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Multiplexed Immunoassays

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1. Introduction

Immunoassay has been widely used for quantification of proteins and small molecules in medical diagnostics, proteomics, drug discovery, and various biological research.¹ ELISA (Enzyme-Linked ImmunoSorbant Assay) is the most commonly used immunoassay format.² Traditional ELISA assays used in clinical settings are laborious and expensive and often consume large quantities of reagents and patient specimen. Meanwhile, armed with the knowledge from genomic and proteomic study, there is an increasing demand for technologies that are capable of extracting high density of bio-information from limited sample volume for better disease diagnosis, prognosis and treatment.³ This demand has driven the development of low-cost, flexible and high throughput methods for simultaneous detection of multiple proteins in parallel in a single assay (multiplexed immunoassay). Although the first concept towards multiplexed immunoassay was already described in 1961 by Feinberg for “microspot” test of antibody-antigen reaction in the thin agar films⁴, it was not demonstrated until 1989 when Ekins described microarray technology principles in the ‘ambient analyte theory’ and envisioned the immense potential application in biomedical research and clinical diagnostics⁵. Since then, a great number of multiplexing platforms have been developed. Particularly, recent advances in technology (*e.g.* fluidics, optics, automated sample handling device) and informatics have enabled a real high-throughput multiplexed immunoassay.⁶⁻⁸ Today, multiplexed immunoassays are becoming popular and have been widely used in basic biomedical research due to their advantages in performing a large number of different assays all in a single reaction vessel from a relatively smaller sample volume with high efficiency. Multiplexed immunoassays are also becoming important for clinical diagnostic purpose by identifying multiple biomarkers for a wide range of diseases. Patterns of several biomarkers have better predictive value compared to detection of single analyte using ELISAs. Although there are still some challenges (*e.g.* complexity, expensive, validation requirement) for these tests, multiplexed protein test panels are now slowly penetrating into clinical diagnostics market and the time of their significant implementation is probably about to come. Several commercial multiplexed immunoassay platforms are available on this emerging market, including Luminex bead based platform, Meso Scale Discovery’s Multi Array Technology, and protein array platforms from Whatman, Arrylyt and others.

This article will provide an overview of multiplexed immunoassays, and will evaluate existing platforms with multiplexing capabilities and their applications in biomedical research and clinical diagnostics, and will also discuss technical challenges and future prospective.

2. Principle of multiplexed immunoassays

Current multiplexed immunoassays are based on multi-marker strategies, in which high-affinity capture ligands (antibodies or proteins/peptides) are immobilized in parallel assays. When incubated with biological samples, target analytes are bound to corresponding capture ligands, respectively. After washing to remove unbound proteins, captured targets are usually detected by using various labeled reporter ligands. Then target analytes are quantified by measuring the signal intensity of the detection label, which is either converted to mass units of target analyte using calibration curves (i.e. quantitative assay) or evaluated using cutoff calibrator (i.e. qualitative assay). Generally, there are two major approaches to realize multiplexed immunoassay: the use of planar microarrays or encoded-microparticle arrays.

2.1 Planar microarrays

Planar microarrays, such as protein microarrays,^{9, 10} are characterized by surface-immobilized capture ligands in microspots onto a two dimensional grid. The identity of capture ligand at each microspot in the array is distinguished by its physical coordinates (x, y) in the grid (**Figure 1A**). Planar microarrays are highly miniaturized and parallelized assays, which consist of high density of microspots (usually $\sim 300 \mu\text{m}$ in diameter; ~ 2000 spots/ cm^2). Recent development of nanotechnology has enabled the fabrication of highly dense protein nanoarray ($\sim 10^6$ spots/ mm^2).¹¹ Fluorescence and chemiluminescence reporters are common used in planar arrays due to their high sensitivity and wide dynamic range. Planar microarray systems are perfect tools for ultra high-throughput screening of proteins due to their simplicity in preparing an array of high density of elements and in subsequent signal readouts. Many companies have produced protein microarrays for research purpose (www.biochipnet.com), and these protein based microarrays have been applied in the detection of many protein biomarkers, such as viral infection, cancers, and auto-immune diseases.¹²⁻¹⁶

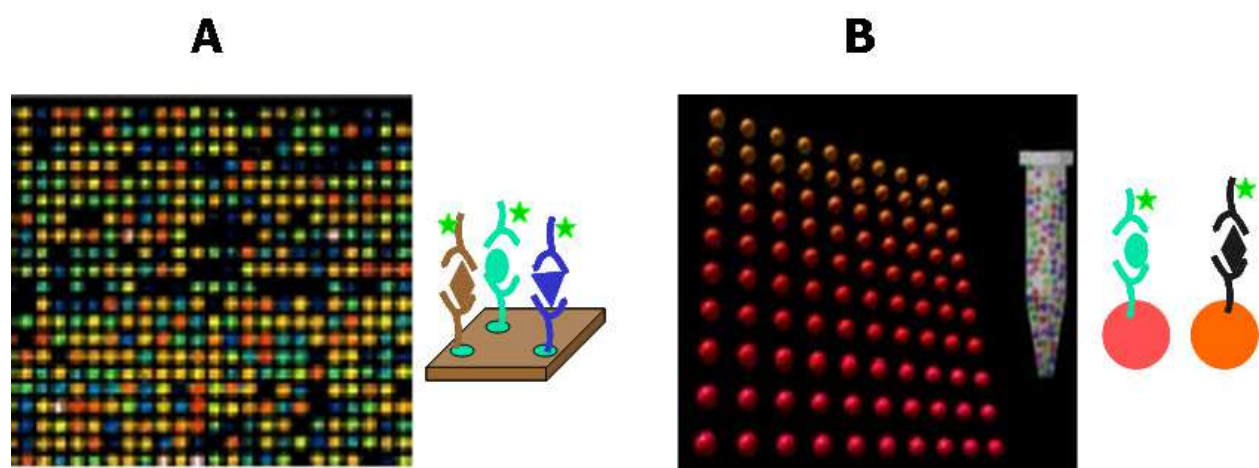


Fig. 1. **(A)** A scheme of planar microarray, which consists of two-dimensional grid of probe molecules (antibodies, peptides, or oligonucleotides). The identity of the probes at each spot in the array is known from its location in the grid. **(B)** An encoded particle array is composed of probe molecules attached to encoded particles. The identity of the probes is revealed by reading the particle code (Reprinted with permission of ref. 17)

However, planar microarrays are limited by some disadvantages, including slow reaction kinetics (due to surface diffusion), problems with localization of capture ligands bound to the 2D chip (due to the use of physical coordinates to indentify), and inflexibility of probe combinations used in an array (due to pre-fabricated flat surface).

2.2 Encoded-microparticle arrays

Encoded-microparticle array based systems (so called suspension arrays) have emerged as a very interesting alternative.^{17, 18} They are composed of encoded microparticles suspended in solution and pre-attached with capture ligands (**Figure 1B**). The nature of the capture ligands attached to each particle is revealed by deciphering the particle code. Encoded-microparticle arrays have several advantages over planar microarrays. First, an encoded microparticle array exhibits greater sensing flexibility where different capture ligands can be mix-and-matched at different combinations at will. Second, suspended particles with curved surfaces benefit from faster diffusion and smaller steric hindrance whereas the reaction kinetics on a planar array is limited by a flat surface. Third, encoded microparticle arrays have greater reproducibility due to the use of hundreds to thousands of replicates for each target molecule in the same assay, which allows for high precision measurements. Accordingly, in this review, we will focus more on encoded microparticle array based multiplexed immunoassay.

Encoded microparticle based multiplexed immunoassays use microparticles as solid phase and therefore require the encoding of microparticle arrays used for the efficient simultaneous measurement of large numbers of biological binding events in a single sample. An ideal microparticle encoding technique must satisfy a number of requirements, it must be: 1) machine-readable (decoding); 2) unaffected by the biochemical reactions; 3) robust, with low error rate; 4) able to encode large numbers of particles, each with a unique code; 5) are compatible with biomolecule attachment; and 6) able to mass production with low-cost. To this end, since the 1990s, different technologies for multiplexing have emerged (optical¹⁹⁻²⁸, graphical²⁹⁻³³, electronic^{34, 35}, or physical^{36, 37} encoding) for different platforms (flow cytometry or fluorescence microscopy). The features of each encoding strategy are listed in **Table 1**.

Of the many encoded technologies developed for multiplexing, optical encoding is the most well established encoding technique, in which the identity of the probe molecules attached to particles is uniquely correlated to the absorbance, fluorescence, Raman, or reflection spectrum of particles. The most common optical encoding method is using polymer microspheres internally doped with one or more fluorescent dyes.^{19, 20} By using fluorescent dyes with different emission spectra and doping at different intensity levels, microspheres of different codes can be obtained (**Figure 2**). The maximum number of codes that can be achieved in this way is determined by the formula: $C = N^m - 1$, where C is the number of codes, N is the number of intensity levels and m is the number of dyes. For a typical multiplexed immunoassay, each set of microspheres with unique combinations of fluorescent spectral is used to attach specific capture ligand and constitutes the platform for specific molecular reaction (like each ELISA microwell). After coupling with the appropriate capture ligands, different sets of encoded microspheres can be pooled and multiplexed immunoassay is then carried out in a single reaction vial. The presence of bound targets to their respective capture ligands on the different microsphere sets can be detected with an

Encoding Strategies	Encoding Materials	Decoding & Detection Methods	Limitations	Ref.
Optical	Fluorescent dye	Fluorescence	<ul style="list-style-type: none"> Limited codes Relied on sophisticated instruments for readouts, Potential interference of encoding and detection spectra 	16,17
	Quantum dots	Fluorescence		18
	Chromophores	Absorption		19
	Multiple wavelength/spatial fluorescence	Fluorescence		20
	Raman tags	Raman		21-24
Silicon photonic crystals	Fluorescence and reflectivity	25		
Graphical	Metal strips	Reflectance and fluorescence	<ul style="list-style-type: none"> Sequential particle synthesis Decoding are time-consuming High throughput limited 	26
	Selective Photobleaching code	Fluorescence; confocal microscope		27
	Structural pattern particles	Physical pattern		28-30
Electronic	Radio frequency memory tags	Radio frequency	<ul style="list-style-type: none"> Size limited Instrument limited Synthesis of the particle are expensive and slow 	31,32
Physical	Particle size	Physical pattern	<ul style="list-style-type: none"> Limited codes Special instrument 	33
	Particle shape	Physical pattern		34

Table 1. Summary of different microparticles encoding methods

antibody conjugate coupled to a reported fluorescent dye. The signal intensities of reported dye are measured, which is used to quantify the amount of captured targets on each individual microsphere. Each microsphere type and thus each binding target are identified using the color code measured by a second fluorescence signal. Flow cytometric principle is the basic technology used in the analysis of optically encoded microsphere based multiplexed immunoassay, which generates robust, rapid, high-throughput, sensitive, and reproducible results for a wide range of biomedical application.

To date, several multiplexed immunoassay platforms that based on optically encoded microspheres have been developed and commercialized. Three major companies market the instruments and materials that required for optically encoded microsphere based multiplexed immunoassay: the xMAP technology by Luminex, the CBA technology by Becton Dickinson BioSciences, and the VeraCode™ technology by Illumina, as summarized in **Table 2**.

The Luminex xMAP (Multi-Analyte Profiling) technology uses 5.5- μm microspheres that are internally doped with two fluorescent dyes (red and infrared) at ten different concentrations to produce up to 100 different sets of microspheres.^{38, 39} Each set of microspheres can then be derivatized by a specific type of capture ligand. As shown in **Figure 3** for a typical sandwiched

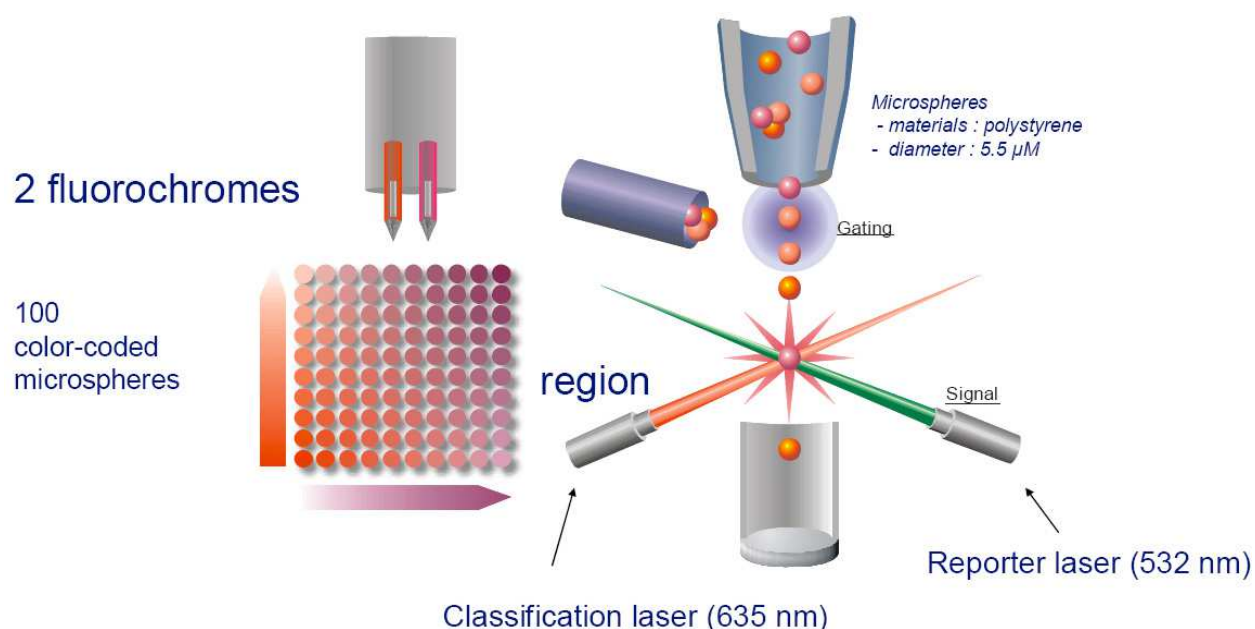


Fig. 2. Optically encoded microspheres: unique microsphere sets are optical coded using a blend of different fluorescent intensities of two dyes and decoded by a flow cytometry. (Courtesy of Luminex Corp.)

immunoassay, binding of target analyte brings reporter molecules to the microsphere surface. A green fluorescent dye (phycoerythrin, PE) is pre-conjugated to the reporter molecules and is used to indicate the occurrence of positive binding and quantify the amount of target analytes. The multiplexing detection is performed on a Luminex analyzer, where two lasers are used to quantitate the green, infrared, and red fluorescence of the individual microsphere as they pass through the sample cuvette. The red laser excites the dye molecules inside the microsphere and classifies the microsphere to its unique set (red-infrared fluorochrome ratio measured by FL1, the classifier signal), and the green laser quantifies the immunoassay on the microsphere surface (reporter signal FL2). Only those microspheres with a complete sandwich will fluoresce in the green part of the spectrum, and the signal is proportional to the amount of capture analyte. The reporter signal is measured as the mean or median fluorescence intensity (MFI) for each microsphere set. And quantitation of capture analyte can be achieved with a built in standard curve in the assay. The Luminex xMAP technology has a unique combination of features: high through-put capacity, analyte quantification over a wide range of concentrations, small sample volume, high reproducibility, and high sensitivity. Multiplexed immunoassays have been designed for up to 100-plexed detection (Luminex 100/200™). Recently, Luminex has developed a new instrument (FLEXMAP 3D™) that can perform up to 500 multiplexed immunoassays by using three internal dyes encoded microspheres. More recently, Luminex also introduced the MAGPIX™ system which was based on the principle of fluorescence imaging, where Light Emitting Diodes (LEDs) and a CCD camera were used to replace the lasers and Photo Multiplying Tubes (PMTs) to deliver a cost-effective, compact, and reliable multiplexing platform. The features of these xMAP systems are listed in **Table 3**. The xMAP technology has been demonstrated as a powerful multiplexing method and has found applications in detection of human cytokines⁴⁰, single nucleotide polymorphisms (SNPs)⁴¹, allergy testing⁴², infectious disease diagnosis⁴³, and biological warfare agent screening⁴⁴.

Instrumentation*	Luminex xMAP (Multi-Analyte Profiling) (www.Luminexcorp.com)	BD™ CBA (Cytometric Bead Arrays) (www.bdbiosciences.com)	Illumina Veracode™/ BeadXpress (www.illumina.com)
Microsphere	5.6 μm Polystyrene/Magnetic	7.5 μm Polystyrene	240 μm (L) X 28 μm (D) Cylindrical silica glass
Bio-conjugation	Covalent: Sulfo-NHS/EDC	Covalent: Sulfo-SMCC	Covalent: Sulfo-NHS/EDC
Fluorescence Detection	Fluorescence signal detector system; PE (phycoerythrin) is normally the reporter molecule	Fluorescence parameters and two size discriminators	Bead identification based on optical “signature” generated by diffraction; four fluorescent dyes can be used for labeling
Multiplexing Capacity	Up to 500	Up to 30	Up to 48 for protein assay
Key Features	Most widely distributed platform; flexible, can test up to 100 analytes simultaneously; commercial kits are available. (New FlexMAP 3D system allows up to 500 multiplexing capability and is compatible with both 96-and 384well plates)	Compatible with conventional flow cytometers and capable of cell-based assays	High-purity silica glass beads are used to minimize fluorescence background, two-color laser system, digital coding enables customizable tracking of multiplex assay markers and critical identifiers such as sample ID, laboratory ID, and reagent kits (up to 24 bits)
Applications	Assays for cytokines, hormones, growth factors, proteinases, cancer markers, cardiac markers, metabolic markers, kinases/ phosphorylated proteins are available; can be used for autoimmune disease diagnostics, infectious disease diagnostics etc.	Assays for complement-derived inflammatory mediators, intracellular signaling molecules, and apoptosis have been described. Kits for viral proteins and cytokines are available	Feasible for up to 384-plex genotyping assays. For protein analysis, only a 10-plex cytokine assay is currently available.

*See Ref.6 for more information

Table 2. Overview of commercially encoded-microsphere based multiplexing technologies

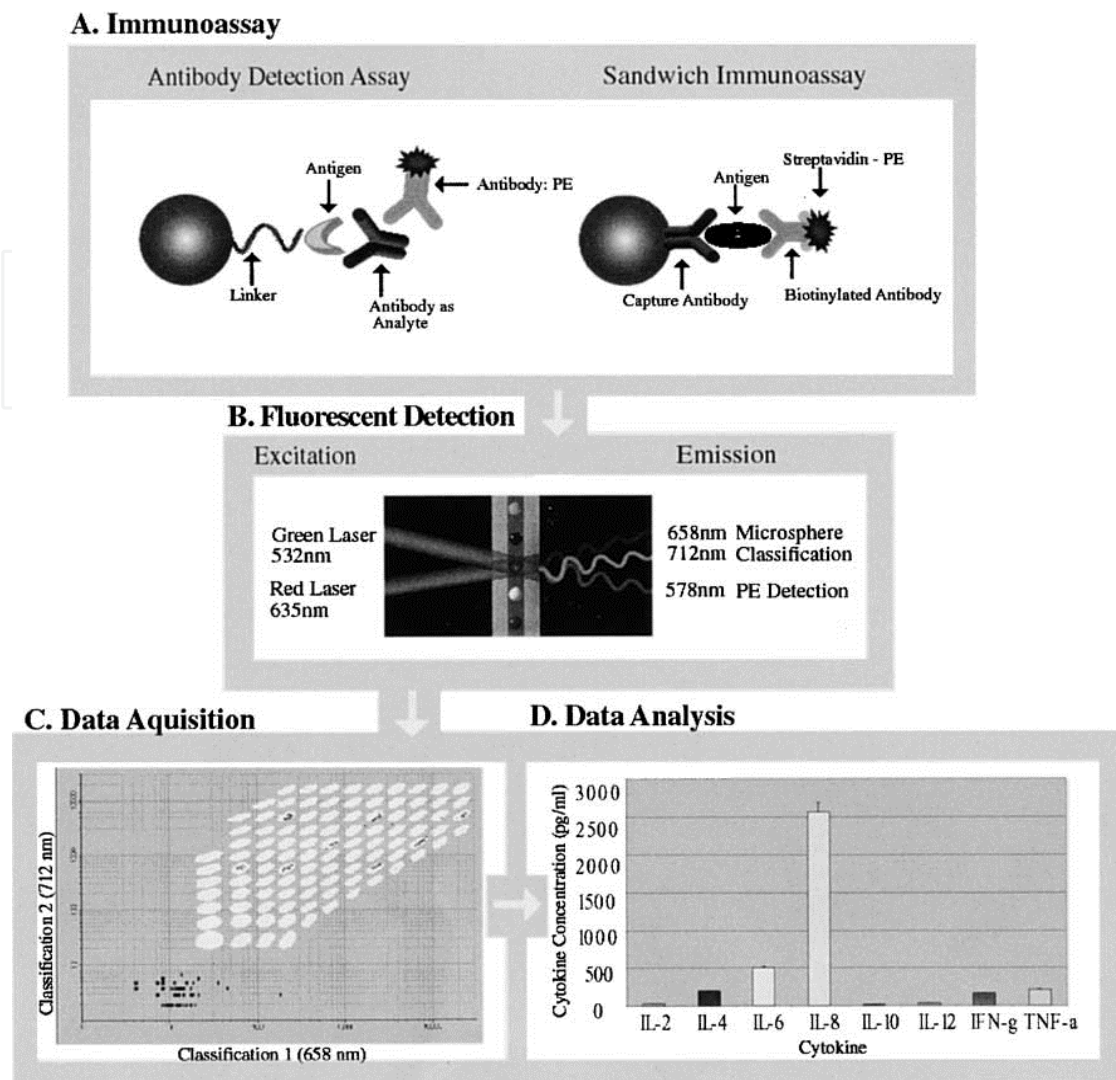


Fig. 3. A schematic illustration of the Luminex xMAP technology: (A) Antibody detection and sandwich immunoassay formats performed on dye encoded microspheres. Any antigen-antibody-based format can be designed with a terminal reporter molecule specific to the laser detection system and distinct from any fluorescence of the microspheres; (B) At the completion of the binding assay, fluorescence intensities associated with each microsphere are measured by flow cytometry (Luminex 100); (C) The dot plot displays 100 distinct regions for each Luminex microsphere population classified on the basis of the intensities of two internal fluorochromes. The instrument was set to acquire at least 100 microspheres each of spectrally distinct populations of microspheres. (D) Multiplexed data from a random sample in an 8-plex assay was calculated. (Reprinted with permission of ref. 44)

Another similar optically encoded microsphere based multiplexing platform was developed by Becton Dickinson BioSciences, named Cytometric Bead Array system (BD™ CBA). This system uses a series of 7.5 μm microspheres doped with one fluorescent dye at different intensities to simultaneously detect multiple analytes from a single sample.^{45, 46} The BD™ CBA combines bead-based immunoassay with sensitivity of amplified fluorescence detection by flow cytometry, creating a powerful multiplexed immunoassay system. The specific capture beads are incubated with tested samples and then mixed with PE conjugated

Features*	Luminex 100/200™	FLEXMAP 3D™	MAGPIX™
Optics Hardware	Lasers/APDs/PMTs Flow Cytometry based	Lasers/APDs/PMTs Flow Cytometry based	LED/CCD Camera Fluorescent Imager
Beads Compatibility	MagPlex® MicroPlex® SeroPlex® LumAvidin® xTAG®	MagPlex® MicroPlex® SeroPlex® LumAvidin® xTAG®	MagPlex®
Multiplexing Capacity	100 (80 for MagPlex®)	500	50
Read Time	~40 mins/96 well plate	~20 mins/96 well plate	~60 mins/96 well plate
Applications	Protein/Nucleic Acid	Protein/Nucleic Acid	Protein/Nucleic Acid
Dynamic Range	≥ 3.5 logs	≥ 4.5 logs	≥ 3.5 logs
Microtiter Plate	96 well	96 well & 384 well	96 well

* For more detail visit www.luminexcorp.com

Table 3. Technical features of three Luminex XMAP systems

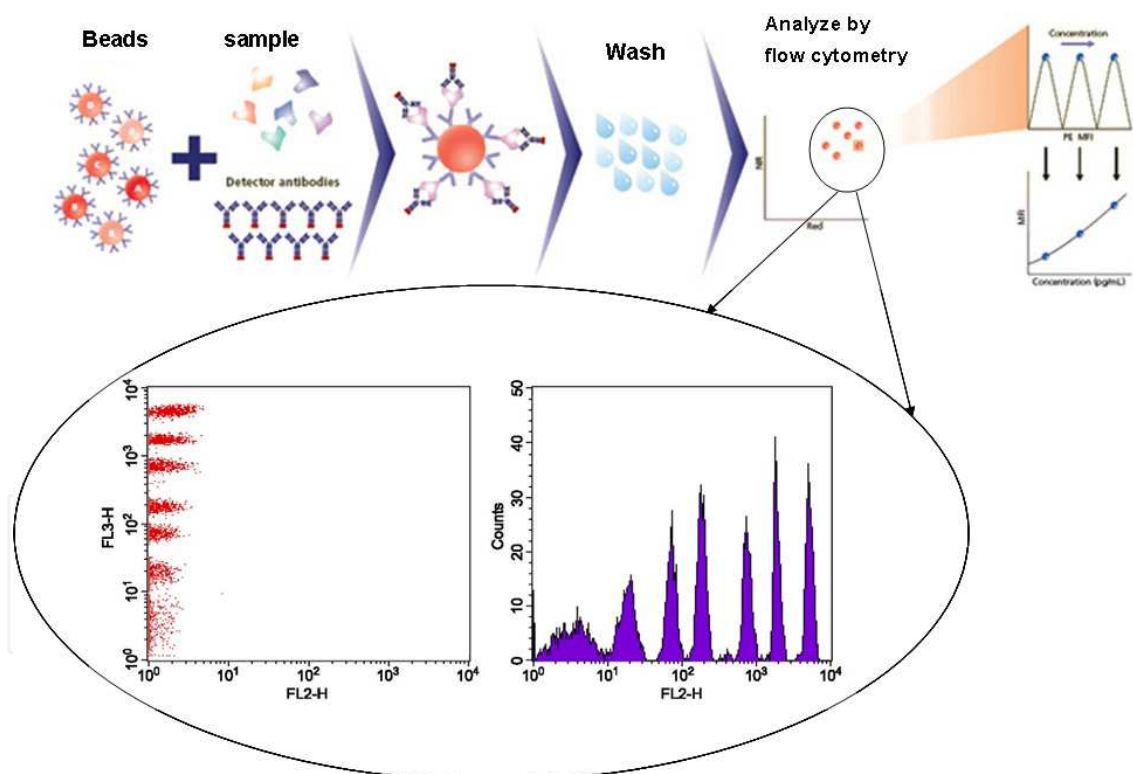


Fig. 4. A schematic illustration of the BD™ CBA technology: After completion of the binding assay, the multiplexing assay was performed on a BD FACSCalibur flow cytometry. Particles (7.5 μm in diameter) were internally labeled with different concentrations of a dye that emits strong fluorescent signals measured in the FL3 channel while displaying minimal fluorescence measured in the FL2 channel as shown in the two-color analysis dot plot (insert left). The non-overlapping nature of histograms for the bead populations are shown on the inset right. (Courtesy of BD Biosciences).

detection antibodies to form sandwich complexes. Sample data is then obtained using flow cytometry. The CBA analysis is designed to run on FACScan and FACalibur flow cytometers, where microsphere population are gated in the forward and side scatter channel (FSC and SSC) to draw an FL3 (bead channel) histogram (**Figure 4**). Each of the fluorescence intensity peaks identified in the FL3 histogram is first classified as an individual assay (one peak represents each type of analyte-bead complex). The corresponding target analyte concentrations are then measured by their fluorescence intensities in the FL2 reporter channel. While the number of assays that can be performed simultaneously (i.e. multiplexing capacity) is limited due to the use of one fluorescent dye for coding, the CBA assays are compatible with any cytometer that is equipped with a 488 nm laser, and capable of distinguishing emissions at 576 and 670 nm. This advantage enables CBA assays to be performed on widely available commercial benchtop flow cytometry. The BD™ CBA systems have commercialized a larger number of multiplexed immunoassay kits for measurement of a variety of soluble and intracellular proteins, including cytokines, chemokines, growth factors, and phosphorylated cell signaling proteins.

The third commercialized multiplexing platform is VeraCode™ technology from Illumina, which is different from above two platforms that are based on fluorescent dye encoded microspheres. It uses cylindrical glass microbead (240 μm in length and 28 μm in diameter) etched with pre-calculated digital holograms. When illuminated with a red laser beam, the holographic elements diffract the beam to produce a unique image code, where each bright stripe represents '1' and dark strip represents "0" (**Figure 5**). By sequential etching holograms onto microbeads, different patterns of codes can be obtained. The surface of the microbead is functionalized with carboxyl groups for covalent binding of capture ligand. Each set of microbead with a unique digital holographic code is used to attach a specific type of capture ligand for target analyte of interest. Consequently, multiplexed assays can be performed by pooling different microbeads embedded with unique "optical signatures" in the same reaction mixture. And the analytes are labeled with standard fluorescent reporters such as PE, Cy3, Cy5, or AlexaFluor dyes. The multiplexed assays are carried out on the Illumina BeadXpress reader, where the microbeads are deposited into a grooved plate so that they are aligned for reading. During analysis, the fluorescence and code are recorded for each microbead by using a red code-readout laser and a green report laser. The holographic code image in each microbead diffracts the incident read laser beam to make up the optical patterns of the bead code and can be detected with a CCD camera. And the green laser quantifies analyte binding. The company has provided carboxylated microbead sets for custom probe attachment.

3. Applications of multiplexed immunoassays

Multiplexed immunoassays allow simultaneous measurement of multiple analytes in a single biological sample, which enables people to obtain high density of biomolecule information with minimal assay time, cost and sample volume. After overcoming the technical hurdles in encoding, functionalizing, decoding, detecting, and improving the limited number of assays to be performed simultaneously, a great number of multiplexed immunoassay platforms, especially optically encoded microsphere based technologies, have been applied in wide range of fields in the biomedical research and clinical diagnostics.

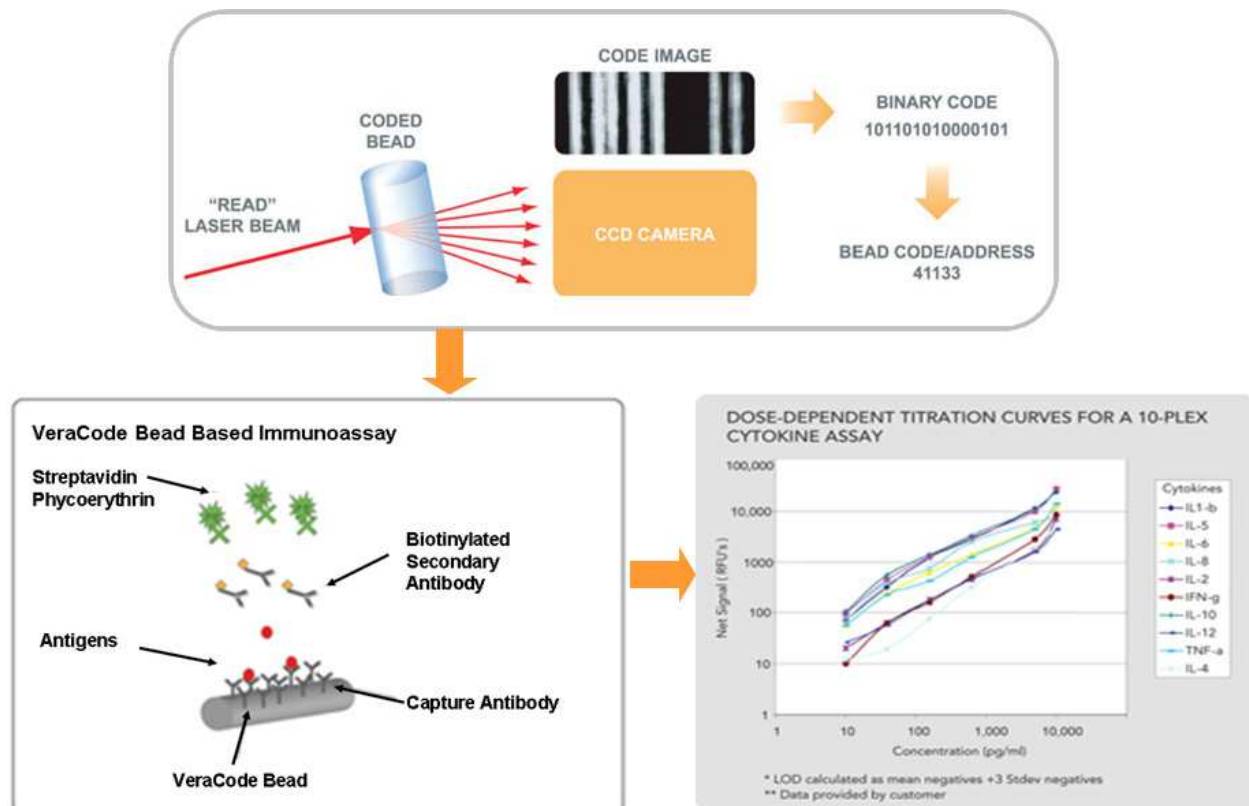


Fig. 5. A schematic illustration of Illumina VeraCode™ technology: a glass bead containing an etched hologram is illustrated with a laser beam producing a code image. Multiplexed immunoassay can be performed by attached specific capture ligand to individual set of bead. (Courtesy of Illumina, Inc.)

At present, the most successful and robust multiplexing technology is Luminex xMAP platform, which combines advanced fluidics, optics, and digital signal processing with proprietary microsphere technology to deliver multiplexed assay capabilities. Importantly, Luminex is an open technology and numerous companies have marketed the Luminex system, including Bio-Rad, Qiagen, Invitrogen, and Millipore *ect.*. And a steadily growing list of ready-to-use multiplexed immunoassays have also been provided by these companies for applications in biomarker discovery⁴⁷⁻⁷⁶, autoimmune disease diagnostics⁷⁷⁻⁸⁰, infectious disease diagnostics⁸¹⁻⁹³, neurological diseases⁹⁴⁻¹⁰¹, HLA testing¹⁰²⁻¹⁰⁴, and drug discovery^{105, 106} (Table 4).

For example, encoded microsphere based multiplexed immunoassays have been used to analyze the expression of cytokines, chemokines and growth factors in diverse samples (serum, plasma, and tissue culture), and therefore serve as a very straightforward approach for biomarker discovery.⁴⁷⁻⁶³ Cytokines, chemokines, and growth factors are cell signaling proteins that mediate a wide range of physiological responses including immunity, inflammation, and hematopoiesis. Changes in the levels of these biomarkers are associated with a spectrum of diseases ranging from tumor growth, to infections, to Parkinson's disease. One of the many commercially available panels for analysis of cytokines, chemokines and growth factors is a 53-plex by Bio-Rad. This panel can simultaneously measure levels of 53 proteins in biological samples using encoded magnetic microspheres, which allows an investigator to take advantage of flexibility of microsphere arrays to develop a customized

Biomedical Research Partners*	Assays/system	Applications
Affymetrix/Panomics (www.panomics.com)	QuantiGene®, Procarta®	Cardiac Markers; Cellular Signaling; Cytokines, Chemokines and Growth Factors; Endocrine; Gene Expression Profiling; Transcription Factors
Bio-Rad Laboratories (www.bio-rad.com)	X-Plex, Bio- Plex®	Cellular Signaling; Cellular Signaling; Cytokines, Chemokines and Growth Factors; Endocrine; Isotyping
Cayman Chemical Company (www.caymanchem.com)		Apoptosis; Cancer Markers; Cytokines; Endocrine
Charles River Laboratories (www.criver.com)		Accute Phase Inflammation; Autoimmune; Cancer Markers; Cardiac Markers; Cytokines and Chemokines; Endocrines; Infectious Disease
EMD Chemicals (www.emdbiosciences.com)	Novagen Widescreen™	Cellular Signaling; Enzymatic Acitivity
Hitachi/MiraiBio Group Indoor Biotechnologies, Inc. (www.inbio.com)	MARIA™	Reagent; Hardware/Software Provider Allergy Testing; Custom Development
Invitrogen (www.invitrogen.com)		Accute Phase Inflammation; Apoptosis; Autoimmune Disease; Cytokines, Chemokines and Growth Factors; Endocrines; Matrix Metalloproteinases; Neuroscience; Signal Transduction
Millipore Corporation (www.millipore.com)	Milliplex™, LINCOpex™, Beadlyte®	Apoptosis; Cancer Markers; Cardiac Markers; Cytokines, Chemokines and Growth Factors; Endocrines; Isotyping; Metabolic Markers
Origene (www.Origene.com)		Genotyping; Transcription Factors
PerkinElmer (www.perkinelmer.com)	Autoplex™	Assay Automation; Hardware/Software Provider
Qiagen, Inc. (www.qiagen.com)		Hardware/Software Provider
R&D Systems (www.rndsystems.com)	Fluoreokine®	Autoimmune Diseases; Cytokines, Chemokines and Growth Factors; Endocrines; Matrix Metalloproteinases
Rules Based Medicine (www.rbmmaps.com)	Human MAPs	Autoimmune Diseases; Cancer Markers; Cardiac Markers; Cytokines, Chemokines and Growth Factors; Endocrines; Infectious Diseases; Isotyping; Metabolic Markers

*Visit www.luminexcorp.com for more information

Table 4.A List of Luminex xMAP biomedical research partners and their application areas

Clinical Diagnostics Partners*	Assays/system	Applications
Abbott Molecular (www.abbottmolecular.com)	Signature	Molecular Infectious Disease
Asuragen (www.asuragen.com)		Human Genetic Testing, Oncology
BMD (www.bmd-net.com)	FIDIS™, CARIS™	Autoimmune Disease
Bio-Rad Diagnostics (www.bio-rad.com)	Bio-Plex®	Autoimmune Disease; Infectious Disease
Eragen Biosciences (www.eragen.com)	MultiCode	Infectious Disease
Celera Diagnostics (www.celeradiagnostics.com)		Human Genetic Testing
Fisher HealthCare (www.fishersci.com)	Prima	Human Genetic Testing; Molecular Infectious Disease
Focus Diagnostics (www.focusdx.com)		Infectious Disease
Innogenetics NV (www.innogenetics.com)	QUANTA Plex™	Alzheimer's Disease
INOVA Diagnostics (www.inovadx.com)		Autoimmune Disease
Inverness Medical Professional Diagnostics (www.invernessmedicalpd.com)	AtheNA Multi- Lyte®	Autoimmune Disease
Microbionix GmbH (www.microbionix.com)	LABScreen, LABType	Custom Development
Multimetrix/Progen Biotechnik GmbH (www.multimetrix.com)		Infectious Disease; Custom Development
One Lambda, Inc. (www.onelambda.com)	LiquidChip™; QIAPlex™	HLA Testing
Qiagen, Inc. (www.qiagen.com)	LifeMatch™	Molecular Infectious Disease
Gen-Probe (www.tepnel.com)		HLA Testing
Zeus Scientific (www.zeusscientific.com)	AtheNA Multi- Lyte®	Autoimmune Disease; Infectious Disease

*Visit www.luminexcorp.com for more information

Table 4.B List of Luminex xMAP clinical research partners and their application areas

multiplex panel for efficient detection of protein of interest. Rules Based Medicine (RBM) also offers a special panel (Human DiscoveryMAP250+) for analysis of up to 250 human biomarkers from human serum sample using minute quantities of sample volume.

Moreover, encoded microsphere based multiplexed immunoassay technology has also been used in cancer biomarker discovery.⁶⁴⁻⁷⁶ Rapid advance in the genomics and proteomics has generated a plenty of candidate cancer biomarkers that could be useful in early cancer detection and monitoring. However, the capacity to verify and validate these candidate

cancer biomarkers is limited, due to the requirement of rigorous testing in a large sample set from many diseases. Recently, Rules Based Medicine has developed a cancer biomarker panel (Human OncologyMAP®) for quantitative measurement of 101 cancer-associated serum proteins. This novel tool is based on encoded microsphere multiplexing technology and offers a powerful tool to aid in the discovery and development of new oncology drugs and diagnostics.

Another example of application of encoded microsphere based multiplexed immunoassay technology is in clinical diagnostics such as autoimmune diseases or infectious diseases. Autoimmune diseases include a wide variety of systemic or organ-specific inflammatory diseases that are characterized by the aberrant activation of immune cells. Many autoimmune diseases are characterized by the presence of specific autoantibody types, which can be used in the diagnosis and classification of autoimmune diseases.⁷⁷⁻⁸⁰ The BioPlex™ 2200 ANA screen (Bio-Rad) and the AtheNA Multi-Lyte™ ANA test system (Zesus Scientific) have obtained marketing approval from the US FDA. These two tests are used in the diagnostics of autoimmune diseases and based on the simultaneous measurement of multiple auto-antibodies. Moreover, encoded microsphere based multiplexed immunoassay technology could improve diagnostics of infectious diseases by enabling the simultaneous detection of antibodies or antigens to multiple infectious pathogens, such as human immunodeficiency virus (HIV), the Hepatitis A, B, C virus, *Mycobacterium tuberculosis*, as well as a large number of other viral, bacterial and parasitic pathogens.⁸¹⁻⁹³ FDA-approved assays for infectious diseases (e.g. EBV, HSV, MMRV, Syphilis, and ToRC assays on BioPlex™ 2200 or Multi-Lyte™) are already available on market.

Encoded microsphere based multiplexed immunoassays are also effective tools for simultaneously measurement of several biomarkers in Alzheimer's disease (AD).^{94, 95} Alzheimer disease is the most common form of age-related neurodegenerative disease, which is a neurodegenerative disorder characterized by accumulation of intracellular neurofibrillary tangles and extracellular amyloid plaques throughout the cortical and limbic brain regions. The development of validated biomarkers for Alzheimer's disease is essential to improve diagnosis and accelerate the development of new therapies. As the list of AD biomarkers is constantly growing, the ability to validate a panel of biomarkers becomes essential. To this end, Innogenetics has offered a multiparameter bead-based immunoassay (INNO-BIA AlzBio3) for the simultaneous quantification of 3 key AD markers in human cerebrospinal fluid (CSF): beta-amyloid 1-42, total tau, and tau phosphorylated at threonine 181.

4. Challenges and limitations of current multiplexed immunoassays

Although hundreds of multiplexed immunoassays are introduced to the research market in recent years, only a limited numbers of them have been approved by the FDA for clinical use. The multiplexed immunoassay, as an emerging technology, is not without limitation. Most FDA approved multiplexed immunoassay platforms are based on encoded microsphere arrays by flow cytometry. Development of robust multiplexed immunoassay required rigorous validation of assay configuration and analytical performance to minimized assay imprecision and inaccuracy. Current limitations associated with multiplexed immunoassay technologies include selection of multiple matched antibodies

pairs; cross-reactivity between antibodies and analytes and assay diluents; interference from matrix effect; the required compromise of the assay parameters when developing multiple assays; and the requirement for pre-labeling reporter molecules for detection

A major challenge of developing multiplexed immunoassay is the need to obtain a large number of highly specific antibodies for a wide range of analytes. Although there are many monoclonal and polyclonal antibodies commercially available, it is difficult to standardize and screen several hundreds of these antibodies to produce reliable assay in a multiplexed format while fulfilling the required assay sensitivity and specificity. Particularly, antibodies suitable for monoplex immunoassays may display cross-reactivity with other analytes in the multiplexed format. Cross reactivity between antibodies and nonspecific analytes limits the number of antibodies that can be used in a given multiplexed assay. To solve this problem, several attempts have been made to optimize antibodies characterization and selection by using high-throughput methods based on multiplexed immobilized proteins or peptides. For example, Poetz *et al.*¹⁰⁷ reported the use of a protein microarray to simultaneously analyze epitope recognition and binding affinity of antibodies to determine their specificity and affinity.¹¹⁴ And Schwenk *et al.*¹⁰⁸ also reported the use of Luminex platform to determine antibody specificity towards up to 100 antigens.

Another major problem is that multiplexed immunoassays are prone to interference due to matrix effect, like any other immunoassay.¹⁰⁹ A matrix consists of all the components in the sample other than the analyte. The interference from matrix would limit the performance of multiplexed assays. Potential sources of interference in the multiplexed immunoassay include endogenous plasma/serum proteins, heterophilic antibodies, soluble receptors, complement components, immune complexes, histidine-rich glycoproteins, lysozyme, fibrinogen, lectins, and some acute phase proteins.¹¹⁰⁻¹¹² To minimize matrix effect, it is important to select suitable blocker, assay diluents and appropriate dilution factor for the sample matrix that mimics real sample during assay development.¹¹³ However, it is a challenge in the multiplexed format to select assay diluents that interact effectively with all the reagents and proteins, because each protein requires specific conditions to maintain its conformation. Minor changes in buffer pH and ionic strength may change the protein structure, thus impairing assay performance.

Multiplexed immunoassays involve two or more capture molecules and allow the simultaneous measurement of different target analytes. In a monoplex immunoassay (ELISA), the assay parameters such as antibody pairs, sample diluents, and conjugate concentration can be easily optimized for a particular assay. However, in a multiplexed format, those parameters would be different from one analyte to another. They have to be adjusted to suit all the analytes within the multiplexed assay. The required compromise of the assay parameters would limit the assay performance of multiplexed immunoassay. Another major issue that limits the assay performance of multiplexed immunoassay is that an increasing number of detection antibodies results in an increase of background noise, which subsequently decreases assay sensitivity. For example, it has been found that the sensitivity of an 11-plex assay decreased by a factor of 1.75-5.0, compared with monoplex ELISA, due to an increasing in background signal in the multiplexed format.¹¹⁴

Current multiplexed immunoassay platforms, based on either planar microarrays or suspended encoding particle arrays, often require an extra labeling step for the detection ligand with reporter molecules (*e.g.* fluorescent dye), which prolongs the assay time and

increases the assay cost. Therefore, challenges in simplifying the tagging process and eliminating the need for pre-labeling reporter molecules for detection still remain. The need to overcome such hurdles has motivated research into the development of a label-free multiplexed assay system, where progress has been made in surface plasmon resonance (SPR) and fluorescent conjugated polymers-based optical detection, nanowire-based electrical or electrochemical measurements, and mass spectrometry (MS)-based high-throughput screening.¹¹⁵⁻¹¹⁸ Conjugated polymers, especially conjugated polythiophene derivatives, can display remarkable changes of optical properties due to conformational changes of polymer chains when binding to biomolecules, therefore offer a potential opportunity as the optical probe for multiplexed assay in a label-free fashion.¹¹⁹⁻¹²¹ We have integrated fluorescent conjugated polymers into metallic encoded nanorods for label-free, multiplexed detection of DNA and cancer biomarkers with high specificity and sensitivity.^{122, 123}

5. Summary and future prospect of multiplexed immunoassay

Multiplexed immunoassays allow simultaneous measurement of multiple proteins in a single biological sample. They have demonstrated comparable sensitivity to traditional ELISAs, making them great potential for both basic research and clinical diagnostics where assays required multiplexing in small sample volume. Currently, a great numbers of multiplexing technologies have been developed and used in the biomedical research and clinical diagnostics. The optically encoded microsphere-based technology is the most advanced multiplexing technology and has been commercialized on the market. Optically encoded microsphere-based technology offers a robust and efficient approach for setting up multiplexed assays and makes multiplexing assays feasible by flow cytometry. However, there are still a number of challenges to be overcome before encoded microsphere based multiplexing platforms can be fully applied in the field of clinical diagnostics. The need to overcome these challenges motivate people to continue to develop robust, sensitive, specific, rapid, and high-through assays with multiplex capabilities that can fulfill the expectations and demands for basic biomedical research and clinical application. Prospective technology development and research direction of multiplexed immunoassays would focus on the following main areas:

5.1 Miniaturization

Miniaturization has been a driver in assay development for many years. The goal is to obtain increasing amounts of molecular information from ever decreasing volumes of sample. Miniaturized multiplexed immunoassays can be regarded as an ideal solution for applications in which several parameters of a single sample with limited volume needed to be analyzed in parallel.¹²⁴ Recent development in the microfluidic system offers great potential in the miniaturized immunoassay.¹²⁵ The most common microfluidic platform relies on networks of enclosed micron-dimension channels, where fluids exhibit laminar flow (i.e. fluidic streams) that flow parallel to each other, and mixing occurs only by diffusion.¹²⁶ Microfluidic immunoassays have several advantages over conventional methods¹²⁷: (1) increased surface area to volume ratios speeds up molecule binding reactions; (2) smaller dimensions reduce the consumption of expensive reagents and precious samples; and (3) automated fluid handling can improve reproducibility and throughput. These advantages can potentially improve assay performance and reduce the operation cost of conventional

immunoassays. Diercks et al. have reported the integration of optically encoded microspheres with microfluidic platform for miniaturized multiplexed immunoassays.¹²⁸ In this work, the encoded microspheres were trapped in a microchannel and imaged using a confocal microscope. They detected four different analytes from a 2.7-nL sample.

5.2 Automation

Immunoassay automation promises to be the most rapidly growing area for research and development in the clinical diagnostics industry.¹²⁹ In the automated assays, all stages of assays, from sample preparation to instrument operation to data processing are highly compatible with robotics and automation. Therefore, automation can reduce labor requirements and reduce testing costs. Quality testing can be achieved with immunoassay automation due to improved assay performance resulting from improved precision, sensitivity, and wide dynamic ranges, as well as from the elimination of sample handling and processing errors. Bio-Rad has developed a fully automated, random access multiplexed testing platform, Bio-Plex™ 2200. It combines the encoded magnetic microsphere with automated liquid handling workstation. This system addresses the needs for high-throughput analysis of clinical samples, which automatically processes up to 100 samples per hour, for a maximum of 2200 reportable results with eight hours of walk-away capability. First results are available in approximately 20–45 minutes (assay dependent), with subsequent patient samples completed approximately every 30 seconds.

5.3 Improved capture ligands

A key step for development of robust immunoassays is generation and characterization of capture ligands. These systems required high quality of capture ligands with high specificity and sensitivity of recognizing target proteins. Current available antibodies may display cross reactivity with other proteins in the multiplexed assay format. To solve this problem, alternative capture ligands such as engineered protein scaffolds and nucleic acid scaffolds are being evaluated to replace antibodies for the specific protein detection¹³⁰⁻¹³². For example, aptamers, highly specific oligonucleic acids or peptide molecules that bind to protein due to their unique three dimensional structure, possess target recognition features as antibodies¹³³

5.4 Improved encoding technology

The development of new encoding technology for microparticles may also offer higher and more stable multiplexing capacity. Recent advances in Quantum dots studies have shown a potential for new alternative optical encoding technology. Quantum dots are photoluminescent semiconductor nanocrystals, which typically consist of a core of cadmium selenide (CdSe) surrounded by a shell of zinc sulfide (ZnS).^{134, 135} Quantum dots have many advantages over traditional fluorescent dyes as ideal fluorophores for wavelength and intensity multiplexing: (1) their emission spectra are tunable by the size of quantum dots, (2) Quantum dots of different emission profiles can be excited simultaneously at the same excitation wavelength, (3) their emission bands are relatively narrow, which allow more emission bands to be resolved with minimal spectra overlap, and (4) Quantum dots have higher quantum yields than most fluorescent dyes and have better photochemical stability

against photo-bleaching. Multicolor optical coding can be achieved by embedding different size of Quantum dots into polymeric microsphere at precisely controlled ratios.¹³⁶⁻¹³⁸ For example, the use 10 intensity levels and 6 colors can generate up to 10^6 codes, which open up new opportunities for gene profiling, high-throughput screening, and medical diagnostics.

5.5 Improved clinical applications

Early stage detection of many diseases requires distinct pattern recognition of various protein biomarkers to identify at-risk individuals with adequate confidence. Multi-biomarker strategies improve medical diagnostic and prognostic information. Therefore, multiplexed immunoassays are becoming more and more important for clinical diagnostics in the future due to their ability of identifying multiple clinical biomarkers for a wide range of diseases. Multiplexed protein test panels for use in cancer, stroke, diabetes, and cardiovascular diseases would be the interest in the future.

6. References

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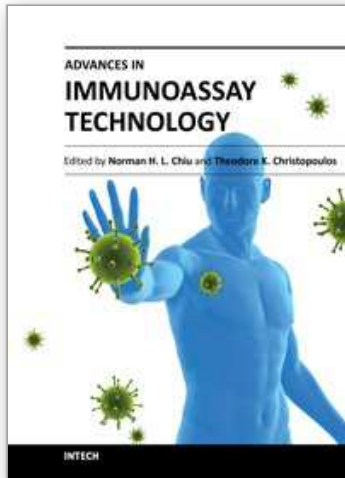
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From the basic in vitro study of a specific biomolecule to the diagnosis or prognosis of a specific disease, one of the most widely used technology is immunoassays. By using a specific antibody to recognize the biomolecule of interest, relatively high specificity can be achieved by immunoassays, such that complex biofluids (e.g. serum, urine, etc.) can be analyzed directly. In addition to the binding specificity, the other key features of immunoassays include relatively high sensitivity for the detection of antibody-antigen complexes, and a wide dynamic range for quantitation. Over the past decade, the development and applications of immunoassays have continued to grow exponentially. This book focuses on some of the latest technologies for the development of new immunoassays.

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