Molecular Tools for Modern Epidemiology: From the Concepts to Clinical Applications

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Abbreviations

ALP	Alkaline phosphatase
BiFC	Bimolecular fluorescence complementation
BSA	Bovine serum albumin
BSE	Bovine spongiform encephalopathy
CEIA	Capillary electrophoresis immunoassay
CMA	Chromosomal microarray analysis
aCGH	Comparative genomic hybridization arrays
cDNA	Complementary deoxyribonucleic acid
CNV	Copy number variants
DNA	Deoxyribonucleic acid
dPCR	Digital polymerase chain reaction
ELISA	Enzyme-linked immunosorbent assay
EtBr	Ethidium bromide
FISH	Fluorescence in situ hybridization
FRET	Fluorescence resonance energy transfer
GFP	Green fluorescent protein
HRP	Horseradish peroxidase
HGP	Human Genome Project
HIV	Human immunodeficiency virus
LIF	Laser-induced fluorescence
LAMP	Loop-mediated isothermal amplification
MHC	Major complex of histocompatibility
MSP	Methylation-specific PCR
MWB	Multiplex western blot
NMR	Nuclear magnetic resonance
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffer solution
PS	Phosphatidylserine
pNPP	P-Nitrophenyl phosphate
PCR	Polymerase chain reaction
PVDF	Polyvinylidene difluoride
qPCR	Quantitative polymerase chain reaction
RFLP	Restriction fragment length polymorphisms
RT-PCR	Reverse transcription polymerase chain reaction
RNA	Ribonucleic acid
SNP	Single-nucleotide polymorphism
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
CFSE	Succinimidyl-carboxyfluorescein ester
TUNEL	TdT dUTP nick-end labeling
UPR	Unfold protein response
WB	Western blot

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Introduction

Since the beginning of the century, the advances in laboratory technologies have allowed the acquisition of valuable molecular information regarding human health [1]. In 2003, the Human Genome Project (HGP) concluded with the publication of over 90% of the DNA sequence in the human genome. Before the HGP, very few loci were associated with diseases. However, the sequencing and annotation of the human genome and further analysis in several diseases allowed the establishment of these associations, firstly with diseases following Mendelian heritage rules and, more recently, with complex diseases which manifest as a result of the combination of several factors, including genetics and environmental cues [2].

Nowadays, it is possible to assess a human genome in order to find disruptions that could be causative of a particular disease [1]. These disruptions range from base pair modifications to

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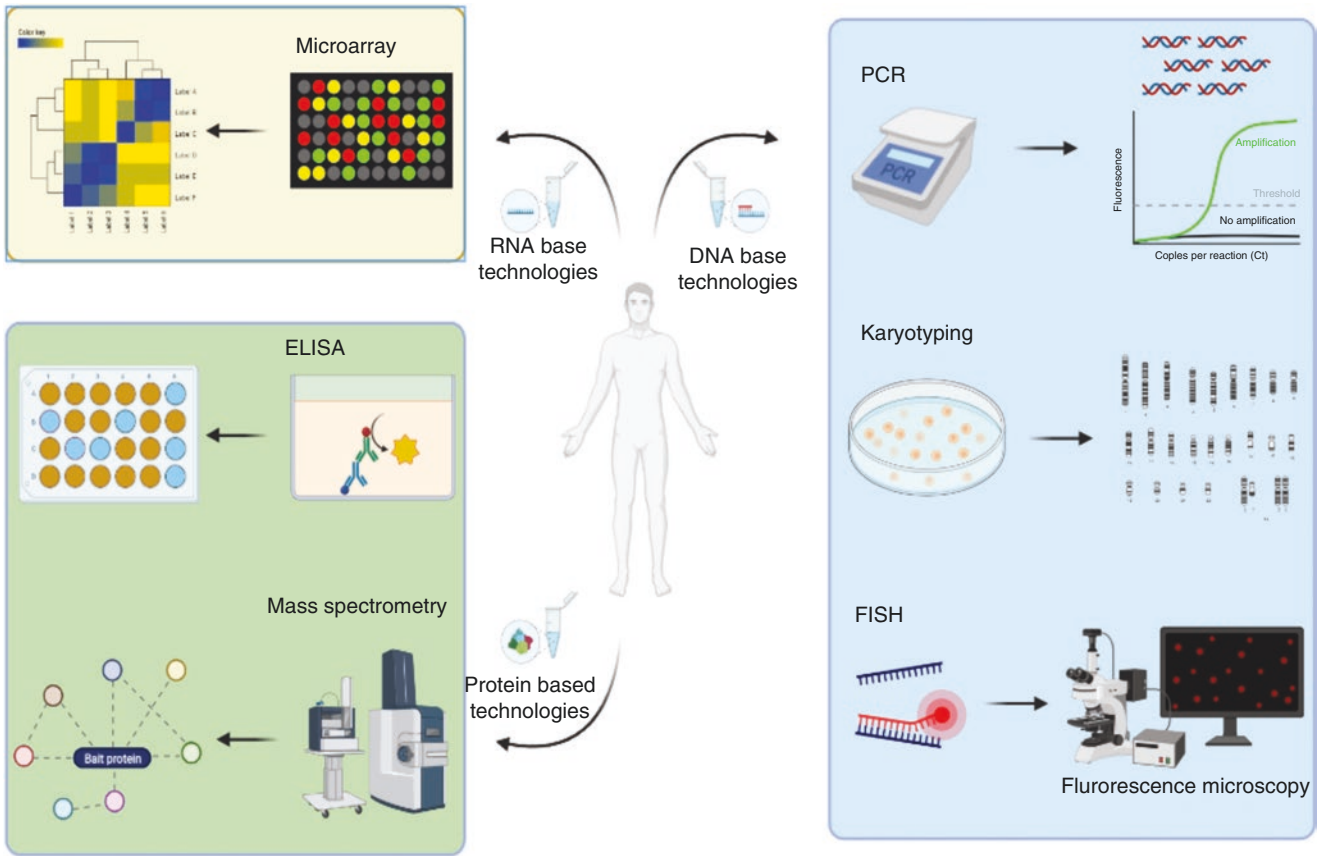

Fig. 2.1 In this chapter we will address the most popular molecular tools that are currently used in clinical practice. For instance, nucleic acids-based technologies such as PCR, microarrays, karyotyping, and FISH; and the protein-based technologies (immunoassays and mass spectrometry)

chromosomal rearrangements and modifications in gene expression [2]. Furthermore, besides chronic and congenital diseases, infectious diseases, such as those caused by bacteria, viruses, or parasites, may be identified rather by changes made in the genome or by the detection of the infectious agent's genome itself. For these reasons, in recent years numerous studies have been done for the discovery of molecular hallmarks in particular diseases for its posterior utilization in clinical contexts.

Molecular tools refer to the whole set of techniques based on properties of nucleic acids, such as DNA or RNA, and proteins for the identification of the causes of a disease. The discovery of molecular hallmarks in a particular disease can permit them to be detected in patients and are useful as a diagnostic tool [1]. Molecular tools have ameliorated the process of disease detection, both by improving efficiency and accuracy of diagnoses, and also its speed, which is of particular interest when the prognosis of the patient depends on the appropriate early diagnosis. Furthermore, molecular tools also allow the collection of useful knowledge for drug development and prevention of diseases, relevant for many of those which have no known cures yet [2].

Although molecular tools have important applications in human disease diagnosis, its utilities go further into the resolution of criminal cases and paternity tests, evolution and population studies of diverse species, microbes identification, and even microbiome studies with the advent of metagenomics. In this chapter, we will review several molecular techniques used in the clinics, with the description of its procedure and medical applications. These encompass PCR, which is commonly used for pathogen identification and deletions or insertions in particular diseases, as well as in forensic medicine and paternal genetic testing; karyotyping, FISH, and microarrays for the detection of chromosomal abnormalities; and finally, immunoassays and proteomics, for the evaluation of aberrant gene expression and protein isoforms or localization in diseases (Fig. 2.1 summarizes the reviewed molecular tools and its applications) [2].

PCR

The polymerase chain reaction, most commonly known as PCR, is a process created in 1983 by Kary Mullis for DNA molecule amplification in vitro [3, 4]. PCR uses polymerases, the enzymes in charge of DNA replication found in all living organisms [4]. Polymerases synthesize DNA by add-

ing the complementary base pair for each position in a whole fragment [3]. To do this, they need a primer, which is a sequence complementary to the DNA fragment but shorter than it: when the correct base pair is incorporated into the growing chain, the 3'-OH molecule exposed in the pentose of the primer carries out a nucleophilic attack to the triphosphate group in the incoming nucleoside triphosphate, forming the phosphodiester bond [3]. Therefore, when performing a PCR, polymerases are required, as well as a pair of primers, deoxyribonucleotide triphosphates (dNTPs), and other substrates, such as buffers and ions, and they are all set in an apparatus called thermal cycler, which raises and lowers the temperature [5]. Since PCR requires heating, a special thermostable polymerase is needed, which is usually the Taq polymerase, the enzyme found in a thermophilic bacteria named *Thermus* aquaticus [5].

The PCR process requires three steps: denaturing, annealing, and amplification. During denaturing, the thermal cycler raises the temperature to 93–95 °C, at which the hydrogen bonds in the double-stranded DNA are broken, resulting in two single-stranded DNA molecules [5]. Next, the temperature lowers to ~60 °C, and the primers designed to flank the extremes of the DNA region to be amplified bind to the single-stranded DNA molecules by base pair complementarity [5]. Finally, in amplification, the temperature again rises to 70–75 °C, and the polymerase incorporates the complementary base pair for every position in the interest sequence, now resulting in two double-stranded DNA molecules [5]. The process is repeated, resulting in 4 double-stranded molecules, 8, 16, and so on, until it is done ~30 times, which yields billions of copies for the interest sequence (~2^{30}) [4].

Finally, in the standard PCR procedure known as endpoint PCR, the amplified fragments are observed in an agarose electrophoresis gel, using ethidium bromide (EtBr) for DNA staining and ultraviolet light. DNA fragments of known length must also be used as markers for the identification of the PCR product with the previous knowledge of its molecular weight. However, there are several PCR variants available.

Types of PCR

Multiplex PCR

Multiplex PCR is a variant that allows the amplification of more than one interest DNA fragment by using more than a pair of primers specific for different fragments [6]. It was first used in 1988 for the detection of deletion variants in the human dystrophin gene, which served as a diagnosis for Duchenne muscular dystrophy. Since several primers are used, it is essential to have extensive knowledge of the sequence in the extremes of the fragment that will bind to them to avoid non-specific amplification [6]. Nowadays, multiplex PCR has several applications, including pathogen identification, genetic diseases diagnoses, and forensic analyses [6].

Real-Time PCR

Real-time PCR or quantitative PCR (qPCR) is a modification that allows the quantification of initial DNA molecules [7]. This type of PCR has the advantage that the agarose gel electrophoresis is not needed; instead, the quantitation of the samples is done, while the reaction is happening [7]. qPCR is done in a thermal cycler able to detect fluorescent signals, and fluorescently labeled probes are included in the reaction [7]. For every cycle, the fluorescence signal emitted is directly correlated with the amount of DNA [4]. Therefore, the more initial concentration of DNA, the faster the fluorescence signal will reach a threshold known as CT, allowing the identification of the cycle in which this is achieved and consequently, the initial DNA volume [4].

Reverse Transcription PCR

Reverse transcription PCR (RT-PCR) allows the amplification of a cDNA molecule originating from an RNA sample. The procedure is essentially the same as in a traditional PCR, with the modification that RNA fragments must be retrotranscribed to cDNA. Usually, the RNA fragments to be amplified come from cellular mRNAs; thus, the primers used for retrotranscription are commonly oligo(dT) molecules that bind to the poly-A tail in the mRNA 3′ end [8]. RT-PCR and qPCR can be combined in a methodology known as qRT-PCR, which allows the quantification of specific RNA molecules [2]. This is particularly useful for the quantification of the viral load for several viral infections caused by RNA genome viruses [2].

Digital PCR

Digital PCR (dPCR) is a PCR variant introduced in 1999 which allows the identification of allelic mutants for a gene or specific locus [9]. dPCR relies on the dilution of DNA to be amplified in wells on a plate, so that there are very few molecules in each well [9]. qPCR is done for every well, and the fluorescent probes used are designed to bind to the different alleles expected, each with a different color [9]. This way, mutant alleles can be identified even in a cell population in which the wild-type allele is predominant — a phenomenon common in certain diseases, such as cancer —thanks to the dilution of the initial sample [9]. The analysis of the fluorescent signal will allow the identification of the proportion of the mutant allele in the cell population, by adding the proportion of mutant and wild-type alleles in every well [9].

Lamp

Loop-mediated isothermal amplification or LAMP is a methodology alternative to PCR, first described in 2000

[10]. In contrast with PCR, LAMP does not require alternating temperatures; instead, all the procedure is carried out at the same temperature, and hence, it does not require a thermal cycler [10]. LAMP is performed at ~60 °C, and it uses several primers, from two to four pairs for each end of the region to be amplified, which highly raises the specificity and lowers the false-positive discovery [10]. Furthermore, LAMP uses a polymerase with strand-displacement activity [10]. The LAMP procedure initiates with a primer targeting the middle region of the 3′ end and amplifying the whole fragment from that starting point [10]. Subsequently, the polymerase begins amplification starting on a primer targeting the outermost region of the 3′ end, and displacing the previously amplified sequence [10]. Since the innermost and the outermost regions of each end are complementary, the newly synthesized strand forms a double stem-loop structure, and this allows new primers targeting the middle region of the 3′ end to anneal to it and amplify again starting from that point [10]. This form of amplification enables multiple copies of an interest fragment to be generated rapidly, as well as with a really high specificity [11]. As well as PCR, LAMP can be done qualitatively and with reverse transcription, which is useful for the detection of viral infections [11] (Table 2.1).

Table 2.1 Applications of different classes of PCR

Type	Application	Principle	Reference
Classical PCR	Forensic medicine	Across the genome, there exist several polymorphisms (over 20,000) that correspond to short repeated sequences in tandem (STRs). The specific amplification of these sequences and its posterior length determination by gel electrophoresis create a "DNA fingerprint" almost unique for every person in the world	[4]
RT-PCR or qRT-PCR	Pathogen detection	Primers are designed for amplification of a pathogen's mRNA, and through electrophoresis, it is possible to determine the presence of the pathogen in the sample. qRT-PCR can also be used to determine the load of the pathogen in the sample	[12]
Allele specific PCR	Point mutations detection	When wanting to distinguish between a point mutation and a wild-type allele in a specific position of the genome, PCR primers are generated so that this position is the last nucleotide of the primer. Since the polymerase needs the 3'-OH, this nucleotide needs to be bound to the fragment to be amplified. If the allele is mutated, there would not be amplification, so it is possible to identify point mutations possibly responsible for a disease	[2]
Classical PCR	ChIP	Chromatin immunoprecipitation (ChIP) is a methodology to identify a region of the genome bound to a specific protein. To do this, the proteins are crossed-linked to the DNA, and the DNA is cut in small fragments. The mixture is divided into two: In one of the mixtures, all the proteins are removed, and PCR is performed for the interest region and for a control region where the protein is known not to bind to. The other mixture is immunoprecipitated with an antibody specific for the interest protein, and only the fragments containing it will be selected. Then, the proteins are removed, and again, PCR is performed for the interest region and for the control region. In the first mixture, amplification is expected for both the control region and the interest region. However, if the control was appropriately selected, in the second mixture there should not be amplification of this region. If the protein is bound to the interest region, amplification will be observed. This methodology works for identification of DNA-protein interactions specifically identified in certain contexts	[3]
PCR-RFLP	SNV detection	Restriction fragment length polymorphisms (RFLPs) are length variants of fragments cut by restriction enzymes (REs) present in the genomes of different people. Some REs recognize a single base pair and cut in it, creating two shorter fragments. If this base pair is mutated, the RE won't cut it, leaving the longer fragment. PCR can be used for amplification of some regions, and the products processed by REs and then run in an agarose gel to identify the different lengths of the fragments. In this way, it is possible to identify the base pair present in a region of the genome in both alleles without sequencing and is a commonly used method for identification of SNPs in mtDNA and in the Y chromosome for ancestry studies	[2]
Common PCR	Insertion and deletion detection	Genetic deletions or insertions can be identified without sequencing by PCR. The methodology consists in using primers flanking the interest region or gene, amplifying it and identifying longer or shorter fragments with a gel electrophoresis. Some diseases, such as cystic fibrosis, are characterized by allele insertions or deletions; thus, this methodology may work as a diagnose tool	[13]
Multiplex PCR	Deletion variants detection	As mentioned previously, the dystrophin gene can present deletions in several exons, leading to the development of Duchenne's muscular dystrophy. 98% of these deletions can be identified by multiplex PCR, working as a diagnose tool	[13]
Methylation-specific PCR (MSP)	Detection of methylated regions of the genome	PCR can be used to detect methylated regions in the genome by first treating the samples with bisulfite, which changes unmethylated cytosine to uracil, and methylated cytosines are left unmodified. Then, primers with guanine are designed to pair with methylated DNA, and primers with adenine are designed to pair with unmethylated DNA. A quantitative PCR can be done for detection of the amount of methylated and unmethylated DNA	[14]
qRT-PCR	Gene expression analysis	A quantitative retrotranscription PCR can be performed to analyze cellular mRNAs with altered expression in certain contexts compared to controls. This is particularly interesting in some diseases that show aberrant gene expression in specific genes, such as cancer	[7]

Principles of Karyotyping

The human genome is arranged in 23 pairs of chromosomes, 22 of them are somatic and 1 of them is the sexual pair [5]. The somatic chromosomes are identical between males and females and are numbered from 1 to 22, the longest and the shortest pairs, respectively [5]. Conversely, the sex chromosomes differ between sexes, females possess two X chromosomes, and males possess an X chromosome and a Y chromosome [5]. The visualization of the arrangement of the chromosomes during mitosis is called a karyotype [12]. A normal karyotype consists of 46 chromosomes and a pair of X chromosomes (46XX) or an X and a Y chromosome (46XY) for females and males, respectively [12]. Karyotypes enable the identification of several chromosomal abnormalities, such as aneuploidies, and also deletions, insertions, duplications, and chromosome rearrangements [2, 15].

Initial karyotyping methodologies, introduced in the 1970s, were based on DNA staining for the identification of abnormalities in the chromosomes [15]. These DNA staining methodologies produced light and dark bands patterns in the chromosomes [2]. One of the most common techniques was the G-banding, in which the chromosomes were treated with trypsin and subsequently stained with a chemical dye named Giemsa [2]. Darker regions observed with G-banding correspond to condensed chromatin, which has low gene density and transcriptional activity. Consequently, G bands have lower CG content [2]. The staining can be observed and photographed under a light microscope, and then the karyotype is constructed by arranging the homologous chromosomes [5].

FISH

Fluorescence in situ hybridization (FISH) is a methodology first described in 1969, which is based on the labeling of a DNA or RNA probe with a fluorescent dye and its hybridization with a sample DNA or RNA [16]. Using a fluorescence microscope, the DNA or RNA hybrids can be observed, and the identification of the position of the probe in the sample is allowed [12]. FISH permitted the location determination of several genes by using entire chromosomes as samples during the Human Genome Project, and it is still useful for organisms whose genome annotation or sequence is not available. For the case of humans, FISH is more commonly used in clinical contexts [17, 18].

FISH can be used for the detection of chromosomal abnormalities, such as deletions, insertions, and rearrangements [18]. Furthermore, in comparison with banding techniques used for karyotyping, FISH has a higher sensitivity and may detect abnormalities more easily than these [18]. For example, FISH can be used to diagnose acute febrile neutrophilic dermatosis, in which the genes BCR and ABL are fused by using two different color dyes in the probes for each gene (e.g., red and green) [16]. The expected FISH results should be two separate spots of each color, but the fusion can be detected if there is a yellow signal [16]. Additionally, a specific type of FISH named Multiplex FISH can be used for the labeling of every human chromosome in metaphase [18]. The probes with different colors are designed for DNA regions in a single chromosome, and after hybridization, only one color should be visible for each chromosome [18]. Translocation events can be detected if chromosomes show color stripes [18].

Microarray

Microarrays can also be used for the detection of chromosomal abnormalities, in a variant known as chromosomal microarray analysis (CMA) [19]. CMAs can detect copy number variants (CNVs) of interest genes or loci, with a much higher resolution than banding methodologies [19]. Furthermore, it can use more probes simultaneously than FISH [20]. There are two existing classes of microarrays useful for chromosomal anomalies detection: comparative genomic hybridization arrays (aCGH) and SNP arrays [20]. aCGH requires DNA isolation from reference and test samples, differential labeling, and their hybridization at interest regions [21]. This approach is useful for the identification of CNVs [21]. If the test sample is labeled in red and the reference sample in green, yellow arrays should be expected for all the interest regions [21]. When the array scanning reveals regions with more or less abundance of test DNA (wells in red and green, respectively), it is possible to spot copy number gains or copy number losses [21]. Conversely, SNP arrays only use test DNA hybridization, and the array results are compared to reference DNA [22]. The probes placed in the array are only around 20 base pairs long, enabling the characterization of small regions of DNA across the whole genome [22].

Immunoassays

Disease diagnosis is crucial for correct patient treatment. The development of proteomic technologies has increased the identification of protein biomarkers involved in the immunogenicity of diseases in body fluids, such as blood, urine, saliva, cerebrospinal fluid, and different tissues (biopsies), to predict the course of the disease, information on cellular signaling pathways, monitoring treatment response, adverse effects and the identification of new diagnostic, therapeutic methods, and new targets[23].

Immunoassay methodologies are the most commonly used tools in protein research, using the properties of antibodies to bind different protein domains and to mark them. ELISA and Western blotting are the oldest methods that changed, adapted, and modernized over time, improving their sensitivity and leading to the appearance of new methods and equipment for biomarker investigation and analysis, with the goal of studying more analytes in a single sample, in a shorter time, and with increased accuracy. The reproducibility and reliability of the results are also a goal pursued by manufacturers [24].

Enzyme-Linked Immunosorbent Assay (ELISA)

The gold standard of immunoassays, ELISA, is a very sensitive diagnostic method used to detect and quantify a large variety of protein biomarkers like antibodies, antigens, proteins, peptides, glycoproteins, and hormones. This technique was developed simultaneously in 1971 by Engvall and Perlmann and Van Weemen and Schuurs, and nowadays it continues to be used as a routine analytic tool. The detection of these products is based on the antigen-antibody interactions, and detection is usually done with the help of an enzyme and a substrate. An antibody is a type of protein produced by an individual's immune system and has a specific region that binds to a protein from a foreign source called "antigen." This binding allows identifying a specific disease biomarker with small amounts of sample [25, 26]

In the ELISAs methodology, the primary and specific antibody only binds to the protein of interest, and the secondary detection antibody is a second enzyme-conjugated antibody that binds the primary antibody and, through the addition of a substrate, generates an observable color that indicates the presence of antigen. The most common substrates available for ELISA are horseradish peroxidase (HRP), whose substrate is hydrogen peroxide and results in a blue color change, and the alkaline phosphatase (ALP) that uses P-nitrophenyl-phosphate (pNPP) producing a yellow color of nitrophenol after room temperature incubation. The new ELISA methodologies have developed fluorogenic, quantitative PCR, nonenzymatic and electrochemiluminescent reporters for signal generation [24].

Currently, four major types of ELISA have played a prominent role in the quantitative and qualitative identification of analytes:

Direct ELISA (Antigen-Coated Plate, Screening Antibody)

The simplest type of ELISA, the primary detection antibody, binds directly to the protein of interest. This method begins with the coating of antigen to the ELISA plates. The first binding step involves adding antigen to the plates and incubate overnight at 4 °C; the next step is to wash the plates of any potential unbound antibody and block any unbound sites on the ELISA plate using agents like BSA, ovalbumin, aprotinin, or other animal proteins to prevent the binding of any nonspecific antibodies and avoid a false-positive result. After adding the buffer, the plate is rewashed to remove any unbound antibody and followed by the addition of a substrate/chromophore (AP or HFP), which results in a color change by the hydrolysis of phosphate groups from the substrate AP or by the oxidation of substrates HRP. The advantages of direct ELISA include eliminating secondary antibody cross-reactivity and quantifying a specific molecule with high sensitivity from a wide variety of samples; it is faster than indirect ELISA, but the signal is less amplified compared to the other types of ELISA, and it has a high cost of reaction [27].

Indirect ELISA (Antigen-Coated Plate; Screening Antigen/Antibody)

Indirect ELISA detection is a two-step ELISA which involves a primary antibody and a labeled secondary antibody. The steps of the indirect ELISA are identical to the direct ELISA, except for an additional wash step and the types of antibody added after the buffer is removed. It requires two antibodies: a primary detection antibody that sticks to the protein of interest and a secondary enzyme-linked antibody complementary to the primary antibody. The primary antibody is added first, followed by a washing step, and then the enzyme-conjugated secondary antibody is added and incubated. After this, the steps are a washing step, the addition of substrate, and detection of a color change. This method has a higher sensitivity when compared to the direct ELISA. It is also less expensive and more flexible due to the many possible primary antibodies that can be used. The only major disadvantage is the risk of cross-reactivity between the secondary detection antibodies and the occurrence of nonspecific signals [28].

Sandwich ELISA (Antibody-Coated Plate; Screening Antigen)

This method appeared to avoid false-positive or false-negative results. Unlike direct and indirect ELISA, the sandwich ELISA begins with a capture antibody coated onto the wells of the plate. The term "sandwich" refers to the way the antigens are "sandwiched" between two layers (capture and detection antibodies). After adding the capture antibody to the plates, the plates are then covered and incubated over-

night at 4 °C. Once the coating step is complete, the plates are washed with PBS, then buffered/blocked with BSA, and finally, the plate is washed with PBS before the addition of the antigen. The plate is rewashed, and the primary detection antibody is added, followed by a buffer wash. The secondary enzyme-conjugated antibody is added and incubated, and the plate is rewashed. Finally, the substrate is added to produce a color change.

The sandwich ELISA has the highest sensitivity and specificity among all the ELISA types. It is suitable for complex samples and has more flexibility to quantify antigens between the two layers of antibodies. Its major disadvantages are the time, the use of expensive "matched pair" (divalent/multivalent antigen), and secondary antibodies [24].

Competitive ELISA (Screening Antibody)

This method is based on a competitive binding process between the original antigen in the sample and the add-in antigen;, the more antigen in the sample, the less labeled antigen is retained in the well and the weaker the signal. It utilizes two specific antibodies, an enzyme-conjugated antibody and another antibody present in the test sample (if it is positive). Combining the two antibodies into the wells will allow for a competition for binding to antigen. The presence of a color change means that the test is negative because the enzyme-conjugated antibody binds the antigens, rather than the antibodies of the test sample. The absence of color indicates a positive test and the presence of antibodies in the sample. The method has a low specificity and cannot be used in dilute samples. However, the benefits are that sample purification is less needed, it can measure a large range of antigens in a given sample, and it can be used for small antigens and has low variability [24].

New Methods

In order to improve the ELISA method, in terms of using smaller quantities of samples, shortening the reaction time, avoiding sophisticated reading equipment, and reducing costs side, new methods have been developed:

The *enzyme-linked immunospot assay (ELISpot assay)* is widely used to evaluate a cellular immune response against viral antigens in allergies, autoimmunity, and vaccine development. The method has a relatively wide quantitative range and offers unique sensitivity by revealing cytokine secretion at the single-cell level. This technique, performed on PVDF membranes, has advantages like specificity, sensitivity, and a wide range of detection [24, 29].

The conventional *single-target assays ELISA-Western blot* are suitable for biomarker validation but could be expensive, time-consuming, and sample limiting. While most of the disease conditions may arise when only one single molecule is altered, more often it is the consequence of the interaction between several molecules within the inflammation milieu; therefore, studying the diseases necessitates a comprehensive perspective [24].

The most recent is the ELISA platform with *ELISA on a chip* (ELISA-LOC), which allows the use of only 5 μl of sample on a miniaturized 96-well plate combined with a CCD camera. The system includes three main functional elements: (1) a reagent loading fluidics module, (2) an assay and detection wells plate, and (3) a reagent removal fluidics module. The ELISA-LOC system combines several biosensing elements: (1) carbon nanotube (CNT) technology to enhance primary antibody immobilization, (2) sensitive ECL (electrochemiluminescence) detection, and (3) a charge-coupled device (CCD) detector for measuring the light signal generated by ECL. This method has greater sensitivities than the corresponding standard manual plate-based ELISAs, and that single samples can be assayed in a minor fraction of the time [30].

Clinical Significance

ELISA testing is an important part of medical care and scientific research. ELISAs can be used in many settings, including rapid antibody screening tests for human immunodeficiency virus (HIV), detection of other viruses, bacteria, fungi, autoimmune diseases, cancer biomarkers, food allergens, blood typing, the presence of the pregnancy hormone hCG, laboratory and clinical research, forensic toxicology, and many other diagnostic settings. Some types of ELISAs and their uses are included in the Fig. 2.2 [24, 27].

Western Blot

The immunoblot or Western blot (WB) is one of the analytical and quantitative techniques mostly used in research laboratories throughout the world for identifying specific proteins in many biological samples, liquid or tissue/cellular homogenates [24]. The WB technique was invented by Harry Towbin and co-workers in 1979. The name "Western blot" was given 2 years later by Neal Burnette, inspired in the earlier name of other blotting methods [28].

In this procedure, crude lysates are first separated based on their molecular weight by SDS-PAGE, transferred to a solid membrane surface (usually nitrocellulose or PVDF) and detected with the help of protein-specific antibodies. The membrane is probed by a specific primary antibody, it binds the specific epitope of the protein, and it is labeled by the addition of a secondary antibody recognizing the primary

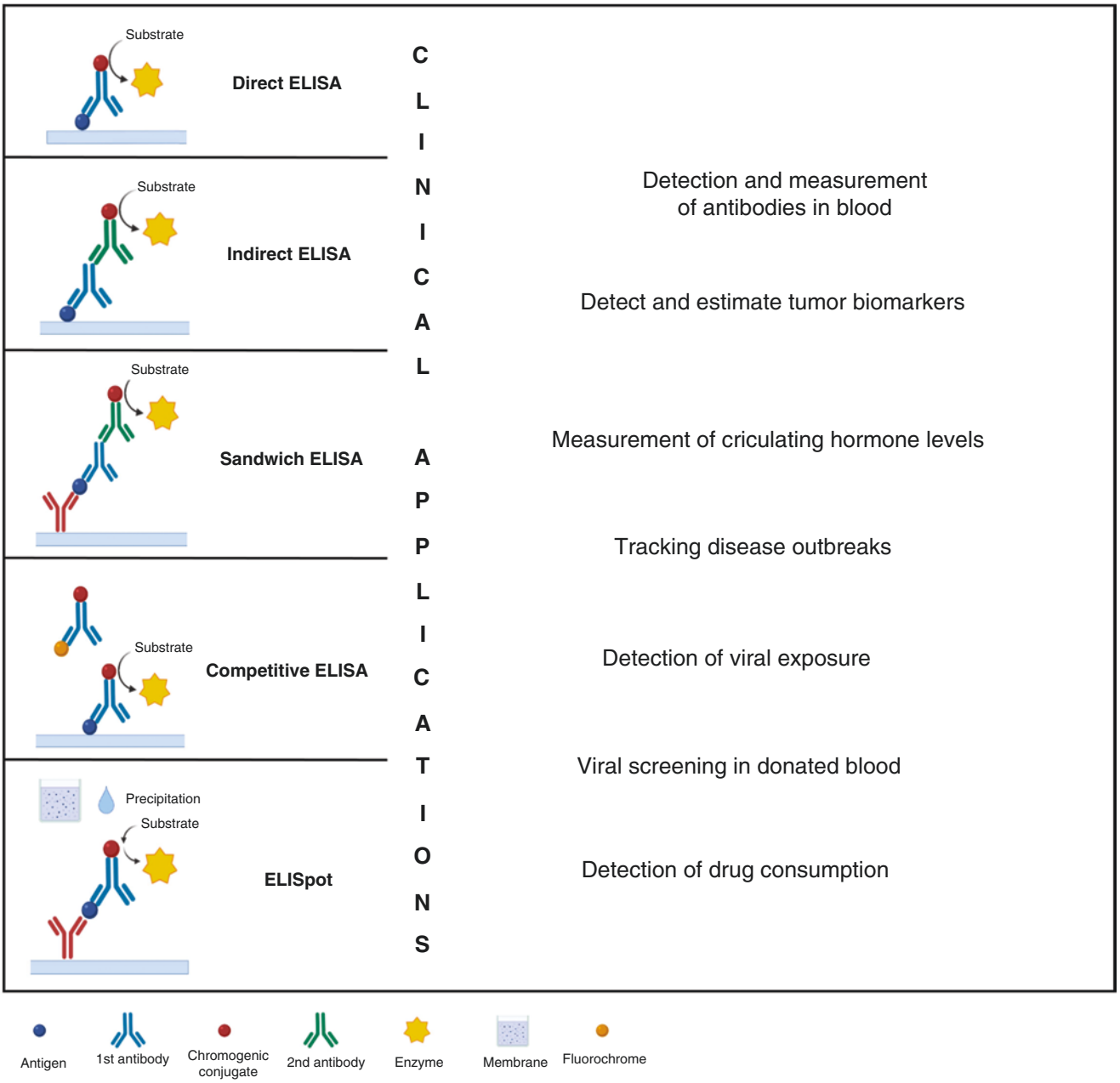

Fig. 2.2 Brief description of ELISA immunoassays and their current clinical applications. In general the working principle of the immunoassays relies on the protein/antigen-antibody reaction, the rest are variations of this principle as depicted in this figure

antibody conjugated with a detection reagent (fluorophore, enzyme, and radioisotope). The visualization is done colorimetric, by chemiluminescence, on X-ray film, or directly in the membrane with the aid of an imaging system [24, 31].

This technique brings concrete and useful information about the amount of protein loaded to independently quantify housekeeping proteins (typically actin, GAPDH, or tubulin). If the target protein present in the sample is altered qualitatively or quantitatively, the band thickness is changed compared to a control being downregulated or overexpressed. The WB results can guide us for a comparison of a target protein expression important in a medical diagnosis or experiment or a genetic investigation in case of partial deletion or duplication in the protein gene [32].

Since WB is a multistep protocol, variations and errors can occur that reduce the reliability and reproducibility of this technique. Also, obtaining maximal sensitivity for the detection of a specific protein remains a fundamental issue, leading to advances in antibody specificity, chemiluminescent formulations, properties of fluorescent molecules and imaging techniques that provide gains in sensitivity, dynamic range, and ease of use. Here we discuss different aspects of

methods based on the Western blotting technique and its contemporary application in epidemiology.

Multiplex Western Blot (MWB)

In the last few years, it has become a necessity to analyze multiple target proteins at the same time, in order to compare the expression of proteins involved in a specific pathology. The MWB method revolutionized medical diagnosis and opened new perspectives in biomedical research. The analysis of several proteins involved in different pathologies reduces the cost and time for analysis.

This method was standardized by Anderson and Davison to study different muscle proteins involved in muscular dystrophies. It allows simultaneous screening of multiple proteins in a biphasic polyacrylamide gel system, which enables the corresponding blot to be probed simultaneously with a cocktail of monoclonal antibodies. The gel is optimized so that large proteins of more than 200 Kd can be analyzed in the top part, while smaller proteins under 150 Kd are separated in the lower phase. This basic system allowed establishing a biomarker profile for each patient, providing valuable information for diagnosis as well as for phenotype-genotype correlations [33].

Capillary Electrophoresis (CE) and Capillary Western Blotting (CWB)

This technique was introduced by Nielsen in 1991, with the concept of capillary electrophoresis immunoassay (CEIA), which uses the capillary electrophoresis (CE) technique to visualize the immunocomplex products that form between an antigen and its corresponding antibody. In this method, a mixture of the antigen and antibody is injected into the end of a capillary to quickly separate according to the size of the immune complex from the free antigen (or free antibody), offering a better resolution. This method decreases the time for analysis and requires a smaller volume for samples compared with classical western blotting. Since it is coupling with laser-induced fluorescence (LIF), it enables the highly sensitive detection of fluorescent molecules in a volume as small as nanoliters of the sample, and it can be used to quantify membrane proteins in extracellular vesicles [34].

Microfluidic Western Blotting

This technology reduces even more the amount of the sample required for WB and also the length of the capillaries from centimeters to microns using microfluidic channels. He and Herr developed this automated immunoblotting method, in which proteins are separated by microchip electrophoresis and can be captured on membranes. This process reduces the separation and reduces time to a few minute glass microfluidic chip to in situ immunoblotting, allowing a rapid protein separation, directed electrophoretic transfer, and high-efficiency identification of proteins of interest using antibody-functionalized membranes [24]. Since this system requires only 0.01–0.5 μg of protein, it has been applied to the detection of specific proteins like GAPDH and β-tubulin from A431 cell lysates [24, 35].

Single-Cell Western Blotting

As the most recent proposal technology, single-cell Western blotting is a combination of microfluidics and conventional Western blotting to achieve protein expression analysis at a single-cell resolution. Due to separation by electrophoresis before the antibody probing, it overcomes the issue of cross-reactions. In single-cell Western blotting, a layer of polyacrylamide gel is coated on a glass and patterned with large-array microwells. Single cells are dropped on the thousands of microwells and lysed in situ, and then proteins are separated by gel electrophoresis, immobilized via photoinitiated blotting, and detected by fluorescent labeled antibodies. Although this technique represents a new technology for single-cell protein expression analysis, it has some limitations, since due to cell loss, thousands of cells are required and have limited detection sensitivity because proteins are easily lost during processing procedures such as cell lysing, protein immobilization, and repeated antibody stripping[36].

Dot Blot

In this method, the samples are applied in small dots directly on the membrane and then spotted through circular templates. After membrane drying, the antibodies are applied. The visualization of a target protein is made as in WB, chemiluminescent, or colorimetric [24]. It is used to test the specificity and antibody concentration used for WB or to evaluate the presence of a target protein in the sample before WB. This methodology has been used for detecting *Sarcocystis* spp.'s antibodies in cattle [37] and analyzing conformational changes in herpes simplex virus entry glycoproteins [38].

Far-Western Blotting

It is used to detect a protein-protein interaction in vitro. Instead of the primary antibody for detecting the protein of

interest, this method uses a nanoantibody protein that binds to the protein of interest. Far-Western blotting detects proteins on the basis of the presence or the absence of binding sites for the protein probe. This method is important in characterization of protein interactions in biological processes such as signal transductions, receptor-ligand interactions, or screen libraries for interacting proteins [39].

Clinical Significance

Western blotting is frequently used for the confirmatory medical diagnosis of infectious diseases such as Lyme disease, HIV infection, bovine spongiform encephalopathy (BSE), hepatitis C infection, syphilis, inflammatory muscle conditions such as myositis, and certain autoimmune disorders (e.g., paraneoplastic disease). For Lyme disease and HIV infection, these are the only two microbial diseases for which an initial borderline or positive ELISA must be followed by a confirmatory Western blot [24, 31].

Flow Cytometry

Flow cytometry is a multiparametric method which analyzes quantitatively characteristics of individual cells within a heterogeneous population, such as size and granularity simultaneously as the cell flows in suspension. The working principle of this tool relies on the information produced on the light scattering of the cells, which is derived from dyes or antibodies coupled to fluorochromes targeting molecules located on the surface or inside the cells [40], as depicted in Fig. 2.3.

Clinical Applications

Flow cytometry may be a cell-specific identification and quantitative technique with a wide spectrum of applications. Particularly, the main clinical applications of this technique are the disease diagnosis (HIV-infected patients) and monitoring disease progression (cancer, leukemia, and lymphoma). As well, flow cytometry is useful analyzing cell proliferation, phagocytosis, and apoptosis. In the following subsections we dissect the most outstanding examples of the flow cytometry clinical applications.

Phenotypic Characterization of Blood Cells

Immunophenotyping or phenotypic characterization of cells consists of both the identification and quantification of a particular cell group within the mixed population, i.e., blood immune cells (T cells, B cells, NK cells, mast cells, basophils, eosinophils, neutrophils, monocytes, among others). This characterization is possible due to the expression of surface proteins specific for each cell type that can be detected by antibodies [44], for instance, human PBMC. Subsequently, PBMC were labeled with the chosen combination of cell surface antibodies as well as anti-CD3, anti-CD4, and anti-CD14. This cell surface staining section and the labeled cells were analyzed by flow cytometry resulting in the separation of each of the populations [45–47]. Beside the characterization of cell population, the current cytometers can split cell populations (*cell sorting*) for further analyses [48, 49].

Intracellular Antigen Expression

Transcription factors and other intracellular molecules can be stained with fluorochrome-conjugated antibodies after fixation and permeabilization of the cells. Flow cytometry, in contrast to classical microscopy techniques, can provide accurate quantification and high-throughput analysis. Expression levels of a protein in >100,000 individual cells can be measured and visualized within a few minutes. However, this internal staining tends to have higher background, whereas optimal fixation and permeabilization methods vary (such as 0.01% formaldehyde, 1–4% PFA or acetone followed by 0.1–1% NP-40 or ice-cold methanol, etc.) [50].

Characterization of Antigen-Specific Responses

Antigen-specific responses are measured by antigen cell stimulation, and with the following characterization of cellular processes such as proliferation, activation, plasticity, or antigen recognition through major histocompatibility complex (MHC) multimers. In vaccination studies, where the identification of multiple cytokines and surface marker are needed to study in parallel, the most used technique is the *intracellular cytokine staining* or cytokine flow cytometry, since this is a combined technique useful for recognizing the antigen-specific T-cell stimulation in complex cellular samples using more than five fluorescent markers [51].

Another method to measure the antigen-specific responses is using the labeled MHC multimers. Usually, MHC multimers are in a monomeric conformation (MHC-I or MHC-II); these are grouped in multimeric arrangements using a biotinylated fluorescent streptavidin backbone. Then the MHC multimers are *loaded* with the antigen leading to the antigen recognition by the T cells, which indicates the amount of response to a particular application; this method is commonly employed in immunogen studies or in cancer diagnosis and prognosis [52].

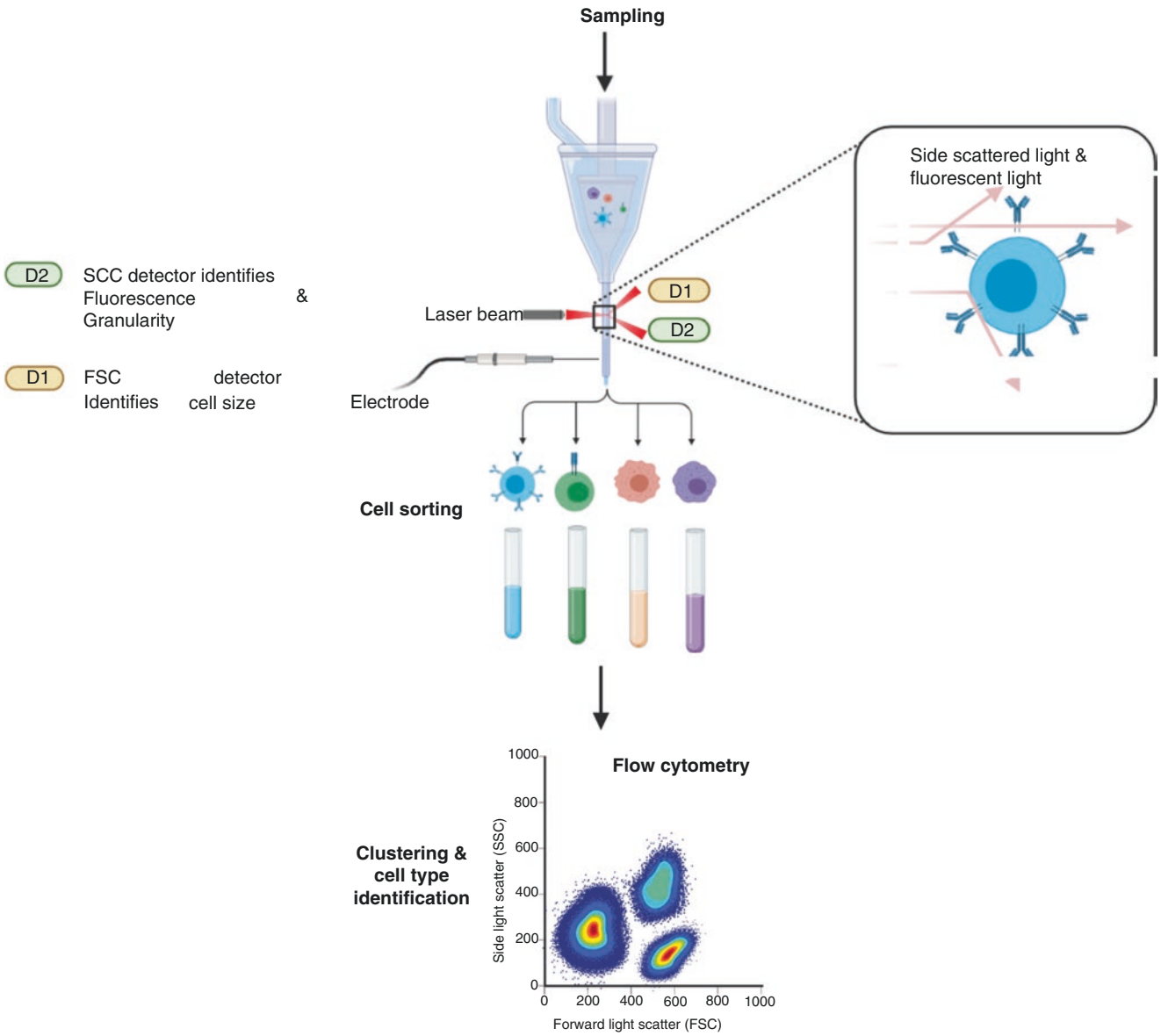

Fig. 2.3 Flow cytometry working principle. Visible light scatter is measured in two different directions, the forward direction (Forward Scatter or FSC) which can indicate the relative size of the cell and at 90° (Side Scatter or SSC) which indicates the internal complexity or granularity of the cell [41]. Light scatter is independent of fluorescence. Samples are prepared for fluorescence measurement through transfection and expression of fluorescent proteins (e.g., Green Fluorescent Protein, GFP), staining with fluorescent dyes (e.g., Propidium Iodide, DNA) or staining with fluorescently conjugated antibodies (e.g., CD3 FITC) [40]. The first flow cytometer was developed to detect the size of the cells; nowadays, these devices are powerful tools capable of detecting up to 14 parameters simultaneously related such as size, shape, complexity, and, of course, any component or cellular function that can be marked with a fluorochrome [42, 43], giving detailed information of cell population in a short period

Cell Cycle Analysis

In cell proliferation, cells may be stained with fluorescent dyes such as succinimidyl carboxyfluorescein ester (CFSE). This dye binds covalently to both intracellular and cell surface proteins and is incorporated equally to the next cell generation (daughter cells) during cell proliferation, with each division CFSE fluorescence decreases twofold leading to identifying up to seven to eight cell divisions accurately [53–55]. Another useful marker to characterize cell proliferation is the thymidine analogs BrdU (5-bromo-21-deoxyuridine) or EdU (ethynyl deoxyuridine), which is similar to the 3H thymidine proliferation assay. The BrdU is a thymidine analog that is incorporated to the newly synthesized DNA and in the subsequent daughter cells; the detection is mediated by the anti-BrdU antibody [53, 56]. Moreover, the use of BrdU is a compatible method is that can be be used simultaneously with other fluorescent markers, and also, both propidium halide (PI) and Hoechst 33342 may be

used to quantify DNA content in each phase of the cell cycle, since the cells that are in S phase are going to be brighter than cells in G1 phase, and cells in G2 phase will be just about two-fold brighter than the cells in G1[57].

Apoptosis

During the early stage of apoptosis, phosphatidylserine (PS) residues that unremarkably exist only within the plasma membrane relocate to the outer surface making such molecules available for PS binding proteins such as annexin V [42]. Since apoptosis is a cascade of events that occurs at different stages, its detection in flow cytometry utilizes multiple targets that bring a complete overview of events related to this process [57]. For instance, additionally to the annexin V labeling, the endonuclease digestion of polymer is identified by TUNEL (TdT dUTP nick end labeling) assay; the caspase activation is targeted by specific fluorescent-coupled antibodies; mitochondrial uncoupling is targeted with dyes that depend on the mitochondrial membrane potential (JC-1, Rhodamine 123 or Mitotrackers), and chromatin granule condensation within the nucleus detected with Hoescht 33,342 [58, 59].

As seen above flow cytometry is a powerful tool that may be employed to characterize a wide range of cellular and biochemical processes simultaneously. Hence, in the following section we enlist the most outstanding clinical applications in which flow cytometry has demonstrated to be a success:

- Leukemia and lymphomas diagnosis, since leukocyte surface antigens may be identified in neoplastic cells [60–62]
- Detection of minimal residual disease (MDR) in leukemia via CD13, CD19, and CD34 identification in blood and bone marrow [63, 64]
- Hematopoietic progenitor cells count in bone marrow transplantation by CD34 identification [65]
- Histocompatibility cross-matching via IgG measurement after incubating donor's lymphocytes with the recipient's serum [66, 67]
- Posttransplantation monitoring via CD3+ T cell counting [68]
- Immunodeficiencies diagnosis via CD4- and CD8-positive cells counting within the blood and other liquid biopsies [69]
- HIV infection diagnosis via CD4-positive lymphocytes count performed in blood samples [69, 70]
- Detection of fetal red blood cells and maternal F cells detection and quantitation the feto-maternal hemorrhage [71]
- Contaminating leukocytes measurement in blood for transfusion [72]
- DNA content to detect malignancies [73–76]
- Auto−/allo-immune diseases diagnosis via IgG and immune serum globulin detection using antiplatelet antibodies and IgG for antineutrophil antibodies [77–79]

As mentioned above, flow cytometry impacts positively in daily clinical practice, since this tool is widely used in both hematology and immunology; this leads to suggest that flow cytometry is a powerful tool for diagnosing, classifying, and determining the prognosis of assorted diseases. However, to improve the methods and expand the applications of flow cytometry, it is vital to strengthen the collaboration between physicians and biomedical researchers.

Proteomics

The proteome refers to the set of proteins present in a cell or organism at any given time. The DNA contains the needed information for the creation of proteins [80]. However, the relationship between the genome and the proteome is complex, since one single gene may encode for more than a single protein by means of alternative splicing [80]. Furthermore, posttranslational modifications and protein cleavage or modifications give rise to the origin of several protein isoforms for each single gene [80]. One astonishing example is the *DSCAM1* gene in *Drosophila melanogaster* (fruit fly), which has over 30,000 identified isoforms [12]. The human protocadherins, encoded in the *Protocadherin* locus, are essential in neural development, and its locus is thought to have a similar number of isoforms as the *DSCAM1* gene [12]. Thus, studying the proteome provides additional information that otherwise we wouldn't notice only studying the genome or the transcriptome [80].

As a general rule, the genome is the same in every cell of an individual [2]. The proteome, however, varies between cell types and conditions, allowing the classification of cells according to their protein expression patterns and the identification of changes in certain contexts, such as diseases [2]. As mentioned before, the phenotype can be better explained by the proteome than by the genome or the transcriptome due to differences in expression levels and to protein modifications [81]. Furthermore, the proteome is more stable and more easily assessed than the metabolome [81]. Thus, proteomics studies are a powerful tool preferentially used in studying disease, development, aging, among others [81].

Studying the Proteome

Usually, proteomics studies involve mass spectrometry analysis, which requires previous protein purification from tissue or cell samples [81, 82]. Two-dimensional gel electrophoresis, liquid chromatography, and capillary electrophoresis are

the most commonly used techniques for this required step [81]. Mass spectrometry subjects proteins to a magnetic and an electric field and calculates a ratio known as *m/z*, which refers to the mass-charge ratio [83]. Finally, the masses of the molecules are plotted as peaks in the mass spectrum. The output of the mass spectrometry is queried against protein databases for the identification and quantification of each specific peptide [83]. Mass spectrometry has the enormous advantage that it is also able to detect modifications in the proteins because these change the behavior of the molecules when exposed to magnetic and electric fields [12].

Protein characterization by mass spectrometry can be coupled with protein-protein interactions and protein structure analysis [84]. Protein-protein interaction analysis reveals proteins that associate with others, and it provides information about its functions, since proteins that interact usually are implicated in similar or interrelated pathways. In accordance to dynamic and context-dependent protein expression, protein-protein interactions adjust to environmental conditions [3]. Therefore, understanding interaction networks — the interactome — in contexts of interest, including diseases, offers a higher level of pathway understanding and effective therapies discovery [85]. Some of the methodologies used for protein-protein interaction assays are yeast two-hybrid (Y2H), bimolecular fluorescence complementation (BiFC),, and fluorescence resonance energy transfer (FRET) [85].

On the other hand, understanding protein structure provides valuable information about protein functions [4]. Proteins acquire their functionality by folding in three-dimensional structures, allowing the formation of channels, binding sites, active sites, among others [86]. However, most peptides are able to fold into millions of different structures, and when misfolding occurs, multiple diseases may arise, by, for example, the formation of toxic aggregates [86]. Cells have a stress response named the unfolded protein response (UPR), which prevents the production of misfolded proteins. However, this response may be altered in diseases, aging, or some viral infections [86]. For this reason, the analysis of misfolded proteins in diseases such as Alzheimer's disease has revealed important molecular hallmarks [86]. Tridimensional structure identification can be done by X-ray diffraction, which is the preferred methodology, combined with others such as nuclear magnetic resonance (NMR) spectroscopy [84]. For a complete review of proteomic, please refer to Chap. 6 in this book.

Concluding Remarks

Routine clinical molecular tools demand rigorous, simple, and most importantly reproducibility procedures that help to characterize accurately biomarkers not only for disease diagnosis but also monitoring the patient's response to clinical interventions. Such characterization may be efficient and performed with the minimal invasion for the patient. However, reaching such a level of success will be only possible if physicians and biomedical researchers collaborate. Hence, with this chapter we aim to encourage such collaboration, since here we bring a brief description of both the most molecular tools employed in clinical screening and the working principle of each one, leading to identify the potential use of such molecular tools in unexplored medicine fields.

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Conflict of Interest Authors declare nonconflict of interest.

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