From Bedside to Bench: Methods in Precision Medicine

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Filipe Cortes-Figueiredo, Vanessa A. Morais, and Helena Pinheiro

12.1 Introduction

The escalating amounts of data in biomedical research have recently arisen as a harbinger of a new approach to medicine—*precision medicine*. Precision medicine aims to consider individual characteristics when diagnosing, treating, and managing the prognosis of a patient by concentrating health interventions (preventive or therapeutic) on those who will benefit from them. Thus, unnecessary side effects may be avoided and the allocation of health resources might be made more efficiently [1, 2].

A key determinant in aiding the advent of precision medicine has been *biomarkers*, which are characteristics that are objectively measured and assessed as indicators of either normal biological processes, pathogenic processes, or pharmacologic responses to therapeutic interventions. Thus, biomarkers may serve numerous functions, namely detecting higher susceptibility/risk to a specific medical condition or

Illustrations by Helena Pinheiro.

F. Cortes-Figueiredo
VMorais Lab—Mitochondria Biology & Neurodegeneration, Instituto de Medicina Molecular João Lobo Antunes, Faculdade de Medicina, Universidade de Lisboa, Lisbon, Portugal

NeuroCure Clinical Research Center, Charité—Universitätsmedizin Berlin, Berlin, Germany
e-mail: filipe.figueiredo@medicina.ulisboa.pt

V. A. Morais (✉)
VMorais Lab—Mitochondria Biology & Neurodegeneration, Instituto de Medicina Molecular João Lobo Antunes, Faculdade de Medicina, Universidade de Lisboa, Lisbon, Portugal
e-mail: vmorais@medicina.ulisboa.pt

H. Pinheiro
EGomes Lab—Cell Architecture, Instituto de Medicina Molecular João Lobo Antunes, Faculdade de Medicina, Universidade de Lisboa, Lisbon, Portugal
e-mail: hpinheiro@medicina.ulisboa.pt

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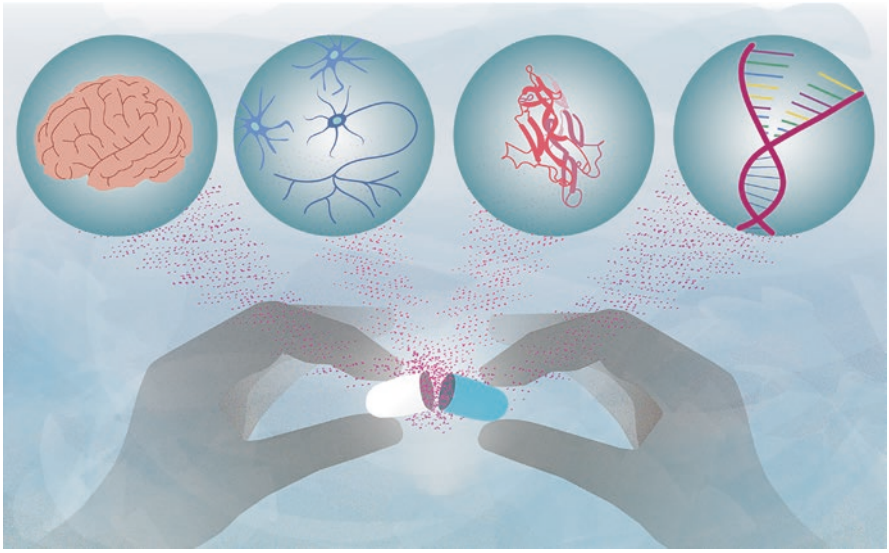

Fig. 12.1 Precision medicine makes use of multiple techniques in a diverse set of biological samples, ranging from organs (macroscopy) to tissues, cells, and molecules (microscopy and nanoscopy)

disease, diagnosing it, monitoring it longitudinally, and anticipating its prognosis. Similarly, biomarkers are pivotal in drug development, as they provide important insights in predicting favorable or unfavorable responses to therapies, assessing pharmacodynamics and safety concerns, and monitoring clinical responses [3, 4].

This chapter aims to peek behind the curtain of laboratorial methodology commonly used in biomedical research and laboratorial clinical settings aimed at identifying known disease biomarkers, as well as uncovering new ones. Precision medicine benefits from a multitude of *in vivo* biological samples (urine, blood, tissue biopsies), where we can go from a macroscopic approach, by analyzing whole organs, to a microscopic/nanoscopic approach, ranging among tissues, cells, and molecules (Fig. 12.1).

12.2 Organs, Tissues, Cells

Apart from autopsies and gross examination of surgical resections, which may assess organs macroscopically, studies in anatomic pathology focus on histologic practices, *i.e.*, the study of tissues and their two interacting components: cells and extracellular matrix. Histologic research is mostly performed *ex vivo*, following a complex process that aims to preserve the cellular architecture observed in the body while avoiding sample deterioration. Afterwards, thin translucent sections of these tissues are cut, allowing microscopic observation [5, 6].

Microscopy is broadly divided into *light microscopy* (*LM*) and *electron microscopy* (*EM*), depending on what interacts with tissue components: light for the former and beams of electrons for the latter. On the one hand, LM encompasses a vast variety of options: *bright-field microscopy*, *fluorescence microscopy*, *phase-contrast microscopy*, *confocal microscopy*, and *polarizing microscopy*. The type of LM will, in very plain terms, mostly depend on the choice of (1) wavelength range and divergence for light source, (2) filter, (3) condenser, and (4) lens system, among others. On the other hand, the beam of electrons in EM has a much shorter wavelength than light, which increases resolution 1000-fold, albeit in shades of black, gray, and white. There are two main types of EM: *transmission electron microscopy* (*TEM*), which provides a sectional view of the sample in shades of gray depending on electro-density (the darker, the denser), and *scanning electron microscopy* (*SEM*), which provides a 3D-like image of the surface of the sample, after a coat of metal ions is applied on the sample [6].

Since tissues and cells are colorless (excluding naturally occurring pigments), one must use a variety of tools to morphologically distinguish different cell types and microscopic components within the samples of interest (Fig. 12.2). This is achieved by [6, 7]:

- *Staining*—Dyes are used more or less selectively, depending on electrostatic linkages with ionizable radicals of macromolecules and acidic or basic compounds. Illustratively, hematoxylin-eosin (H&E), very commonly used, is composed by hematoxylin, a basic dye that binds to basophilic components such as DNA, RNA, and glycosaminoglycans, and eosin, an acidic dye that interacts with acidophilic components such as mitochondria, secretory granules, and collagen.
- *Autoradiography*—Radioactively labeled metabolites are provided to living cells in order to be incorporated into macromolecules of interest. After sample processing and sectioning, radioactivity is detected by silver bromide crystals and microscopic slides (LM or TEM) are developed photographically.
- *Enzyme histochemistry or cytochemistry*—The minimally processed tissue sections are subjected to an enzyme of interest (phosphatases, dehydrogenases, peroxidase) after the sections have already been exposed to their specific substrate. Afterwards, a marker compound that detects the enzymatic reaction is added and

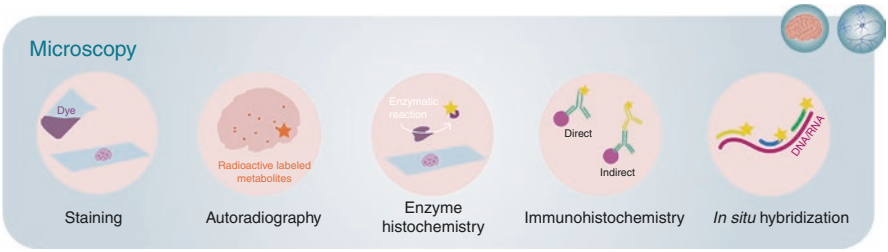

Fig. 12.2 Different techniques allow colorless cells to become visible under a microscope

its precipitation is detected by LM or EM, thus pointing out the sites of reaction in the cell.

- *Immunohistochemistry*—Antibodies are used to specifically target an antigen of interest, mostly proteins with high molecular weight [8–10], and are able to label them either directly through a tagged antibody (*direct immunohistochemistry*) or indirectly through a tagged secondary antibody that targets an untagged primary antibody, which, in turn, targets the antigen of interest (*indirect immunohistochemistry*). These tagged antibodies may be combined with different labels: fluorescent compounds for fluorescence microscopy, enzymes for enzyme histochemistry, and gold for TEM, thereby making use of established approaches to both LM and EM microscopy.
- *In situ hybridization* (*ISH*)—A single-stranded DNA- or RNA-tagged probe is used to bind specific complementary strands of interest (genes, viruses, among others). Probes may be tagged with radioactive nucleotides (autoradiography is used), with a compound, such as digoxigenin which interacts with peroxidase-labeled antibodies against digoxigenin (immunocytochemistry is used), or with a fluorochrome (fluorescence microscopy is used). ISH with a fluorescent probe is called fluorescent ISH (FISH).

The use of antibodies and fluorescence has revolutionized histologic research, but it has also ushered in a new era for cell biology, where microscopy may be complemented by indirect detection of specific cellular markers. Two main examples are *flow cytometry* and *mass cytometry*. Flow cytometry, commonly used in immunology and hematology, allows for the identification of cells or their intracellular components depending on the detection of fluorescent antibodies or dyes after excitation by a beam of lasers. As cells are in a laminar flow and each antibody or dye will have a different emission spectrum detected by different filters, it is possible to collect the many different spectra emitted per cell and, indirectly, assess various cellular components at once [11]. Similarly, mass cytometry uses antibodies but these are tagged with rare heavy metal isotopes. As cells in a single-cell suspension are nebulized and their heavy metal reporter ions are released, the time-of-flight (TOF) of each one, which depends on the mass of each atom, is detected by an atomic mass cytometer, allowing the decoding of the exact composition of metal atoms on each cell. Since emission spectra in flow cytometry have significant overlap, mass cytometry is able to analyze more parameters per cell at once [12, 13].

Currently, new efforts are being made towards expanding these technologies. Taking fluorescence microscopy as an example, which is commonly employed in imaging live cells, new extensions to its basic functioning have been created: *fluorescence nanoscopy*, which has attained resolutions below 50 nm [14]; *multiphoton microscopy*, which has allowed noninvasive *in vivo* cell imaging in humans [15]; and *imaging flow cytometry*, which has provided fluorescent cell imaging to flow cytometry [16].

Focus on Stroke

The *neutrophil-to-lymphocyte ratio* (*NLR*) might be used as a clinical marker of inflammation, by establishing a ratio between the balance of neutrophils, cells in the innate immune system associated with the phagocytosis of bacteria and direct tissue lesion, and lymphocytes, which orchestrate a directed and more adequate response in the adaptive immune system [17]. NLR has been linked to an increased risk to multiple cardiovascular diseases, including stroke [18], and it has also been shown to have a predictive role in assessing the risk of hemorrhagic transformation in ischemic strokes [19]. Far from the traditional methods of using hemocytometers and manual cell counting, currently most white blood cell (WBC) counts are performed automatically with a large variety of techniques, including *flow cytometry* and *cytochemistry* [20–24].

12.3 Proteins

Proteins are the largest contributor to a cell's dry mass and are the most complex macromolecules in the body. On the one hand, they are key pieces in cellular architecture, providing a "cellular skeleton", and, on the other hand, they are the main elements in the complex biochemical interactions that constitute life [25–27].

Proteins are translated from mRNA in a ribosome, forming a polypeptide chain of various amino acids. They then assume their structure, which is key for the protein's functions within the cell, through a sequence of increasingly complex post-translational modifications, such as folding, covalent modifications (*e.g.*, methylation, acetylation, phosphorylation, lipidation, and glycosylation), cleavage, and assembly into multi-subunit proteins [28, 29].

12.3.1 Protein Purification

Prior to any analysis with a focus on a specific protein or set of proteins, it is necessary to find ways of purifying our proteins of interest out of a cell homogenate with thousands of other proteins and macromolecules.

Firstly, different cell components might be separated by centrifugation: *differential centrifugation*, where varying levels of centrifugal force allow the separation of particles based on their sedimentation rates, or *density gradient centrifugation*, where a solution with a density gradient is able to separate another solution after a centrifugal force is applied, based on either the particles' size and mass (*rate zonal*

centrifugation) or their density (*isopycnic centrifugation*) [30, 31]. Another approach is *selective precipitation*, where protein differential solubility is explored by altering the pH/salt balance or by adding precipitating agents such as ethanol, acetone, and polyethylene glycol, among many others [32]. *Immunoprecipitation* uses antibodies to precipitate a specific protein of interest [33].

12.3.2 Protein Separation

Chromatography offers a very versatile approach of separating a mixture of different proteins into its different constituents. Regardless of its various versions, chromatography relies on a *stationary phase* and a *mobile phase*, which do not mix and compete for the components in the mixture, depending on differing properties determined by the method chosen. The mobile phase serves as a medium of moving the proteins across the stationary phase, where they will move at varying speeds as they interact with both phases (Fig. 12.3) [27, 34]:

- *Column chromatography*—A vertical container made out of glass, plastic, or stainless steel (the column) is filled with a stationary phase (inorganic materials, synthetic organic polymers, or polysaccharides). The protein mixture is placed

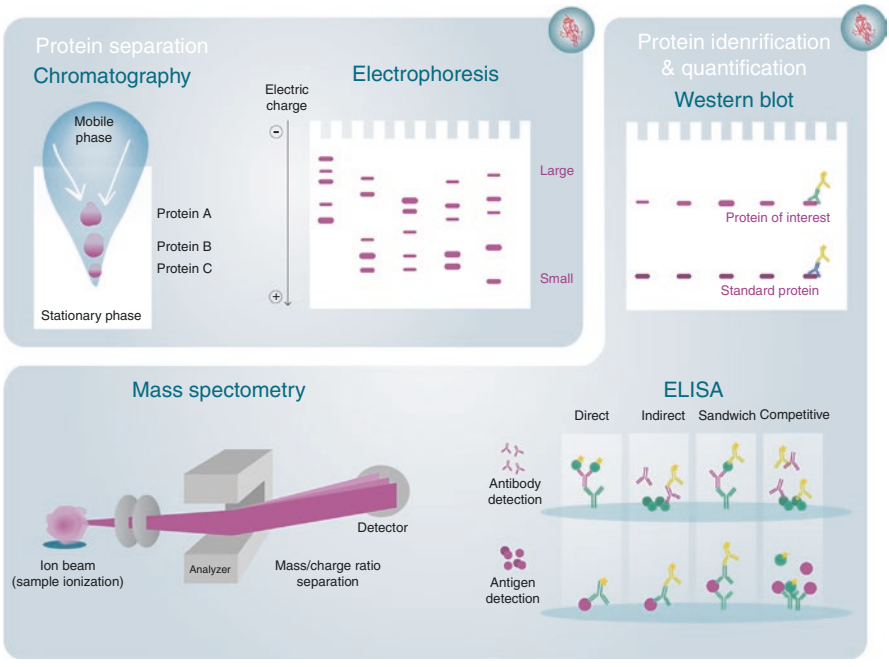

Fig. 12.3 Proteins are crucial macromolecules in cell biology which may be separated, identified, and quantified through a variety of techniques

atop the column and a mobile phase moves the proteins along the stationary phase at different speeds according to their adsorption or dispersion properties.

- *High-performance* (*high-pressure*) *liquid chromatography* (*HPLC*)—This variation of standard column chromatography uses much smaller beads as the stationary phase, usually silicon based, and stainless steel columns able to withstand much higher pressures of mobile phases passing through, which significantly increases the scalability and speed of the separation process.
- *Size-exclusion* (*gel-filtration*) *chromatography*—The column is filled by porous beads (the stationary phase) that will filter the proteins differently according to their molecular size. As the mobile phase moves the proteins along, the proteins small enough to go through the pores will travel faster than the ones excluded from them, which are forced to move around the beads. The most common stationary phases are dextran-based and agarose gels.
- *Ion-exchange chromatography*—The proteins will move along the stationary phase depending on the electrostatic interaction they have with it: stationary phases with positive groups, called *anion exchangers*, will make more negatively charged proteins move slower, retaining them, while stationary phases with negative groups, called *cation exchangers*, will do the exact opposite.
- *Hydrophobic interaction chromatography*—The stationary phase components are coated with hydrophobic groups that will retain proteins with a similar hydrophobic surface, while nonadherent proteins move freely with the mobile phase, usually of high ionic strength.
- *Affinity chromatography*—In contrast to the other nonspecific chromatography methods, this method is based on selective properties between the stationary phase, embedded with ligands for our protein of interest, and the protein we are interested in retaining. A few examples of common interactions are between antigen and antibody, enzyme and substrate, and hormone and receptor, among others.

Chromatography methods can also be a mix of several approaches such as high-performance affinity chromatography (HPAC), combining HPCL and affinity chromatography, which is commonly used in biomedical research [35, 36].

Gel electrophoresis is another method that separates proteins by applying an electric field to a solution of proteins in a semipermeable gel (*e.g.*, polyacrylamide, agarose) at different currents according to their net charge (Fig. 12.3). When the proteins are kept in their native structure, this is called a *native gel electrophoresis*. However, as protein structure might greatly impact their migration, proteins are subjected to sodium dodecyl sulfate (SDS), an anionic detergent; 2-mercaptoethanol, a reducing agent; and heat, which denature the protein and disassemble multimeric proteins, while increasing the protein's negative charge proportionally to its molecular weight. Thus, in *polyacrylamide gel electrophoresis* (*SDS-PAGE*) proteins will migrate according to their molecular weight in the polyacrylamide matrix. Proteins can be visualized with a dye such as Coomassie Blue [27, 34].

Isoelectric focusing (*IEF*) is another method that uses an electrical field to separate proteins according to their isoelectric point (pI), *i.e.*, when the net charge of the protein (sum of negative and positive charges at the amino acid chain regions) is null. To achieve this, a pH gradient is created within a polyacrylamide matrix using

ionic buffers and an electric field that will allow proteins to migrate to a point where their pI is null [27, 34].

If one combines both methods by running an IEF first, followed by a SDS-PAGE, proteins with very similar molecular weights will be first separated by their pI and afterwards by their size. This technique is known as *two-dimensional gel electrophoresis* (*2DGel*) and it is very useful in analyzing complex protein mixtures [27, 34].

12.3.3 Protein Identification and Quantification

Mass spectrometry (*MS*) is a high-throughput technology that is able to detect and, in particular setups, also quantify an enormous variety of macromolecules, including proteins. This highly versatile technique has been responsible for the "-omics" revolution in molecular cell biology, giving rise to the fields of proteomics [37], lipidomics [38], and metabolomics [39]. Despite the many variants of *MS*, its functioning revolves around the following (Fig. 12.3) [27, 34, 37, 40]:

- Following protein purification and separation, proteins are digested into smaller peptides and undergo *molecular ionization* that fragments and charges the molecules. This creates a gaseous phase of intact ions.
- Afterwards, the ionized molecules are separated according to either their *mass-to-charge* (*m/z*) *ratio* or *TOF* in a *mass analyzer*, through magnetic, electric, or electromagnetic fields.
- Finally, the separated ionized molecules reach a *detector* that plots the results in a *mass spectrum*, with the *m/z ratio* plotted against signal intensity. This allows both the identification of various amino acids per the mass spectra and their quantification per the signal intensity.

There are three main MS-based protocols in biomedical research [34, 41]:

1. *Liquid chromatography-mass spectrometry* (*LC-MS*), which uses liquid chromatography to separate proteins prior to MS analysis and *electrospray ionization* for molecular ionization of peptides, through the electron-charged dispersion of a liquid mixed with volatile components.
2. *Matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry* (*MALDI-TOF*), which uses MALDI for molecular ionization, through a laser beam that excites a liquid matrix that absorbs light and serves as a source of protons to the preprocessed peptides.
3. *Tandem mass spectrometry* (*MS/MS*), which uses two or more MS steps performed sequentially (*e.g.*, two mass spectrometers connected to each other, two mass analyzers in the same instrument), through a first step that separates peptides according to their mass and a second step that isolates a peptide of interest and further dissects it into its mass spectra.

Another common method of protein quantification is *enzyme-linked immunosorbent assay* (*ELISA*), which combines antibodies directed towards proteins of interest and

linked to an enzyme. A substrate is then added to produce a change in color, luminescence, or fluorescence which is objectively assessed with a spectrophotometer or fluorometer, thus quantifying our proteins of interest proportionally to signal intensity. Depending on what is controlled in the experiment, it is possible to quantify either the antigens or the antibodies: by providing known antigens we may quantify antibodies and by providing antibodies for a specific antigen we may quantify the antigen. This technique is widely used in laboratorial research and has different variations (Fig. 12.3) [34, 42, 43]:

- *Direct ELISA* - It derives from the *direct* link between an enzyme-linked antibody and a specific antigen.
- *Indirect ELISA* - It derives from the link between a *secondary antibody*, which is enzyme-linked, directed towards *primary antibodies*, which, in turn, are linked to an antigen.
- *Sandwich ELISA* - It derives from the link between three antibodies: a *capture antibody* directed towards an antigen, the *primary antibody* linked to the captured antigen, and the *secondary antibody*, which is enzyme-linked and will react with the substrate. This is the most sensitive form of all ELISA approaches.
- *Competitive ELISA* - It derives from the competition between an enzyme-linked antibody or an enzyme-linked antigen and the antigen-antibody interaction we are interested in quantifying. In contrast to the other methods, the signal coming from the enzymatic reaction will be inversely proportional to the protein we are interested in studying, since higher signal intensity will correspond to the enzyme-linked protein (an antigen or an antibody) and not the antigen-antibody interaction we are interested in quantifying.

Western blot is a method commonly used for the *relative* quantification of proteins, *i.e.*, the levels of our protein of interest will be relative to another known standard protein. A Western blot starts with a gel electrophoresis (details provided in Sect. 12.3.2) followed by a transfer to a porous membrane (made of nitrocellulose or polyvinylidene difluoride) through an electrical current. After *electrotransfer*, the detection of specific proteins is done through antibodies: the membrane is *blocked* first to prevent nonspecific antibody binding; afterwards *primary antibodies* against our proteins of interest are added and incubated, followed by the addition of a *secondary antibody* targeting the host IgG of the primary antibodies previously added, which will be either radiolabeled or enzyme linked (Fig. 12.3). Proteins are then quantified by colorimetric, chemiluminescent, radioactive, or fluorescent detection [34, 44].

Due to its limited scalability, more high-throughput versions of Western blots have been developed [34, 45, 46]:

- *Reverse-phase protein lysate microarrays* (RPA)—it uses a microarray with a nitrocellulose slide to detect proteins of interest by immunochemistry, without, however, separating the peptides by molecular size.
- *Micro-Western arrays* (*MWA*)—which further expands on the technology of RPA and adds the possibility of separating the peptides by molecular size.

12.3.4 Protein Sequence and Structure

Prior to the advent of MS-based technology, protein sequencing was achieved through the *Edman method*, which is able to determine the amino acid composition and order of a polypeptide from its N-terminus. In it, proteins are first denatured to a polypeptide chain and phenyl isothiocyanate, the *Edman reagent*, is added. Upon addition of an anhydrous acid that breaks the peptide bond between the N-terminus amino acid and second amino acid, the first amino acid is released. Afterwards, the released amino acids are extracted sequentially, being further separated by HPLC [27, 34].

Proteins, however, are much more than a sequence of amino acids, the *primary structure* of a protein. Through covalent and noncovalent bonds (*e.g.*, hydrogen bonds, electrostatic attractions, van der Waals attractions, and hydrophobic clustering force) protein *conformation* determines the role it will execute in the cell. In addition to the primary structure of a protein, there is also the *secondary structure* (α-helices and β-sheets), the *tertiary structure* (the three-dimensional organization of a polypeptide chain), and the quaternary structure (a protein composed of various polypeptide chains). A few tools may be used to decipher these levels of protein structure: *circular dichroism* (*CD*), *nuclear magnetic resonance* (*NMR*) *spectroscopy*, *X-ray crystallography*, and EM [26, 34].

Focus on Stroke

The *B-type natriuretic peptide* (*BNP*) is synthesized by cardiomyocytes in the heart ventricles in response to mechanical stretch, leading to a reduction in blood pressure and circulating volume, thus reducing cardiac preload and afterload. During its processing, both BNP and an inactive N-terminal proBNP (NT-proBNP) are released in equal amounts [47]. BNP and NT-proBNP have been linked to an increased risk of ischemic stroke, particularly cardioembolic strokes, as well as, for stroke patients, worse functional outcomes and higher mortality [48–52]. BNP was initially entitled *brain natriuretic peptide* because it was identified in the porcine brain in 1988, through precipitation, chromatography, and sequencing with the Edman method [53]. Currently, however, various *immunoassay techniques* (which are based on the principles of immunochemistry) are used instead [48, 49, 51].

12.4 Nucleic Acids

Human cells hold their genome, the entire set of genetic instructions, in ~3 billion base pairs (bps) of double-stranded *deoxyribonucleic acid* (*DNA*) molecules, densely packed in 46 chromosomes. *Genes* are transcribed into single-stranded *ribonucleic acid* (*RNA*), a much more unstable but extremely versatile macromolecule that controls *gene expression* in a plethora of ways [26, 54]:

- *Messenger RNAs* (*mRNAs*) - Coding RNAs that might be translated into proteins.

- *Ribosomal RNAs* (*rRNAs*) - Basic elements of ribosomes and responsible for protein translation.
- *Transfer RNAs* (*tRNAs*) - Responsible for bridging amino acids and RNA translation into polypeptide chains.
- *Small nuclear RNAs* (*snRNAs*) - Responsible for alternative splicing of mRNAs, producing various protein isoforms.
- *microRNAs* (*miRNAs*) *and small interfering RNAs* (*siRNAs*) - Responsible for inhibiting gene expression at the RNA level.

12.4.1 Nucleic Acid Extraction

Both DNA and RNA extractions involve cell lysis with a detergent, protein digestion with proteases, centrifugation, and precipitation. While DNA extraction is very straightforward with a large variety of different kits due to its stability, extracting RNA involves careful handling of the samples due to ubiquitous enzymes that degrade RNA—*RNase* [7, 55, 56].

12.4.2 Nucleic Acid Visualization and Quantification

Akin to the Western blot for protein quantification (details provided in Sect. 12.3.3), *Southern* and *Northern blots* are used to visualize DNA and RNA, respectively. A gel electrophoresis separates fragments according to size, as nucleic acids are negatively charged. The gel is then transferred to a membrane or paper and a complementary labeled probe is added, thus revealing the location of the fragments of interest through hybridization (details provided in IHS, in Sect. 12.2). For Southern blot, DNA is digested with *restriction enzymes* that cut it into fragments prior to gel electrophoresis and must be denatured prior to membrane transferring to become single stranded. By using restriction enzymes that cut at specific locations, it is possible to *genotype* different samples that differ at *restriction sites*. For Northern blot, this technique may be used for relative quantification of gene expression, since the presence of more transcripts of interest will produce a band with increased signal [57, 58].

An absolute quantification of DNA and, indirectly, of RNA through cDNA, obtained by reverse transcriptase, can be attained with *quantitative polymerase chain reaction* (*qPCR*). This approach is based on the biological method for DNA replication, where a thermostable DNA polymerase, two short oligonucleotide probes called primers designed to flank the gene or region we are interested in amplifying, and free nucleotides exponentially replicate DNA molecules through a cycle of denaturation of the double-stranded molecules, annealing of the primers, and elongation of the polynucleotides. Afterwards, the products of each cycle will serve as templates for the following cycles. By adding fluorescent dyes or probes that interact with the PCR products, it is possible to quantify the *in vitro* DNA production and, thereby, the initial concentration of DNA. This can be done both

relatively, regarding a gene we view as our baseline, and absolutely, by performing a standard curve with serial dilutions and using it as a reference. *Genotyping* is also possible by analyzing the *melt curves* of fluorescent probes, since different genotypes will have different affinities to the same probes [7, 59].

Another approach to relative quantification of gene expression or genotyping is *DNA microarrays*. DNA microarrays are plated with numerous probes for specific genes or single-nucleotide polymorphisms (SNPs) of interest. Then, a labeled DNA/cDNA is added, usually using fluorochromes, and hybridization to the sequences of interest is recorded by fluorescence intensity. Thus, genes with higher fluorescence have more copies in our cDNA and SNPs with recorded fluorescence are present in our DNA samples. Usually, however, a comparison between a control and a sample of interest is performed. In this case, samples are labeled with different fluorochromes and the pattern of fluorescence is used to determine the number of differentially expressed genes and differing SNPs [7, 60].

Genome-wide analysis studies (*GWAS*) mostly use DNA microarrays to interrogate SNP differences in patients and controls and between different populations [61]. Shortly after the initial draft of the human genome was published [62], the search for different haplotypes in the human population, *i.e.*, a collection of SNPs that tend to be inherited together, launched the International HapMap Project [63] aiming to collect, organize, and make freely available the SNPs of multiple human ancestries. After the first study in 2002 [64], GWAS grew exponentially, uncovering 71,673 variant-trait associations from 3567 publications in 2018 [65].

12.4.3 Nucleic Acid Sequencing

Despite the 2004 publication of the Human Genome Project's final version of the human genome [66], the reference genome has been subjected to a variety of reviews, with its latest release in 2017 [67]. Similarly, sequencing methods have suffered a revolution since its origins with *Sanger sequencing* (Fig. 12.4).

Sanger sequencing uses *dideoxyribonucleoside triphosphates* (*ddNTPs*), which, contrary to normal nucleotides, lack the 3′ hydroxyl group and block the elongation of a DNA strand being actively replicated. Following PCR amplification with a specific primer, exact copies of our DNA molecule of interest are divided into four tubes, each one with one of the ddNTPs and the remaining three nucleotides, plus a DNA polymerase. As we amplify the DNA molecules through PCR, we obtain fragments of different sizes that terminate at the ddNTP of each tube. Afterwards, by loading each tube and performing a gel electrophoresis, one is able to reconstruct the original DNA fragment sequence. Since this gel electrophoresis is very labor-intensive, the method was adapted to an automated capillary gel electrophoresis with fluorescently labeled ddNTPs, where each ddNTP has a different color (Fig. 12.4) [26].

Massively parallel sequencing (*MPS*) or *next-generation sequencing* (*NGS*) offers a completely different approach to Sanger sequencing by producing numerous *reads* that can be aligned to a consensus sequence bioinformatically. Despite significant differences between various platforms, the process overall starts by *library preparation* with DNA fragmentation and adapter ligation. Afterwards, each

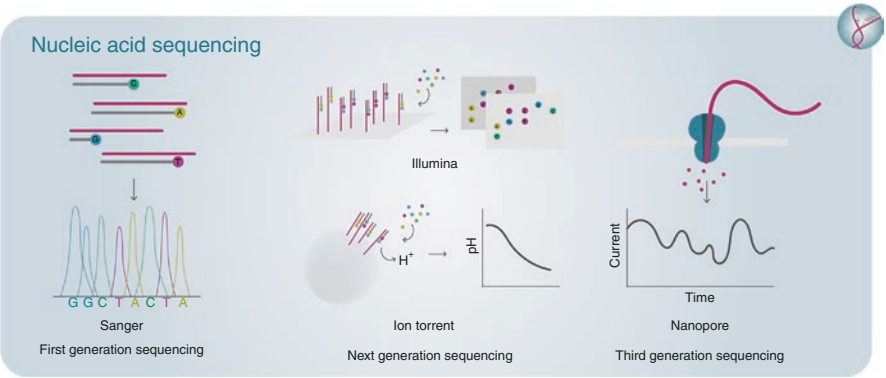

Fig. 12.4 Nucleic acid sequencing has suffered a revolution since its origins in Sanger sequencing with the advent of next-generation and third-generation sequencing approaches

DNA fragment with its unique adapter is amplified in a solid support that allows copies of the same oligonucleotide to be sequenced in *clusters*. Numerous clusters are then sequenced in parallel. The most common approaches are the following (Fig. 12.4) [26, 68, 69]:

- *Illumina sequencing*—The reaction is performed on a microarray, through sequencing-by-synthesis. It uses nucleotides labeled with different fluorochromes that block DNA elongation, similarly to ddNTPs in Sanger sequencing. However, the tags can be removed enzymatically and a further tagged nucleotide may be added afterwards. Thus, DNA sequences are determined by reading the sequence of different fluorescent colors.
- *Ion torrent sequencing*—The reaction is performed on DNA-covered beads spread throughout multiple wells, through semiconductor sequencing. Each bead is covered with the same DNA fragment after PCR enrichment. As the well is filled with a specific nucleotide, a voltage-sensitive semiconductor chip detects changes in pH, since a proton is released when a nucleotide is incorporated. If the pH changes, the nucleotide filling the well is registered as having been incorporated and the sequence is successively determined.

A completely alternative sequencing approach to the aforementioned NGS approaches is the *nanopore sequencing*, sometimes classified as a *third-generation sequencing technology*. Nanopore sequencing is able to sequence a single-DNA molecule through a stationary DNA polymerase, by measuring the time nucleotides with different removable fluorescent dyes reside on the polymerase. Nucleotides who linger the most before losing their dye are the ones incorporated by the polymerase (Fig. 12.4). Despite achieving reads with up to ~10,000 bps, error rate is significantly increased in this method [68, 70, 71].

NGS technologies have revolutionized the field of genomics, allowing the sequencing of whole genomes and exomes (the coding regions only), and launching the fields of transcriptomics (RNA-seq) and epigenomics (methylation sequencing), as well as the 1000 Genomes Project, which aims to collect a diverse set of whole genomes and make them public [7, 69, 70, 72–74]. As NGS prices continue to

decrease [75], these initiatives will surely continue to thrive and further expand into clinical applications [54, 69].

Focus on Stroke

The *rs2107595 SNP* is a regulatory region variant located at 7p21.1 in the histone deacetylase 9 (*HDAC9*) locus which may be found in around 17% of the global population with an increased prevalence in Asian populations [76, 77]. It has very consistently been linked to an increased risk in ischemic stroke due to large vessel disease [78–81]. This most likely stems from the SNP's effect in increasing *HDAC9* gene expression through E2F3/Rb1 complexes, ultimately leading to an augmented pro-inflammatory response that promotes carotid plaque and carotid intima-media thickness (IMT) [82–84]. The rs2107595 SNP was found through a combination of *DNA microarrays*, *qPCR*, and *MALDI-TOF* [78–80].

The *miR-106b-5p microRNA* is expressed from 7q22.1 and has been found to be upregulated in patients with ischemic stroke [85–87]. Data from experimental models suggests that this might be caused by an enhancement in glutamate-induced apoptosis and increased oxidative stress [88]. The miR-106b-5p was found through *DNA microarrays* and *qPCR* [85, 86].

12.5 Conclusion

This chapter summarizes the main methods used in biomedical research, with a primary focus on the ones applicable to medicine. Although methods were presented in a somewhat simplified fashion, in reality, many of the techniques are far more complex. Nonetheless, the promise of *precision medicine* and the discovery of novel biomarkers are dependent on reliable and robust scientific methods in biomedical research. Having a strong and broad knowledge of the available techniques in histology and cell and molecular biology fields is important not only for current scientific progress, but also for future endeavors in science, ultimately leading to scientific breakthroughs.

Regarding stroke per se, at present, the distinction of stroke mimics from transient ischemic attacks (*TIA*), ischemic strokes, and hemorrhagic strokes is made by combining a patient's medical and family history with clinical examination by an experienced neurologist and brain imaging. Similarly, the choice of treatment, etiology assessment, and prognosis prediction are based on the accrued clinical data, symptom severity, and time elapsed since the first symptoms [89–93]. Due to the complexity of this process, novel biomarkers that would be able to objectively and quickly ascertain stroke risk, subtype, etiology, and/or prognosis would be highly valuable in the clinical setting. Currently, however, single biomarkers, although promising, have failed to provide an effective solution to this problem [94–97]. Thus, panels of multiple biomarkers are actively being explored in clinical trials [98–100].

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