Chapter

Multiplex Technology for Biomarker Immunoassays

Haseeb Ahsan and Rizwan Ahmad

Abstract

The simultaneous measurement of different substances from a single sample is an emerging area for achieving efficient and high-throughput detection in several applications. Although immunoanalytical techniques are established and advantageous over alternative screening analytical platforms, one of the challenges for immunoassays is multiplexing. While ELISA is still commonly used to characterise a single analyte, laboratories and organisations are moving towards multiplex immunoassays. The validation of novel biomarkers and their amalgamation into multiplex immunoassays confers the prospects of simultaneous measurement of multiple analytes in a single sample, thereby minimising cost, time and sample. Therefore, the technological advancement in clinical sciences is helpful in the identification of analytes or biomarkers in test samples. However, the analytical bioanalysers are expensive and capable of detecting only a small amount or type of analytes. The simultaneous measurement of different substances from a single sample called multiplexing has become increasingly important for the quantification of pathological or toxicological samples. Although multiplex assays have many advantages over conventional assays, there are also problems that may cause apprehension among clinicians and researchers. Hence, many challenges still remain for these multiplexing systems which are at early stages of development.

Keywords: biomarkers, multiplex assays, immunoassays, autoantibodies, ELISA, analytes

1. Introduction

An early and accurate diagnosis of a specific disease plays an important role in its effective treatment, especially in an emergency where an immediate decision needs to be made (such as in stroke or sepsis) for the treatment of patient, and the rapid and precise identification of the pathological condition is vital. However, in many instances, the clinical evidence based on a single analyte or biomarker is not adequate for an appropriate diagnosis of a disease or monitoring of its treatment. The biomarkers have a pathophysiological significance and clinical application which may have a profound impact on the diagnosis and treatment of the patient. While contemporary singleplex techniques such as enzyme-linked immunosorbent assay (ELISA) and biomarker kits are able to accurately analyse a single analyte, the monitoring of more complex, multifactorial diseases such as cancer and autoimmune and neurodegenerative diseases requires the analysis of multiple biomarkers in order to implement optimised therapeutic regimen [1]. In addition, it is advantageous to screen various analytes simultaneously for a rapid, cost-effective and

reliable quantification [2, 3]. The development of technologies for the analysis of whole genome (genomic) and total proteins (proteomic) has ushered in a new era of biomarker discovery, which has yielded numerous new biomarkers. In the future, they will have a significant impact on clinical diagnostics and therapeutics [2]. Since the advent of the proteomic era, multiplex immunoassays now constitute valuable tools for efficiently harnessing information available to expedite observation, monitoring and treatment of diseases. While the availability and implementation of commercial multiplex immunoassays for research applications is expanding rapidly, incorporation of the technology within routine clinical diagnostics is in infancy due to operational and quality control issues such as robust automation, time constraint, operational cost, etc. [1]. The multiplex assays are now replacing conventional ELISAs to save time, material and labour costs and allow efficient handling of a large number of samples to enhance the overall throughput. With increasing running costs, a major focus of immunoassays has shifted towards cost-effectiveness and convenience of handling a large number of samples rather than results and reliability of the assay.

Most of the diagnostic methods rely on immunoassays or enzymatic reactions and strongly depend on the sample (e.g. size of sample, patient-to-patient variation) and assay conditions (e.g. temperature, humidity, etc.) [3]. An ideal device for emergency testing should offer high performance, sensitivity, multiplexing capability, short turnaround time, low system complexity, low-cost fabrication and minimised user intervention [3]. Therefore, the multiplexed diagnostic devices capable of high-throughput analysis of several parameters have recently become important in the last couple of decades which are able to analyse different markers simultaneously, e.g. RNAs, metabolites, proteins, cells, etc. [3]. Multiplex immunoassays confer several advantages over widely used singleplex assays including increased efficiency, greater output per sample volume and higher throughput prediction leading to detailed diagnosis facilitating personalised medicine. Nonetheless, relatively few protein multiplex immunoassays have been validated for in vitro diagnostics in clinical settings [1]. The emerging need and demand for novel biomarkers (e.g. aptamers) or targets (e.g. circulating RNAs and DNA, tumour cells, miRNAs, etc.) and their applications for diagnostic, prognostic and therapeutic implications, including therapeutic drug monitoring, will shape the future of multiplex systems [3]. The validation of novel biomarkers and their incorporation into multiplex immunoassays confers the prospect of simultaneous measurement of multiple analytes in a single patient sample, thereby minimising assay costs, time and sample volume. Moreover, the advent of multiplex technology complements the progressive shift towards personalised medicine with holistic, molecular analyses of diseases through the identification and characterisation of biomarkers to accommodate greater diagnostic resolution between closely related disease phenotypes. The multiplex immunoassays will continue to garner popularity and secure a mainstream role in research and eventually clinical settings [1].

The singleplex or conventional ELISA immunoassays have assumed a ‘workhorse’ role in the highly sensitive qualitative and quantitative detection of analytes within heterogeneous samples for over half a century. Moreover, the advent of hybridoma technology as a means of generating monoclonal antibodies (MAbs) has facilitated the generation of highly robust, antibody-based assays for standardisation and automation [4]. Both the singleplex and multiplex ELISAs adopt a common ‘sandwich’ format (capture antibody-analyte-detection antibody). The multiplex ELISA adopts chemiluminescent/fluorescent reporter systems as enzymatic reporters are chemically incompatible for simultaneous analysis of multiple localised targets. The concept of immunoassay for diagnostics was conceived in 1963 by Joseph G. Feinberg and A.W. Wheeler when they developed a ‘microspot’ technique

as a means of detecting autoimmune antibody and tissue antigens, whereby thyroglobulin immobilised in a microspot on cellulose acetate strips were incubated with serum from autoimmune thyroiditis patients [5]. The microspot assay has the ability to detect low levels of both autoantibody and antigen; has the advantage of being simple, sensitive, objective and quick; and requires minute quantities of serum and antigen. In 1989, Ekins postulated the miniaturisation of immunoassays (i.e. reduction of the capture antibody concentration) and outlined the fundamental microarray multiplex technology principles and envisioned their potential application in research and clinical diagnostics [6]. The large-scale screening in the postgenomic era has encompassed applications, ranging from functional analysis of unknown genes to identification of disease-related gene products, screening in drug discovery and clinical diagnostics [1].

2. Principle of multiplex assays

The multiplex assays require complex technology such as PCR, ELISA, microarrays, gel electrophoresis, capillary electrophoresis, Sanger sequencing, etc. The fluorescence spectroscopy measurements are important due to its compatibility with biochemical assays, small size of sample, ease of conjugation to potential molecules, affordability, stability, robustness and detection with less expensive optical instruments [7]. Mass spectrometry (MS) can identify molecules without separation. For example, matrix-assisted laser desorption/ionisation mass spectrometry (MALDI-MS) is employed in hospitals to characterise antigens [1]. However, these bioanalysers are bulky, expensive and capable of detecting only a small amount or type of analyte [1]. Thus, there are still many challenges of these systems due to their cost or their detection capability [1, 2].

Multiplexing as a new technology emerged a few decades ago for the detection and quantification of first nucleic acids and then proteins. Using this technology, a range of biomarker molecules can be identified and quantified for health and disease. Multiplexing is a significant technology for the analysis of thousands of analytes with high reproducibility, miniaturised protocols and increased output. In spite of the advantages of ELISA and PCR, the multiplex immunoassay allows for a large number of analyses in a short amount of time through simultaneous measurement of expression of genes in a single sample by reducing time, labour and cost. The omics profiling using whole genome, epigenome, transcriptome, proteome and metabolome may also offer detailed information and aid personalised medicine [8]. Precision medicine is based on advanced omic technologies, such as next generation sequencing, protein and gene microarray, laser capture microdissection in the correlation of genomics, epigenomics, proteomics and metabolomics with the clinical phenotypes of the individual patient. The development of multiplex genotyping technologies and high-throughput genomic profiling allows the analysis of the patient genome from peripheral blood or biopsy samples [9].

Assaying for soluble antigens and antibodies as biomarkers of various diseases has always been an invaluable diagnostic and research tool. Currently, ELISA has potentially replaced agglutination, complement fixation, precipitation and immunodiffusion in diagnosis and research. The possibility of automation of the test procedure is one of the main reasons in transition from the classical serological tests to ELISA. Despite its advantages, ELISA is capable of measuring one analyte or a few analytes at a time, which may be a limiting factor where multiple markers need to be evaluated. Although the original idea of multiplexed assays involves antigen-antibody interaction, a vast knowledge comes from planar DNA microarrays. The

chemical synthesis of oligonucleotides, DNA sequencing and PCR are major breakthrough technologies which greatly accelerated the accumulation of genomic and transcriptomic data. These key technologies also enabled the construction of DNA microarrays with the aid of bioinformatics, optics and microfluidics [10]. Systems biology research demands a complete snapshot of measurable parameters possible at a time in order to analyse and describe biological systems. The DNA microarray fulfils the requirement in the detection of cellular changes such as genetic polymorphisms, mutations, methylation patterns and transcript abundancies. However, these features usually give an indirect assessment of the cellular physiology. A more concrete and detailed picture of cellular machinery may be depicted by means of high-throughput analysis of proteins and metabolites. The DNA microarray involves surface immobilisation of DNA probes with sequences deduced from either nucleic acid or protein database. It has always been an intricate task to fabricate high-throughput multiplexed protein arrays compared to DNA arrays since proteins are physicochemically more diverse, complicated and often fragile in nature. Today, 60-plex cytokine measurement panels are available, in contrast to thousands of DNA probes in one planar microarray [10–14]. The real benefits of multiplexing assays may be achieved by miniaturisation of immunoassays. The real potential of microspot mediated analyte detection was discovered by Roger Ekins [6], in which the number of capture molecules can be attached onto a large macrospot surface far exceeding a tiny microspot surface. Consequently, macrospots consume analyte molecules in the sample to yield higher total signal intensity; even after all analyte molecules have been consumed, the available binding sites still remain on the assay surface. In contrast, decreasing spot size enhances the occupancy of capture molecules with analytes. Therefore, capture molecule concentration is directly related to assay surface area and total signal intensity and inversely related to signal density [15].

Multiplexed in situ tagging (MIST) is a reliable strategy which makes use of convertible DNA antibody barcoded arrays. It assays hundreds of molecular targets in a single cell, with high throughput and sensitivity. MIST technique was created to overcome the limitations of prevailing microfluidic-based methods [16]. One of the common limitations of conventional microfluidic-based single-cell protein assays was low multiplexity, which is often linked to fluorescence spectrum overlap, due to the phenotypically similar cell populations exhibiting a large degree of intrinsic heterogeneity [17]. Recently, technological advancements in single-cell proteomics have allowed highly multiplexed measurement of multiple parameters simultaneously by using barcoded microfluidic ELISAs and mass cytometry techniques [18]. It was achieved by integrating a multicolor, multicycle molecular profiling technique with barcoded microbead antibody arrays and a DNA-encoded antibody library [15, 16].

3. Planar and suspension multiplex immunoassays

The multiplex assays use immunoassay principles in which high-affinity capture ligands are immobilised in parallel assays. The systems use either antibodies or proteins/peptides as binding molecules to capture circulating proteins or autoantibodies. The multiplex immunoassays are divided into two classes: planar assays and suspension microsphere assays. In planar assay, the capture ligands are immobilised on a two-dimensional support and the fluorescent or chemiluminescent signals identified. In suspension assay, the capture ligands are immobilised on colour- or size-coded microspheres, and flow cytometry is used to detect assay-specific fluorescent signals (**Figure 1**) [19]. Planar arrays can be produced in two formats,

either slide based or microtitre based. The slide-based format supports various layouts where repeated or individual assays composed of specific sets of antibodies are printed robotically upon the activated slide surface. The microtitre-based immunoassays harbour antibodies within the wells of a standard protein-binding plate as in conventional ELISA. The suspension immunoassay employs thousands of micrometre-sized plastic microbeads infused with a chemiluminescent/fluorescent dye and a functionally activated surface, prior to linking with a specific capture antibody. The detection antibodies are individually labelled with a single chemiluminescent/fluorescent reporter added upon completion of incubation and washing stages. Each bead accommodates a 'sandwich' consisting of the captured target analyte and the cognate reporter-conjugated detection antibody. The bead analyte reporter constructs are subjected to analysis in a flow chamber bead separation in which the lasers excite the chemiluminescent/fluorescent reporters and emitted light is collected by a series of detectors for quantitative analysis (**Figure 2**) [1].

In the planar microarray format, the capture molecules are spotted on the modified glass surface to form a grid, and micrometric-sized beads may also be used as assay surfaces. A mixture of beads with a library of capture molecules is called 'bead array' or 'liquid array'. A suspension of the bead library is allowed to interact with the sample and reporter molecules [20]. Flow cytometry is used to measure the assay signals in the beads. Multiplex bead array assays (MBAA) offer multiple analyses at a time, but there may be discrepancies in measurements of certain cytokines by using ELISA or MBAA. In many experimental setups, it was not possible to test the same pair of capture and reporter antibodies in both tests which may originate from multiplexing itself. Possible cross-reactivity between the target analytes and other interfering substances present in the sample may cause matrix effect. Therefore, precaution is required in multiplexing assays that work perfectly in monoplex systems [15].

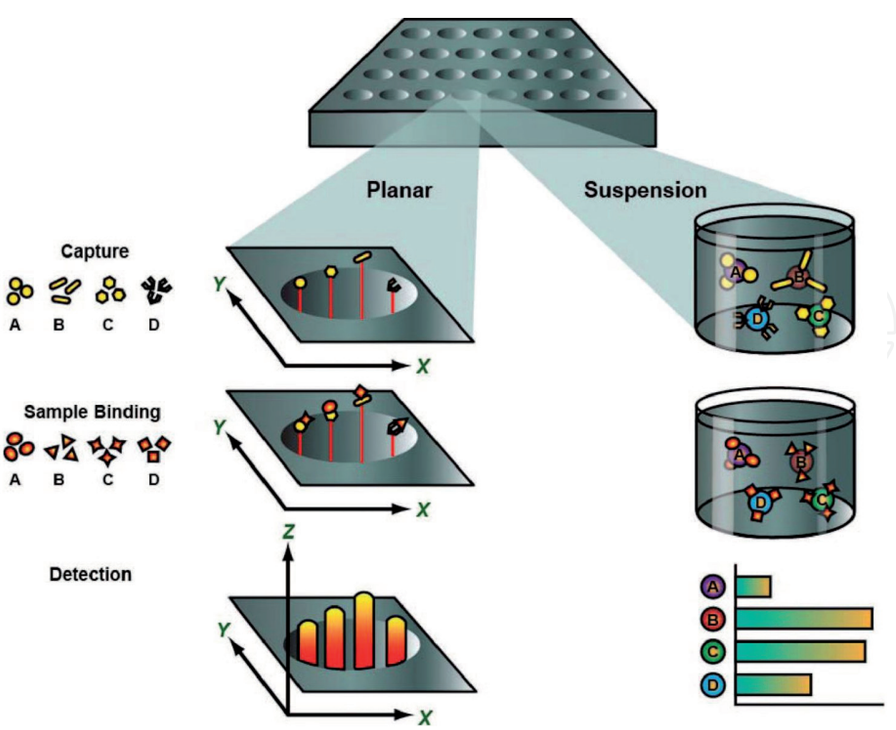

Figure 1.
Planar and suspension multiplexed immunoassay formats. In planar assays, capture ligands are immobilised on a rigid two-dimensional support and probed with sample. In suspension assays, capture ligands are immobilised on colour- or size-coded microspheres. Assays are distinguished by coding attributes, and flow cytometry is used to detect assay-specific fluorescent signal (adapted and reproduced from [19], with permission).

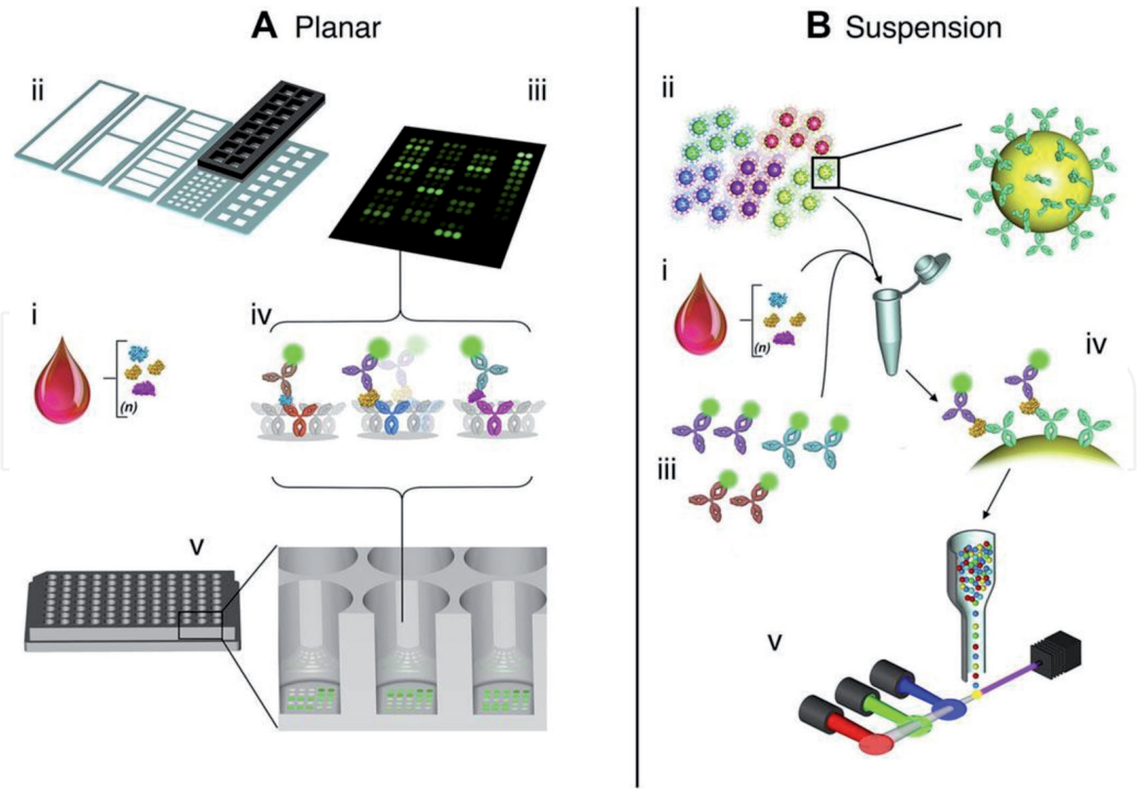

Figure 2.
Multiplex assays include planar-based assays and suspension-based assays. (A) Planar arrays (B) Suspension assay. Both assays us the serum sample extracted from blood as the starting point. (adapted and reproduced from [1], with permission from WILEY-VCH Verlag GmbH & Co).

4. Multiplex immunoassays for autoimmune diseases (AD)

A substantial percentage of the population carries detectable levels of circulating autoantibodies without developing clinical symptoms. Autoantibodies are also present in the sera of patients with systemic autoimmune diseases, such as systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), etc. many years before clinical disease onset. The detection of antinuclear antibodies (ANA) has long been an important tool in the early diagnosis of autoimmune connective tissue diseases [21–25]. Antinuclear antibodies are detected in a substantial population, yet few individuals are diagnosed with the autoimmune disease, although some ANA-positive healthy individuals eventually develop clinical autoimmunity [26]. The correct use and interpretation of serologic testing for diagnosing autoimmune diseases present a challenge to clinicians for several reasons: (a) the sensitivity and specificity of laboratory tests for autoimmune disease and (b) detection of autoantibodies using different techniques such as indirect immunofluorescence (IF) or MBAA give different results. Multiplex microarray-based ELISA assays provide consistent results when compared with ELISA-based tests with the added advantage of reduced labour and the complete autoantibody profile in a single test. Autoantibody biomarkers assist in diagnosis and monitoring of disease activity, predicting disease onset, classifying disease subsets and defining prognosis. Despite various methods being used for autoantibody profiling, new techniques continue to be developed to facilitate diagnosis and therapeutic monitoring in connective tissue diseases [26, 27]. It is critical to evaluate new methods along with those being used in laboratories in order to assess their performance as well as to identify deficiencies of methods that are in current use such as methodology based on ELISA, western blot immunoassays, etc. [27].

Immunoassays are generally not considered as multiplex assays even though double immunodiffusion or immunoprecipitation can detect many specific autoantibodies in a single run [28]. Multiplex technology is considered to be beneficial for the simultaneous detection of different autoantibodies related to autoimmune diseases. The autoantibody profiling of patients may be useful for determining the concentration of specific autoantibodies, which may display different trends over time, both for diagnosis and prognosis, e.g. celiac disease, anti-phospholipid syndrome (APS), etc. which are characterised by the presence of autoantibodies of different isotypes. Nowadays, multiplex technology has achieved high analytical accuracy and provides results comparable or superior to the manual and automated monoplex technology [29]. Multiplex autoantibody assays can detect many specific autoantibodies in a single run, whereas the traditional ELISA uses a single antigen to detect only a single autoantibody. Thus, in a multiplex assay, a combination of native antigens or antigenic peptides is used to detect many autoantibodies. The multiplex assays include line immunoassay (LIA), MBAA, and solid-phase antigen microarray (protein microarray). LIA is similar to the dot blot or western blot (immunoblot) in which a diluted serum is incubated with a strip that has several specific antigens in different areas on a strip. In MBAA, beads of different sizes and fluorochromes with different colours or intensities are coated with specific antigens for the detection of specific autoantibody. In antigen microarrays, different specific antigens are coated on a slide/membrane, and the strips, mixture of beads or slide/membrane with multiple antigens are incubated with the diluted serum, and many specific autoantibodies can be determined simultaneously. While new multiplex immunoassays have certain advantages over conventional assays, using them without complete understanding or validation against classic or standard assays may lead to concerns, confusions and conflicts in autoantibody immunoassays in clinical settings [28].

5. Conclusion

Although immunoanalytical techniques are established and advantageous over alternative screening analytical platforms, one of the challenges for immunoassays is multiplexing. The simultaneous on-site measurement of different substances from a single sample called multiplex testing has become increasingly important for in vitro quantification of pathological or toxicological samples. The multiplex assays have recently gained importance for clinical diagnostics, with emerging applications in the developing world. The multiplex assays have several advantages such as performing many reactions on the sample and the ability to provide more information from the sample in a fast and efficient manner. Hence, the technological advancements in clinical sciences are helpful in the identification of analytes or biomolecules in pathological samples. While ELISA is still commonly used, many laboratories and organisations are moving towards multiplex immunoassays. The ELISA formats are able to accurately diagnose and characterise a single analyte; however, their amalgamation into multiplex immunoassays confers the prospects of simultaneous measurement of multiple analytes in a single sample, thereby minimising cost, time and volume.

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