero by subtracting the offset after a robust linear regression utilizing the *lmrob()* function. Subsequently, negative first derivatives of the *smooth.spline()* function fitted data of the refMFI were plotted against the temperature ($-\Delta\text{refMFI}/\Delta T$).

We found that the melting curve shapes were identical between both machines (not shown). Analysis of melting peaks, however, revealed different melting temperatures (Fig. 19a). The iQ5 thermal cycler reported about 80, 76, 80.5, 78 °C whereas the HCU about 82, 78, 82.5, 80 °C for *avim*, *VIM 1*, *VIM 2* and *MLC-2v* (Fig. 19b), respectively. Thus an estimated static offset of 2 °C between the two machines was found, independently of the sequence used. The difference is presumably due to slight technical differences. Since the offset appears to be constant there is no limitation in the usefulness of the HCU.

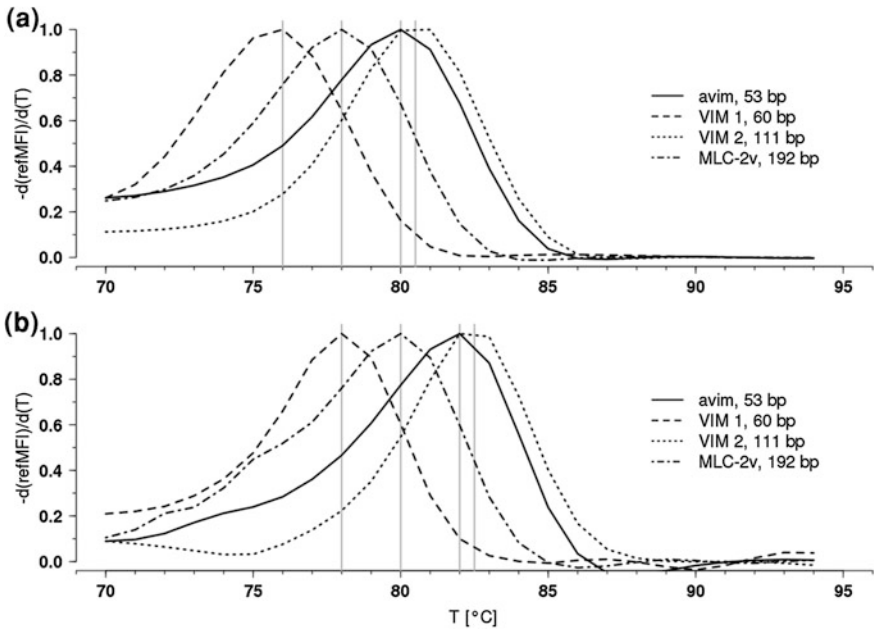

Fig. 19 Comparison of the melting peaks of the **a** i Q5 thermal cycler (*top*) and the **b** VideoScan HCU (*bottom*). Negative first derivatives of the refMFI were plotted versus the temperature. For better comparison, data were normalized to the maximum fluorescence intensity. Both systems report identical melting peak shapes but with an offset of about 2 °C. Note: *MLC-2v* reported a different T_M compared to the qPCR experiments. This was due to different buffers and the fact that the synthetic sequences contained no dUTP in contrast to PCR products amplified with the Maxima PCR kit (Fermentas)

In conclusion, the VideoScan HCU allows real-time PCR experiments including melting curve analysis, broadening the spectrum of applications significantly.

6.3 Multiplex Melting Curve Analysis on the Surface of Microbeads

A desirable advance of the VideoScan platform is a real-time PCR microbead assay that would require a melting curve analysis on the microbead surface. Being able to perform multiplex melting curve analysis would also help to understand fundamental hybridization processes which are influenced by the interaction between capture probes and analytes under different reaction environments, incubation times and temperature schemes [78, 79]. Generally, multiplex melting curve analysis requires easily distinguishable FRET pairs or probe immobilization on defined positions [13]. To perform multiplex melting curve analysis on the

Table 8 Capture probes and analytes for the analysis of microbead surface reactions

Target	Capture probe	Analyte
VIM-cap	p: bt-ACCTGCTCAATGTTAAGATGGCCCTTGA CATTGAGATTGCC D: BHQ2-ACCTACAGGAAGCTGCTGG-P	CCAGCAGCTTCCTGTAGGTgGC AATCTCAATGTCAAGGGCCATCTT AACATTGAGCAGGT
GAPDH-cap	p: NH_2-$(dT)_{10}$TGCACCACCAACTGCTTAGC	GCTAAGCAGTTGGTGGTGCA
MLC-2v-cap	p: NH_2-$(dT)_{20}$TCGAACCCTTGCCACTCATCTTCC	CCAAGGGCAGCAAAGGTGTCTCT
poly$(dA)_{20}$-cap	p: NH_2-AAAAAAAAAAAAAAAAAAAA	TTTTTTTTTTTTTTTTTTTT
aCS-cap	p: NH_2-$(dT)_{20}$GAAATGAGAGAGGCCCAGCTACTGC	GCAGTAGCTGGGCCTCTCTCATTTC
HPRT1-cap	p: NH_2-$(dT)_{20}$CCTTTTCACCAGCAAGCTTGCGACCT	AGGTCGCAAGCTTGCTGGTGAAAAGG
SERCA2-cap	p: NH_2-$(dT)_{20}$TCGAACCCTTGCCACTCATCTTCC	GGAAGATGAGTGGCAAGGGTTCGA

D: Detector probe (compare Fig. 16) for *VIM*. The capture probe (p) was 5′ amino (NH_2) modified or biotinylated (bt) with an additional poly$(dT)_{10/20}$ region

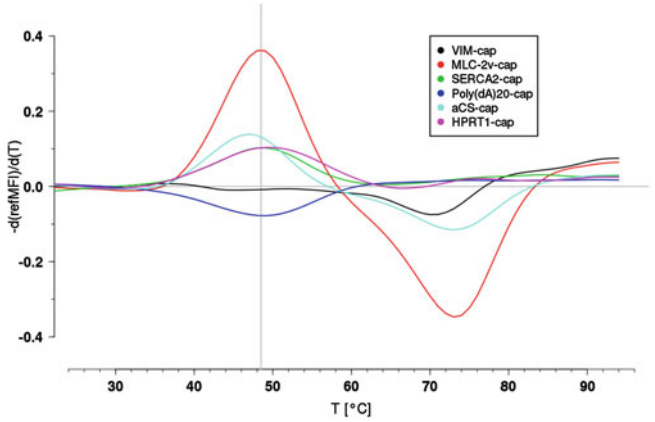

Fig. 20 Multiplex melting curve and melting peak analysis on six microbead populations. At about 48 °C *poly(dT)$_{20}$* and *poly(dA)$_{20}$* start to melt followed by the gene-specific probes at temperatures above 60 °C

surface of microbeads, we employed direct hybridization (Fig. 7) and hybridization probe (Fig. 16) chemistry. Only one ligand channel was used for interrogation of the poly(dT)$_{20}$ region and the gene-specific regions. Six microbead populations were loaded with capture probes *VIM-cap*, *MLC-2v-cap*, *SERCA2-cap*, *poly(dA)$_{20}$-cap*, *aCS-cap*, and *HPRT1-cap*. The poly(dT)$_{20}$ regions were interrogated. The capture probe *VIM-cap* (Table 8) was immobilized using the biotin–streptavidin interaction and the remaining via amide bonds as described in [3]. Microbeads were immobilized as described in Sect. 3.1. Targets were hybridized in PCR buffer (2.5 mM $MgCl_2$) as described in Sect. 5.2. Excess of non-hybridized target was removed by washing two times with 100 µL PCR buffer. Samples were added to 20 µL PCR buffer, sealed with 25 µL mineral oil, and placed in the HCU. The HCU was operated by stepwise heating (1 °C/step) between 25 and 95 °C with a dwell time of 15 s. Fluorescence data were processed with *RKWard* [27] as described in Sect. 6.2.

Using the VideoScan HCU it was possible to measure eight melting temperatures on six microbead populations simultaneously. A first melting effect was measured at about 48 °C by a decrease of the refMFI (Fig. 20) of *poly(dT)$_{20}$-cap* regions of *MLC-2v-cap*, *SERCA2-cap*, *aCS-cap*, *HPRT1-cap*. The slight variances observed are presumably due to the covalent immobilization. The same applied to the *poly(dA)$_{20}$* probe where the release of the *poly(dT)$_{20}$* quencher lead to an increase of refMFI. As a control, *VIM-cap* without a *poly(dT)$_{20}$-cap* region remained unchanged. With increasing temperature melting of *VIM-cap* (70.5 °C), *MLC-2v-cap* (72.5 °C), and *aCS-cap* (74 °C) were measured. *SERCA2-cap* and *HPRT1-cap* were left with no complementary quencher oligonucleotide (negative control) and remained unchanged.

In conclusion, VideoScan HCU is useful for determining melting temperatures of immobilized DNA fragments and is therefore useful (i) for basic analysis of

hybridization processes on microbead surfaces and (ii) for multiplex melting curve analysis in a future real-time VideoScan assay format.

6.4 Multiplex Hybridization Temperature Optimization on a Microbead Surface

Diagnostic applications based on nucleic acid hybridization require rapid reaction kinetics to deliver fast results, especially in a clinical context. To achieve fine tuning of the critical parameters, a multiplex approach is favorable. One of the main strength of VideoScan is its ability to assess kinetics of multiplexed samples both in solution and on the surface of microbeads.

The rate, stringency and efficiency of a hybridization reaction are crucial for optimal assay development and performance. To investigate multiplex hybridization events in solution, the number of available FRET pairs or filter sets limits the multiplexing level (e.g., in qPCR cyclers). The DNA hybridization kinetics for spatially resolved microbeads was done under precisely controlled temperature conditions and high microbead redundancy (compare [80, 81] for planar arrays). VideoScan can be used to study the multiplex hybridization at customizable temperatures, either in solution or solid phase under identical conditions requiring only one FRET pair. During each measurement cycle, microbeads are tracked individually. Our aim was to determine the optimal hybridization temperature in a multiplex format.

The Atto 647N fluorescence dye-labeled capture probes (Fig. 7) *SERCA2-cap*, *GAPDH-cap*, and *MLC-2v-cap* (Table 8) were coupled to microbeads according to Armstrong et al. [24]. Prepared microbeads were pooled in 20 µL PCR buffer [3] (2.5 mM $MgCl_2$) including quencher-labeled target probes for *GAPDH-cap* and *MLC-2v-cap* at a final concentration of 55 nM and sealed with 25 µL mineral oil. The VideoScan HCU was set to denature the samples for 30 s at 95 °C and to subsequently heat the samples at a defined temperature. Measurement was done at indicated time intervals.

Two capture probes (*MLC-2v-cap*, *GAPDH-cap*) were tested for optimal hybridization conditions in the presence of control for non-specific hybridization (*SERCA2*). It was found that the optimal hybridization temperature is close to 50 °C for *MLC-2v-cap* and *GAPDH-cap* (Fig. 21). Both capture probes had similar hybridization efficiencies of 75% but *MLC-2v-cap* reached its plateau after 20 min while *GAPDH-cap* needed more than 60 min hybridization time. Despite the fact that a higher hybridization temperature of 65 °C initially resulted in a quicker hybridization, the final hybridization efficiency was drastically lower ($\sim$20%) than at 50 °C. This behavior is expected due to the start of melting processes (compare Fig. 20 for *MLC-2v-cap*). The negative control *SERCA2-cap* remained unchanged.

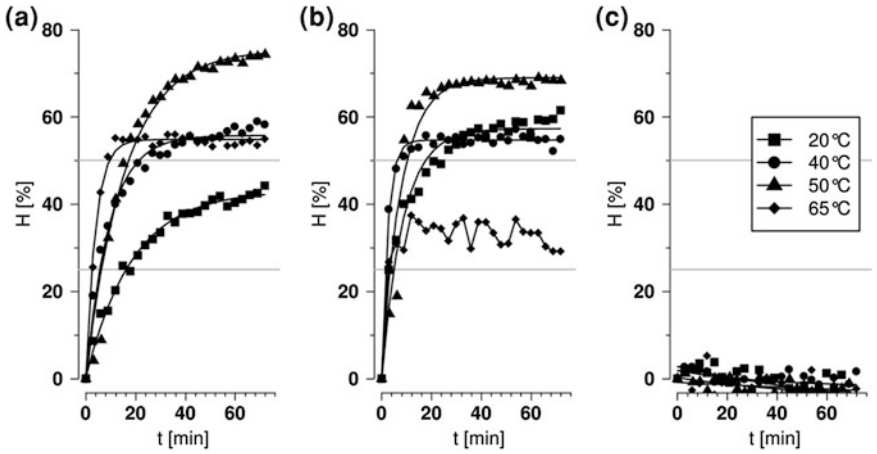

Fig. 21 Determining the optimal hybridization temperature using the VideoScan HCU. **a** *GAPDH-cap*, **b** *MLC-2v-cap* and **c** *SERCA2-cap* as negative control were simultaneously analyzed at 20 °C (■), 40 °C (●), 50 °C (▲) and 65 °C (◆). The hybridization efficiency H [%] (Eq. 3) is plotted versus the time in minutes

In conclusion, the critical parameters rate, stringency and efficiency of hybridization reactions can be measured using VideoScan, thereby allowing the optimization of diagnostic DNA detection assays.

7 Conclusions

VideoScan is a microscope-based technology platform suitable for real-time, multiplex and multipurpose bioanalytical analysis. It can perform real-time PCR including melting curve analysis—two features that turn the VideoScan into a real-time PCR machine. Microbead assays using nucleic acid or protein/peptide capture probes can be performed in a multiplex format allowing the simultaneous measurement of several analytes, thus reducing reagent and sample consumption and also processing time. Temperatures can be precisely modulated throughout the experiment using the HCU, enabling kinetic measurements of DNA hybridization. On the other hand, its image processing capabilities enlarge the spectrum of applications for VideoScan allowing high content screening of autoimmune antibodies or bacterial colony formation on the surface of eukaryotic cells (data not shown). From an operator's perspective, the possibility of using standard equipment and cheap consumables might be as much appreciated as the ease of designing and adapting VideoScan assay formats for individual needs. It is this adaptiveness and versatility that gives VideoScan a competitive edge over other bioanalytical technologies. Considering all the applications described, VideoScan is a versatile technology platform for the multiplex real-time detection of biomolecules.

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Platform Technologies for Molecular Diagnostics Near the Patient's Bedside

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Abstract It is believed Lab-on-Chip systems will become a mainstream technology within the next centuries. Especially because of new findings in molecular medicine and global trends such as the changing global population in third world countries and an ageing population in industrial countries, the need for fast and reliable diagnostics is rising tremendously. Hence, diagnostics have to become more frequently and more easily available. In this regard, technologies have to be found that enable the cost-effective production and hence an affordable price. In a joint-project between seven Fraunhofer institutes a Lab-on-Chip system was developed which consists of a credit-card-sized cartridge and a base station. The cartridges contain besides the reagents necessary for a specific assay also functionalities such as pumping or heating enabling a self-contained system without any fluidic interfaces, which tend to be error-prone. Because of the modularity of the system it is possible to integrate an optical sensor as well an electrochemical sensor into the cartridge. Hence, the system can be customized to serve the needs of the particular assays. This chapter will describe the system including generic design rules for such Lab-on-Chip systems, the development of these rules into a modular Lab-on-Chip system, the integration of biomedical assays, and the production possibility of this system.

Contents

On behalf of the Fraunhofer ivD-consortium

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1 Introduction

The huge research activities of the last two decades relating to genome and proteome research led to a deeper understanding of the molecular basis of diseases, their occurrence, development, and cure [1–3]. In consequence of this knowledge, more suitable therapies are on the horizon and are discussed widely as "personalized medicine" [4]. Molecular diagnostics will be an integrated part of this concept, since medication, success of treatment but also early occurrence of specific biomarkers for early detection or even presymptomatic diagnosis will move into the focus of medical treatments. Also genetic markers for risk screening and all aspects of companion diagnostics which defines medication according to the genetic constitution of a patient will help to serve for an improved therapy [5].

Therefore, the market for molecular in vitro diagnostics can forecasted asvery attractive.this to a producible and cost Especially combined with point-of-care testing (POCT)—sometimes better described as point-of-need—in vitro diagnostics might help in gaining great benefit from molecular knowledge [6]. Biochip- and Lab-on-Chip (LoC) technologies designed for routine application present the opportunity of performing complex and multiparameter analysis on a small scale. Lab-on-Chip systems have the potential to transfer molecular diagnostics to the point-of-need [7, 8].

Despite the fact that several POCT products are already available on the market, widespread use in routine or day-to-day diagnostics has not been achieved. There are several reasons for this related to the present need for investment in POCT: Besides well-trained personnel several machines for sample preparation (e.g., centrifuges), microarray spotting and readout, washing, and also incubation stations (with well controlled temperature) have to be made available.

Miniaturization and integration of electronic, microfluidic, mechanical, and optical components will help to overcome these limitations. For this reason Lab-on-Chip systems have to be built that have been especially designed for POCT applications in medical care and in vitro diagnosis.

The Lab-on-Chip concept is based on microfluidic structures, which should integrate all necessary steps of a biochemical assay including sample preparation, isolation or separation, and finally quantitative analysis of the components of interest. All this should be done in a device as small as a credit card. Ideally the use of the LoC does not need more than the application of the sample, for example a drop of blood from the finger tip, and readout of the signal as the given result, all

steps in-between are fully automated. This feature allows for routine use by the doctor in his office while the patient is still present as well as during intensive care monitoring, monitoring at emergency stations of a clinic or in external emergency situations where the performance of laborious assays would be prohibitive or simply impossible. Also in quite different settings such as nutrition and food control, agriculture or environmental analysis point-of-care diagnostics, which do not require any hands-on time, would be beneficial.

To fabricate such LoC devices several technical and technological hurdles have to be overcome. The production of LoC devices has to be compatible with mass production; otherwise the market will never accept the prices necessary to produce LoC today. Therefore, standardized processes have to be implemented for the production of POCT devices based on LoC. Another technological challenge comes from all aspects of nano-micro integration: Nanostructuring of surfaces for sensors, the integration of small-scale low-cost electronics, as well as the analysis of biocompatibility of the microchannels and reaction chambers and the homogeneous surface coverage of the whole device.

Hence, new technologies have to fulfill many requirements to enable their use in this new way diagnostics can be performed. To sum up, the four key challenges for the technologies involved are (i) to deliver more data in less time (ii) to be closer to the point-of-need (iii) to provide more complex information, and (iv) to be mass producible.

2 ivD-Platform

2.1 Technical Design

In a joint project between seven Fraunhofer institutes a more generic Lab-on-Chip system was developed [9]. The key design rules for the Lab-on-Chip system were as follows to match the above-mentioned challenges:

(i) High degree of integration for highest possible miniaturization of the system;
(ii) Open and modular system to adapt many different biomedical assays;
(iii) Possibility for serial production.

The developed system consists of a credit-card-sized cartridge which is used as a consumable and a processing and read-out unit which enables the fully automated processing of the cartridges once inserted. Depending on the types of analytes to be detected the read-out unit can be as small as possible to be used as a handheld device especially for protein markers or small molecules (immunoassays) or it can be a bench-top device for the detection of nucleic acids including PCR on-chip.

Within the development process a generic workflow was established. It includes all steps necessary to describe the value chain from a biomarker to a marketable

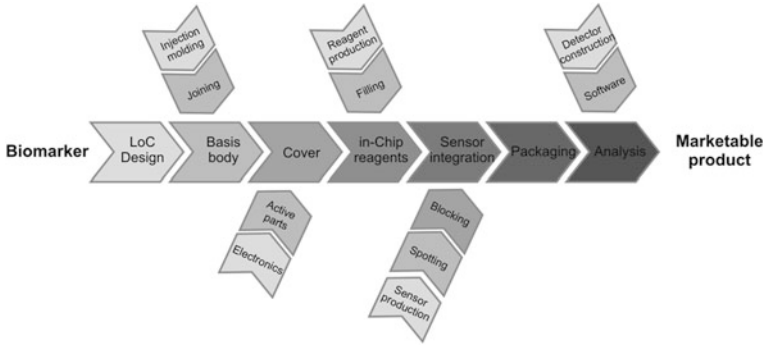


Fig. 1 Separable steps for production and development of the Fraunhofer ivD-platform

product (Fig. 1). This workflow is divided into vertical and horizontal lines. While the horizontal line represents the production of the cartridge, the various vertical lines represent steps of development or preproduction steps.

Starting from a biomarker, the first essential step is the development of an assay which determines the specific biomarker. After having established this in a normal laboratory workflow, for example as an Enzyme-Linked Immunosorbent Assay (ELISA), the assay has to be transferred into the LoC design. Hence, the basis body has to be treated to match the needs of the specific assay. Further developmental steps are then reagent production and sensor manufacturing combined with spotting of the capture molecules on the sensor and blocking for the reduction of un-specifically bound analyte molecules (all shown as vertical lines in relation to the horizontal production line). Finally, before obtaining a marketable product the detector and the software also have to be adapted to the specific assay to provide results in an appropriate design for the specific application.

Because of the open and modular form of the systems the workflow has many degrees of freedom. Depending on the application and specifically depending on the targeted analytes (i.e., proteins, DNA, or small molecules) different techniques for surface treatment, different reagents, different analytes, or also a different sensor have to be chosen. In particular, the different degrees of freedom includes sample preparation, clean up and amplification, labeling (e.g., fluorescence or redox-cycling), synthesis of probe and binding molecules, spotting, printing, immobilization, hybridization, incubation, microfluidics, detection, data collection, and data analysis. In this regard many steps can be modified to match in all cases the requirements of the assay enabling a detection of the analyte in the medically relevant range.

2.2 Cartridge

The core of the ivD-platform is a cartridge the size of a credit card exhibiting an outer diameter of 85 × 45 mm and a height of 3.8 mm. The cartridge mainly

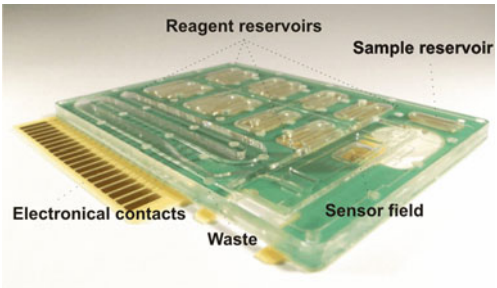


Fig. 2 Representation of the ivD-cartridge including functionalities

consists of two layers, a printed circuit board (PCB), and an injected-molded part, which are assembled in production via different tapes and membranes. Nevertheless, the materials used were chosen to be cheap enabling manufacture through serial production to gain large economies of scale.

Matching the need for a high degree of integration the cartridge was developed to contain functionalities to perform as many steps as possible on-chip. In respect of this, pumps, heating, reagents, as well as different sensors can be integrated into the cartridge [10, 11]. For that, the base layer is a PCB which is used to supply the current needed for the activation of in-chip pumping or for heating. The upper layer is an injection-molded part made from a polymer for cost-effective production. Within this injection-molded part all necessary reservoirs for sample as well as reagents and different microfluidic channels are integrated. Also the different sensors can be attached via different tapes to this part enabling their easy assembly. Between these two layers different tapes and membranes are located. The membrane is of particular importance for the pumping. The pumping principle is based on the electrocatalytic generation of a gas which inflates the membrane into the reservoirs [10, 11]. With this movement the reagents or the sample are pushed into the microfluidic channel or the sensor field where immobilized capture molecules are located. This principle is not only be used for liquid handling. The inflation of a membrane by means of electrocatalytic gas generation can also be used to function as valves. This is in huge contrast to other systems that have mostly passive components and external pumping. The pumps within the Fraunhofer ivD-platform have a nonlinear deflection but according to a pumping protocol constant flow rates between 0.1 and 1 μL/s can be set.

The actual design of the cartridge is chosen to provide eight reservoirs for reagents and washing buffer and one reservoir for the sample (Fig. 2). Four of the eight reservoirs have a size of 150 μL, the other 75 μL, and the sample reservoir a maximum of 45 μL to assure a small sample volume for example used when blood from the finger tip is taken. To reduce the risk of contamination also a waste reservoir is provided which can hold all processed liquids.

Even if the cartridge is not a prototype in the sense of materials (since the materials used can be later applied in serial production) the size of the system is a prototype. With eight reservoirs of the above-mentioned sizes, many different complicated assay sequences can be performed (Fig. 3). One example could be the washing steps of a DNA-microarray with buffers of different stringency. Nevertheless, depending

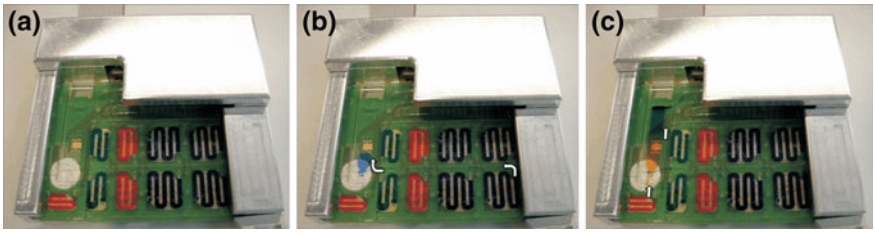


Fig. 3 Pumping sequence of the different reservoirs. **a** Cartridge prior to pumping. **b** *Arrows* indicating flow of liquids from reservoir to sensor field. **c** Pumping of sample reservoir into sensor field

on the application not every reservoir is used and hence a decrease in size is possible reducing both costs and waste.

To sum up, the self-contained active microfluidic cartridge offers various advantages compared to other systems. They are as follows:

- Small sample and reagent volumes;
- Adaption of standard biomedical assays such as immunoassays;
- No error-prone external interface for pumping and reagents required;
- Just an electrical current is needed for the activation of pumping.

2.3 Possible Sensors: Optical or Electrochemical

Within the concept modularity was one of the main design aspects. The design was chosen to be open for various types of sensors. Hence, the cartridge offers two different "free spaces" for the integration of, so far, an optical and an electrochemical sensor (Fig. 4).

Both sensors require an immobilization of capture molecules for the particular application. In most cases these will be antibodies specific to an analyte but also antigens, DNA or artificial receptor molecules can be immobilized on the various surfaces. These sensors have a heterogeneous sandwich assay type, meaning an immobilized capture molecule binds the analyte. After a washing step a secondary antibody is flushed over the sensor which can bind from the opposite side of the bound antigen. Depending on the assay a detection antibody labeled with a dye or an enzyme is often used which binds to the secondary antibody and hence "shows" the amount of bound analyte. Besides the detection of antigens as described by an immobilized antibody also other assay types such as serological assays or DNA hybridization experiments can be performed on the surface of the sensors.

The optical sensor is based on measuring a fluorescence signal of a microarray which is deposited on a substrate. To maintain the cheap producibility of the whole chip it was decided to make the substrate out of a polymer. After various material

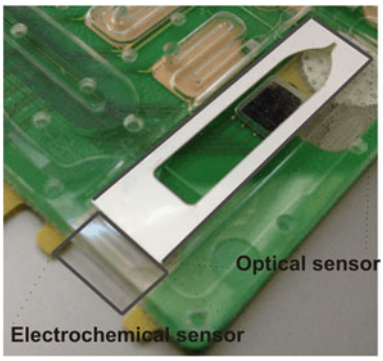


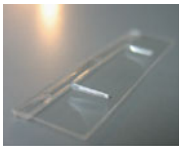
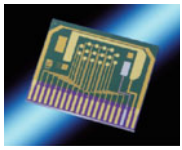
Fig. 4 Different "free" spaces within the cartridge for sensor integration. Sensor integration will be performed via a connecting layer (in *white*: protective layer)

tests cyclic olefin copolymer (COC) seemed to be appropriate due to its ability to be formed within injection-molding processes and especially due to its low autofluorescence. For integration into the optical sensor system the polymer film has a size of 10 × 40 mm and a height of 0.2 mm. This film acts simultaneously as a wave-guiding element and as the substrate for the capture molecules. To act as a waveguide the polymer film has two integrated prisms in which laser light from a light source within the processing unit is coupled in and out. The effect of guiding the light into the polymer film is that an evanescent field is created which illuminates just a volume 100 nm next to the surface. Hence, the fluorescence signal arising from the dye-labeled detector antibodies has a much better signal-to-noise ratio due to a smaller background signal. In this regard, the used total internal reflectance fluorescence-principle (TIRF) is able to deliver a higher sensitivity compared to normal fluorescence detection. As dye-labels in general many different fluorescence dyes can be used. In the specific case, the use is limited to one dye with one defined excitation wavelength of 630 nm to simplify the need of a laser and detector within the read-out unit.

In addition to the optical read-out also an electrochemical sensor can be chosen. In this case the substrate made in a seven-step fabrication process is an electronic chip with a size of 8 × 10 mm having 16 gold electrodes for the immobilization of capture molecules, a gold counter electrode, and an iridium/iridium oxide reference electrode. The electrodes have a diameter of 350 μm and capture molecules are immobilized that are specific to the desired analyte. The concentration of the analytes can be determined via a secondary and detection antibody which is conjugated with an enzyme. In many cases an alkaline phosphatase or a beta-galactosidase is used as the enzyme which converts a substrate into an electrochemically active substance. This can then be determined locally by measuring the current in a three-electrode set-up. While temperature stabilization during the binding of analytes to the capture molecules in the case of the optical sensor is performed with a heater on the cartridge itself, the electrochemical chip has an additional heater.

Concerning the fabrication process of both sensors the electrochemical one seems to be much more complicated. As stated above seven fabrication steps are

Table 1 Comparison of features of both possible sensors

	Optical	Electrochemical
Format		
Size	10 × 40 mm	10 × 8 mm
Number of parameters	Up to 500	Up to 16
Cost	~1 €	~1 €
Principle	TIRF	Single-electron redox cycling

necessary: (i) thermal oxidation of the base layer, (ii) vapor deposition for gold and iridium metallization, (iii) photolithography, (iv) lift-off processing for metal structuring, (v) plasma-enhanced chemical vapor deposition of silicon nitride as an insulating layer, (vi) photolithography, and (vii) dry etching for the structuring of silicon nitride. However, for the fabrication silicon wafers of 6- or 8-inches are used. Hence, in the case of the 8-inch wafer about 400 electrochemical chips can be fabricated at once thus maintaining a small production price (Table 1).

The choice of which sensor is more suitable for a certain application is quite difficult to answer. In general, both transducing types are well known and are used in many applications. Nevertheless, because of the above-mentioned features the choice of sensor is more dependent on price, number of parameters etc. than on performance itself.

2.4 Read-Out and Processing Unit

Besides the cartridge in which many steps are integrated a read-out and processing unit has to be used. The features to be delivered by this base station include:

- all circuitry necessary for pump and valve control;
- the hardware for the chosen read-out;
- a user-friendly interface for displaying results;
- patient identification via a barcode scanner;
- and in special cases: temperature control.

In general, the base station has to deliver all compounds for performing the assay completely automatically with a high focus on user-friendliness and ease of production to allow low-cost serial production.

As already mentioned the choice of base station is dependent on two parameters. Basically one parameter is the chosen sensor type; the other is the nature of the chosen analyte which can be DNA, proteins, or small molecules.

Depending on the type of sensor the hardware has either to provide optical or electronic parts. For the optical read-out mainly optical parts are used within the base unit [12]. For an excitation of the microarray a high-power LED with a wavelength peak of 627 nm (bandwidth 26 nm) is installed. Before reaching the polymeric wave guide the laser beam is shaped with a collimator and cylindrical lenses to fit in size and shape with the prism. Rectangular to the polymeric waveguide an uncooled CCD-camera is located which detects with varying integration times a picture of the illuminated microarray. In the current set-up a read-out area of 6×10 mm^2 was chosen which is equal to 240 spots with a pitch of 500 μm. To avoid interference with the light used for excitation an interference filter with a bandwidth of 663–737 nm was chosen. By varying the integration time the sensitivity of the assay can be tuned. This is especially important for multiparameter microarrays hence the assay performance of two parameters can differ. Despite the fact that it is possible to vary the integration times up to one and a half minute, for normal immunoassays integration times between 0.3 and 5 s are sufficient.

For data analysis, the differently integrated pictures are used. Through guiding spots on the array a grid is automatically fitted to the pictures and each spot can be analyzed. After normalization of the determined values by means of integration time and their comparison with a calibration curve values can be given in terms of medical units. Of special importance in this case are the calibration curves which have to be lot-specific and will be transmitted with every new batch of reagents and cartridges.

Comparably to the optical base unit the electrochemical base unit has to include the hardware necessary for the detection and measurement of the signal generated by redox cycling [13]. In this regard, a potentiostat is inserted which is responsible for measuring the redox-active species. To do so the potentials of the electrodes are switched in a certain frequency and the integrated potentiostat carries out chronoamperometric measurements. For data analysis, the measured current which rises over time is recorded and the slope used for the quantification.

Both systems can be used for the detection of a binding event which is the case for antigen measurements or serological analysis. Nevertheless, the detection of DNA from a sample includes an additional step since the amount of DNA in a sample is too low for its direct analysis. Hence, an amplification step is necessary to gain a sufficient concentration for its detection within a microarray. In this regard, a polymerase chain reaction (PCR) has to be carried out. A normal PCR includes various heating and cooling steps. The main challenge for the technology is to provide the heating and cooling rates necessary for a PCR-reaction. Hence, a heating and cooling on chip is not sufficient. Therefore, the read-out device was expanded to include in addition to the detection function also the possibility for delivering the temperature rates necessary. This is obtained within the base unit by a peltier element which is lowered down onto the PCR-chamber (located on the

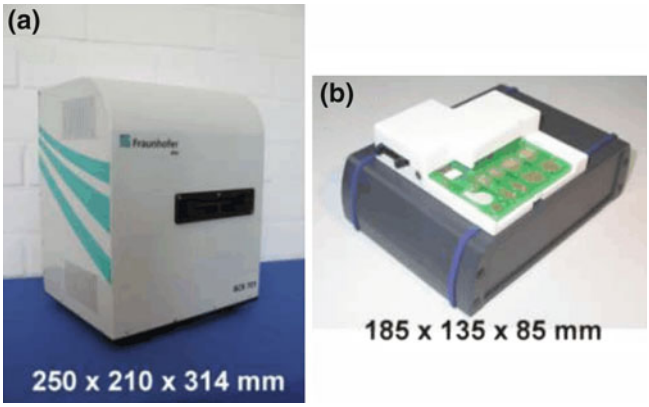


Fig. 5 (**a**) Base station for optical sensor including heating for DNA amplification; (**b**) electrochemical base station (Reprinted from [9], permitted)

cartridge). With a maximum heating power of 9.5 W and a heat spreader made from aluminum the heating is realized. The cooling is performed via a block of copper, a heat sink, and a fan. Hence, the set-up allows the fast and accurate temperature profiling necessary for a PCR-reaction to take place.

In summary, especially for antigen and serological assays the base unit has just to hold the hardware necessary for the specific read-out. Hence, the size can be as small as possible enabling its use as a handheld device. For example, the current base unit for electrochemical measurements has a size of 185 × 135 × 85 mm. For the detection and amplification of DNA the current device including the optical read-out has a size of 250 × 210 × 314 mm (Fig. 5).

2.5 Development and Performing an Assay

Beside the technology also the biochemistry plays an essential role for such as system. In general, there are many different methods for integrating biology into chips proposed in the literature. One of the most common methods is the use of particles or beads. In these cases, the reaction between the capture molecule and the analyte in solution takes place on the surface of such a particle. The main advantage is that by using magnetic beads separation steps are done very fast and efficiently. The disadvantage is that starting from a common ELISA which is performed in a microtiter plate the surface chemistry can be different and can be more versatile. Hence, within the Fraunhofer ivD-platform microarrays on planar surfaces are used to target a fast transfer of already established assays into the Lab-on-Chip system.

For that a whole value chain has to be considered starting from the right choice of surface chemistry, the right recognition element, the set-up of the sandwich

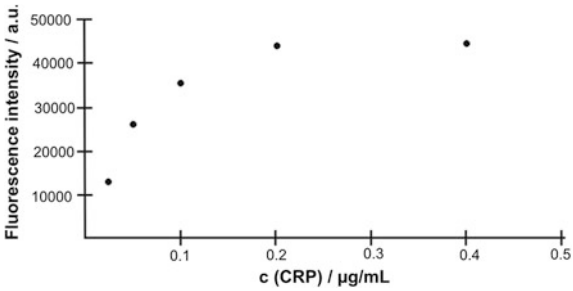


Fig. 6 Concentration dependency of CRP in a miniaturized microarray format

assay, and the choice of detection method. In addition, the processing of the assay within the chip has to be optimized. Here, incubation times and flow rates can be varied for an effective optimization of the assay.

As an example, the detection of C-reactive protein (CRP) by means of a common antibody assay and the optical read-out will be described. CRP is an acute phase protein and its level arises due to inflammation.

As a first step the substrate has to be modified to enable a covalent binding of the capture antibody to the surface. In this experiment, surface modification using 3-glycidyloxyproyltriethoxysilane was chosen. On that surface, the capture antibody was deposited by contact-free spotting techniques. For CRP detection a monoclonal mouse anti-CRP-antibody was used. The monoclonal antibody was chosen to be as specific as possible in the first reaction step avoiding cross-reactivity with other inflammation markers. Besides the capture antibody also marker spots (containing just the labeled detector antibody) and negative control spots containing just the spotting buffer were deposited onto the substrate. Prior to incubation with CRP the surface was blocked with an appropriate blocking agent, in this case 3 % bovine serum albumin. After incubation of the CRP and different washing steps a polyclonal rabbit anti-CRP-antibody was used as a secondary antibody. This is able to bind from the opposite side of the CRP. For the last step a polyclonal goat anti-rabbit-antibody labeled with Cy5 was chosen to make the optical detection of the bound analyte possible. Choosing in both cases a polyclonal antibody leads to signal amplification because more than one antibody is able to bind to the same target. The microarray was optimized to show its use as a diagnostic tool. In Fig. 6 the concentration dependency is shown from 50 to 400 ng/mL.

To optimize the microarray different washing solutions (maybe with use of detergents) and a variety of different blocking agents can be used. Besides these optimization steps also the number of washing steps and the incubation time can be varied. In the context of the Lab-on-Chip system incubation times can be controlled by the flow rate.

A typical flow protocol for an immunoassay within the ivD-platform includes different washing and incubation steps. As a first step, PBS buffer is pumped over the sensor field to wet the microfluidic channels. Then the sample is rinsed over the microarray followed by washing steps and the application of the secondary and

detection antibody. Prior to detection, a last washing step is performed to remove any unbound detection antibody. This process, including in total seven different steps has an assay time of about 15 min.

2.6 Production

As one of the key aspects of the design its production in terms of serial production was considered. With this in mind, the materials used for the cartridge design were chosen to be easily available in large number, i.e., PTB as the base layer, an injection molded part as the last layer and in-between a layer with different tapes and membranes. Although these parts can be supplied by different companies their assembly is a process to be defined and designed in an appropriate production line. Owing to the need for a high modularity of the cartridge also the production line has to have certain degrees of freedom to serve production requirements. For example, depending on the application either electrochemical or optical sensors have to be introduced into the cartridge or different assays will require various surface modifications. Hence, also the concept of the production and assembly line was designed to be modular.

In this way, the concept was to have different stand-alone units in which specific steps such as assembly, filling or coating are performed. These modules are connected via a conveyor belt with a piece carrier on which the cartridges are located. Hence, a modular assembly can be performed for every cartridge.

Besides the modularity of the system which matches the requirements of different cartridges the advantage is that in an implementation phase a hybrid approach using automated as well as manufacturing steps can be chosen. In this way, in early stages costs can be decreased and the details of every production step can be learned and investigated.

3 Summary and Further Developments

The described Fraunhofer ivD-platform was developed to provide a modular system which can be adapted to different application areas and match their special requirements. Within this chapter its use for immunoassays was described. Nevertheless, on-going research targets DNA-preparation, purification, and amplification. In this way, the ivD-platform could be used as a general platform for routine diagnostics in many areas such as emergency units, hospitals, or even in the physician's office.

From a more technical point of view, the ivD-platform is a great example that a combination of different technologies (covered by the seven Fraunhofer institutes involved in this project) can give added value in terms of highly innovative

inventions. Especially the merging of material sciences, microfluidics, and biology can serve as a door opener to better diagnostics for physicians, patients, as well as society as a whole.

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Microfluidic Technology for Molecular Diagnostics

Tom Robinson and Petra S. Dittrich

Abstract Molecular diagnostics have helped to improve the lives of millions of patients worldwide by allowing clinicians to diagnose patients earlier as well as providing better ongoing therapies. Point-of-care (POC) testing can bring these laboratory-based techniques to the patient in a home setting or to remote settings in the developing world. However, despite substantial progress in the field, there still remain many challenges. Progress in molecular diagnostics has benefitted greatly from microfluidic technology. This chapter aims to summarise the more recent advances in microfluidic-based molecular diagnostics. Sections include an introduction to microfluidic technology, the challenges of molecular diagnostics, how microfluidic advances are working to solve these issues, some alternative design approaches, and detection within these systems.

Keywords Microfluidics · Lab-on-a-chip · Immunoassays · Pre-treatment · Paper-microfluidics · Detection · Lensless · Label-free

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1 Introduction

Microfluidic technologies have produced a broad range of applications for chemistry and biology in the past 20 years, covering such topics as chemical synthesis, separation of compounds, and analysis of biological samples, e.g. living cells [25, 46, 98, 112]. Integrated components for sample manipulation, separation and detection on a single microfluidic chip have led to the terms 'micro total analysis system' (μTAS) [49, 63] and the more general 'lab-on-a-chip'. The first decade of research saw the development of new materials and fabrication techniques along with new detection methods. First applications focussed on the downscaling of analytical methods and the use of microsystems for fluidic handling, e.g. to deliver the sample to a sensor [74, 96]. The first glass microfluidic platforms were made for capillary electrophoresis (CE) and demonstrated advanced separation efficiencies [17, 120]. Later, microchips for DNA amplification by polymerase chain reaction (PCR) were fabricated and showed promising performance [50, 52, 119]. Microfluidic methods for DNA analysis [16] as well as cell analysis [32] were then introduced. These proof-of-concept studies in the first years raised the hope to revolutionise analytical chemistry and related fields, including diagnostics. Advances and simplifications in the fabrication technology of microfluidic chips, mainly driven by the use of cheap polymers such as poly(dimethylsiloxane) (PDMS), have made the technology accessible to more than just microsystems engineers. As a result, during the past few years, microfluidics has found its way into many analytical, biological, and chemical laboratories. More recently, the technology has been successfully implemented in chemical synthesis [94], cell studies [29, 80, 89, 98], proteomics [1, 26], pharmacological screening [28], and point of care (POC) diagnostics [31, 38, 44].

Although there has yet to be the so-called 'killer' application that many hope will lead to a global market for miniaturised systems [6], there have been a number of high-impact publications in the field, often focussing on specific questions in academic sciences [14, 19, 52, 72, 113, 117]. However, microfluidic technology has the potential to be used in routine diagnostic applications because it can address and solve the many technological challenges. Figure 1 shows what an idealised diagnostic device containing microfluidic technology might look like. In this case, a sample would be placed on the disposable end, where it is pretreated and directed to microfluidic channels. The target molecule(s) then would be captured and the signal electronically recorded by a reusable section. Such a device would offer high sensitivity within minutes from low volume samples, all at low manufacturing costs.

In the following section, the major benefits of microfluidics are described. After giving a brief summary of the needs for molecular diagnostic, including POC devices, we present and discuss recent advances in microfluidic technology as well as detection methods adapted for microfluidics that may be important for future developments.

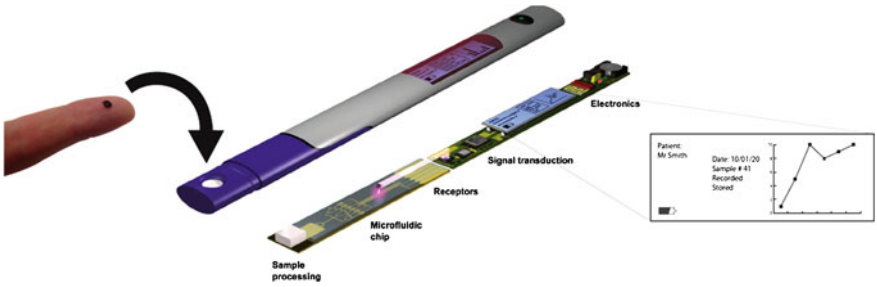

Fig. 1 A concept diagnostic device built around microfluidic technology. Adapted from [34]

2 Microfluidics in Brief

Microfluidic platforms are so called because they typically deal with fluidic reagents contained within micrometre-sized channels or chambers. Nano- or picolitre volumes are transported around these microchannels where they are filtered, mixed, reacted and detected—ideally on the same device or 'chip'. Fluid transport is often induced by applying a pressure difference by means of syringe pumps or pressure-driven systems. Alternatively, electro-osmotic flow or capillary forces can be used to move fluids. The latter is particularly interesting for POC devices because no further external devices are required. Figure 2 depicts a number of different microfluidic methods, including microchambers arrays that are commonly used to direct fluids to specific positions on a microchip. Additionally, open systems based on paper or specifically prepared surfaces are interesting alternatives to direct fluids along predefined pathways; these will be discussed later in this chapter.

Fluid flow can be characterized by the dimensionless Reynolds number, which provides the ratio of inertial to viscous forces within a system. Microfluidic systems usually have Reynolds numbers much less than 100, meaning that the viscous forces dominate. As a result, fluid flow is laminar and without turbulence. In order to carry out on-chip reactions, the reagents must first be mixed quickly and efficiently. In a laminar flow system, mixing occurs via diffusion alone. Therefore, in microchannels that are typically tens or hundreds of microns wide, proteins will only take seconds to diffuse and mix compared to minutes in a macroscopic system. Although this is not enough to resolve fast kinetics, it is sufficiently fast for diagnostic tests, which usually involve simple binding reactions. Using microfluidic technology may enable future devices to provide fast readouts of results. Moreover, because additional components to promote mixing are not required, it eliminates any expensive fabrication step.

Advantages. Systems that use microfluidic technology have several advantages over the more traditional methods for chemical analysis, bioanalysis, and diagnostics, which can be directly derived when simply considering the changes in the volume and surface areas when a system is scaled down.

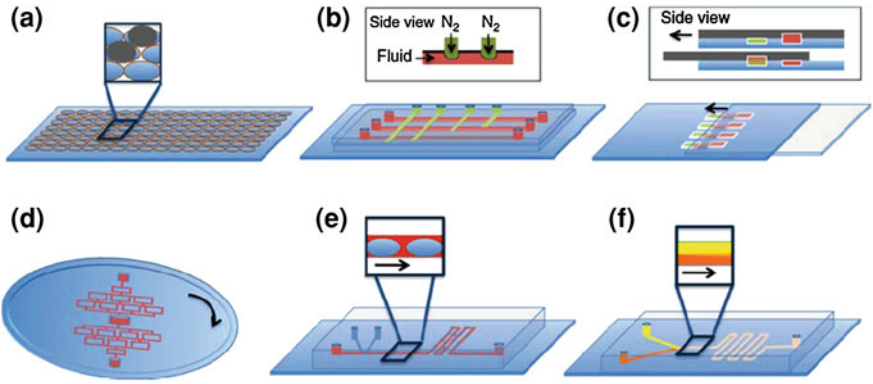

Fig. 2 Fluid handling using microfluidic systems. **a** Picolitre volumes in microarrays. **b** Pneumatic valves to isolate microchannel sections. **c** Microchambers and microchannels can be combined by moving separate layers. **d** Rotating devices use centrifugal forces to direct fluids. **e** Water microdroplets within a moving oil phase. **f** Continuous flow where mixing is due to diffusion [59]. Reproduced with permission of Informa Healthcare

(i) The small size of microfluidic devices makes them highly portable, thus allowing them to be easily transported and used in the most remote locations. (ii) Perhaps the most important advantage is the reduced cost that comes from the small fluid volumes contained within the channels. Compared to the conventional benchtop systems that typically use micro or millilitres, microfluidic systems only require nano- or picolitre volumes to fill the channels. Not only does this reduce the cost of the regents involved, but it also means that diagnostic tests can still be performed efficiently when only limited amounts of the sample are available, ideal for POC applications. (iii) Faster transport times can be realised by having short distances, and therefore integrated processes can be performed very quickly. (iv) The volume and surface area scale with the cube and square of the edge length, respectively, resulting in an increased surface-to-volume ratio in microfluidic channels. Therefore, surface-based biochemistry, e.g. immunoassays, can be performed very efficiently. Scaling down also provides the additional benefit of increased thermal transport, allowing the small volumes to be quickly heated and cooled to provide thermal gradients or precise temperature regulation during a reaction. This is also an important aspect for applications that require an electrical field, which may generate heat, e.g. electrophoresis. (v) Additionally, the dimensions of the microchannels are ideally suited to the handling and analysis of cells and cell organelles.

Fabrication and materials. Although microsystems in the range of a few hundred micrometres can usually be manufactured with conventional instruments and milling machines, further downscaling to dimensions below $\sim$300 μm requires alternative fabrication processes. Most devices in these dimensions are made by means of photolithography. This requires clean room facilities and expensive equipment to achieve good quality structures. For further reading on fabrication methods, the textbook by M. Madou is recommended [60].

In academic laboratories, rapid low-cost prototyping is often performed by so-called soft lithography [68]. For this, a master mould is produced in the clean room and a cheap polymer, most often PDMS, is used for casting of the final device. This last step can be performed numerous times in a standard laboratory atmosphere. For commercial applications, however, PDMS may not be the best candidate because surface modifications are not permanent and channel stability can be compromised under high pressures. Devices made from injection moulding thermoplastics, on the other hand, are more suited to commercial applications because they do not suffer from the above issues and can be produced in high-throughput, thereby reducing the price per chip [14]. Alternative materials are glass or silicon. In particular, glass devices are very useful because they provide a stable surface where well-known surface chemistry can be applied for modifications. Furthermore, it is biocompatible and transparent (required for optical detection methods). The main disadvantages are the more elaborate fabrication (etching of channels) and the difficulty in implementing movable modules into the channels. More recently, paper-based microfluidics has emerged as a possible candidate for commercialising microfluidic diagnostic devices [68]. Such devices would take advantage of the low-cost and biocompatibility of standard chromatography paper.

Detection. Because of the open architecture of microchannel networks, the majority of detection methods so far have focussed on traditional optical techniques, particularly, fluorescence microscopy. Although this is an ideal method for designing and testing new microfluidic systems in a laboratory, it is often not practical for POC diagnostics. There are many alternative detection methods available that have been successfully demonstrated, including UV absorption, surface enhanced Raman spectroscopy, surface plasmon resonance SPR, electrochemical and mass spectrometry (MS) [2]. It is even possible to integrate detection components within the chip, thus taking us closer to the original concept of a μTAS. For commercial applications, however, it may be more suitable to interface the microchannels with permanent external detection modules so that the sample-containing parts can be disposed after use. Moreover, many of the fluorescent assays that provide the high sensitivity currently used in enzyme-linked immunosorbent assays (ELISA) can be implemented in a microfluidic system.

3 Challenges of Molecular Diagnostics

Some health care settings lack the proper equipment for diagnostics. These include home care, emergency first response, and health care for the developing world. In these situations, access to fully working laboratories is either impractical or uneconomical. The World Health Organisation (WHO) has stated that diagnostic devices for developing countries should be “ASSURED”: affordable, sensitive, user-friendly, rapid and robust, equipment free, and deliverable to end-users [79].

Full working laboratories that perform molecular diagnostics use bench-top systems such as PCR cyclers and separation columns that are simply impractical for POC applications. To make them mobile, they therefore need be scaled down but still retain the sensitivity and specificity of the industry standards.

Home care and developing world applications should require minimal or no user training. In most cases, the person performing the test will have little or no experience with medical diagnostics. Therefore, the test must be quick and simple to perform so that it can be used at home or by a healthcare worker in the field. Ideally, it would provide a basic yes/no result to avoid any user misinterpretation.

Economic factors also play an important role in choosing the next diagnostic platform. Any portable device needs to be mass produced cheaply and require minimal peripheral equipment. The operation of these devices must also be kept low-cost by using small reagent volumes to pretreat and handle the samples. The costs can be further reduced by isolating the peripheral components, such as readers and temperature controls from the samples. This eliminates the need for cleaning because the element containing the sample is simply disposed of after use.

Microfluidic systems have the potential to meet all of these needs and become widely used in the field of diagnostics. The following section highlights some of the more recent developments aiming to meet these requirements.

4 The Challenges in Molecular Diagnostics Being Met by Microfluidic Technology

Pretreatment of the sample. Biological samples are a complex mix of many different molecules. Before a sample can be analysed, it must first be pretreated to separate the target analytes from the rest of the unwanted ones. In the case of blood, the conventional laboratory solution is to use centrifugation to provide purified plasma, but this can be a lengthy procedure and is also not feasible in resource limited-settings. Therefore, microfluidic solutions have been developed in order to perform on-chip sample preparation. These include capillary electrophoresis, dielectrophesis, liquid chromatography, optical and magnetic fields, filtration by micro-structures, diffusion, and centrifugation. Several good reviews exist that discuss the current state of microfluidic separation [64, 115] and the following will summarise some of the more recent developments.

The microfluidic H-filter, first developed in the 1990 s by [10], works by exploiting the purely laminar flow within microchannels as shown in Fig. 3a. As smaller molecules diffuse faster than larger ones, they move across the width of the channel and are therefore separated from the mixture at the outlets. Applications include the extraction of small analytes from whole blood [41] and saliva[43].

Inertial forces within microchannels can be used to separate analytes from cells in whole blood samples as demonstrated recently by Wang et al. [110]. Similarly to lateral flow POC devices, the sample is first filtered using a paper film at the inlet to remove up to 90% of the cells from the plasma. The cells and analytes

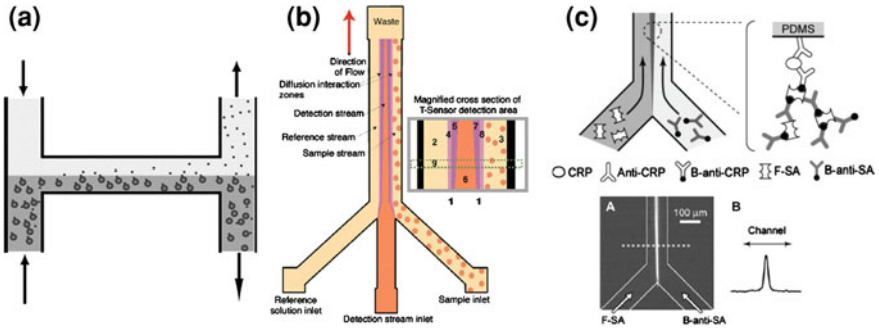

Fig. 3 Microfluidic separation by diffusion. **a** The H-filter Reprinted from [90]. **b** The T-sensor Reprinted with permission from ref. [113]. **c** An immunoassay of human CRP based on the T-sensor. Reprinted with permission from [45] Copyright (2007) American Chemical Society

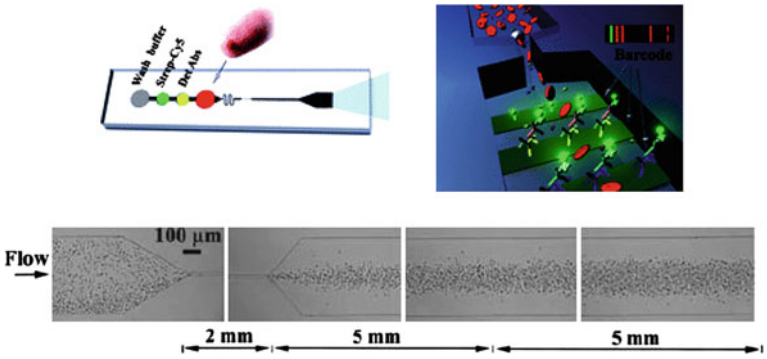

Fig. 4 Analyte separation using the inertial forces generated by flow within microchannels. Reproduced from [110] with permission of The Royal Society of Chemistry

enter a 500 µm wide chamber containing the captured antibodies via a narrow 10–20 µm microchannel. The fluid flow generated in this configuration produces inertial lift forces that constrict the large cells to the centre-line of the chamber (Fig. 4). The theory behind this phenomenon has been studied in detail previously [13, 122]. The result is that the small target molecules can diffuse out into the chamber and the cells do not interfere with the protein assay. The method was successfully shown to extract and detect 11 proteins from whole blood.

Pretreatment can also be achieved using centrifugation microfluidic platforms. These are typically fabricated on round structures which are spun at high speed to generate centrifugal forces that drive the fluids through the channels. They are often in the shape of compact discs, which makes them cheap and easy to operate [62]. Moreover, their geometry allows for multiple parallel assays and easy integration with optical detectors [61]. There are a number of examples of microfluidic centrifugation for separation applications [37, 40, 55, 100]. A fully automated

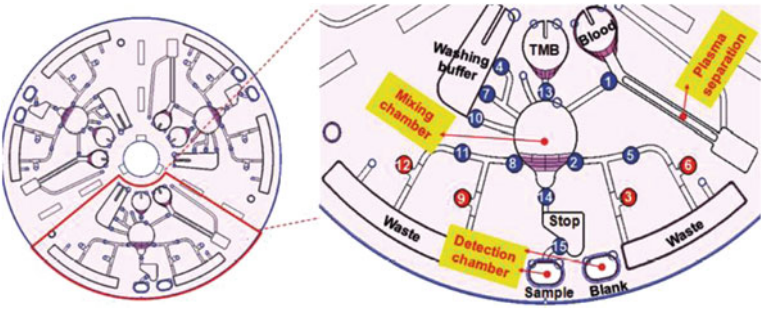

Fig. 5 On-chip centrifugation for pretreatment of whole blood. Reproduced from [55] with permission of The Royal Society of Chemistry

device capable of detecting the antigen and antibody of the Hepatitis B virus (HBV) was recently shown by Lee et al. [55]. After a whole blood sample is introduced into the circular chip, it is inserted in a device and spun-up, separating the plasma from the cells (Fig. 5). Control of fluids was realised using valves fabricated from ferromax (a mixture of paraffin wax and iron oxide nanoparticles), which can be melted with a laser to allow the fluids to pass. Using optical absorbance, the device was able to achieve a limit of detection down to 8.6 mIU mL^{-1} in less than 30 min, making it applicable for POC use.

Microfluidic immunoassays. The high sensitivity and specificity of immunoassays makes them a powerful tool in molecular diagnostics. The current 'gold standard' ELISA, while proven to be highly robust and reliable, suffers from large sample requirements, long diffusion times, and hence, long incubation times [97]. Moreover, it requires a specific enzyme-fluorophore complex that does not interfere with the reaction. In recent years, much research has focussed on combining the classic ELISA approach with microfluidic technologies [48]. Such devices enable quantitative immunoassay results by integrating sensitive optical, electrical, or mechanical detection. The main advantages of a microfluidic immunoassay compared to conventional approaches are: (i) fluidic control can be automated to increase throughout and reproducibility (ii) lower consumption of precious samples and expensive reagents and (iii) the increased surface-to-volume ratio of the microchannels can speed up the antibody-antigen binding reactions. The following section will highlight some of the more recent advances in microfluidic immunoassays.

The microfluidic T-sensor, which can be exploited for use in immunoassays, has been around since the late 1990s [113]. Like the H-filter, it takes advantage of the laminar flow in microchannels and separates out the target antigen from the sample based on diffusion (Fig. 3b). The sample containing the antigen, the antibody solution and a reference solution are all introduced separately into the device where they meet in the main channel and begin to diffuse and mix [113]. Large molecules such as blood cells will not significantly diffuse into the other streams; therefore, the smaller target molecules will move into the antibody stream

and the resulting binding can be monitored by fluorescence microscopy. The reference solution can then be used to calculate the concentration of the analyte.

More recently, the idea has been extended by Hosokawa and co-workers to perform a sandwich immunoassay for detecting C-reactive protein (CRP) in human serum [45] (Fig. 3c). A microfluidic device with a Y-shaped channel configuration was used to exploit the fast lateral diffusion of small molecules. Firstly, the channel was coated with anti-CRP followed by a blocking solution to reduce nonspecific absorption. Human serum containing CRP was then flowed down one of the inlet channels, where it could diffuse across the interface and bind to the anti-CRP. A dendritic amplification method was used to increase the limit of detection by sequentially flowing biotinylated anti-CRP, FITC-labelled streptavidin and biotinylated anti-streptavidin. The resulting fluorescent signal allowed detection of 0.15 pM of CRP in only 0.5 μl of sample. A rapid diffusion-based immunoassay for detecting theophylline (a therapeutic drug for asthma treatment) in human serum was recently reported by Tachi et al. [104]. This simple design also used a Y-shaped configuration and involved introducing the sample in one inlet and fluorescent tracer-antibody complex in the other. Mixing occurred downstream and the theophylline competes for binding of the antibody. Because the tracer-antibody complex has a higher mass compared to the tracer alone, the amount of theophylline could be monitored using fluorescence polarisation with a relatively simple optical setup. This fluorescence polarization immunoassay (FPIA) allowed detection of the analyte in the therapeutic range in 65 s. The advantage of this rapid microchip-based FPIA system is that a complete assay, from a blood sample collection to detection, can be performed within a few minutes compared to approximately 30 min with conventional FPIA systems.

Surface immobilised immunoassays in microchips typically involve physical adsorption or chemical binding of antibodies/antigen onto the surface, which can be glass PDMS, poly(methyl methacrylate) (PMMA), silicon nitride, polystyrene or cyclic polyolefin. The sample is then flowed over the surface to allow binding of the target molecule and the rest are simply washed away downstream. The binding activity, however, can be reduced due to an unwanted interaction with the surface. This problem can be overcome by attachment of the antibody via linkers such as dextran, lipids, DNA and PEG [73]. Wen et al. recently reported a novel technique for immobilising antibodies on PMMA surfaces that enhances capture efficiency and activity [114]. Using biotin-poly(L-lysine)-g-poly(ethylene glycol) (biotin-PLL-g-PEG) as a surface linker, they were able to maximise the repulsive force between the surface and the antibody, in this case protein A. Firstly, the PMMA surface is activated using an oxygen plasma, then UV-induced copolymerisation is used to graft poly(acrylic acid) to produce functional carboxyl groups (Fig. 6). To separate out the final immobilised antibody, a mixture of both PLL-g-PEG and biotin-PLL-g-PEG was introduced, and the final biotinylated protein A was linked via NeutrAvidin bridges. This method increased the efficiency and the detection compared to using a PEI linker. This work shows a significant improvement to on-chip surface immobilisation and could be applied to other immunoassays. Furthermore, as immobilisation efficiencies continue to improve, there will be less

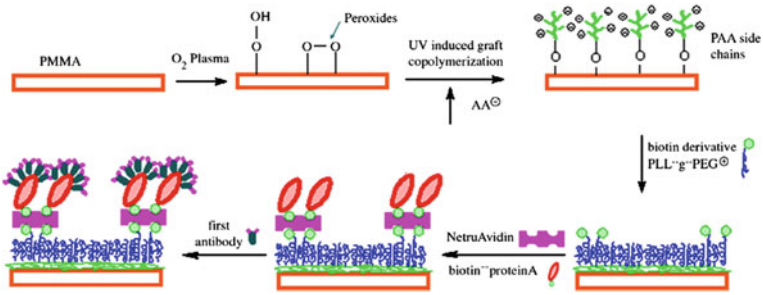

Fig. 6 Antibody immobilisation on the PMMA surface of a chip. Reprinted from [114] with permission from Elsevier

need for sophisticated detection components, moving microfluidics closer to practical POC devices.

By basing a system around microfluidic technology, one can design a complete integrated solution for portable diagnostic testing. An early example, named the "POCKET immunoassay," was presented in 2004 by the Whitesides group [97]. Its primary goal was the detection of HIV antibodies, which it achieved by overcoming a detection problem when using microchannels: the short optical path length of the channels makes measurements of optical absorbance difficult, especially in a continuous-flow format. An assay was developed in which the secondary antibody is conjugated to gold colloids and was then exposed to a silver reducing solution. The reduced silver atoms form an opaque film where the absorbance is a function of the analyte concentration. The PDMS microchip was integrated with a compact, low-cost, battery-powered detection system comprised of a laser diode and a photodiode costing just $45 (in 2004). Using the sera from HIV-1-infected patients, it was able to successfully detect and quantify anti-HIV-1 antibodies in 10 min. Although the system still required sample pretreatment, it offered an alternative approach to ELISA in a simple low-cost portable format.

Chin et al. recently reported a complete microfluidic immunoassay that incorporates all the steps of ELISA to detect HIV in whole blood [14]. The 'mChip' (Fig. 7) integrates multiple microfluidic technologies: (i) high-throughput manufacturing, (ii) automatic delivery of multiple reagents and (iii) a microscope-free signal amplification immunoassay. The chips comprising the microfluidic channels were fabricated in transparent polystyrene (the same material used in ELISA multiwell plates) and cyclic olefin copolymer using injection moulding. This reduces the cost compared to conventional PDMS-based chips and allows high throughput production, with one chip being made every 40 s. With the aim of simultaneous detection of HIV and syphilis, the researchers needed to develop an assay with multiple steps. This required sequential delivery of multiple reagents into the channels without an expensive external pumping system. Their solution was to use a simple bubble-based fluid delivery technique that involves sequentially drawing fluid plugs into the tubing by hand using a syringe. The reagents are

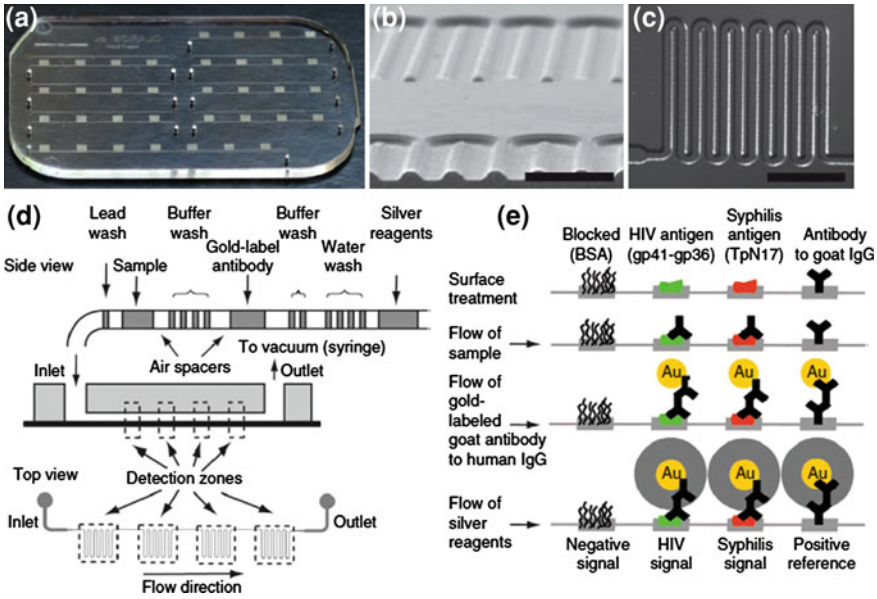

Fig. 7 The mChip. Reprinted by permission from Macmillan Publishers Ltd: Nature Medicine [14] copyright (2011)

kept separate from each other by allowing air to space out the fluids (Fig. 7d). The tubing is then attached to the inlet of the chip and flowed through the channels using a syringe attached to the outlet. This can easily be performed by local workers in remote settings with minimal training. The capture proteins on the surface of the chip (Fig. 7e) can be spotted manually by pipetting or in an automated fashion using robots. An envelope antigen (gp41–gp36) was used for capturing HIV-specific antibodies and an outer membrane antigen (TpN17) was used for capturing treponemal-specific antibodies (the causative agent of syphilis). One region was coated with BSA to serve as a negative control and another with antibodies to goat IgG to provide a positive control. Signal amplification was realised by the reduction of silver ions onto gold nanoparticles. Gold-labelled antibodies were introduced after the sample, followed by silver reagents. The resulting silver formation over a 5-min. time period could then be monitored by eye or quantified by measuring the optical absorbance using a simple low-cost device made from LEDs and photodetectors. To evaluate its performance in the field, the device was taken to Rwanda and 70 patients with known statuses were tested for HIV using less than 1 μl of whole blood. The results showed 100% sensitivity and 96% specificity, rivalling current lab-based techniques. Similar accuracy for HIV was also obtained for patients who were positive for hepatitis B and C. The combined HIV-syphilis test was performed using the serum and plasma samples of Rwandan patients and achieved a sensitivity of 100 and 94% and a specificity of 95 and 76% for HIV and syphilis, respectively. The advantages of

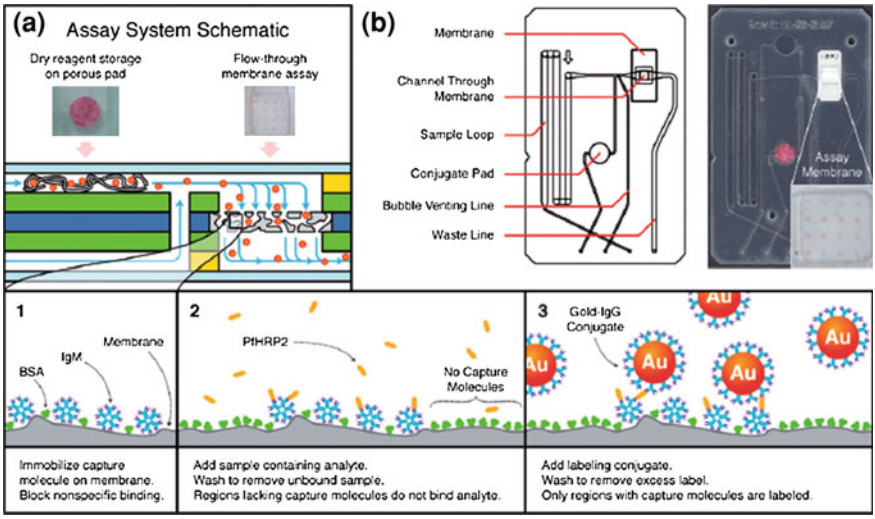

Fig. 8 Dry reagent storage technique. Reproduced from [102] with permission of The Royal Society of Chemistry

this work are that it can be conducted with minimum training, with no external infrastructure or electricity, and the costs are extremely low. The price of the reader was reported to be $74.50, the regents were $0.20 per test and each cassette cost less than $0.10 to manufacture (in 2011).

The long-term storage of reagents within POC devices is a challenge for diagnostics. Approaches to prolong the functionality of the immobilised proteins include using sugars [21, 102], sol-gels [109] and desiccants [35]. The mChip has a shelf life of 6 months, if it is maintained at room temperature [14]. For settings where this might not be feasible, Stevens et al. developed a microfluidic system that solves this problem by storing the reagents in a dry form [102]. The labelling reagents are dried in a preservative sugar matrix within a fibrous pad encased within a microchannel at the time of device fabrication. To perform the assay, a buffer is simply flowed through the pad to introduce the reagents to a porous membrane containing the target analyte (Fig. 8). They showed that functionality could be preserved over a 60-day period at 4, 20 and 45 °C, suitable for settings where the temperature cannot be controlled.

5 Alternative Approaches to Microfluidic Design

Microfluidic systems typically involve fluids being transported through closed channels, but there are alternative techniques for fluid handling that can be applied to molecular diagnostics (Fig. 9). Droplets containing the reagents can be manipulated using electrowetting platforms [8, 47, 69, 82, 99, 116]. By changing

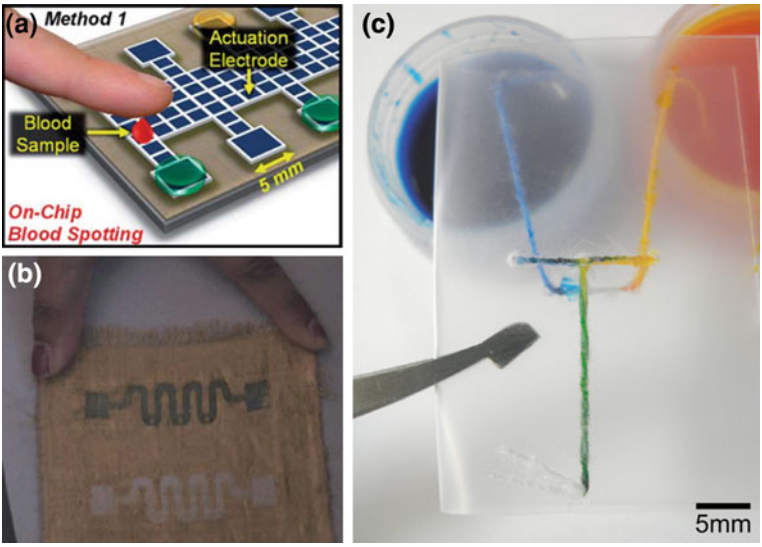

Fig. 9 Alternative techniques for microfluidics. **a** Electrowetting platforms [47]. **b** Fabric-based systems [5]. Both reproduced with permission of The Royal Society of Chemistry. **c** Thread-based systems. Reprinted with permission from [3] Copyright [2011], American Institute of Physics

the voltage of integrated electrodes, the wetting behaviour of the droplets can be changed, which allows them to be transported, split, and merged for analysis. Such a device, also known as 'digital microfluidics,' was recently used in tandem with MS to quantify amino acids in dried blood samples [47]. This method is also applicable to immunoassays as demonstrated by Wheeler and co-workers [69]. The device was able to detect the model analyte Human IgG over a dynamic range of one order of magnitude, faster than macroscopic approaches. Although the sample consumption is low, the current design of these devices involves external electronic instrumentation that is not suitable for cheap mass production.

Often, advances in technologies are accelerated by necessity. The global outbreak of the pathogenic avian flu virus H5N1 in 2004 provided the drive to find a low-cost, easy-to-use, portable diagnostic test. In 2007, Pipper et al. reported such a device based on the manipulation of microdroplets by electromagnetic forces [81]. The superparamagnetic particles contained within the droplets can be remotely controlled by magnets located off-chip, permitting low-cost tests in single-use disposables. Moreover, the magnetic interaction is unaffected by temperature effects, making it suitable for decentralised testing. The researchers were able to combine RNA sample preparation and real-time PCR to detect H5N1 in 28 min, considerably faster than the 4 h achieved by marketed tests.

Other low-cost microfluidic solutions include fabric-based [5] and thread-based microfluidic systems [3, 56, 85, 86] (Fig. 9). However, these methods are still in the early stages of development. The most promising low-cost emerging technique is

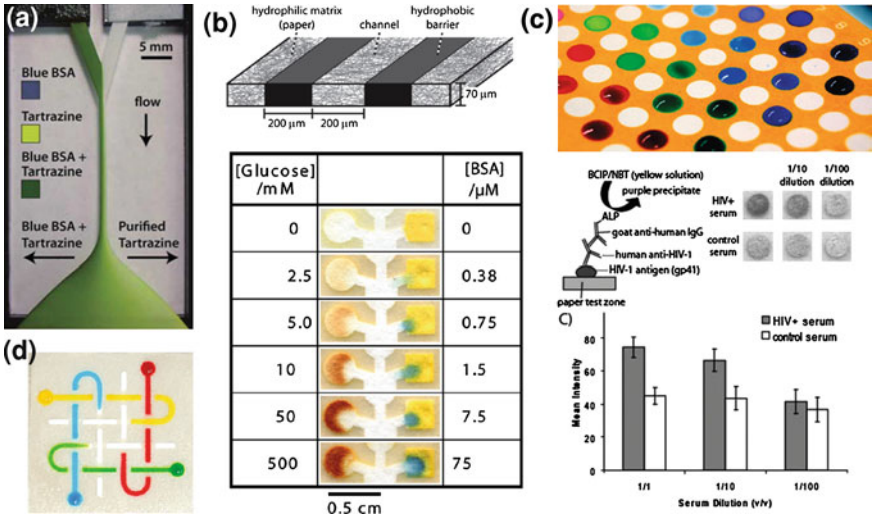

Fig. 10 Paper-based microfluidics. **a** Paper-based H-filter. Reproduced from [75] with permission of The Royal Society of Chemistry. **b** Colorimetric urine analysis Reprinted with permission from [67]. Copyright (2009) American Chemical Society. **c** Microzone paper plates with ELISA. Reprinted with permission from [11]. Copyright (2009) American Chemical Society. [12]. **d** Three dimensional networks in paper [66] Copyright (2008) National Academy of Sciences, U.S.A

paper-based microfluidics (Fig. 10) [67]. Here the fluids are contained within hydrophilic channels made of chromatography paper bounded by hydrophobic barriers (Fig. 10b). Fabrication of the patterned structures using wax printing or photolithography techniques is relatively straightforward [67]. Chromatography paper is widely available and inexpensive, resulting in a cost of less than $0.01 per device. They require no external pumping systems because the natural wicking property of the paper passively transports the fluidics along the channels. Moreover, paper is already well established in lateral-flow immunoassays, providing detection of labelled antibodies or analytes [80, 83]. Building on this pre-existing technology, the progress in paper-based microfluidic research has accelerated in the last few years.

Recently the H-filter has been shown to work in paper-based microfluidic devices [75]. Here the principle is the same, but the fluidic channels are replaced by a nitrocellulose membrane cut into channels. The fluids containing the molecules absorb into the material and move down the length of the channel network via the membrane pores. Figure 10a shows how it is possible to separate the dye tartrazine from Blue-BSA [75]. The main channel widens at the end to maximise the interdiffusion zone to further spatially separate the different-sized molecules. Content of the two regions can be analysed by cutting the paper, vortexing them in water, and measuring the absorbance of the solutions. In this case, an enrichment factor of 64 times was achieved. Apart from the reduced costs, the other advantage of a paper-based pretreatment system is that pumps and external power are not

required, which greatly simplifies the system. A disadvantage may be the speed at which the test can be performed, but a recent report by Rezk et al. demonstrated increased mixing in paper microchannels using surface acoustic waves [87].

Performing a paper-based test requires the addition of 0.1–1 μl of reagent to the test zone, either by hand or using an inkjet printer and then allowing it to dry. One of the first applications was the analysis of glucose and protein content of urine [65] (Fig. 10b). Martinez et al. used a well-known colorimetric assay in which the intensity of the colour is a function of the analyte concentration enabling quantitative measurements. Detection can be performed either by a desktop scanner or a digital camera and the intensity of the reflected colour is then compared to a calibration curve. The device was able to simultaneously detect glucose and BSA in approximately 11 min with sensitivity comparable to commercial dipstick assays. More recently, it has been shown that it is possible to improve the limit of detection of a colloidal gold label using a gold enhancement solution [33]. The model target was biotinylated-BSA, which bound to a streptavidin-gold conjugate. The size of the gold nanoparticles was then increased using an enhancement solution and an average signal amplification ratio of 5.9 was achieved. Other methods of detection compatible with paper-based systems include electrochemical detection [27] and chemiluminescence [20, 111].

Paper microzone plates have been shown to be low-cost alternatives to plastic plates [11] (Fig. 10c). Here only a few microlitres of the reagent are added to the paper test zones, reducing the sample consumption costs. The solution evaporates quickly due to the large surface-to-volume ratio, which increases the sensitivity by approximately 40 times. The cost of each plate is approximately $0.05 and they can be easily transported and stored. Both 96- and 384-microzone plates fabricated from paper are compatible with conventional microplate readers, but they can also be analysed with a scanner or camera. Paper microzone plates can also be designed to incorporate connecting microchannels between the test zones, which allows replicate assay and mixing of reagents to develop assays. ELISA for the detection of antibodies to the HIV-1 envelope antigen gp41 can be performed using paper microzone plates [12] (Fig. 10c). Using human serum, Cheng and co-workers showed that a positive result could be detected in a 10-fold dilution in less than 1 h. The limit of detection was determined to be 54 fmol/zone using rabbit IgG and a colorimetric readout, which is approximately 10 times lower than conventional ELISA using absorbance.

Multiple paper layers can be stacked to form 3D microfluidic networks (Fig. 10d) [66]. The advantage is that each layer can be made from different types of paper, thus providing multiple functionalities, i.e. sample filtration before detection. It is even possible to assemble these designs using the principles of origami [57]. So far, 3D paper-based platforms using colorimetric assays have been used to detect glucose and BSA simultaneously [66] and hepatitis B in serum using ELISA [58]. Although the current paper-based ELISA is faster and less expensive, it fails to match the sensitivity of conventional ELISA. With the majority of paper-based microfluidic research focussed on diagnostic applications, this is likely to improve in the near future.

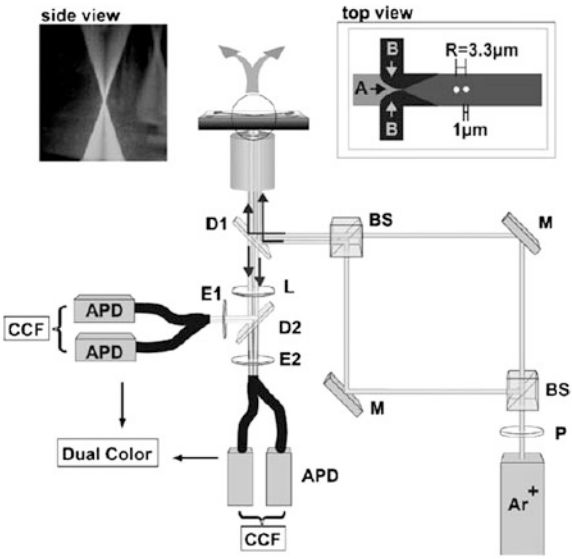

Fig. 11 Single molecule detection in microfluidic devices. This scheme depicts the optical setup used to detect a DNA cleavage reaction using FRET. At the same time, the flow velocity was measured by dual-beam FCS. (Reprinted with permission from [23] with kind permission from Springer Science and Business Media)

6 Detection Methods for Diagnostics

The majority of detection within microfluidic systems uses fluorescence due to its high sensitivity, specificity, and the availability of a wide range of different fluorophores [2]. Methods such as total internal reflection microscopy, fluorescence lifetime imaging microscopy, fluorescence correlation spectroscopy, fluorescence polarization and Förster resonance energy transfer have successfully been used for the detection and analysis of molecular interactions within microsystems [22–24, 42, 54, 88]. Combining these detection methods in a single optical setup can maximise the amount of information extracted from a reaction (Fig. 11).

These techniques represent the current state-of-the-art in detection but normally require additional labelling steps, bulky external instrumentation to achieve a high sensitivity (laser, lamp, microscope, camera, PMT, expensive electronics) and trained personnel, making them unsuitable for resource-limited resource settings or home healthcare. To this end, there have been significant advances in compact detection methods [48, 71, 73], aided by the increasing availability of low-cost LEDs, diode-pumped solid-state lasers, photodiodes, and CCDs. This has broadened the availability of excitation wavelengths without compromising sensitivity. Technologies such as miniaturised integrated microscopes, recently developed by Ghosh et al., could, in principle, be used with microfluidic platforms but currently lack the required resolution [36].

Detection for POC devices should not only be affordable and low maintenance, but the signal should be converted to a simple readout for easy user interpretation. For these reasons, the future may well see increased use of simple absorbance methods, such as those used in current lateral flow assays, that can easily be seen

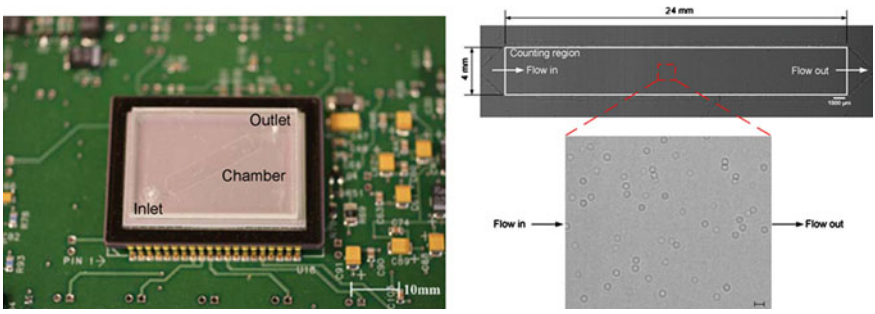

Fig. 12 Imaging $CD4^+$ T-lymphocytes on-chip using a lensless system. Reprinted from [70] with permission from Elsevier

with the naked eye [14]. However, if the application demands higher sensitivity, then alternative methods will need to be explored. An emerging topic that is gathering more and more interest is optofluidics [9, 84]. Here, microfluidic technology is combined with optics to realise highly compact and integrated devices. The following will focus on the recent developments in integrated on-chip detection methods that are suitable for molecular diagnostics.

Lensless systems. Monitoring the cell counts of patients in resource-limited settings is currently performed using fluorescent activated cell sorting systems. Although effective, these systems are expensive and require an experienced operator [118]. The combination of optical technologies with microfluidic platforms has led to the recent development of integrated lensless systems for POC diagnostics [39]. The aim is to provide high resolution images of cells from a small sample volume using a low-cost portable device. Applications of this emerging technology include wide-field cell monitoring arrays [91–93, 103], optofluidic microscopy [9, 18, 77], and lensless on-chip microscopy [76, 107].

A lensless system integrated with a microfluidic chip has been used to count $CD4^+$ T-lymphocytes for monitoring HIV [70]. In this work, the anti-CD4 antibody was immobilised on the surface on an optically clear microchannel situated above a CCD sensor (Fig. 12). A 10 μl blood sample was diluted in serum, centrifuged and the extracted serum was introduced into the chamber. The unbound cells, such as red blood cells and unwanted $CD4^+$ monocytes, were then washed away with a buffer solution. The remaining $CD4^+$ T-lymphocytes were imaged using a lensless shadow imaging technique. White light from a guided light source passes through the semi-transparent cells and casts a shadow on the sensor. The light is partially scattered, creating a holographic shadow that can be processed using the equivalent graphics-processing power found on a mobile phone. The reconstructed images had sufficient resolution to distinguish the cells from the background and allowed cell counting in 3 s. In nine separate devices, the results showed an $88.8 \pm 5\%$ capture specificity for $CD4^+$ cells and a $70.2 \pm 6.7\%$ capture efficiency. Moreover, when compared to the criterion standard of flow cytometry, the overall performance was $83.5 \pm 2.44\%$. The small 2.44% standard

Fig. 13 Telemedicine using smartphones. Reproduced from [107] with permission of The Royal Society of Chemistry, and reprinted from with permission from [127] copyright (2011) American Chemical Society

deviation means that the cell count can be corrected to give a clinically acceptable count error. This integrated platform offers a low-cost solution that can provide CD4 counts for monitoring HIV within 10 min. without the need for a skilled labourer. The same lens-free platform could also be used for high-throughput whole blood analysis in resource-poor settings. Seo et al. demonstrated automatic counting of red blood cells of densities up to $\sim 0.4 \times 10^6$ cells/μL from whole blood samples [91]. The spatial resolution of this lensless technology is sufficient to enable measurements of the volume of individual red blood cells as well as being able to differentiate between three types of white blood cells: granulocytes, monocytes and lymphocytes.

The fluid flow generated within microfluidic channels can be exploited to image cells and organisms with sub-micron resolution without the need for bulky external optical setups [18, 126]. The technology, known as optofluidic microscopy, involves passing the object over a CMOS sensor array using the laminar flow within the microchannel. The chamber is illuminated from above using an LED light source and the object casts shadows as it passes over the array. The series of image slices acquired over time can be used to calculate an image of the original object with a resolution comparable to conventional microscopy [18]. Yang and co-workers first demonstrated this method by imaging *Caenorhabditis elegans* with a resolution of 0.9 μm using a gravity-driven flow system. They also showed that by controlling the flow electrokinetically, it was possible to image cells with a similar resolution. More recently, the method was optimised by implementing a pixel super-resolution algorithm to reconstruct images with a $\sim$0.75 μm resolution [126]. High-resolution imaging using a low-cost disposable device could find its application in imaging blood cells or parasite morphologies for healthcare diagnostics in the developing world.

The small size of microfluidic systems has allowed researchers to combine them with mobile phones to drive telemedicine applications [105] (Fig. 13). It is predicted that by 2015 more than 86% of the world's population will own a mobile phone, many of which are in the developing world [4]. It is easy to see that using

smartphones is an attractive prospect to the field of POC diagnostics. This approach would involve using the existing camera and CPU of the mobile phone to acquire an image of a sample, which can then be transmitted wirelessly to a remote location such as a hospital or clinic. The Ozcan group have demonstrated a lens-free microscope that can be installed on a mobile phone and is capable of imaging red blood cells, white blood cells, platelets, and a waterborne parasite (*Giardia lamblia*) [107]. Here the sample is loaded into the device attached to the back of the smartphone (Fig. 13a) and illuminated using an LED light source. The camera sensor records a holographic shadow cast by the sample, which is then processed by the CPU of the phone to reconstruct the image. This method does not require bulky lasers or optical parts, making it lightweight and compact. More recently, the same group reported fluorescence microscopy on a mobile phone [127] (Fig. 13b).

Label-free. There is an increasing interest in label-free sensing technologies that can be implemented with microfluidic systems. In the contexts of molecular diagnostics, there are several advantages to using label-free detection. These include the reduced time and cost of preparing the sample, and the possibility of detecting target molecules that cannot be labelled. We will now discuss three label-free detection options that can be combined with microfluidic systems: SPR, interferometry and electrochemical detection.

SPR is the most commonly used label-free detection method used in microfluidic platforms [121]. In this approach, the light of a specific incident angle and wavelength is used to excite surface plasmons in a thin film of metal. The evanescent wave produced is highly sensitive to refractive index changes at the metal-liquid interface. Therefore, when a ligand binds to immobilised antibodies at the surface, a change in the SPR angle is detected. There have been a great number of SPR immunosensors developed since its introduction in the 1990 s [95]. Recently, Estmer Nilsson et al. used a microfluidic reagent handling system in combination with SPR for detecting influenza virus [30]. By immobilising recombinant HA proteins on the dextran matrix of a sensor chip and using SRP, they were able to increase the sensitivity (detection range 0.5–10 μg/ml) compared to the standard method. SPR imaging or SPRi, in which the reflected light is recorded on a CCD camera, can be used to detect molecular species in a microarray format. Krishnamoorthy et al. recently showed that it was possible to selectively deliver samples to a single row in a microarray using electrokinetic flow focusing and SPRi for high-resolution detection [53]. This approach therefore offers label-free detection of binding events in parallel.

Interferometric label-free measurements have also been demonstrated in microfluidic channels. Here, a coherent light source is split into two beam paths that are directed into the sample. Binding of the analyte is directed to the region containing only one of the beams, causing a refractive index change and therefore a difference in phase shift with respect the reference beam. This shift can then be detected in a projected interference pattern. Ymeti et al. showed that a Young interferometer configuration can be combined with a microfluidic immunoassay [124]. Here, microchannels served as optical waveguides for the beams, which

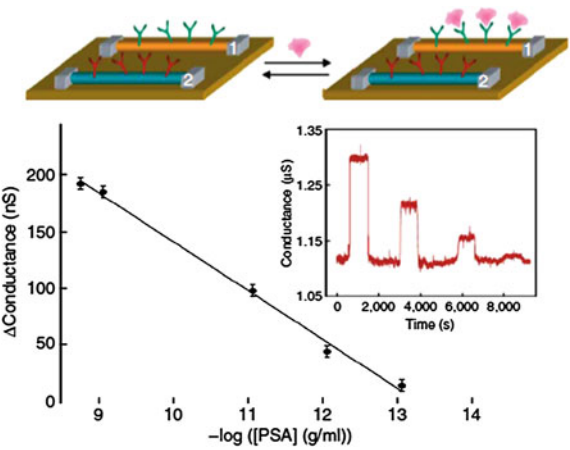

Fig. 14 Electrochemical detection for label-free immunoassays. Reprinted by permission from Macmillan Publishers Ltd: Nature Biotechnology [125], copyright (2005)

were split into four separate channel and then directed onto a CCD camera. Three of the channels contained antibodies immobilised at the surface and the remaining channel provided the reference. Binding of the antigens interacted with the evanescent wave at the surface causing a change in the refractive index and hence a change in the interference pattern. Using this device, they were able to detect herpes simplex virus in the femtomolar range. More recently, the interferometric principle has been extended to detect binding events in free solution using a method called back-scattering interferometry by Bornhop et al. [7]. Here, the walls of a rectangular microfluidic channel were used provide the interference pattern and the scattered light was directed to a CCD array. Using this label-free method the researchers were able to measure binding events of a range of binding partners including protein A with IgG and calmodulin with calcium ions. The device was even able to measure the picomolar dissociation constant of interleukin-2 with its antibody. For POC applications, this method would require a pre-filtering step as the measurement can suffer from refractive index problems caused by a complex sample.

Electrochemical detection has been widely used for detection in microfluidic immunoassays. Here the analyte concentration can be monitored via the change in conductivity measured by an electrode fabricated within the microchannel. Field effect transistors made from silicon nanowires offer high sensitivity without the need for labels. These function as semiconductors where the antibody binding results in a change in the dielectric environment of the nanowire and hence a change in the conductance. An early application demonstrated the detection of four cancer markers in serum with femtomolar sensitivity [125] (Fig. 14). This approach is known to suffer from high levels of background signal in whole blood samples, but recently a label-free electrochemical detection has been demonstrated under physiological conditions [101]. Stern et al. used a microfluidic purification chip to isolate antigens from whole blood and achieve quantitative detection of two cancer markers in less than 20 min using only 20 μl of whole blood.

Additionally, quartz crystal microbalances (QCM) can provide an electrical signal when molecules bind to their surface via the piezoelectric effect. Recent advances have led to the detection of the following biomarkers using QCMs; immunoglobulin E [123] CRP [51], and prostate specific antigen [108]. Combined with the fluid handling abilities of microfluidic systems, QCM may provide highly sensitive label-free signals of target analytes for future POC devices [78].

7 Future Goals and Perspectives

We have presented the latest developments in microfluidic technology for molecular diagnostics. Currently, the main goal for microfluidics is to combine the steps required for isolation and detection of the target analyte(s). This full integration on a single chip is still difficult to achieve due to the high complexity of the sample to be analysed (e.g. blood), which also differs from patient to patient. Continued research will be maintained through the funding provided from public and private sources, and there are also a number of companies who have turned microfluidic technology into commercial products aimed at POC diagnostics (see a recent review by Chin et al. [15]).

To date, there have been very few reports of field testing of microfluidic devices. If microfluidic technology is to provide a solution for global health care, it is up to researchers to provide thorough testing of future devices and demonstrate their robust operation in the field. The same is true for home-testing diagnostic devices. Furthermore, cheap and easy-to-use readout and periphery instruments, such as the camera of a mobile phone and simple readout software, will allow untrained operators to use the devices. A broader acceptance will only be successful if medical doctors are already involved in the research at the early developmental stage.

Microfluidics technology can also address the challenges of personalised medicine, which will continue to grow in the future as the average age of the population in the developing world increases. In this case, microfluidics provides a promising perspective as the diagnostic functions of a microchip could be combined with the therapeutic consequences. Here, the diagnostic result would be transferred into the delivery of an optimised drug combination, which could be administered by the same microfluidic device. Although the main applications differ from the devices aimed at low resource settings, they share many of the same goals, such as successful patient trials and feedback, which will need to be achieved before effective integration into the healthcare system.

Based on the innovations presented here and those we will see in the next few years, it is expected that microfluidic diagnostics will have an impact on global healthcare in the future.

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Biosensors for Diagnostic Applications

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Abstract Biosensors combine a transducer with a biorecognition element and thus are able to transform a biochemical event on the transducer surface directly into a measurable signval. By this they have the potential to provide rapid, real-time, and accurate results in a comparatively easy way, which makes them promising analytical devices. Since the first biosensor was introduced in 1962 as an "enzyme electrode" for monitoring glucose in blood, medical applications have been the main driving force for further biosensor development. In this chapter we outline potential biosensor setups, focusing on transduction principles, biorecognition layers, and biosensor test formats, with regard to potential applications. A summary of relevant aspects concerning biosensor integration in efficient analytical setups is included. We describe the latest applications of biosensors in diagnostic applications focusing on detection of molecular biomarkers in real samples. An overview of the current state and future trends of biosensors in this field is given.

Keywords Biomarker · Biosensor · Detection · Diagnostics · Test format

Contents

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1 Introduction

Diagnostic applications include the detection of disease-related biomarkers, e.g., metabolites or proteins, in human body fluids. Biomarker concentrations can be used to define disease type, state, or progress as well as the patient's response to therapy. Research on disease-related biomarkers is an ongoing process [1]. It is undeniable that established biomarkers exist which are significant for certain diseases, such as increased levels of blood glucose typically indicate diabetes [2]. However, other biomarkers reported to be disease-related are still under investigation regarding their significance in clinical diagnostics [1]. This applies, for instance, for biomarkers which are used to specify subtypes of diseases, such as markers used for classification of a tumor into subsets, which could be a useful tool in a more efficient medical treatment [1, 3]. Furthermore, particularly in the latter case, more than one biomarker has to be determined to allow efficient diagnosis, leading to marker profiles or "molecular signatures" [3, 4]. Although these markers might not be fully established in clinical routine, owing to strict requirements regarding human health, the number of clinical tests is still rising, resulting in an increasing demand for suitable and effective analytical instruments [5].

Currently, testing for biomarkers is typically performed in centralized laboratories using large automated clinical analyzers based on DNA or protein microarrays including immunoassay methods [6, 7]. They usually allow multiplex detection of several analytes, but also require trained staff and increased time and effort [5]. On the other hand, portable analytical instruments for determining blood glucose levels [8, 9] or blood coagulation [10] for patient self-testing are available. They provide results within minutes, enabling independent dosage of medication in the patient's self-management. These devices usually determine only a single analyte or a few analytes, but this is sufficient for the applications they are designed for. However, between the multianalyte high-throughput arrays used in centralized laboratories and the single-analyte devices used by the patients themselves there is a huge gap. For detection of marker profiles, e.g., at a physician's office, user-friendly instruments are required. They should allow rapid detection and quantification of several analytes without the need for specialized staff. For this testing scheme outside the central laboratories, i.e., near the patient, the term "point-of-care testing" (POCT) has been adopted. Although many different technologies and systems have been developed in this area, a clear leader has not yet been established in clinical routine [5, 11].

Biosensor setups have also been considered as POCT devices. According to the definition by the International Union of Pure and Applied Chemistry (IUPAC), a biosensor is an integrated receptor–transducer device, i.e., it combines a biochemical recognition system (receptor) with a detector (transducer). A biosensor transforms the biochemical (biological) response into a measurable output signal [12]. Therefore, biosensors should not require additional processing steps or reagents between sampling and signal output, and hence are most promising when it comes to quantitative, fast, and economic measurements [3–5, 11, 13].

Biosensors based on electrochemical detection have already found their way into POCT devices, with most of them being used as glucose meters [2, 8]. However, compared with the numerous publications and patents available, there is a huge gap between scientific output and commercial success, the latter implying availability and acceptance in clinical routine [13, 14].

One reason for this discrepancy may result from the sample matrix, which is more complex in clinical samples (e.g., serum, containing numerous proteins) than in laboratory samples prepared to demonstrate the system's performance (e.g., buffer solution, containing only the target analyte). The complex sample matrix can interfere with the biosensor signal response, which therefore has to be investigated in addition to the system's performance shown with buffer solutions [13, 14]. Furthermore, considering commercialization, a powerful biosensor instrument requires both a high-performance biosensor component and a user-friendly and efficient instrumental housing [15]. However, in many cases only individual aspects of biosensor instruments were thoroughly investigated, i.e., only single components were optimized, disregarding potential system integration. Finally, not least owing to biomarker profiles still under investigation, there is a need for versatile sensing technologies which can easily be adapted to the respective application, including multiplex sensing as well as miniaturization and low costs [13, 14].

In the following we will describe biosensor transduction principles and biorecognition layers as well as test formats with regard to their potential use in diagnostic applications. We will outline relevant aspects concerning biosensor integration in an efficient analytical device. This will be followed by an overview of biosensor applications in diagnostics focusing on serum biomarkers in cardiovascular diseases (CVDs), cancer, and autoimmune diseases as well as biomarkers in cerebrospinal fluid (CSF) for neurodegenerative diseases. We will describe the latest developments and give an overview of future trends of biosensors in this field.

2 Biosensors and Biosensor Systems

A biosensor is an analytical device which combines a biorecognition element with a transducer [12]. Whereas the biorecognition element determines the degree of selectivity or specificity of the biosensor, the biosensor's ability to detect low concentrations is mainly influenced by the transducer, as it transforms the biological or biochemical response into a quantifiable output signal [16]. Biosensors can be classified by the transduction principle, i.e., the detection, or by the type of biorecognition layer, which defines the type of specific analyte binding [12]. Table 1 gives an overview of the most common types of biosensors following these classifications. It is obvious that, for instance, immunosensors can be designed using optical or electrochemical detectors, acoustic biosensors can be based on antibody or aptamer coating, etc. Terms such as "micromachined"

Table 1 Classification of biosensors

Biosensors by transduction principle	Biosensors by biorecognition element → alternative biosensor term[a] (if available)
Electrochemical	Enzyme → enzyme sensor
Optical	Antibody → immunosensor
Acoustic or gravimetric	Aptamer → aptasensor
Thermal or calorimetric	Oligonucleotide → DNA sensor, genosensor
Magnetic	Cell → whole (microbial) cell biosensor
	Molecularly imprinted polymer

[a] Instead of (biorecognition element) biosensor

biosensors are sometimes used to describe the manufacturing method and therefore may include several types of biosensors, regardless of the transduction or biorecognition principle [17].

2.1 Biosensor Transduction Principles

2.1.1 Overview

According to the categories typically used, the "enzyme electrode" introduced by Clark and Lyons [18] in 1962 was an amperometric biosensor. This milestone in biosensor development was followed by other electrochemical biosensors, but it was not until 10 years later that biosensors based on other transduction principles were reported. The first gravimetric biosensor was a quartz crystal microbalance (QCM)-based immunosensor for protein detection; it was reported in 1972 [19]. This was followed by the first thermal biosensor for enzyme–substrate studies, called an "enzyme thermistor", in 1974 [20]. The first optical biosensor based on total internal reflectance fluorescence (TIRF) used fluorescein-labeled antibodies for detection of small molecules (haptens), and was also reported at the beginning of the 1970s [21, 22]. The first fiber-optic-based biosensor—which was dedicated to glucose detection just like the first electrochemical biosensor—was reported not until a decade later [23]. The first biosensors exploiting magnetism as a transduction principle for a variety of analytes were introduced in the 1990s [24–26].

Biosensor development has been driven by analytical demands, but it was the technological progress in related fields, such as communication and optics, as well as in manufacturing, which enabled the state of development of the current devices [27]. Electrochemical biosensors, for instance, are now comparatively easy to miniaturize, which is one of the reasons for their widespread availability [28]. In fact, detectors for biosensors used today depend mainly on electrochemical transduction, followed by optical and acoustic effects [4]. Thermal transduction is less frequently used, as are magnetic effects. However, the latter are increasingly employed as a separation tool in bioanalytical assays [29]. The following sections will outline current transduction principles to give the reader an idea of potential methods which would allow particular diagnostic applications. For a detailed

description of the respective measurement setups, including detection limits and commercialization status of available devices, the reader may refer to the cited references.

2.1.2 Electrochemical Transduction Principles

Electrochemical biosensors are typically supplied as electrode setups. They are generally divided into three major categories, depending on the underlying measurable parameter. These are current for amperometric biosensors, potential or charge accumulation for potentiometric biosensors, and conductive or resistive properties for conductometric or impedimetric biosensors, where both of the latter are usually treated as one category [28, 30].

Amperometric biosensors are typically used to detect small molecules by means of an enzyme, e.g., a peroxidase, catalyzing a redox reaction [31]. The amperometric biosensor for the detection of glucose by means of glucose oxidase was not only the first biosensor principle introduced, it is still, after several improvements, the most widespread biosensor [28]. The principle of potentiometric measurements is familiar to most from the commonly used pH electrode. Similar to amperometric biosensors, potentiometric biosensors are best suited to detect small molecules [32]. Detectors for potentiometric biosensors include the family of field-effect transistors, which have been gaining interest in recent years owing to newly developed nanomaterials, such as carbon or silicon nanotubes and nanowires. They allow label-free detection, i.e., do not require labeling with enzymes or nanoparticles to get a signal response, which reduces the expense [28]. Impedimetric biosensors are the youngest among the electrochemical biosensors and also offer label-free detection of biomolecules. They allow the investigation of a large variety of analytes, ranging from small molecules to proteins and even cells [33]. Ongoing research in the field of electrochemical biosensors is mainly focused on new materials, e.g., graphene [34] and diamond [35], as well as on miniaturization and parallelization of the devices, e.g., by screen-printed electrodes [28, 36].

2.1.3 Optical Transduction Principles

Optical biosensor detectors can generally be divided into labeled and label-free. The labels are, e.g., fluorophores or nanostructured materials such as nanoparticles, quantum dots, or carbon nanotubes [37]. Detectors using labels typically use the evanescent field outside a waveguide resulting from total reflectance within the waveguide. This evanescent field can excite, for instance, fluorescent or chemiluminescent labels outside the waveguide [38]. An example of this is the first optical biosensor which was based on TIRF [22]. Chemiluminescence can also be induced electrochemically; it is then called electrochemiluminescence [37]. With labels, small molecules as well as large molecules, such as proteins, can be detected.

The evanescent field can also be used for label-free detection, which is then based merely on refraction. The penetration depth of the evanescent field is typically only a few hundred nanometers; the refractive index of the medium determines the propagation of the radiation. Hence, it is advantageous if large analyte molecules have to be detected, such as proteins, as they lead to a higher change in the refractive index. Furthermore, when the sensing layer is being prepared, it has to be ensured that analyte binding occurs within the penetration depth of the evanescent field, otherwise the binding cannot be detected by the transducer and therefore will not be transformed into a quantifiable signal. The best examined and most widespread evanescent field technique uses surface plasmon resonance (SPR) on gold surfaces [39]. The respective systems have been further developed continually [40], including related techniques, such as SPR imaging [41] and localized SPR [42]. Other waveguiding methods make use of grating couplers or prism couplers; the latter was introduced as a so-called "resonant mirror" [43]. Furthermore, evanescent field techniques have been combined with interferometric principles, as realized in Mach–Zehnder and Young interferometers [39, 44, 45]. Recently developed evanescent field techniques for label-free optical detection for biosensors include use of Bragg gratings, photonic crystals, and optical ring resonators [44, 45].

Apart from refractometry, label-free optical detection can also employ reflectometry. In this case, signal response changes result from changes in the optical thickness, i.e., the product of the refractive index and the physical thickness, where changes of the latter prevail. Hence, the evanescent field does not have an impact here, and binding events do not necessarily have to be near the surface [39]. Again, large molecules are preferred for detection as they lead to greater thickness changes. Detection methods using reflectometry include ellipsometry, which is based on polarized light, and reflectometric interference spectroscopy, which can be performed with white light, which is why reflectometric interference spectroscopy may be considered as a simplified version of ellipsometry [46].

In addition to the numerous optical detectors which are already available, ongoing research is motivated by the aim of introducing new label-free transduction principles for biosensors that allow, in particular, the label-free detection of low concentrations or small molecules or both. Currently under investigation are, e.g., terahertz spectroscopy [47] and surface-enhanced Raman spectroscopy [48].

2.1.4 Acoustic Transduction Principles

Gravimetric (mass-sensitive) transduction principles use mechanical acoustic waves which are typically generated by means of piezoelectric materials. Signal response changes mainly result from mass changes in the biorecognition layer; hence, it is advantageous if large molecules have to be detected [49, 50]. Acoustic detection is usually label-free, but the use of nanoparticles as mass labels to increase mass sensitivity has been reported [51].

The best known acoustic biosensors are QCM, surface acoustic wave (SAW), and cantilever biosensors [49, 50]. QCM-based biosensors were the first mass-sensitive biosensors reported and are still the most commonly used acoustic biosensors. They use thickness shear modes of the piezoelectric substrate and hence are bulk acoustic wave devices [50, 52]. In contrast, mechanical waves of SAW biosensors propagate along the surface of the piezoelectric substrates. This allows higher resonance frequencies than with QCM devices, which is beneficial, because the mass sensitivity increases with increasing frequency [53]. Cantilever biosensors originate from the cantilevered sharp tips used in atomic force microscopy. If they are operated in the dynamic resonant mode, mass increase on the cantilever surface leads to a decrease of the resonant frequency, similar to QCM biosensors. If they are operated in the static deflection mode, mass increase causes a deflection of the cantilever which can be measured, e.g., optically, or, provided that a piezoresistive coating was used, electrically [54, 55]. Aside from miniaturization and parallelization of the devices, ongoing research is focused on other nanomechanical resonators, e.g., trampoline-like resonators, as a future development aiming at higher mass sensitivity of the resulting biosensors [51].

2.1.5 Thermal Transduction Principles

Thermal transduction relies on the principles of calorimetry, which requires the measurement of temperature changes. Thermal biosensors typically use thermistors, i.e., temperature-dependent resistors, as the detector unit. Similar to the term "enzyme electrode" used for the first amperometric biosensor, the first thermal biosensor was called an "enzyme thermistor". Thermal transduction offers the possibility of label-free detection of small molecules, but measurements using enzyme labels have also been reported. Still, thermal biosensors have rarely been used for biosensor measurements. One reason for this may be the difficulty to deal with nonspecific temperature changes, e.g., arising from dilution or solvation effects, requiring additional means, such as reference channels [56, 57]. More recent research suggests the use of thermopiles instead of thermistors as this would simplify the ambient temperature control of the system and hence the miniaturization of the device [58].

2.1.6 Magnetic Transduction Principles

Magnetic transduction principles for biosensors usually require magnetic nanoparticles as labels, because the analytes to be detected are typically nonmagnetic. The most commonly used magnetic effect for signal transduction in biosensors is giant magnetoresistance. Magnetoresistance is the change in the resistance of a material due to the application of a magnetic field. If alternating layers of ferromagnetic and nonmagnetic metals are used, this change is much greater than expected owing to quantum interferences resulting in giant magnetoresistance [59–61]. However,

giant magnetoresistance biosensors—as well as other biosensors based on magnetic transduction principles—are less common than biosensors based on electrochemical,s optical, or acoustic detection. We think this is because the magnetic principles require more sophisticated readout electronics than the methods mentioned above. On the other hand, magnetic labels are very attractive for use as a separation and purification tool as there is usually no magnetic background in biological samples. Depending on the type of magnetic label, they can still be used for detection afterwards, e.g., using electrochemical or optical transduction principles [29, 59].

2.1.7 Radioactive Transduction Principles

The first immunoassay developed by Yalow and Berson [62] in 1959 was a radioimmunoassay (RIA) for detection of insulin. Radioisotopes were not only the first labels used, for a long time they also offered one of the most sensitive detection methods for immunoassays. As it is not possible to differentiate radioactivity of bound radionuclides from that of free radionuclides, they must be separated. Therefore, all RIAs follow heterogeneous test formats requiring an immobilization step on a surface [63]. Hence, transduction principles exploiting radioactivity should almost be predestined for use in biosensor measurements. However, using radioisotopes is also accompanied by several disadvantages, particularly regarding technical and safety issues [63]. This may be the main reason why—to the best of our knowledge—radioactivity has not found its way into transduction principles for biosensors.

2.2 Biorecognition Layers

2.2.1 Requirements

To turn a transducer into a biosensor, it has to be coated with a biorecognition element. Examples are given in Table 1. Enzymes [64], antibodies [65], oligonucleotides [66], and, more recently, aptamers [67, 68] are examples of biorecognition elements commonly used for diagnostic applications. Biosensor coatings consisting of whole cells (microbial biosensors) [69, 70] or of molecularly imprinted polymers [71] and other synthetic binders [72] as well as coatings using lipid bilayers or liposomes for embedding, e.g., membrane proteins [73], are currently only marginally used in this area and therefore will not be covered here.

The immobilization procedures for biorecognition elements depend mainly on the underlying device material and hence on the chemical environment available [74], but they are largely independent of the transduction mechanism. However, considering transduction processes near the surface, such as electrochemical principles based on electron transfer through electric double layers [33], evanescent field techniques [39], or SAWs [75], the thickness of the complete sensing

layer, i.e., biorecognition element including any intermediate layers above the transducer, must be taken into account. For instance, the thickness may be required not to be too great, particularly if diffusion through the layer is hindered. Otherwise, with increasing distance from the transducer surface, analyte binding to the biorecognition element might lead to a reduced effect on the near-surface signal transduction and hence to reduced signal changes [74].

Ideally, sensing layers should be stable, robust, and possess a certain lifetime. Surface-bound binding sites must be accessible and able to bind the analyte to be detected. On the other hand, nonspecific binding on the transducer surface has to be inhibited to prevent false-positive signals. This applies in particular for nonanalyte molecules in complex sample matrices, such as serum, as used in diagnostic applications. However, this also applies, for example, for secondary detection antibodies, which may be used for signal amplification (for details, see Sect. 2.3), i.e., for any nonanalyte molecule involved in the measurement which may interfere with the biosensor signal response. Strategies to avoid nonspecific binding include a high surface density of the surface-bound biorecognition element, if permitted by its type and availability, and additional intermediate layers with nonstick properties as provided, e.g., by hydrogels. Furthermore, the use of blocking solutions which contain surfactants, proteins (such as bovine serum albumin), or even analyte-free serum (if available) is recommended. These solutions are supposed to occupy the nonspecific binding sites; however, the binding efficiency might be reduced and the blocking proteins themselves might interact with the sample components. Another strategy is to dilute the serum sample to minimize nonspecific binding. However, this also dilutes the concentration of the analyte to be detected and therefore might not be suitable if the analyte concentrations which are expected are too low [76].

In the following sections typical immobilization strategies for biorecognition elements—including nonstick intermediate layers—will be outlined.

2.2.2 Noncovalent Immobilization

Direct adsorption of the biorecognition elements on the surfaces would be the easiest way; however, owing to unavoidable denaturation of the biomolecules and insufficient shielding against nonspecific binding of sample matrix components to the substrate, this method is generally not recommended. Electrostatic binding of, for instance, oligonucleotides might not be stable enough for use in biosensors, as in this case potential shifts in the solution's pH value or ion strength may lead to detachment of biorecognition elements from the surface. Therefore, immobilization protocols based on covalent coupling procedures are typically preferred [74]. To ensure the binding ability of enzymes, however, it has proven to be useful to encapsulate them, e.g., via hydrogel, sol–gel, or lipid bilayers, instead of coupling them covalently to the surface [28, 31].

2.2.3 Covalent Immobilization

The transducer surfaces typically provided are metals (e.g., gold), oxidic layers (e.g., metal oxide or quartz/glass), and polymers. First, functional groups have to be introduced. In the case of gold layers, suitably substituted thiols forming self-assembled monolayers (SAMs) are used as adhesion linking layers for further coupling. A chain length of at least ten carbon atoms is recommended to obtain well-defined and stable SAMs of high density. If oligonucleotides or aptamers provided with thiol substituents are available, they can be coupled directly to the gold surfaces, which will lead to complete biorecognition layers. On oxidic layers, SAMs can be formed by treatment with silanes. Frequently used silanes are 3-aminopropyl triethoxysilane and 3-glycidyloxypropyl trimethoxysilane, providing amino groups and epoxy groups, respectively, for further coupling procedures [74]. If the transducers expose polymer surfaces, they can be activated by oxidative treatment via wet etching or plasma treatment and processed directly [74] or silanized similarly to the oxidic layers [77]. In the next step, biomolecules or hydrogels serving as intermediate layers can be coupled to the transducer surfaces via the functional groups of the SAM.

As mentioned earlier, minimizing nonspecific binding on biosensor surfaces is crucial for biosensor measurements in complex media. In this context, it was shown that sensing layers consisting of antibodies coupled on gold surfaces by means of 16-mercaptohexadecanoic acid can be used for analyte detection in serum samples, because nonspecific binding was effectively reduced [78]. If gold surfaces are not at hand, it is often advantageous to couple the biorecognition elements via an additional hydrogel layer, as these layers are known to effectively prevent nonspecific binding on sensor surfaces. Furthermore, they enable mild reaction conditions, so the biomolecules will retain their physiological structure and hence their functionality in the subsequent modification step [74]. The most commonly used hydrogels featuring these properties are functionalized dextrans, particularly carboxymethyl dextran, and poly(ethylene glycol)s [39, 74].

If these hydrogels provide carboxyl groups, antibodies, enzymes, and other protein-based biorecognition elements can easily be immobilized covalently via their amino groups by means of carbodiimide. This coupling procedure is regarded as flexible, simple, and robust and provides high coupling yields, which is why it is the most frequently employed immobilization method [74]. If the transducer (hydrogel) surface provides amino groups itself, this method can still be applied, because the amino groups can easily be converted to carboxyl groups, e.g., by means of dicarboxylic acid anhydrides [74, 79]. One drawback of the carbodiimide coupling procedure is the fact that a protein's amino groups are distributed all over the molecule; hence, the protein will be coupled in a random orientation to the surface, resulting in a potential blocking of the protein's binding sites. A way to overcome this problem is the affinity binding of the biorecognition elements via corresponding capture molecules. Examples are the coupling of biotinylated species of biorecognition elements via streptavidin and the coupling of antibodies via protein A or protein G. However, these capture molecules might increase the

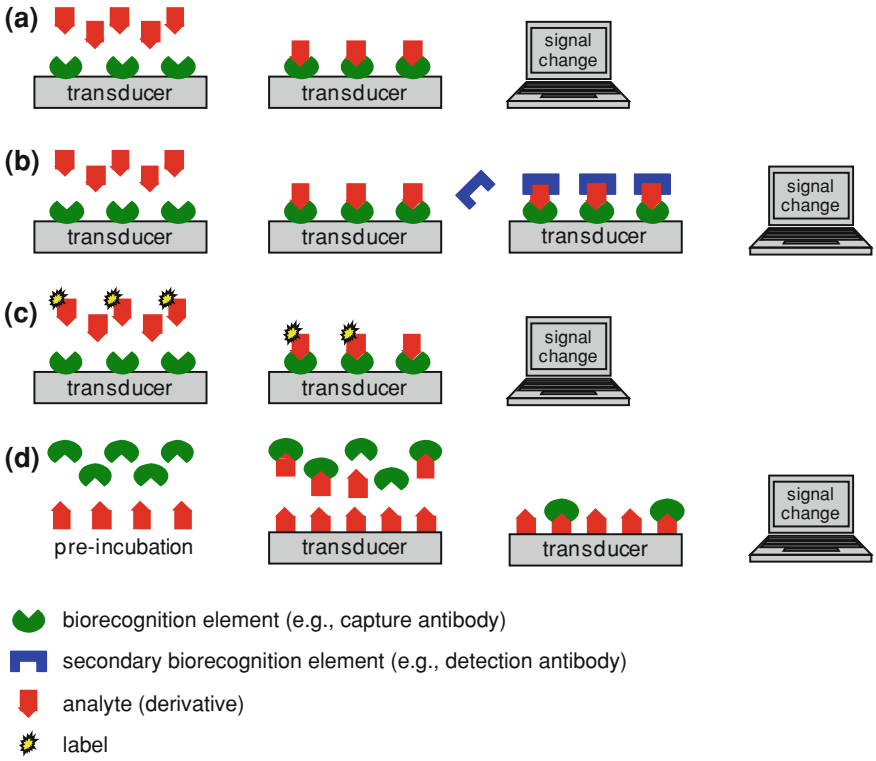

Fig. 1 Biosensor test formats. **a** Direct detection, label-free: quantifiable signal response obtained by binding of analyte. **b** Sandwich assay, label optional: quantifiable signal response obtained by binding of a secondary biorecognition element subsequent to analyte binding. **c** Competitive assay, label required: quantifiable signal response obtained by binding of a labeled analyte derivative competing with the analyte for the surface-bound binding sites. **d** Binding inhibition assay, label optional: after a preincubation step, in which equilibrium between analyte molecules and unbound biorecognition elements is achieved, the remaining free biorecognition elements are detected via immobilized analyte (derivative), allowing calculation of the original analyte concentration

degree of nonspecific binding on the sensing layer [74, 80]. For potential problems arising from the increased sensing layer thickness in the case of near-surface transduction principles, see Sect. 2.2.1.

2.3 Biosensor Test Formats

2.3.1 Test Formats Derived from Assay Formats

Figure 1 gives an overview of test formats derived from assay formats applied in biosensor measurements. In principle, four categories of test formats are available:

1. Direct detection (Fig. 1a)
2. Sandwich assay (Fig. 1b)
3. Competitive assay (Fig. 1c)
4. Binding inhibition assay (Fig. 1d).

By definition, biosensors provide immobilized biorecognition elements; hence, biosensor test formats are per se heterogeneous. Therefore, they can generally be derived from test formats used for traditional immunoassays, such as RIA and enzyme linked immunoassay (ELISA), which include the use of labels [81, 82]. However, instead of a "passive" substrate, such as a microplate, a biosensor provides an "active" transducer on which the biorecognition element is immobilized. Hence, biosensors permit an additional test format, i.e., the direct and label-free detection of the analyte (Fig. 1a). In this case, provided a label-free transduction principle is used, the signal response changes as soon as the analyte binds to the biorecognition element immobilized on the transducer surface. It is a one-step process without any other reagents which even allows real-time monitoring of binding to the biosensor surface, if required. The simplicity, speed, and the inexpensiveness of this approach are the major advantages of this procedure and are among the driving forces for the development of label-free transduction principles. However, the performance in the detection of low concentrations or of small molecules (or both) may be limited. Furthermore, when one is working with complex sample matrices, such as serum, the simplicity of the direct approach might turn out to be a considerable disadvantage, because nonanalyte components from the sample matrix may also bind to the transducer surface, leading to false-positive results. This has to be strictly avoided [45, 83, 84]. To prevent nonspecific binding, specific procedures for the preparation of biosensor recognition layers are required which have to ensure that only the analyte to be detected will bind on the biosensor surface. Details are described in Sect. 2.2.

Widely used labels for biosensors involve enzymes, nanoparticles, and fluorescent or electrochemiluminescent probes [82, 83]. Details about corresponding transduction principles are described in Sect. 2.1. If labeled test formats are used, undesired effects resulting from nonspecific binding of sample matrix components are supposed to be reduced. This is because the final biosensor signal response is usually determined by the labeled compound, whose binding behavior is assumed to be largely independent of the matrix components. However, nonspecific adsorption of labeled compounds will also lead to false-positive results and therefore has to be avoided as well. An important advantage of labeled test formats (including the use of transduction principles requiring labels) is the higher potential for the detection of lower analyte concentrations due to the amplification afforded by the respective label or label-induced reaction, such as a fluorescent signal or an enzyme-catalyzed redox reaction [83]. However, a significant disadvantage of labels is that they can interfere with the analyte binding, leading to distorted results. In the case of TIRF or other fluorescence-based transduction principles, autofluorescence of biological samples can be a problem as well [85]. Furthermore, the need for labeled compounds

usually implies higher operation costs than for direct label-free detection, as the costs of these compounds have to be added. Another point to consider is the fact that real-time monitoring of the analyte binding to the surface is not possible [45, 82–84]. Finally, when it comes to deciding whether to use labels or not, the transduction principle also has to be taken into account, as some of the transduction principles require the use of labels. Examples are transduction principles based on fluorescence, which would require the use of fluorophores. On the other hand, a label-free gravimetric transduction principle, for instance, can be used without a label, but a compound labeled with a nanoparticle as a mass label may help to increase the signal respone, if required [51].

In a sandwich assay (Fig. 1b), the analyte-related signal response is obtained when a second biorecognition element binds to the analyte after the analyte has bound to the biosensor surface. This requires analyte molecules which are large enough for two independent biorecognition elements to bind. Whether the second biorecognition element has to be labeled or not depends on the transduction principle. It is also possible to use a third biorecognition element binding to the second one [51] ("indirect sandwich"). The use of more than three biorecognition elements is not common [81]. In a competitive assay (Fig. 1c), the analyte and a known concentration of the labeled analyte (derivative) compete for the surface-bound analyte-specific binding sites provided by the biorecognition element. This test format including the corresponding transduction principles requires labels. In the binding inhibition assay (Fig. 1d), a preincubation step, in which the analyte and a known concentration of the unbound biorecognition element equilibrate, precedes the actual measurement. Again, whether the biorecognition element has to be labeled or not depends on the underlying transduction principle. After equilibrium has been attained, the binding sites of the remaining free biorecognition elements are detected with a biosensor providing an immobilized analyte (derivative), allowing one to derive the original analyte concentration in the sample [82]. This method is particularly suitable for label-free detection of small molecules, as the signal response is achieved by binding of the corresponding biorecognition element. The latter is usually a protein of higher mass and size and hence should create a higher signal response [86].

2.3.2 Test Formats Based on Molecular Switches

Today, 85% of all biosensors sold are glucose sensors [8]. The reason for this success story might be that this is one of the very few biosensor types in which the interaction between the analyte and the biorecognition element creates a reagent which can easily be detected: in this process the chemical transformation of glucose by glucose oxidase results in the formation of hydrogen peroxide, which can be monitored amperometrically [2, 28, 87]. In contrast, other biosensor formats, such as those mentioned earlier, are mainly based on physical effects near the transducer surface, i.e., no reagent is released, and there are few significant conformal changes supporting the signal response. From a technical point of view, the

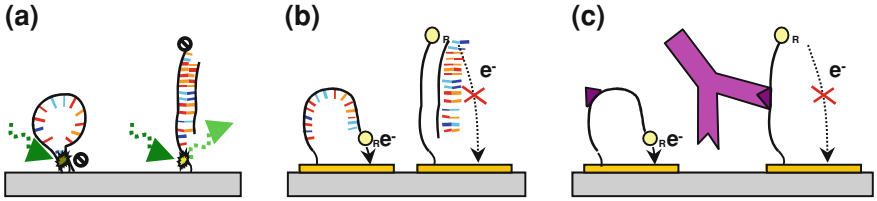

Fig. 2 Proposed sensing mechanisms of molecular switches. **a** The classic fluorescent molecular beacon. The proximity between a fluorescent dye and a quencher is broken as a consequence of a binding reaction (hybridization); thus, the quenched fluorescent signal becomes visible. **b** An electrochemical molecular switch. The proximity of a redox species *R* to a current-collecting electrode is broken as a consequence of a binding reaction (hybridization); thus, the electrons are not able to drift to the electrode anymore, resulting in a decrease of the current. **c** The same mechanism as in **b** can also be used to detect larger molecules, such as antibodies, by means of an aptamer which will change its conformation once the antibody is bound

fact that analyte binding to the biorecognition element does not create a measurable species per se, such as emission of electrons or photons or the like, is the main problem for biosensors. Still, there may be a change in conformation which can be used for the detection. On the basis of this thought, a new type of biosensor was developed in which the interaction between the analyte and the biorecognition element results in a significant conformation change. To allow simplified detection, the biorecognition element was designed in a way that the conformation change will induce an additional response which can easily be measured. These sensors are called "folding-based" biosensors, most of them making use of molecular switches (Fig. 2). They can be ranked as a further development of aptasensors. The easiest and earliest cases of such switches were reported for the detection of nonhybridized DNA by means of a complementary single-stranded DNA (ssDNA) bound to a surface (see Fig. 2a). These biosensors used the fact that ssDNA can be designed in a way, e.g., by matching complementary bases at both ends of the DNA strand, that a fluorescent dye and a quencher matching the dye are in close proximity. In this case, incoming light will not result in visible emission because of quenching. If the complementary ssDNA is detected from the sample, the DNA hybridizes and the proximity between the dye and the quencher is broken, thus allowing visible emission of light as a consequence of the binding. The first fluorescent biosensors of this kind were reported as "molecular beacons" in 1996 [88]. These principles were later transferred to electrochemistry [89, 90], suggesting the mechanism depicted in Fig. 2b. In this setup a redox species (e.g., methylene blue) is tagged to the end of the ssDNA, which is immobilized on a current-collecting electrode. In the nonhybridized state the redox label will dwell near the surface, allowing the electrons created during the oxidation process to drift to the electrode, resulting in a current. In the hybridized state the resulting double-stranded DNA (dsDNA) will stiffen, thus bringing the redox label away from the surface. As a result, the registered current will be

reduced. Similar mechanisms can also be used to detect affinity binding systems such as antigen–antibody reactions [91] or protein binding to a (small) recognition element [92], both cases using deformation of an aptamer carrying a redox label in a way that the measurable current will be changed (Fig. 2c).

The strength of this approach is in the fact that the actual binding itself triggers the signal, thus the reference to a molecular switch: if no binding occurs, the switch will not be triggered. This principle is robust even in very complex backgrounds (cell culture medium, serum, etc.) as it is not affected by nonspecific adsorption on the transducer surface. As will be shown in the next part of this chapter, applications of biosensors in real (serum) samples are comparatively rare, despite the need for easy-to-use protein detection systems and despite the potential of biosensors in this field. This new biosensor type relying on a new test format has already been used for antibody detection and for protein detection in serum samples [91, 92]. Vascular endothelial growth factor is a regulator of both physiologic and pathologic angiogenesis. With a folding-based biosensor, a detection limit of 5 pM (190 pg/ml) in 50% blood serum could be achieved, which is in the clinically relevant concentration range [92].

2.4 Biosensor System Setup and Microfluidic Integration

The biosensor is the heart of every biosensor system. Without this component no signal can be generated. However, in most cases this is not sufficient. To be suitable for handling by the user, the biosensor needs to be provided in a way that ensures the device's integrity during handling and prevents damage to the device during operation. This is usually guaranteed by means of embedding the device into a biosensor housing. The combination of a biosensor in its housing is typically referred to as "biosensor chip". The physical embedding comes with the disadvantage that the biosensor may no longer be directly accessible for sample delivery or signal readout. This is why the biosensor housing usually has to be supplied with features other than mere physical protection [15].

There are several aspects to keep in mind when developing a housing strategy for a biosensor. Foremost, ease of handling the component has to be ensured. However, it may be equally important to keep in mind the additional costs caused by the biosensor housing and the processes required for the embedding. If the biosensor system is to be commercialized for the mass market, compatible manufacturing techniques have to be chosen. In such a scenario, the biosensor housing would usually be designed as a low-cost component created in an industrially available polymer such as poly(methyl methacrylate) or polystyrene. Furthermore, polydimethylsiloxane has become popular in recent years, especially if active microfluidic structures (such as

microvalves or micropumps) are to be embedded next to the biosensor [15]. Polymers allow the use of cheap mass-market replication techniques, such as hot embossing and injection molding, at low cost. Keeping manufacturing costs low will also allow a potential use of the resulting biosensor chips as single-use components, which are usually preferred in biomedical and clinical applications [13].

Another important aspect to consider for biosensor integration is the fact that usually more than one analyte may have to be detected, and thus the combination of several biosensors into biosensor arrays would be beneficial to allow multiplex detection in one measurement cycle. This is a task that can usually be fulfilled by the biosensor housing. Two potential application scenarios have to be considered here: more than one analyte (target) is to be detected in one sample ("single sample–multiple target" biosensor array) or one marker is to be detected in multiple samples ("single target–multiple sample" biosensor array). In both cases the physical protection of the individual biosensors may be provided by the same biosensor housing, but the individual biosensor chips have to be separated fluidically. This is required not only for the experiment but also for the application of the sensing layers.

The most commonly found example for the housing strategy in single sample–multiple target arrays is the creation of more than one transducer structure on a single substrate and their (potentially permanent) fluidic separation by means of a polymeric flow cell. Such examples can be found amply in the literature [93]. This strategy is also used for numerous commercialized biosensor systems, such as the SPR biosensor system of Biacore [94] and the SAW biosensor system of SAW Instruments [95]. This setup is especially advantageous if the physical structures required for biosensor elements (such as electrodes) can be manufactured by cheap techniques on planar substrates. Potential manufacturing techniques include screen-printed electrodes [28, 36] or electrodes made by spotting or printing of conductive polymers [96].

If the biosensor has to be created as a single component (maybe because a nonplanar substrate, such as a fiber, is used), the biosensors are embedded individually and assembled in chip format into biosensor arrays. Recently, a strategy was suggested allowing the embedding of individual biosensors into separate polymer housings and assembling them into versatile biosensor arrays [97]. For this, a disposable polymer microfluidic chip is used which defines the number of individual biosensors in the array. In the same work, it was also demonstrated that such a strategy can be complemented by integration of such an array into a microfluidic environment [97]. Especially suitable for such applications is the use of indirect microfluidic systems that allow an overall biosensor system array to remain an "all-disposable" setup, as the active components responsible for driving the fluids in the system, i.e., pumps and valves, will not come into contact with the sample [98].

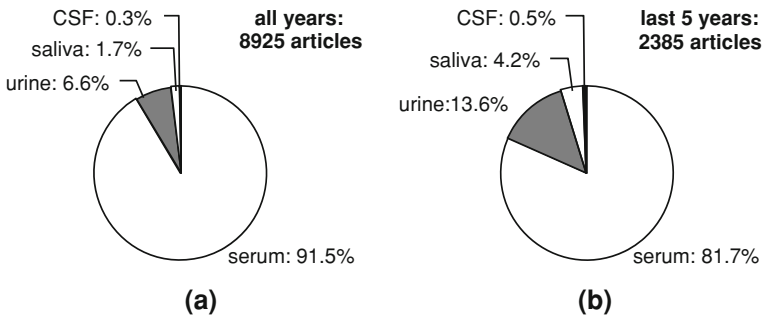

Fig. 3 Number of publications on biosensors in combination with different sample matrices, ISI Web of Knowledge, August 15, 2011. The keyword combination was "biosensors AND [sample type]". The article counts are shown as a percentage of the sum resulting from the four keyword combinations. **a** All years (database starts 1926); **b** last 5 years

3 Biosensors for the Detection of Biomarkers in Real Samples

3.1 Real Samples for Diagnostic Applications

When it comes to the choice of material for disease diagnosis, human body fluids instead of tissue are generally preferred, mainly because of the lower invasiveness. This goes well together with biosensor measurements, which typically require liquid samples. Provided that the disease markers to be detected are contained, samples seem the more attractive the easier they can be collected and processed with minimum time and effort [1]. The most commonly used sample type for biosensors in diagnostic applications is serum, but in the last 5 years other body fluids, such as urine and saliva, have increasingly been investigated (Fig. 3), which is because they can also be collected by noninvasive sampling. Furthermore, they allow a closer investigation of the state of their origin. Urine samples, for instance, may provide more information about the current state of kidney and bladder than serum samples [99]. Salivary samples, aside from detection of viruses, drug abuse, and some cancer biomarkers, allow, in particular, the monitoring of oral diseases [13, 100]. Biosensor measurements using CSF have also been gaining interest in recent years owing to potential biomarkers in CSF allowing early detection of neurodegenerative diseases, such as Alzheimer's disease [101]. All the real sample matrices have in common that they supply a complex protein background which may nonspecifically bind to the biosensor surface, resulting in false-positive results. For strategies to overcome this problem, see Sect. 2.2.

In the following sections the use of biosensors for the detection of disease-related biomarkers is demonstrated. Sample dilution has often been reported as means to reduce nonspecific binding, despite the fact that mostly small biomarker concentrations have to be detected. This leads to high demands regarding the biosensor systems.

Four application areas were chosen to show the performance of biosensors dealing with real samples:

- Cardiac biomarkers in serum. They may be a sign of, e.g., myocardial infarction and therefore require a fast and reliable detection.
- Serum biomarkers for cancer and autoimmune disorders. Chronic diseases require early diagnosis as well as regular control of certain biomarkers, i.e., a reliable and low-cost detection method would be beneficial.
- Biomarkers in CSF for neurodegenerative diseases. Early diagnosis and treatment would allow delay of severe symptoms, i.e., a reliable and low-cost detection method would be beneficial.

3.2 Detection of Cardiac Biomarkers

3.2.1 Cardiovascular Disease Diagnostics

Globally, CVDs are the main cause of death [102]. Among acute coronary syndromes, myocardial infarction, commonly referred to as "heart attack," is one of the most life-threatening forms, as it quickly causes irreversible damage to the heart. Therefore, it has to be diagnosed as soon as possible, e.g., by means of an electrocardiogram or by determination of concentrations of cardiac specific biomarkers, such as troponin and myoglobin. C-reactive protein (CRP) may also be detected; however, this marker is rather used for monitoring the risk of recurrence of myocardial infarction [103]. As previously reviewed, commercially available POCT devices exist which are able to determine the acute phase biomarkers within 5–20 min assay time in the required concentration ranges. Most of them are based on immunoassay or immunochromatographic test methods using fluorescence or chemiluminescence detection allowing the determination of several cardiac biomarkers within one measurement cycle. However, the integration of those devices in clinical routine—and thus their acceptance—is still low, mostly due to cost restraints and because clinical cutoff values have not yet been established [103]. Therefore, as biosensors provide the possibility for low-cost analysis, they could still find their way in this field of application. This will be demonstrated in the following by examples of biosensors for the detection of the cardiac biomarkers troponin, myoglobin, and CRP. The biosensors presented are immunosensors, i.e., antibodies were immobilized as analyte-specific biorecognition elements on the transducer surfaces.

3.2.2 Cardiac Biomarkers in Serum

Troponin and Myoglobin

Cardiac troponins occur in human blood only when the myocardium is directly damaged. Although increased levels are not observable before 3–4 h after the myocardial infarction, because of the lack of other markers, cardiac troponin I (cTnI) and cardiac troponin T (cTnT) are currently considered as the "gold standard" in myocardial infarction diagnosis. The cutoff levels are in the ranges 0.01–0.1 ng/ml (cTnI) and 0.05–0.1 ng/ml (cTnT), respectively. In contrast to the cardiac troponins, increased levels of myoglobin can be detected 1–3 h after the myocardial infarction; the cutoff levels were specified in the range 70–200 ng/ml. However, increased levels of myoglobin are not specific for myocardium damage. Therefore, in the case of a suspected myocardial infarction, it would be beneficial to monitor the concentrations of both troponin and myoglobin during the first few hours for early and efficient diagnosis [103].

Commercially available POCT devices using electrochemical biosensors are provided by Abbott Point of Care (i-STAT series), including a test cartridge for cTnI. This amperometric biosensor setup allows the determination of cTnI in the range 0–50 ng/ml. In contrast to the POCT devices mentioned in Sect. 3.2.1, this biosensor setup detects only a single biomarker [104]. Direct detection of cTnT in human serum samples obtained from cardiac patients could also be performed with a label-free capacitive immunosensor [105] and an SPR immunosensor [106]. In both cases, the concentrations were determined prior to the biosensor measurements by means of commercially available electroluminescence immunoassays. cTnT in serum could be detected with the capacitive biosensor in the concentration range 0.07–6.83 ng/ml [105], and with the SPR biosensor in the range 0.83–3.16 ng/ml [106].

An interesting approach was reported for direct detection of myoglobin with an impedimetric biosensor. The sensing layer was based on half-antibody fragments which could be immobilized in a well-defined orientation, resulting in very dense layers on the biosensor surface. Serum was spiked with myoglobin over the concentration range 10^{-14}–10^{-7} M, corresponding to 0.178 pg/ml to 1.78 μg/ml (M_r myoglobin 17,800) and could be detected with the biosensors in this wide concentration range [107]. Finally, an SPR immunosensor using a SAM based on 16-mercaptohexadecanoic acid was successfully used to detect myoglobin and cTnI in undiluted serum. (Actually, bovine serum was used for the measurements; nevertheless a complex serum sample matrix was represented.) The limit of detection of myoglobin was reported to be 0.9 ng/ml, and that of cTnI was 0.7 ng/ml; in both cases linearity was reported to be up to 50 ng/ml [78].

C-reactive protein

CRP is a classic acute phase protein; however, it is a nonspecific biomarker which indicates all kinds of tissue damage, bacterial infections, and other acute

inflammatory events, including even autoimmune diseases. Furthermore, CRP is a predictive marker of future risk of CVD. There is no defined clinical cutoff value, but concentration ranges for risk prognosis are suggested: CRP concentrations below 1 μg/ml mean low risk, concentrations in the range 1–3 μg/ml indicate intermediate risk, and concentrations in the range 3–15 μg/ml show a high risk [103]. Owing to the comparatively high concentrations in blood, CRP is one of the most commonly used model analytes for testing and demonstrating the performance of a biosensor. Therefore, numerous approaches with all kinds of transducer principles and biosensor coatings have been reported. CRP biosensors were recently reviewed in connection with other cardiac biomarkers, [103].

Furthermore, CRP was detected in human serum by means of TIRF using two test formats, i.e., sandwich assay and binding inhibition assay. Samples were diluted 1:100. The working range of the sandwich assay was 0.044–2.9 μg/ml (detection limit 0.13 μg/ml), and the working range of the binding inhibition assay was 0.13–22.9 μg/ml (detection limit 0.055 μg/ml). These values represent concentrations in undiluted serum, i.e., both methods allow the detection of CRP in the low/intermediate risk range [108]. An impedimetric biosensor using a so-called biogenic nanoporous silica membrane as a layer for each electrode was designed for direct and label-free detection of CRP and another cardiovascular biomarker, myeloperoxidase, in human serum. In human serum samples, for both proteins a large dynamic range from 1 pg/ml to 1 μg/ml was obtained [109]. Admittedly, in the case of CRP, quantification of concentrations below 1 μg/ml is usually not required. However, in this case sample dilution for minimizing the potential risk of nonspecific adsorption could be performed without further ado.

3.3 Detection of Cancer Biomarkers

3.3.1 Cancer Diagnostics

Besides CVD, cancer is globally a major cause of death [110]. There are more than 200 cancer-related diseases and all have one thing in common, i.e., an early detection and classification is crucial for a positive outcome for the patients [4, 7]. Since 1958, tumor staging has been standardized via the TNM system, which based on information about tumor size or depth (T), lymph node spread (N), and the presence or absence of metastases (M) [3, 111]. This anatomical-based staging system has increasingly been complemented by molecular markers to allow further classification of the tumor into subsets, targeting improved diagnosis, prognosis, or prediction of therapy [3]. This includes monitoring of cancer treatment or remission cycles, which is easier to perform if serum biomarkers can be used instead of tissue-related biomarkers [1, 13].

In the case of cancer, efficient diagnosis demands the detection of more than one single biomarker. Instead, a complete marker profile (“molecular signature”) has to be determined, both on the genomic and on the proteomic level [3, 4],

Table 2 Cancer biomarkers

Marker	Type	Source	Cancer type	Threshold	References
HER2/neu	Protein	Serum, tissue	Breast	15 ng/ml	[111, 112]
PSA	Glycoprotein	Serum	Prostate	4 ng/ml	[7]
			Breast	1 pg/ml	[113]
CEA	Glycoprotein	Serum	Colon	3 ng/ml	[7, 111]
hCG	Glycoprotein	Serum, urine	Testicular, ovarian	5 IU/l	[7, 111]

HER2 human epidermal growth factor receptor 2, *PSA* prostate-specific antigen, *CEA* carcinoembryonic antigen, *hCG* human chorionic gonadotropin

consisting of 30–100 biomarkers [83]. This rough estimation of the required number of biomarkers results from the fact that these marker profiles have not been established yet and are still under investigation [1, 4]. Still, a great number of biomarkers have already been linked to cancer types, which leads to enhanced diagnostic information than obtained using the TNM system only [3, 111]. Currently, clinical, quantitative detection of tumor markers is typically performed by means of immunoassay based methods [7]. Considering reduced time and effort, biosensors could offer an alternative, provided that the demands resulting from the respective diagnostic application are met. This means that the biomarkers have to be detected in clinically relevant concentrations in real samples. Furthermore, the determination of multiple concentrations in one measurement cycle would be preferable. In the following, examples of the current state of biosensor detection of cancer biomarkers will be shown. The biosensors presented are immunosensors, i.e., antibodies were immobilized as analyte-specific biorecognition elements on the transducer surfaces. The cancer biomarkers chosen are listed in Table 2, including the properties required because of the respective diagnostic application. The biomarkers chosen are FDA-approved [111].

3.3.2 Cancer Biomarkers in Serum

Human Epidermal Growth Factor Receptor 2

Breast cancer is the most common cancer among women worldwide. Human epidermal growth factor receptor 2 (HER2)/neu, an epithelial transmembrane protein, is mainly regarded as prognostic or as a therapy-predictive marker for this type of cancer [114]. The extracellular domain of HER2/neu is shed from cancer cells into the circulation and is therefore measurable in serum. Critical values for HER2/neu are above 15 ng/ml [112].

Immunosensors for direct and label-free detection of the HER2 extracellular domain in serum samples were recently reported. For detection by optofluidic ring resonators, serum samples were spiked with HER2 biomarker. The device was able to detect HER2 concentrations in serum in the range 13–100 ng/ml [112]. For detection by means of microcantilevers, diluted patient samples were used

(dilution 1:40), and the real concentrations were determined by a commercially available ELISA kit. The microcantilever allowed quantification of final serum concentrations above 2 ng/ml [115]. Both label-free biosensors allowed HER2 detection in the range of clinically relevant concentrations.

Prostate-Specific Antigen

Prostate-specific antigen (PSA), a serine protease produced by the prostate epithelium, is clinically used for the detection and screening of prostate cancer [116]. Critical values for PSA are above 4.0 ng/ml total PSA in blood [7, 116]. Actually, specificity of PSA for prostate cancer is low, resulting in the need for a marker profile to allow accurate diagnosis [4, 116]. Still, owing to its widespread use as screening parameter, PSA was commonly used as a model analyte for testing the performance of biosensors designed for diagnostic applications, which has led to numerous publications. Biosensors for PSA detection were recently reviewed [116]. The latest developments include mainly methods for enhancing the biosensor signal response to allow determination of low PSA concentrations, preferably in the picogram per milliliter range. This should allow early diagnosis of prostate cancer recurrence [116]. Furthermore, increased levels of PSA (above 1 pg/ml) are considered as a parameter for the detection of breast cancer [113].

An amperometric biosensor was used for PSA detection, where signal enhancement was obtained by labeling the secondary antibody (in addition to the peroxidase label) with nitrodopamine-functionalized iron oxide nanoparticles. Serum samples containing 1–10 ng/ml were tested with this biosensor sandwich assay and with ELISA. The correlation coefficient was 0.992, i.e., the results were in good agreement. A comparison of the performance of several electrochemical immunosensors for PSA detection was also given in this work [117]. A QCM biosensor was used for PSA detection in 75% serum. Signal enhancement was obtained by labeling the secondary antibodies with gold nanoparticles. In this sandwich assay, a linear range for PSA concentrations between 0.29 and 150 ng/ml was obtained, corresponding to 0.39–200 ng/ml in 100% serum [118]. Finally, a so-called localized surface plasmon coupled fluorescence fiber-optic biosensor was used to detect PSA in tenfold diluted normal women's serum. This sandwich immunoassay combined with the localized surface plasmon technique allowed the detection of 1.8 pg/ml PSA in diluted serum [113].

Carcinoembryonic Antigen

The glycoprotein carcinoembryonic antigen (CEA) has less specificity to a certain type of tumor than reported for HER2 or PSA (see earlier). Hence, it is associated with several types of cancer, in particular colorectal cancer, but also pancreatic cancer, liver cancer, and gastric cancer to name but a few [3, 7]. Critical values for CEA are above 3.0 ng/ml [7].

An SPR biosensor using signal enhancement by a secondary antibody (sandwich assay) was reported. Samples were spiked with the respective CEA

concentrations and diluted 1:10. CEA serum concentrations were determined in the range 25–800 ng/ml, i.e., only increased CEA levels could be detected [119]. Furthermore, two amperometric immunosensors were used for CEA detection via sandwich assay. The first amperometric biosensor did not use additional means for signal enhancement. Serum samples from colon cancer patients were tested, i.e., the serum CEA concentration was at least 3 ng/ml. The results were in good agreement with those from ELISA tests performed with the same samples [120]. The second biosensor used signal enhancement by labeling the secondary antibody with a gold nanoparticle carrying several peroxidase molecules. In spiked serum samples, concentrations in the range 1–40 ng/ml CEA could be determined. This means that CEA concentrations near and below the threshold value could be detected. Again, the results were confirmed with ELISA tests [121].

Human Chorionic Gonadotropin

The level of the glycoprotein hormone human chorionic gonadotropin (hCG) is increased in the case of pregnancy, and the threshold value recommended for a reliable diagnosis is 14.3 mIU/ml in serum [122]. In the field of cancer diagnosis, hCG is used as a biomarker for testicular and ovarian cancers. In this case, the threshold value is 5.0 mIU/ml, i.e., it is lower than in the case of pregnancy [7].

Direct detection of hCG was performed with a label-free capacitive immunosensor. Clinical serum samples were diluted 1:2 and tested both with the immunosensor and with RIA. Serum concentrations were in the range 18–450 mIU/ml hCG. The results obtained with the immunosensor were in good agreement with those obtained with RIA tests [122].

3.4 Detection of Biomarkers for Autoimmune Disorders

3.4.1 Autoimmune Disease Diagnostics

Autoimmune disorders are pathological conditions in which the immune response is directed against the patient's own cells and tissues. They can become manifest in a single organ or tissue (localized or organ-specific autoimmune diseases), such as type 1 diabetes, or can affect multiple organs and tissues (systemic autoimmune diseases), such as systemic lupus erythematosus (SLE). More than 80 autoimmune diseases have been described [123, 124]. All of them have in common that they are typically chronic, and an early diagnosis of disease type or disease flare-up is required to enable early treatment and avoid the risk of severe symptoms [125]. Autoimmune diseases are mainly characterized by autoantibodies, i.e., antibodies which are directed against self-proteins. Epitope specificity of autoantibodies as well as disease-related molecular profiles to identify the disease type and state have been widely investigated [123, 126].

3.4.2 Disease-Related Autoantibodies in Serum

Type 1 Diabetes

In type 1 diabetes, the autoimmune response is directed against the insulin-producing β-cells of the pancreas. Diabetes-related autoantibodies include insulin autoantibodies (IAA) and antibodies to tyrosine phosphatase-like protein IA-2 [76, 127]. For both autoantibodies, SPR biosensor assays were developed. For IAA detection, an "indirect competitive immunoassay" was performed using biosensors with immobilized insulin. Each serum sample was divided into two portions. One half was spiked with insulin, similar to the preincubation step in a binding inhibition assay, as the insulin would block potentially available IAA binding sites. The other half of the sample was just diluted (buffer), so the sample dilution in both portions was 1:2. In a measurement cycle, first the sample without insulin was applied on the biosensor surface. Autoantibody potentially bound on the biosensor surface was detected with a second antibody to enhance the signal response (sandwich assay). After a regeneration step, the sample with insulin was applied, including the subsequent detection with a secondary antibody. The difference between the signal responses was used as a measure of the IAA concentration in the sample. The cutoff value of the biosensor was determined to be 6 U/ml, i.e., IAA concentrations below that level would be detected as IAA-negative. This value was reported to be similar to the RIA cutoff value. We think this method describes an interesting alternative to deal with nonspecific effects, which may alter from serum to serum. By blockage of potentially present IAA in one half of the sample with insulin, an inherent reference was created which is consistent with the respective real sample [76]. A more traditional way for biosensor assay was used for the detection of IA-2 autoantibodies in human serum with QCM and SPR biosensors. In both cases a synthetic peptide of 15 amino acids derived from IA-2 molecules was immobilized on the biosensor surfaces. Measurements were performed using samples diluted 1:100 and spiked with antibody. A minimal detected antibody concentration of 0.2 mM in 1% serum was obtained with the SPR biosensor; the QCM biosensor was not sensitive enough [127].

Rheumatoid Arthritis

Rheumatoid arthritis is characterized by chronic inflammation of the joints; sometimes, systemic complications may be involved. Currently, antibodies against citrullinated peptides (citrulline-containing peptides) are the most specific serological markers for diagnosing rheumatoid arthritis [128]. Carbon nanotubes were used to detect those antibodies. First, a QCM biosensor was coated with single-walled carbon nanotubes to allow higher binding capacity owing to the three-dimensional structure, then citrullinated peptide was immobilized. Measurements were performed with serum samples diluted 1:300. Serum samples obtained from rheumatoid arthritis patients could be distinguished from serum

samples obtained from healthy individuals . The observed accuracy obtained with the QCM biosensor was even greater than that obtained by ELISA, i.e., with the QCM biosensor more rheumatoid arthritis patients with antibodies to the citrullinated peptide were identified [129]. In another approach, composites of multiwalled carbon nanotubes and polystyrene were used in an amperometric biosensor setup. Multiwalled carbon nanotube–polystyrene mixtures were applied on metal electrodes. The peptide was covalently coupled on these composite-based electrodes. Samples were diluted 1:100 and 1:200, respectively. Peroxidase-labeled secondary antibodies were used to allow detection (sandwich assay). Only a few patient samples were tested in preliminary tests, but they could be distinguished from samples from healthy donors [128].

Antiphospholipid Syndrome

Antiphospholipid syndrome is an autoimmune disease which is mainly characterized by recurrent thromboses. According to the name, antibodies against phospholipids are produced, such as anti-β2-glycoprotein I [126]. SPR biosensors with covalently immobilized β2-glycoprotein I were used to test serum samples which were diluted 1:100. Distinguishing between antiphospholipid syndrome patients and healthy individuals was possible [130].

Systemic Lupus Erythematosus

SLE is a systemic autoimmune disease which may affect almost any organ as well as connective tissues and blood vessels. Furthermore, SLE may lead to neurological and hematological disorders [126]. Several autoantibodies are used for diagnosis, mostly anti-chromatin, antibodies against nuclei, i.e., antinuclear antibodies (ANA), and anti-dsDNA. In particular, the latter may also be important as a diagnostic parameter for other autoimmune diseases. An amperometric biosensor was reported to detect anti-chromatin antibodies. Electrodes were screen-printed on a poly(vinylidene fluoride) membrane impregnated with chromatin. Serum samples were diluted 1:50 and applied on the sensors, followed by peroxidase-conjugated secondary antibody (sandwich assay). Serum samples obtained from SLE patients could be distinguished from serum samples obtained from healthy individuals [131]. For the detection of ANA in serum, a fiber-optic evanescent wave biosensor modified with colloidal gold has been developed. Extractable nuclear antigens were immobilized on the fiber. The ANA content detected in samples diluted 1:100 was in good agreement with that determined by ELISA tests [132]. Detection of anti-dsDNA in serum samples has been performed with potentiometric [133, 134], QCM [135], and SPR [136, 137] biosensors. Potentiometric biosensors were made of glassy carbon electrodes coated by electropolymerization of aniline [133] or phenothiazine dyes (methylene blue and methylene green) [134] and subsequent immobilization of dsDNA. Serum sample dilution in the range 1:20–1:50 was found to be appropriate to achieve the best

results. Sensor responses obtained with samples from SLE patients could be distinguished from those of healthy donors [133, 134]. Furthermore, a QCM biosensor was modified with dsDNA. Serum samples were diluted to a final protein concentration of 0.1 mg/ml. As serum contains 60–80 mg/ml protein [76], this equates to a dilution factor in the range 1:600–1:800. The results regarding antibody content were reported to be comparable to those from ELISA measurements; however, the QCM results were more affected by nonspecific binding, i.e., the biosensor signal response for the healthy donor sample was increased [135]. Finally, an SPR biosensor modified with oligodeoxynucleotide allowed serum samples obtained from SLE patients and serum samples obtained from healthy individuals to be distinguished [136]. Furthermore, the results were reported to be satisfactory compared with classic immunoassay methods [137].

Celiac Disease

Celiac disease, also referred to as celiac sprue, is an autoimmune disease which is characterized by a disorder of the small intestine triggered by wheat gluten. Serological markers include autoantibodies directed against tissue transglutaminase [138]. Screen-printed gold electrodes were coated with transglutaminase and used as impedimetric immunosensors. Sample signal responses were enhanced by a peroxidase-labeled secondary antibody with subsequent biocatalytic oxidation of the corresponding substrate (sandwich assay). Patient samples could be distinguished from samples of healthy donors [139]. Furthermore, an amperometric immunosensor was developed for detection of anti-transglutaminase. Transglutaminase was covalently coupled on gold electrodes. Antibodies in serum (dilution factors 1:100 and 1:400) were detected via a peroxidase-labeled secondary antibody (sandwich assay). Patient samples could also be distinguished from samples of healthy donors [140].

Cancer Autoantibodies

Circulating antibodies to cancer-related antigens are considered to be present in early stages of disease; therefore, autoantibodies are also increasingly being investigated as cancer biomarkers [141]. Autoantibodies against CEA (see Table 2), for instance, were detected with an SPR biosensor modified with CEA. Both direct detection and detection via a secondary antibody (sandwich assay) allowed patient samples to be distinguished from negative controls which were obtained from healthy individuals [142].

3.5 Detection of Biomarkers for Neurodegenerative Diseases

Neurodegenerative diseases, such as Alzheimer's disease and Parkinson's disease, are mainly characterized by progressive loss of functions of the nervous system.

The diagnosis of such a neurodegenerative disease is required in an early stage to allow early treatment and thus delay of severe symptoms. Current research in this field includes the investigation of disease-related biomarkers in CSF. Sampling of CSF is less invasive than sampling of brain tissue, and owing to its proximity to the brain, it should be better suited than serum to reflect processes in the brain [101].

SPR biosensors have been used to investigate biomarkers in CSF aiming at diagnosis of Alzheimer's disease. Amyloid β peptides in CSF have been reported as potential biomarkers for Alzheimer's disease. A sandwich assay based on an SPR immunosensor was developed to determine the concentrations of two different amyloid β peptides in CSF. The secondary antibody was required as a means of signal enhancement because of the low (subnanomolar) concentrations of amyloid β peptides in CSF. CSF samples from patients diagnosed with Alzheimer's disease could be distinguished from CSF samples from healthy donors [143]. Furthermore, it was suggested that the mitochondrial enzyme 17β-hydroxysteroid dehydrogenase type 10 (17β-HSD10) binds to amyloid β peptides. Therefore, the 17β-HSD10/amyloid β peptide complex could also serve as an Alzheimer's disease biomarker. To detect concentrations of this complex, antibody against amyloid β peptide was immobilized on SPR biosensors. After patient CSF samples (dilution factor 1:2) had been applied, antibody against 17β-HSD10 was applied as a secondary antibody (sandwich assay). An increase in signal response should only be obtained when the 17β-HSD10/amyloid β peptide complex binds to the surface, and not when the pure amyloid β peptide binds. The results were in good correlation with results obtained from ELISA tests performed with the same samples. Samples from patients diagnosed with Alzheimer's disease could be distinguished from control samples obtained from patients suffering from noninflammatory neurological diseases [144].

4 Conclusion

Biosensor development started around 50 years ago with the development of an "enzyme electrode" for monitoring glucose in blood. Since then, biosensor development has been ongoing, with clinical applications still being one of the main driving forces. However, despite the fact that biosensors are promising devices when it comes to fast and easy detection of analytes, their use has not yet been established in clinical routine, in contrast, for instance, to immunoassay techniques.

There is no doubt that numerous publications and patents dealing with biosensors have been published. However, looking at the material published in the last few years more closely, we realized that only a comparatively small part of the work reported was performed using real samples. One reason for this discrepancy might be that the problem of nonspecific binding arising from complex sample matrices has not been completely solved. Strategies commonly used to deal with nonspecific binding in a typical biosensor measurement usually have to be adapted

for the specific application and hence are not easily transferable to other applications. We think that biosensors based on molecular switches offer a promising tool to solve this problem, because in this case it is only the analyte binding which is able to generate a signal response (provided that cross-reactivity is excluded). Molecular switches have been reported for optical and electrochemical transduction principles, which are currently the most commonly used transduction principles for biosensors.

A powerful biosensor system requires a high-performance biosensor component as well as a user-friendly instrumental setup. However, biosensor setups have to be adapted to specific applications. As disease-related marker profiles are still under investigation, specifications regarding a suitable biosensor instrument can hardly be given at the moment. But, almost certainly, such a device should permit multiplex analysis to determine a marker profile in a sample in one measurement cycle. Therefore, a biosensor array would be required. At least as long as these profiles are not established, it could be useful to keep these arrays flexible. This would be supported by a packaging strategy in which each biosensor element is integrated in a single, array-compatible housing allowing user-defined combination. This would make the arrays potentially adaptable to the respective application and hence make the underlying biosensor instrument more versatile.

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Planar Protein Arrays in Microtiter Plates: Development of a New Format Towards Accurate, Automation-Friendly and Affordable (A^3) Diagnostics

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Abstract A technology for protein microarrays in 96 well microplates has been developed as a widely adoptable platform for multiplexed protein based diagnostics. Procedures for protein microarray manufacturing, immobilization, assay optimization and optical detection methods were developed. Using a clever combination of state of the art technologies, a versatile platform that works with peptides, proteins and antibodies as capture agents in a scalable format for planar arrays is described.

Keywords Biosensor · Diagnostics · Microarray · Non-contact spotting · Test format

Contents

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1 Introduction

Proteins are involved in all functions of organisms and they show a much greater variety and complexity compared to genes. For years and decades scientists have been committed to the investigation of proteins, its structure and function. In life sciences and especially in medicine and diagnostics a better knowledge of protein–protein-interactions may lead to a better understanding of diseases, the development of new diagnostics, targeted drugs and therapy monitoring tools. Substantial progress in technology developments demonstrated that microarrays have become an important tool for investigating proteins. However, major challenges have to be dealt with when investigating protein–protein-interactions [1]. Proteins are a heterogeneous group of molecules and it is difficult to preserve their full functionality in experimental environments. This challenge refers to both: the physical handling of proteins with pipettes or automated arrayers and to experimental conditions such as appropriate buffers or washing procedures [2]. High resolution detection devices and corresponding labelling methods are needed [3]. Sophisticated analysis software tools are essential to receive distinctive results. Finally, investigations should be time saving and as cost effective as possible to be successful in the market.

Protein Microarrays have become an important high throughput tool to investigate protein–protein interactions because they provide a parallel, fast and straightforward analysis of as many proteins as can be potentially immobilized on a given surface [4–7]. For diagnostic applications typically a chosen subset of capture agents is deposited on planar substrates, to allow the parallel analysis of two or more interactions in a single reaction [8, 9]. Today, typical applications include autoimmune diseases, infectious diseases or cytokine analysis and a growing number of further applications or specifications are under development. In screening experiments of disease markers not only the expression and/or the amount of the proteins may be of interest but also their post-translational modifications e.g. phosphorylations or glycosylations [10].

2 Substrates for Protein Binding

Depending on the actual sample format and the diagnostic application, various routes can potentially be taken to generate, incubate and read out any protein array. For the majority of diagnostic applications one key requirement is to produce the multiplexed test format as cost effective as possible—a requirement that already has to be considered during research and development. While planar protein arrays have historically been developed using inexpensive membrane formats [5, 6] a significant number of publications described the use of glass slides for capture probe immobilisation. The latter offer the advantage of fluorescent detection schemes, which provide an improved signal to noise ratio when compared to auto-fluorescent

membrane materials. Another advantage of glass slides is that they can be coated and thus allow for efficient protein binding. Although used for some commercial products [11] now for many years, the slide format has several disadvantages in molecular diagnostics approaches based on protein arrays. Two main reasons have driven many researchers to look for alternatives to glass slides. Reason number one is, that planar and coated high quality glass slides are still considered expensive with prices of 10 Euro per slide being more the rule than the exception. Reason number two is the format of slides itself and its difficult handling when it comes to automation: Slides can be easily handled manually, but automating all required handling steps in an array experiment for 10,000 s of slides per day is still challenging and according to the authors knowledge there is no solution available at the time of editing.

There are several alternatives to glass slides as carriers for protein arraying that have been published in the past couple of years. This includes a number of different biosensors [12, 13], mostly developed for label free detection schemes. Although these items can be mass produced, still their size is something that is typically unknown in diagnostic laboratories and often the interface to the real laboratory environment needs more and better ergonomic design.

GeneOhm was one of the first companies to combine (DNA) biosensing devices with standard diagnostic formats in the form of microplates (http://www.slas.org/education/standards/ANSI_SBS_1-2004.pdf). Other formats of arrays produced on a specific surface and brought into tubes or plates commonly used in diagnostic laboratories were developed by Affymetrix [14] and German Clondiag that later became a part of Alere [15].

Protein arrays in microplate structures are available as products for autoimmune diagnostics for some years now. Toronto based SQI Diagnostics was the pioneer for these developments [16]. To array the capture antigens a planar glass slide with a microplate footprint is used, to which a “motherstructure”, providing 96 microplate chimneys, gets connected with a specific gasket. After connection, 96 individual reaction wells are available for sample incubation in a standard microplate footprint. Albeit this format is very amenable to automation and provides very good optical quality for fluorescent detection, still the costs of goods for precisely manufactured glass surfaces in microplate format, required gaskets and related polymer structures are still significant and for a diagnostic product are unproportionally high.

3 Direct Printing into Microplate Wells

Multiwell or microtiter plates cover a broad spectrum of biological, chemical and pharmacological laboratory uses. For high throughput screening as well as diagnostic methods such plates are mostly produced in one piece by injection moulding. Especially as a result of high throughput applications auxiliary automatic and robotic instrumentation has been developed and the dimensions of the

plates were standardised. Some analytical or diagnostic applications of multiwell or microtiter plates involve attachment or immobilisation of biological agents or probes to a surface within the wells, mostly the bottom surface, and the performance of one or more reactions in the wells is followed by some sort of quantitative and/or qualitative analytical process. For example, the biological agents or probes, such as DNA or proteins, are immobilised on the bottom surface of each well in form of a microarray, i. e. as multiple spots each containing the same or different probes. The putative target(s) to be analysed are mostly fluorescently, colorimetrically, or chemiluminescently labelled and brought into contact with the microarray of probes under highly stringent conditions. Alternatively, the hybridised or bound unlabelled target is detected by a labelled secondary agent. Subsequently, target incubation is determined via an appropriate detection method. Thus, if different probes are used for each spot a multitude of reactions can be performed in only one well.

Direct printing into 96 well microplates is a potentially straightforward and inexpensive way to support a diagnostic platform. Unfortunately the immediate advantages of an inexpensive polymer substrate, well known to most diagnostic users, its easy automation and its surface modification properties comes together with several challenges: with high fluorescent backgrounds for a number of polymers, electrostatic charging of the surfaces and therefore making spot deposition very difficult as well as long manufacturing times due to the requirement of always doing a z-movement into the well being the most obvious ones.

For microarray based applications robotic instruments have been developed to process the plates fully automated. Although the standard structure of the plates has facilitated such automated processing, at the same time this structure presents challenges with regard to certain procedures. For example, the deposition of biological agents by printing them as microarray spots onto the bottom surface of a well is done by robotic liquid-handling systems, so-called arrayers. These depositions require the pipetting head or printing pin of the arrayer to move significantly up and down (z-axis) as it spots one well of a microtiter plate after the other with small drops of liquid comprising only about 0.1–4 nl. Depending on the kind of microarray printed it might be necessary to move the pipetting head over 100 times into one well which makes this process very time consuming. Furthermore, these movements increase the risk of damaging the pipetting or spotting head.

3.1 Contact Printing Versus Non-Contact Printing

Although it is theoretically possible to print 96 identical arrays into microplates using contact or pin printing, this technology plays no role in diagnostic manufacturing. This is mainly due to proteins being destroyed due to mechanical stress when contact spotted to the surface and the high CVs in the print process, which are mostly based on the inefficient transfer of the fluids from the print tips end to the surface. Fig. 1 shows in detail the transfer of a protein dissolved in spotting

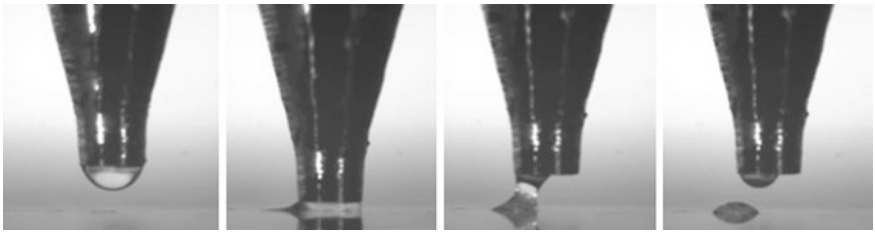

Fig. 1 Details of protein printing using stainless steel, blunt end pins

buffer onto a glass slide. From left to right the loaded pin is moving down towards the glass surface. When the liquid at the end of the pin reaches the surface, the protein solution gets transferred to the surface. Then the pin moves up again. Interesting, not all of the liquid is transferred and the spot is not centred below the pin. This unprecise locating of the spot combined with varying spotting volumes, results in uneven spotting patterns with a great variety in spot diameters, making a downstream array analysis unnecessarily difficult. Deposited volume shown in Fig. 1 is between 1.5 and 2 nL

Other disadvantages include that high volumes of source solutions to be spotted have to be provided (e.g. 96 times 30 μl, so a total of 2.88 ml spotting solution of a single capture agent, provided to load 96 pins in parallel), which unnecessarily increases dead volume and the manufacturing costs dramatically.

Non-contact printing potentially provides a mild printing of the capture agents to the surface or bottom of a microplate well. Figure 2 is the graphical representation of a non contact actuated system that shows a glass tube surrounded by a piezoelectric element. Upon application of a signal with a typical height of 50–150 V and a duration between 10 and 50 μs the piezoelectric elements starts expanding and compressing, thereby creating an overlay of waves inside the glass tube. On the right side the end of such a tapered glass tube is shown. Using stroboscopic imaging technology droplets can be visualized, clearly showing that a free flight of droplets forms the basis for a non contact spotting technology. In the picture shown in the right hand part a volume of exactly 300 pL is deposited.

The printing process has very low CVs and with repetition rates of up to 1000 Hz is potentially very fast which is shown in Fig. 3. Using different combinations of actuation parameters in terms of pulse height and pulse length, different drop volumes can be generated with non contact dispensing technology using an identical dispenser. The graph shows the dispensed volumes of 10,000 drops at different settings using a single dispenser or tip. Dispensed volumes were determined gravimetrically and optically, where both methods resulted in CVs of much less than 3 %.

Depending on the desired throughput in diagnostic manufacturing, samples can be aspirated from several nanolitres to 1 ml, thereby providing a stock of liquid that can form between 20 and 2,000,000 arrays.

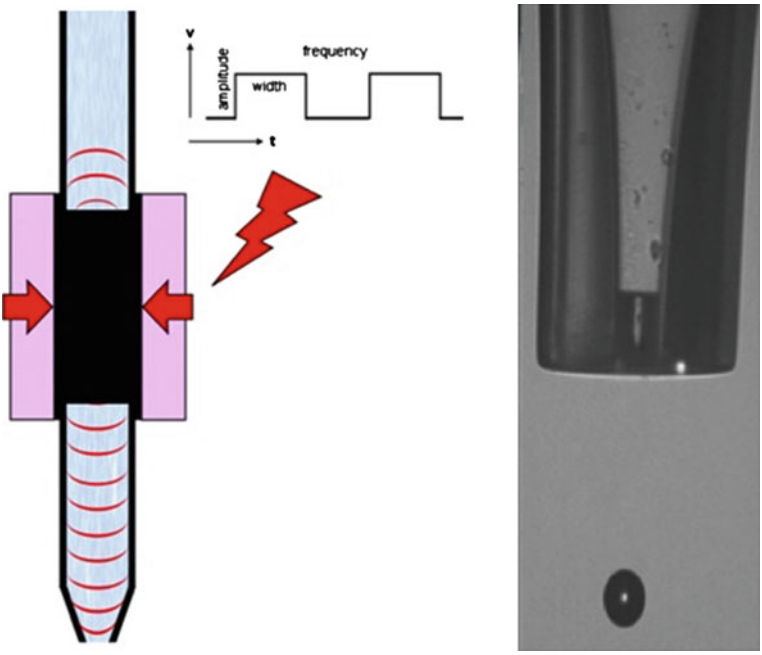

Fig. 2 Non-contact spotting. Free fly of droplets allow very small CVs in the amounts deposited onto a surface. The printing process has very low CVs and with repetition rates of up to 1000 Hz is potentially very fast which is shown in fig. 3

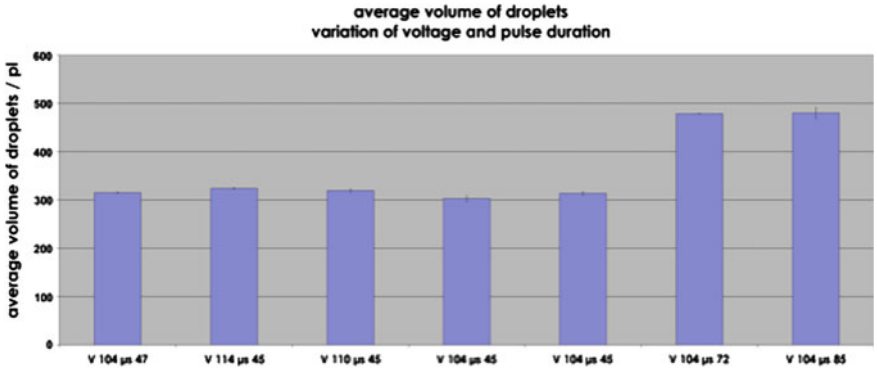

Fig. 3 Determination of droplet volumes using piezo driven non-contact dispensers

One immediate drawback of non-contact spotting procedures becomes obvious when the z-movement of the spotting head has to be minimized to allow for fast production: electrostatic charge as present in most polymer plates creates a field which influences the trajectory of a deposited protein-containing droplet. The resulting array will show spots that have all an identical size, but will be placed in

the well bottom very irregularly and show significant changes in the spot to spot distances. An elegant solution for this initial drawback is beside de-ionisation of the polymer microplates the usage of shielding plates. These conductive plates inserts, either provided as a part of the microplate or as a separate metal plates, are acting as effective faraday cages and allow a field free deposition of the protein droplets onto the surface, when ejected from above the microplate. When printing is finished the shielding plates are just taken of the spotted microplate and can be re-used [17] Fig. 4 shows a metal shielding plate providing 96 discrete chimneys (top) can be inserted into a standard 96 well plate (middle) and non contact spotting can be done from above the plate (bottom), thereby increasing print speed significantly.

As mentioned before, a movement along the z-axis with the spotting head. It can be used for highly accurate deposition of capture probes to the bottom of wells, too. Typical spotting heights vary from 200 to 500 μm. Although time consuming, this mode of direct printing provides a robust method to array flexible protein capture array patterns on a given substrate Fig. 5 shows Direct printing into microplate wells with two non contact dispensers. Each dispenser creates a separate array in separate wells. For visualisation purposes a blue dye was used to illustrate a precise sample deposition.

3.2 Microarrays with Up to 1400 Spots Per Well

The number of spots that can be printed into a single well of a 96 well plate depends on the shape of the well (round or squared) and varies from single spots having a printing volume between 100 pL and 100 nL to print patterns that accommodate more than 1400 spots per well (Fig. 6). Using non-contact printing technology more than 1000 spots per well can be generated. The picture shows >1400 spots deposited with a sciFLEXARRAYER S11 (Scienion AG, Berlin, Germany).

Nevertheless, most applications developed for diagnostic purposes have between 30 and 300 spots, where 10–100 analytes are printed as triplicates (three replicates). Replicates give some level of security if one spot isn't displayed adequately or shows an effect that is not foreseeable. In addition replicates are a very good QC tool for analysing array results, since principally they should show identical behaviour.

Microplate formats have been used for ELISA purposes for many years [18] and proven that the polymer materials work very nicely for well coating purposes. When array spotting is applied, surface defects in the plate's bottom become clearly visible immediately, when a spot is located in such an area. Thus, using a defect free high quality microplate is essential before starting any array based diagnostic developments. Figure 7 shows a microarray printed into a well of a microplate with an unwanted result. Very poor protein binding, combined with non uniform spots and unwanted particles in a microplate do not allow for proper analysis of an experiment .

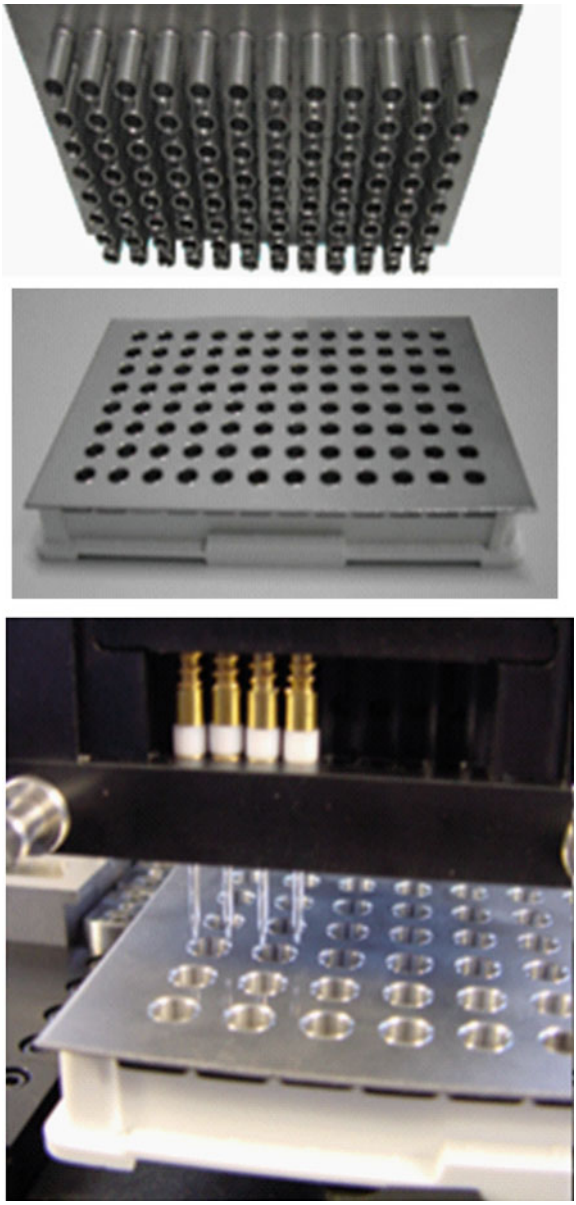

Fig. 4 Sample deposition in microplate cavities without z-movement due to the use of shielding plates

In addition to conventional 96 well plates, that always provide 96 reaction vessels simultaneously, strip well plates can be printed and used as a variable platform, too. Using strips of 8 or 12 vessels that can be mounted in a frame that again has a 96 well footprint enables cost efficient use of this technology, where not always 96 samples have to be analysed in parallel. Even single breakable strips are available, that allow any number of up to 96 samples to be analyzed in parallel

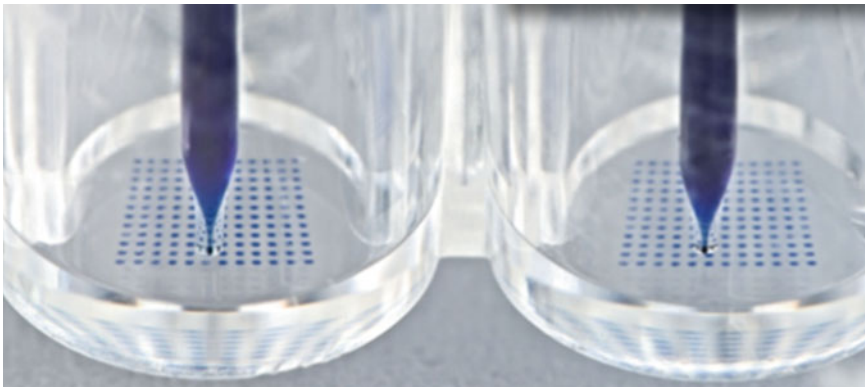

Fig. 5 Direct non-contact spotting into a 96 well plate

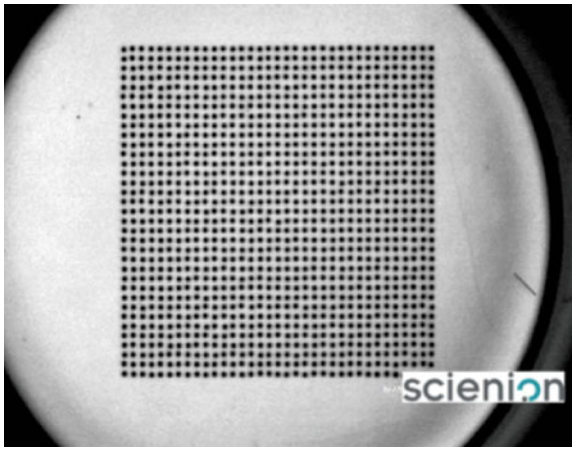

Fig. 6 Microplate well loaded with 1400 spots

in a microplate compatible frame. The SBS compatible microplate formats shown in Fig. 8 are sciPLEXPLATES (Scienion AG, Berlin, Germany). These plates have a minimised number of surface defects, allow for an efficient protein capture probe binding to the surface and are available in two formats. While in the strip well plate (left) always eight individual wells are connected, single wells can be used with a specific holder to allow for a single breakable strip (right).

4 Colorimetric Protein Array Detection

Fluorescent labelling technologies have a widespread use in several protein array formats. For the detection of the incorporated or adopted fluorescent dyes typically sophisticated and expensive detection devices have to be used. Colorimetric

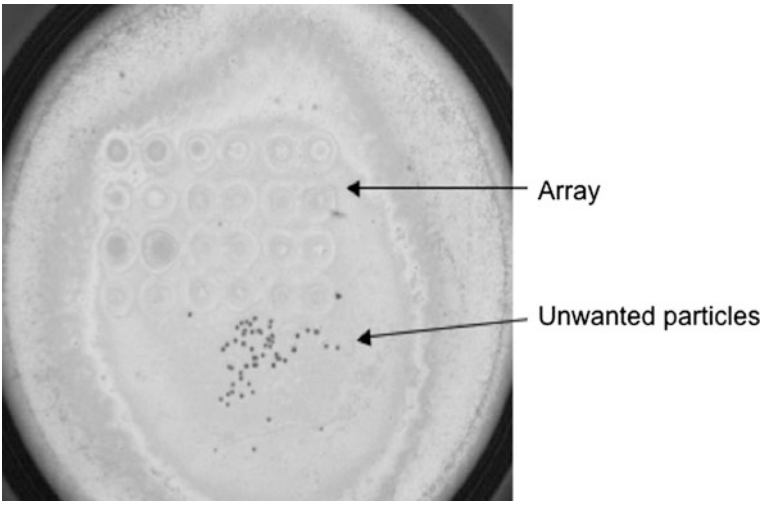

Fig. 7 Initial results of an array in plates research programme

Fig. 8 Ninety-six well plates in different formats developed for precise non-contact spotting

staining of arrays has been shown as a genuine alternative to fluorescent detection with the advantage to use less sophisticated and therefore less expensive detection devices, as costs can represent a real entry barrier for diagnostics markets. Analysing protein microarrays using a colorimetric nanogold probe coupled with silver enhancement (gold-silver detection) has been described Liang and co-workers [19]. In this method, the gold nanoparticles are introduced to the microarray by the specific binding of gold-conjugated antibodies or streptavidins and then coupled with silver enhancement to produce a black image of microarray spots, which can be easily detected with a conventional CCD camera. The method showed good detection sensitivity (1 pg of IgG immobilised on slides or 2.75 ng/ml IgG in solution) and a good linear correlation between the signal intensity and the logarithm of the sample concentration. The examination of this method in analysing a demonstrational ToRCH antigen microarray developed showed identical results when compared to fluorescent detection schemes. These results suggested that the colorimetric gold-silver detection method had potential applications in proteomics research and clinical diagnosis. Nevertheless it seems that detection systems based on silver reduction are very sensitive to environmental changes (e.g. temperature) and timing, making it somewhat difficult to use in routine analysis.

The majority of colorimetric (also called chromogenic) detection for miniaturised ELISAs is based on colorimetric substrates for ELISA development with alkaline phosphatase (AP) or for horseradish peroxidase enzyme (HRP). Compared to the Gold/Silver detection they are typically less expensive and have been used for conventional ELISAs for years. Typical reagents commercially available from http://www.piercenet.com/browse.cfm?fldID=EEB28337-5056-8A76-4E7C-1FA2CA04F788) do include:

p-Nitrophenyl Phosphate, Disodium Salt (PNPP) is a widely used substrate for detecting AP in ELISA applications. PNPP produces a yellow water-soluble reaction product that absorbs light at 405 nm. PNPP is available either as a crystalline powder, 5 mg tablets or as a ready-to-use formulation.

2,2′-Azinobis [3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt (ABTS) is used to detect HRP and yields a water-soluble green end reaction product. The green product has two major absorbance peaks, 410 nm and 650 nm. ABTS is less sensitive than the OPD and TMB substrates (see below) for HRP detection. Colour development is slow (approximately 20 min) which may be advantageous if unacceptable background results from the use of the OPD or TMB substrates due to higher sensitivities. ABTS is available in either tablet or a ready-to-use for formulation.

o-phenylenediamine dihydrochloride (OPD) is used to detect HRP and yields a water soluble yellow-orange reaction product. The reaction product has an absorbance maximum of 492 nm. OPD is available in either powder or tablet forms and easily prepared by dissolving in Stable Peroxide Substrate Buffer or buffered hydrogen peroxide solution.

3,3′,5,5′-tetramethylbenzidine (TMB) soluble substrates yield a blue colour when detecting HRP. The major absorbance maxima or the reaction product are 370 nm and 652 nm. The colour then changes to yellow with the addition of

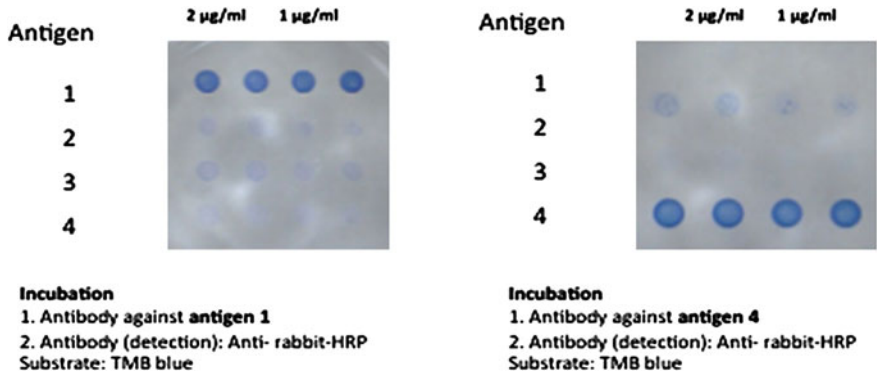

Fig. 9 Example of a 4plex miniaturised ELISA with colorimetric detection

sulfuric or phosphoric acid with maximum absorbance at 450 nm. TMB is very sensitive and may produce significant background signal if too much protein or antibody is used. TMB is more quickly oxidized than other HRP substrates, resulting in faster colour development.

The development of the experimental conditions for colorimetric detection in multiplex assays using multiple analytes in a single well as capturing agents typically needs several rounds of optimisation, especially when the agents show potential cross reactivity. Very essential parameters do include the actual concentration of the printed analyte, the buffer composition, the spotting volume, blocking reagents and times as well as timings for the individual incubation steps and their respective reagents concentration. The development of a multiplexed panel with TMB readout is illustrated in Fig. 9. This figure shows the printing of four different antigens in two different concentrations onto the bottom of a sciPLEXPLATE. Each concentration is deposited in neighbouring duplicates. After incubation and washing of two different antibodies in two separate wells detection of the specific interactions was easily visible although some background in the non specific binding spots is visible. Using an integration of the spots signals and the introduction of a threshold clearly distinguishes specific from unspecific binding (data not shown) .

Typically colorimetric detection is thought to be single colour detection only, but recent developments have shown that a dual colour detection scheme is compatible with microplate based read-outs. In Fig. 10 different colour TMB substrates developed by Seramun (Heidesee, Germany) were applied in a dilution series and gave good signal to noise ratios. Two different arrays in two different wells of a 96 well sciPLEXPLATE were printed with various volumes of capturing agents. From left to *right* the spotting volumes in each row are 1-2- 3 and 4 nL. While incubation in the left well was finally done with TMB blue, in the right well a similar chemical called TMB green was applied resulting in a green colour for detection .

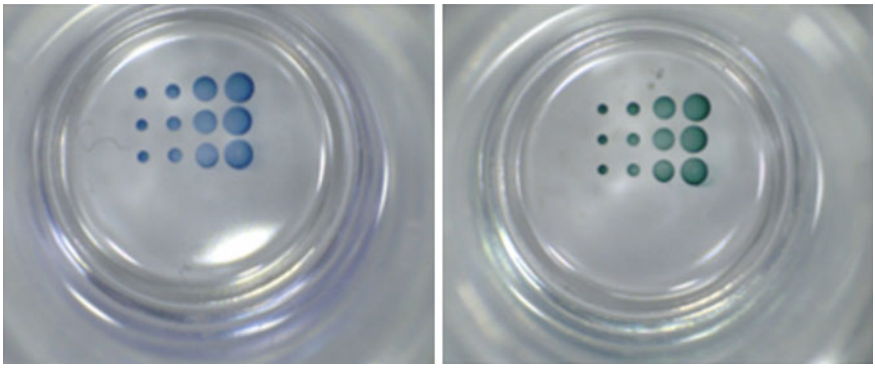

Fig. 10 Dual colour colorimetric detection

4.1 Detection Devices for Colorimetric Array Readout

For the detection of colorimetric assays typically quite inexpensive detectors might be used, especially when compared to fluorescent detectors. Whereas in fluorescent detectors specific excitation light sources are required, colorimetric detection of a well requires just an even lighting from a white lamp, e.g. a halogen lamp, being much less expensive when compared to laser based excitation systems. Dependent on whether transparent or non transparent surfaces are used translucent or reflective lighting is required. For detection conventional CCD cameras can be used. If for some reason long exposure times are required to detect meaningful signals, these CCD cameras should be cooled. If a single colour has to be detected, monochrome CCDs are sufficient, whereas in the multi-colour detection scheme shown above a colour CCD detection is required. Most array analysis programs require at least 10×10 pixels for an accurate spot analysis. Consequently a protein array with spot sizes of 300 micron needs a resolution of at least 30 micron, whereas larger spots tolerate even less resolution while smaller spots, e.g. a 100 micron spot, require at least 10 micron resolution. Although office flat bed scanners might be suitable for the colorimetric detection of arrays on slides or membranes, their usability for detection of arrays in microplates is somewhat limited due to the uneven lighting resulting from the chimneys of the microplates.

5 Sophisticated Software Tools are Needed for Array Production and Analysis

Protein microarrays offer enormous potential for a wide range of applications but a major bottle neck remains the automated analysis of the biochips. The analysis of protein microarrays is still in their infancy in comparison to nucleotide

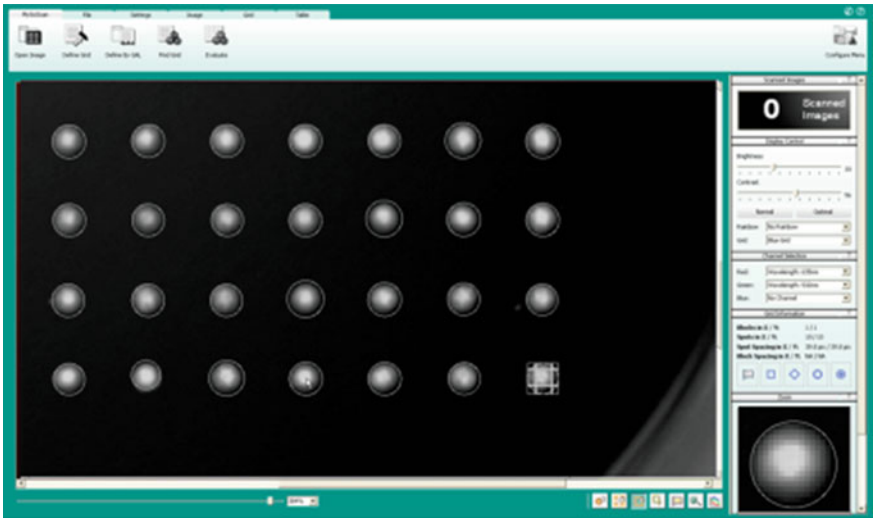

Fig. 11 Software screenshot after automatic grid finding (sciANA 4.1, Scienion AG, Berlin, Germany). Shown is an inverted picture of a protein array spotted in a sciPLEXPLATE. Circles show the areas that will be taken for downstream data analysis

microarrays. Although there are some software packages for protein microarray data analysis available like ProCAT [20], rapmad [21] or PASE [22] these tools have severe limitations compared to their more mature DNA microarray analysis counterparts. Protein microarrays have several specific features making their analysis more sophisticated than the analysis of nucleotide microarrays. The most important factors relate to the variances in spot positioning and protein concentrations due to protein properties and the resulting technical limitations of the array production process discussed in the previous paragraphs.

There are two main application areas for protein array analysis software: quality control in the production process and the analysis of the processed biochips. The reliable automated evaluation of the protein arrays is the most important requirement for both application areas.

5.1 Determination of Spot Position and Grid Alignment

Computational processing of microarray data begins with the acquisition of a digital image representing the signal intensity of the protein spots on the microarray. An essential requirement for grid alignment is the transfer of spotting information from the spotting device to the analysis software. Most convenient for this purpose is an interface enabling the automated transfer of the spotting information to the analysis application. The spot placement is constrained by the configuration of the printer, and by the organization of the spotting pattern in the original plates.

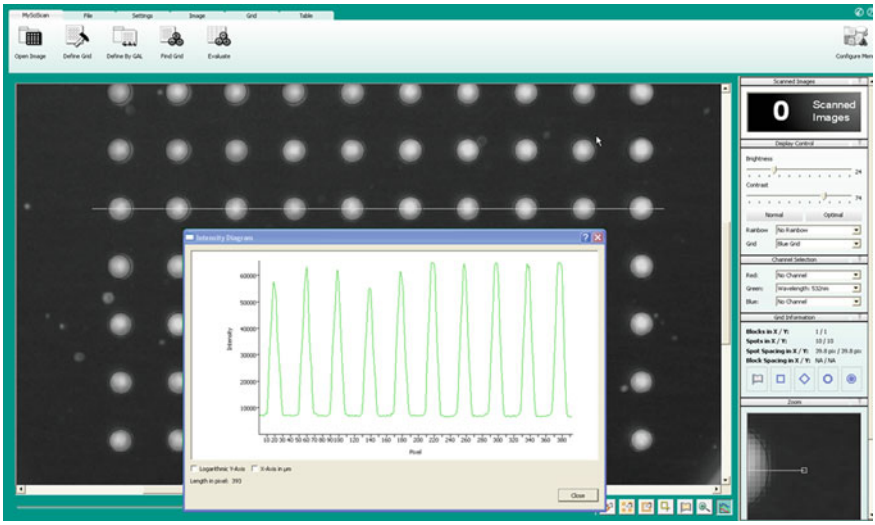

Fig. 12 Intensity line diagram of intensity values for replicated spots (sciANA 4.1, Scienion AG, Berlin, Germany)

Currently, determining the spot locations within each image requires operator assistance, but this process could become fully automated with the inclusion of reference spots. The inclusion of spots for correct orientation and positive and negative controls enable the automated alignment of the grid, the automated control of array quality and the control of the array evaluation. Having suitable reference spots at hand automatic grid finding algorithms can be adjusted to a wide range of image formats and grid types as displayed in Fig. 11. Shown is a software screenshot after automatic grid finding (sciANA 4.1, Scienion AG, Berlin, Germany). Additionally analysis software with automatic grid finding algorithms is a valuable tool for quality control in the protein array production process.

5.2 Determination of Spot Intensity

Signal intensities are generally quantified by subtracting the foreground intensities with background intensities. The intensity of a specific spot can be estimated by integrating over a fixed spot area or by using flexible radii for each spot. For the determination of the spot intensity the local background intensity has to be measured and used for intensity correction and noise reduction. The background intensity can be estimated either by a number of empty spots within the image or as background signals from regions immediately surrounding the spot of interest.

In protein microarrays the local background regions are often distorted by local artefacts or strong positive signals in the neighbourhood resulting in over shining

effects. In both cases, the measurement for that spot will be inaccurate. Local artefacts can increase the local background and thus reduce the intensity of the signal resulting in lower protein quantification. Over shining effects can have an effect on the positioning of the grid and thus increase the variance of the same protein at replicate experiments. To overcome these problems the grid and radius can be fitted manually to each individual spot which takes a lot of time and is prone to errors. Much more efficient is an automated detection of distortions on the protein microarray and the labelling of the artefacts and over shining effects for the subsequent evaluation process. Figure 12 shows intensity line diagram generated with software sciANA 4.1. The green intensity plot displays the signal intensities of 10 with identical peptide concentration of equal spotting volume along the line given in the inverted b/w picture.

6 Conclusion

In contrast to closed systems for biochips that have been promoted for a number of years and been successful (e.g UNIgene® Arrays from Protagen (Dortmund, Germany), IgXPLEX™ celiac qualitative assay from SQI Diagnostics (Toronto, Canada) the approach described here offers selectable combinations of state of the art technologies for the production and analysis of microarrays with any content in scalable formats at significantly reduced manufacturing costs. This toolbox approach allows for any adoptions to individual needs and formats that are scalable from research and development to diagnostics manufacturing purposes without a technology switch—where the presented non-contact printing technology, the consumables and detectors covers many aspects of the technology, but needs to work hand in hand with surface chemistry, assay development, alternative detection technologies and algorithms for data analysis.

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