SMGroup

# Overview of Multiplex Immunoassays

**Amit Kumar Pal***

Department of Oral Biology, The Maurice and Gabriela Goldschleger School of Dental Medicine, Tel Aviv University, Israel

***Corresponding author:** Amit Kumar Pal, Department of Oral Biology, The Maurice and Gabriela Goldschleger School of Dental Medicine, Tel Aviv University, Tel Aviv, Israel, Email: amitpal07@gmail.com

**Published Date:** January 15, 2015

## ABSTRACT

The ability to measure the multiple proteins precisely and simultaneously in a single biological sample has revolutionized the diagnostic and experimental research. The value of multiplex measurement is slowly and progressively establishing itself in the studies of comprehensive proteomic surveys, protein networks and pathways, validation of genomic discoveries and in the development of clinical biomarker. Currently, a great numbers of multiplexing technologies have been developed and used in the biomedical research and clinical diagnostics. This chapter will provide an insight of multiplexed immunoassays and will evaluate the current and future platforms with multiplexing capabilities along with their applications in biomedical research and clinical diagnostics.

**Keywords:** Multiplex immunoassays; Planar microarrays; Bead-based microarrays

## INTRODUCTION

For the last four decades, immunoassays have revolutionized the diagnostic field. Immunoassays allow for sensitive and specific detection of various target structures in complex biological sample and are widely accepted tool in hospitals, laboratory medicine and research to improve the health and well-being of humans as well as in animals. Since their introduction in early 1960s (RIA; Radio Immuno Assay) or 1970s (ELISA; Enzyme Linked Immuno Sorbent Assay), have become an indispensable analytical tool in wide range of applications including clinical diagnostics and

has shortened hospital stays and decreased the severity of illness by identifying and assessing the progression of disease, thereby leading to improved therapeutic choices. Its application in research and diagnosis as well as also been widely used in industries as analytical and quality control tool such as detection of contaminants in food, water and monitoring specific molecules during product processing. The impact of these assays on research and clinical diagnosis is enormous, with almost 10,000 studies published per year seen in two synonymic words; "enzyme immunoassay" or "enzyme-linked immunoassay" [1].

Although conventional immunoassay has great advantage in identifying one or two target proteins, but some drawbacks such as labor intensiveness, requirement of large amount of sample and procedure is laborious, and above all still very expensive for the number of developing and undeveloped nations to use it . This led to the discovery of low-cost, flexible and high throughput methods for simultaneous detection of multiple proteins in parallel in a single assay (multiplexed immunoassay). In 1989, Ekins described microarray technology principles in the ambient analyte theory which lead to opening of new door in the field of diagnostic and research. Ekins's theory stated that a tiny spot of purified antibody (or any other macromolecule) provides substantially better sensitivity than when used in large amounts. Fueled by large-scale genome sequencing projects, DNA microarray technology became the first application of this theory and has been widely used in gene expression profiling [2-6]. However; biological functions are carried out primarily by proteins rather than nucleic acids. Furthermore, RNA expression levels do not always correlate with protein expression levels, and it is almost impossible to predict biochemical properties of a protein encoded by a given gene simply based on its expression profiles. Therefore, by focusing on studies of protein structures functionalities and protein–protein interactions one can more directly characterize biological function of a given gene [7].

Now days, increasing awareness of the multifactorial nature of various diseases and pathological states *viz*. cancer, sepsis, etc. really calls for simultaneous, time-saving and cost effective measurement of multiple analytes. This multi-marker strategy could be then translated into more robust diagnostic algorithms, better-fitting prognostic models and more effective population screenings. Of note, multiplex immune analysis is perfectly suited for diagnostics of immune system disorders (autoimmune diseases) and allergies. Being widely used in basic research, multiplex methodologies are slowly penetrating in to the *in vitro* diagnostics market [8]. Hence, time of their significant implementation is probably about to come.

This chapter is an effort to endow with us the capabilities of multiplex immunoassay and will discuss the capabilities of multiplex immunoassay.

Currently, two main streams of multiplex immunoanalysis exist: planar microarrays (protein chips) and bead-based microarrays (suspension arrays). These approaches vary greatly in many aspects, but primarily in the way of the individual analyte identification (position vs. bead characteristics).

## BASIC OF MULTIPLEXED IMMUNOASSAYS

Multiplexed immunoassays are based on traditional immunoassay principle, except that in these methods high-affinity capture ligands are immobilized either in planar format (flat surface) or on microspheres in suspension that binds to target analaytes of sample. Subsequently, multiple analytes can be analyzed in a single reaction set up. Currently two major approaches were used for multiplex assay one is use of planar microarrays second the use of Suspension array (encoded-micro particle arrays).

## PLANNER/ ANTIBODY MICROARRAY

The best way to describe the Planner microarray is array of multiple ligands immobilized on two dimensional grid containing microspots. Each spot or group of spots represents a unique target binding site. It is one of the emerging proteomic technologies and recently becoming critical tools in biochemistry and molecular biology. Currently two classes of protein microarrays are available (1) analytical and (2) functional protein microarrays. Analytical protein microarrays are mostly antibody based. This became one of the most powerful multiplexed detection technologies. Functional protein microarrays are being increasingly applied to many areas of biological discovery, including studies of protein interaction, biochemical activity and immune responses. Great progress has been achieved in both classes of protein microarrays in terms of sensitivity, specificity and expanded application.

These assays are generally also known as protein chip assay. They are highly miniaturized and contain small amounts of purified proteins in a high-density format and allow the simultaneous determination of a great variety of analytes from small amounts of samples within a single experiment. This property makes it as one of the advance tool in the field of bio-medical research [9].

These assays are typically prepared by immobilizing proteins onto a microscope slide using a standard contact spotter. After proteins are immobilized on the slides, they can be probed for a variety of functions/activities. Finally, the resulting signals are usually measured by detecting fluorescent or radioisotope labels. The typical image of protein microarrays is shown as Figure 1.

### Working Principle of Analytical Microarray

Analytical protein microarrays are usually composed of well-characterized biomolecules with specific binding activities, such as antibodies; to analyze the components of complex biological samples (e.g., serum and cell lysates) or to determine whether a sample contains a specific protein of interest. They have been used for protein expression profiling, biomarker identification, cell surface marker/glycosylation profiling, clinical diagnosis and environmental/food safety analysis. In this array the matrix is a patterned surface where the recognition molecules are immobilized by microprinting or microstructuring processes (Figure 1a). An optical matrix on microparticles is also possible by mapping with a chromogenic code. The 2D readout is achieved by scanning or

imaging the patterned surface (Figure 1b) by means of a sensor array, by parallel arrangement of segmented channels or by parallel detection of two bits of optical information (e.g. two different fluorescence emission wavelengths) encoded in microparticles. Quantitative results with an analytical microarray are obtained by calibration with standardized analytes (Figure 1c). Data analysis of an analytical microarray cab be achieved with software for image processing [10].

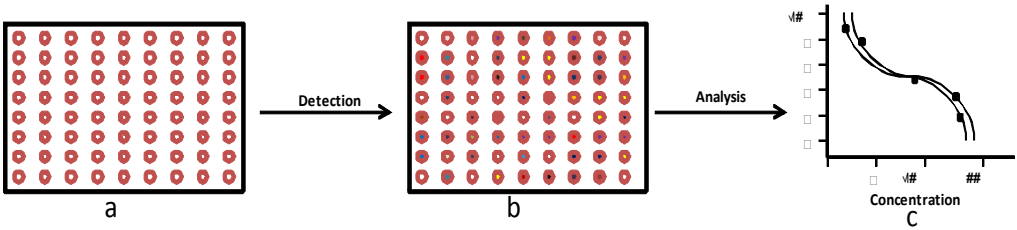


**Figure 1:** Schematic diagram of (a) immobilized matrix (b) Detection after sample processing and (c) data analysis for a quantitative analytical microarray.

## Working Principle of Functional Microarray

On the other hand, functional protein microarrays are constructed by printing a large number of individually purified proteins and are mainly used to comprehensively query biochemistry properties and activities of those immobilized proteins. In principle, it is feasible to print arrays comprised of virtually all annotated proteins of a given organism, effectively comprising a whole proteome microarray. Functional microarrays have been successfully applied to identify protein-protein, protein-lipid, protein-antibody, protein small molecules, protein-DNA, protein-RNA, lectin-glycan and lectin-cell interactions to identify substrates or enzymes in phosphorylation, ubiquitylation, acetylation and nitrosylation as well as to profile immune responses.

These microarrays are composed of full length functional protein or protein domain. These protein chips are used to study the biochemical activities of an entire proteome in a single experiment. Capturing protein molecules with high specificity and selectivity are the essential prerequisite for the design and fabrication of fuctional microarrays [11-13].

# SUSPENSION ARRAY

Suspension arrays of microspheres analysis using flow cytometry offer a new approach to multiplexed assays for large-scale screening applications. By optically encoding micron-sized polymer particles, suspension microarrays can be created to enable highly multiplexed analysis of complex samples. Each element in the array is comprised of a subpopulation of particles with distinct optical properties and each array element bears a different surface receptor. Nucleic acids, proteins, lipids or carbohydrates can serve as receptors to support the analysis of a wide range of biomolecular assemblies and applications in genomic and proteomic research are being developed. Coupled with recent innovations for rapid serial analysis of samples, molecular

analysis with microsphere arrays holds significant potential as a general analysis platform for both research and clinical applications.

Suspension array employs encoded microspheres as array elements that bear specific receptor molecule with distinct optical properties, such as light scatter or fluorescence from an internal dye are used as solid supports for a variety of molecular analyses. By careful adjustment of these intrinsic optical properties, it is possible to prepare arrays of microspheres in which individual microsphere subsets can be identified and used to perform multiplexed analysis. Conceptually, microsphere arrays are similar to flat-surface microarrays, with distinct quanta of an intrinsic optical parameter substituting for physical location on a surface. Beyond this similarity, microsphere arrays and flat-surface arrays differ significantly in their implementation. The use of optically encoded microspheres as array elements affects issues such as array preparation, density and flexibility.

## Working Principle of Suspension Array

Similar to ELISA, a majority of assays are designed according to a capture sandwich immunoassay format**.** In case of suspension arrays, micrometre-sized solid particles known as beads are used for attaching probe biomolecules, such as antigens for immunoassays. The probes are attached by reacting different particles (in suspension) with solutions of different probes, thus reducing the problems associated with attaching molecules to microarrays. For a typical assay, a selection of different particles is used, each with a different probes attached. A suspension of such particles is reacted with the sample and the binding measured. Unlike a microarray, where the molecular probe is identified from its location in a matrix, each particle must carry a unique identifier, i.e. a tag or code, as shown in Figure 2. After reaction every particle is analyzed for (a) fluorescence indicating binding of (antibody) and (b) the unique code. The combination of these two allows identification of the probe molecule and therefore the antibodies present in the sample.

Particles can be analyzed in microfluidic systems, providing a high throughput platform, which can be integrated into low-cost devices for biochemical research and point-of-care medical diagnostic applications [15-16]. Potentially particles could be directed post analysis into different reaction vessels, for split-and-mix synthesis where molecules such as a peptides or oligonucleotides are progressively and combinatorial synthesized on the beads [17]. The number of codes required for a bead-based assay technology depends on the assay; some immunoassays only require the identification of a few tens of antibodies in the sample [14]. By contrast, assay studies of biological processes such as gene expression and protein–protein interaction can require very large numbers of codes (around $10^4$ – $10^5$) Other assays that involve combinatorial synthesis of large numbers of compounds, such as drug discovery assays, require similarly large numbers of codes.

A microparticle encoding technique must satisfy a number of requirements: It must be

machine-readable by non-contact methods; suitable for encoding particles of tens of microns in size; unaffected by the biochemical reactions; robust, with low error rate; able to encode large numbers of particles, each with a unique code; implementable on materials which are compatible with biomolecule attachment and amenable to low-cost mass production (particularly important for diagnostic applications). A large number of techniques have been proposed for encoding beads, some of which have been reviewed elsewhere [18,19] and research in this area has been extensive.

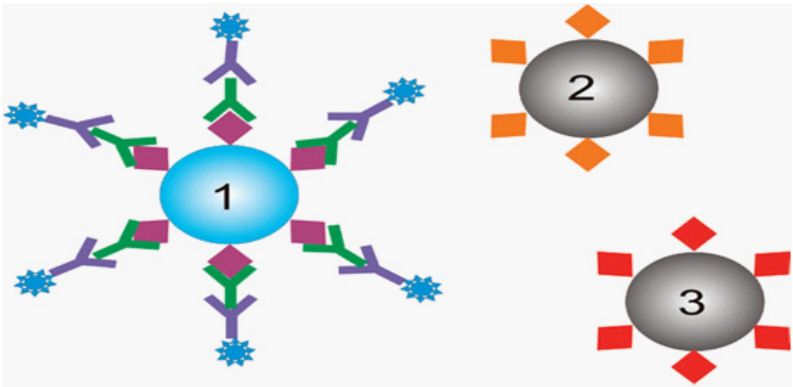


**Figure 2:** Suspension Array **(**bead based assay). The probe molecules being attached to different site on a slide, each probe is attached to a different bead. Since the beads do not have a well defined position, each must have some kind of code which allows it to be identified after the reactions have taken place.

## A COMPARISON OF PLANAR MICROARRAYS AND SUSPENSION BEAD ARRAYS

Capture analytes in both cases are bound to a solid substrate. In the case of planar micro arrays, the capture analytes are covalently bound to the surface of the microarray. They cannot interact with each other in solution, as can capture analytes attached to beads in suspension. The miniaturization of printed microarrays provides a platform where the detecting proteins in solution are in excess concentration over the capture analytes bound to the microarray. Increased concentration means faster binding kinetics and increased sensitivity to provide more sensitive and reproducible results. Planar microarray data quality is checked by careful examination of the raw data. Bead array flow cytometer detection instruments do not provide any raw data. Bead suspensions depend on redundancy in the system to provide data. Bead array platforms cannot use label-free detection methods, whereas microarray spot locations define and identify the capture analytes and only a single image scan is required. Exposure to light damages beads to deliver misleading results. Beads cannot multiplex at both the antigen and antibody level as can only be done by planar microarray. In bead suspension systems, only single beads that are in suspension are counted. All beads aggregated at the bottom of the well or attracted or bound to

other beads are not interrogated to contribute data, leading to becoming a false-negative result [20].

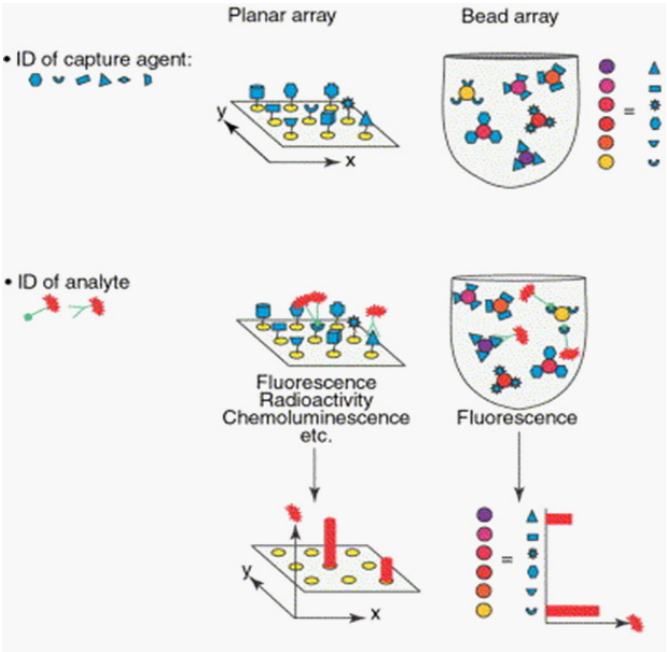


**Figure 3:** Schematic representation of Planner and Suspension microarrays. Either planar microarrays or bead-based arrays can be employed for multiplexed ligand-binding assays. Planar microarrays can be generated with hundreds and thousands of different capture spots whereas multiplexing in bead based arrays is limited to the number of distinguishable beads. The separation of beads is performed *via* colour- (Luminex) or size-coding (BD) of capture beads. In planar arrays analyte spots are easily distinguishable by their xy-coordinates in the array. Detection on planar arrays is performed using either chemoluminescence, radioactivity, mass spectrometry or fluorescence. The latter is the preferred detection method for bound analytes in bead-based microarray assays. (Courtesy: Dieter Stoll *et al*, 2004; Drug Discovery Today: 2004, 9(24 Suppl):S10-7.)

## APPLICATION OF MULTIPLEX IMMUNO ASSAY

The biochemistries of thousands of proteins can be characterized and quantified in a parallel format through the use of multiplexed immunoassays. Unlike the early assays functional microarrays provide a flexible platform that allows detection of a wide range of protein biochemical properties. This technology enables the researcher and clinical diagnostic people to get more information from the biomolecule with minimal assay time, cost and sample volume. Robinson (2002) shows that antibody arrays to be very promising tools to study auto antigen and autoantibody responses in autoimmune diseases [21]. Till date, well-developed assays include detection of various types of protein-ligand interactions, such as protein-protein, protein-DNA,

protein- RNA, protein-lipid, protein-drug, protein-glycan and identification of substrates of various classes of enzymes. Application of these assays has had a profound impact on a wide range of research and diagnostic areas. A number of multiplex assays have been developed for the diagnostic market. Currently multiplexing technology Luminex xMAP platform is most commonly used in commercial assay. It combines advanced fluidics, optics and digital signal processing with proprietary microsphere technology to deliver multiplexed assay capabilities and the most important thing with this technology is that it is open technology make it access to leading diagnostic companies to use this technology [39].

Companies like Bio-Rad, Qiagen, Invitrogen, Millipore, EMD, Origene, Perkin Elimer are launching their diagnostic kits based on this technology for biomarker discovery, drug discovery, infectious disease and HLA testing (Table 1).

Suspension 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 [22-30]. 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 and leads to Parkinson's disease [31-39].

**Table 1**: Examples of commercially available planar multiplex assay accessible for cytokine profiling, protein profiling, protein interaction studies and for reverse screening comparative.

| S.No | Product Name | Application | Manufacturer |
|---|---|---|---|
| 1 | Panorama™ Ab Microarray Cell Signalling Kit™ | Protein analysis | Sigma -Aldrich. |
| 2 | BD Clontech™ Ab Microarray | Protein analysis | BD Biosciences. |
| 3 | Rolling circle amplification technology (RCAT™) | Protein profiling | Molecular Staging Inc. |
| 4 | RayBio™ Cytokine Arrays | Cytokine profiling | RayBiotech Inc. |
| 5 | Zyomyx Protein Profiling Biochip System | Cytokine profiling | Zyomyx Inc. |
| 6 | ProVision™ HCA | Cytokine profiling | Schleicher & Schuell Bioscience. |
| 7 | SearchLight™ Arrays | Cytokine profiling | Pierce Biotech Inc. |
| 8 | ZeptoMAR™ CeLyA Cell Lysate Arrays | Reverse screening | Zeptosens AG. |
| 9 | SELDI Protein Chip® technology | Reverse screening | Ciphergen Biosystems Inc. |
| 10 | The Yeast ProtoArray™ | Protein interaction studies | Protometrix Inc. |

Recently Bio Rad Company develops a multiplex immunoassay named 53-plex. This multiplex assay can simultaneously detect 53 different kind of protein in sample. This help the investigator for better diagnose of the subject.

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 [36-38].

Proteomic research, high-throughput drug compound screening and diagnostic applications will lead to a growing demand for multiplex immunoassay technologies. In medical research, these assays can significantly accelerate immune diagnostics by enabling the analysis of all relevant diagnostic parameters simultaneously. In addition, the reduction of sample volume is of great importance, in particular on occasions when the samples are limited as in the analysis of multiple tumor markers from biopsy material. Screening of large well-defined sample collections will allow the identification of panels of biomarkers related to disease predisposition, progression and response to treatment. This technology will help accelerate the drug development process and uncover new possibilities with respect to patient monitoring during clinical tests, disease treatment and therapy.

## 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.

## ACKNOWLEDGEMENT

The author thanks to Dr. Madhukar Saxena, Babasaheb Bhimrao Ambedkar University, Lucknow, India for giving me opportunity and inspiration for writing the above chapter and Dr. Vijay Raju Boggula Ariel university, Ariel, Israel for his kind support.

### References

1. Lequin RM. Enzyme immunoassay (EIA)/enzyme-linked immunosorbent assay (ELISA). Clin Chem. 2005; 51: 2415-2418.
2. Pease AC, Solas D, Sullivan EJ, Cronin MT, Holmes CP. Light-generated oligonucleotide arrays for rapid DNA sequence analysis. Proc Natl Acad Sci U S A. 1994; 91: 5022-5026.
3. Schena M, Shalon D, Davis RW, Brown PO . Quantitative monitoring of gene expression patterns with a complementary DNA microarray. Science. 1995; 270: 467-470.

4. DeRisi JL, Iyer VR, Brown PO. Exploring the metabolic and genetic control of gene expression on a genomic scale. Science 1997, 278: 680–686.

5. Schadt EE, Monks SA, Drake TA, Lusis AJ, Che N. Genetics of gene expression surveyed in maize, mouse and man. Nature. 2003; 422: 297-302.

6. Morley M, Molony CM, Weber TM, Devlin JL, Ewens KG. Genetic analysis of genome-wide variation in human gene expression. Nature. 2004; 430: 743-747.

7. Hu S, Xie Z, Qian J, Blackshaw S, Zhu H. Functional protein microarray technology. Wiley Interdiscip Rev Syst Biol Med. 2011; 3: 255-268.

8. Lueking A, Cahill DJ, Müllner S. Protein biochips: A new and versatile platform technology for molecular medicine. Drug Discov Today. 2005; 10: 789-794.

9. Chen CS, Zhu H. Protein microarrays. Biotechniques. 2006; 40: 423-427.

10. Seidel M, Niessner R. Automated analytical microarrays: a critical review. Anal Bioanal Chem. 2008; 391: 1521-1544.

11. Hall DA, Zhu H, Zhu X, Royce T, Gerstein M. Regulation of gene expression by a metabolic enzyme. Science. 2004; 306: 482-484.

12. Zhu H, Bilgin M, Bangham R, Hall D, Casamayor A. Global analysis of protein activities using proteome chips. Science. 2001; 293: 2101-2105.

13. Hall DA, Ptacek J, Snyder M. Protein microarray technology. Mech Ageing Dev. 2007; 128: 161-167.

14. Pang S, Smith J, Onley D, Reeve J, Walker M. A comparability study of the emerging protein array platforms with established ELISA procedures. J Immunol Methods. 2005; 302: 1-12.

15. Ligler FS. Perspective on optical biosensors and integrated sensor systems. Anal Chem. 2009; 81: 519-526.

16. Yager P, Domingo GJ, Gerdes J. Point-of-care diagnostics for global health. Annu Rev Biomed Eng. 2008; 10: 107-144.

17. Cavalli G, Banu S, Ranasinghe RT, Broder GR, Martins HF. Multistep synthesis on SU-8: combining microfabrication and solid-phase chemistry on a single material. J Comb Chem. 2007; 9: 462-472.

18. Wilson R, Cossins AR, Spiller DG. Encoded microcarriers for high-throughput multiplexed detection. Angew Chem Int Ed Engl. 2006; 45: 6104-6117.

19. Braeckmans K, De Smedt SC, Leblans M, Pauwels R, Demeester J. Encoding microcarriers: present and future technologies. Nat Rev Drug Discov. 2002; 1: 447-456.

20. Lea P, Keystone E, Mudumba S, Kahama A, Ding SF. Advantages of multiplex proteomics in clinical immunology: the case of rheumatoid arthritis: novel IgXPLEXâ„¢: planar microarray diagnosis. Clin Rev Allergy Immunol. 2011; 41: 20-35.

21. Robinson WH, DiGennaro C, Hueber W, Haab BB, Kamachi M. Autoantigen microarrays for multiplex characterization of autoantibody responses. Nat Med. 2002; 8: 295-301.

22. Carson RT, Vignali DA. Simultaneous quantitation of 15 cytokines using a multiplexed flow cytometric assay. J Immunol Methods. 1999; 227: 41-52.

23. Prabhakar U, Eirikis E, Davis HM. Simultaneous quantification of proinflammatory cytokines in human plasma using the LabMAP assay. J Immunol Methods. 2002; 260: 207-218.

24. de Jager W, te Velthuis H, Prakken BJ, Kuis W, Rijkers GT. Simultaneous detection of 15 human cytokines in a single sample of stimulated peripheral blood mononuclear cells. Clin Diagn Lab Immunol. 2003; 10: 133-139.

25. Olsson A, Vanderstichele H, Andreasen N, De Meyer G, Wallin A. Simultaneous measurement of beta-amyloid(1-42), total tau, and phosphorylated tau (Thr181) in cerebrospinal fluid by the xMAP technology. Clin Chem. 2005; 51: 336-345.

26. de Jager W, Rijkers GT. Solid-phase and bead-based cytokine immunoassay: a comparison. Methods. 2006; 38: 294-303.

27. Maier R, Weger M, Haller-Schober EM, El-Shabrawi Y, Theisl A. Application of multiplex cytometric bead array technology for the measurement of angiogenic factors in the vitreous. Mol Vis. 2006; 12: 1143-1147.

28. McDuffie E, Obert L, Chupka J, Sigler R. Detection of cytokine protein expression in mouse lung homogenates using suspension bead array. J Inflamm (Lond). 2006; 3: 15.

29. Dunbar SA, Vander Zee CA, Oliver KG, Karem KL, Jacobson JW. Quantitative, multiplexed detection of bacterial pathogens: DNA and protein applications of the Luminex LabMAP system. J Microbiol Methods. 2003; 53: 245-252.

30. Chen R, Lowe L, Wilson JD, Crowther E, Tzeggai K, et al. Simultaneous Quantification of Six Human Cytokines in a Single Sample Using Microparticle-based Flow Cytometric Technology. Clin Chem. 1999, 45: 1693-1694.

31. Fulton RJ, McDade RL, Smith PL, Kienker LJ, Kettman JR Jr. Advanced multiplexed analysis with the FlowMetrix system. Clin Chem. 1997; 43: 1749-1756.

32. Kayal S, Jaïs JP, Aguini N, Chaudière J, Labrousse J. Elevated circulating E-selectin, intercellular adhesion molecule 1, and von Willebrand factor in patients with severe infection. Am J Respir Crit Care Med. 1998; 157: 776-784.

33. De Freitas I, Fernández-Somoza M, Essenfeld-Sekler E, Cardier JE. Serum levels of the apoptosis-associated molecules, tumor necrosis factor-alpha/tumor necrosis factor type-I receptor and Fas/FasL, in sepsis. Chest. 2004; 125: 2238-2246.

34. Reinhart K, Bayer O, Brunkhorst F, Meisner M. Markers of endothelial damage in organ dysfunction and sepsis. Crit Care Med. 2002; 30: 302-312.

35. Bozza FA, Salluh JI, Japiassu AM, Soares M, Assis EF. Cytokine profiles as markers of disease severity in sepsis: a multiplex analysis. Crit Care. 2007; 11: R49.

36. Ewis AA, Zhelev Z, Bakalova R, Fukuoka S, Shinohara Y. A history of microarrays in biomedicine. Expert Rev Mol Diagn. 2005; 5: 315-328.

37. Nambiar S, Mirmohammadsadegh A, Bär A, Bardenheuer W, Roeder G. Applications of array technology: melanoma research and diagnosis. Expert Rev Mol Diagn. 2004; 4: 549-557.

38. Bustin SA, Dorudi S. Gene expression profiling for molecular staging and prognosis prediction in colorectal cancer. Expert Rev Mol Diagn. 2004; 4: 599-607.

39. Weiming Zheng, Lin He. Multiplexed Immunoassays. Norman HL Chiu, Theodore K Christopoulos (eds). In: Advances in Immunoassay Technology. Croatia: Intech. 2012; 143-164.

40. Stoll D, Bachmann J, Templin MF, Joos TO. Microarray technology: an increasing variety of screening tools for proteomic research. Drug Discov Today. 2004; 9: S10-17.