9

Multiplexed Bead Immunoassays: Advantages and Limitations in Pediatrics

Emma Burgos-Ramos, Gabriel Ángel Martos-Moreno,
Jesús Argente, Vicente Barrios*
Department of Endocrinology, Hospital Infantil Universitario Niño Jesús, Instituto de Investigación La Princesa, Department of Pediatrics, Universidad Autónoma de Madrid, Madrid, and Centro de Investigación Biomédica en Red de Fisiopatología Obesidad y Nutrición (CIBERobn), Instituto de Salud Carlos III, Spain

1. Introduction

The development of flow cytometric bead-based technology has afforded new perspectives in basic and clinical investigation, allowing for the simultaneous measurement of multiple analytes in biological samples. In this chapter, we will analyze this innovative technology based upon the use of small fluorescent particles coated with highly specific antibodies.

When performing an immunoassay, a specific antibody is used to identify and quantify the concentration of target molecules or analytes in complex samples, such as serum or urine. The use of monoclonal antibodies, highly specific, was a great improvement in these assays. Currently, the combination of these specific antibodies with different fluorophores and novel detection technologies has allowed the achievement of higher sensitivity and several improvements in this technique.

This review will be focused on the multiplexed bead immunoassay (MBIA), which has emerged as a powerful tool to simultaneously quantify several analytes in limited sample volumes. This is of great interest in Pediatrics, given the difficulty to obtain biological samples, particularly in newborns and the clinical interest of this specific type of analysis has increased in recent years (Lee et al., 2008). In fact, MBIA presents several advantages over the classically used immunoassays in pediatric samples, showing better reliability and consistency in the measurements, what is of great interest in longitudinal studies and clinical trials (Bomert et al., 2011).

This multi-analyte analysis method is a solid-phase immunoassay sandwich that uses a capture monoclonal antibody for every molecule aimed to study, that are joined to a specific microsphere with unique features. This microsphere combines two fluorescent compounds that allow for the discrimination from other particles in the assay. A second antibody recognizes another epitope, and detects each analyte bound to the complex, by using several

* Corresponding Author

detection methods, such as secondary or tertiary antibodies with fluorescent probes or the use of phycoerytrin-streptavidin, among others (Chandra et al., 2011). A flow cytometer, based in X-map technology, recognizes and integrates the emission of the last signal discriminating it according to the specific emission of each microsphere (Vignali et al., 2000). Currently, the color-coded microspheres allow the simultaneous performance of up to 100 determinations.

However, the development of assays focused on the diagnosis in pediatric disorders is still sparse, with new antibody detection panels needed, especially in growth and pubertal disorders. The acceptance of MBIA depends on the acquisition of comparable results to those achieved by using classical techniques, such as radioimmunoanalysis (RIA) or enzyme-linked immunosorbent assay (ELISA), accepted as "gold standards" to date. Although some studies have comparatively quantified the measurement of some hormones by using classical inmunoassays and MBIA (Liu et al., 2006), further comparisons are needed, especially for analytes present at low concentrations, that show significant differences in their values according to the methodology used. Therefore, the establishment of reference values for the pediatric population and the improvement in the detection of those parameters present in a low concentration in samples constitute new challenges for both, the investigators and the MBIA manufacturers.

2. Multiplexed bead immunoassays: principles and technology development

The limited sample volume and time-saving gains of the MBIA have made it an election technique for studies involving multiple factors, such as cytokines and pituitary hormones, among others (Djoba-Siawaya et al., 2008). In fact, these complex profiles require an arduous procedure and high volume samples when numerous analytes were analyzed by classical methods. Nevertheless, this laborious process has been improved by the introduction of fluorescent bead assays.

2.1 General considerations

The analytes measured by the immunoassays are extensive, including proteins and low molecular weight molecules, and have been widely utilized in the diagnosis for over forty years (Tetin & Stroupe, 2004). Immunoassays were applied initially to the determination of hormones and the antibodies used were polyclonal (Yalow & Berson, 1959). Its routine use led to a revolution in the field of endocrinology, as well as to the introduction of new tests for the diagnosis and management of endocrine disorders. Subsequently, the use of this technique was extended to other areas, such as biochemical disorders and oncology, by the determination of enzymes, vitamins and tumor markers.

Nevertheless, these classical assays show several limitations. Probably the most important, is that they only measure the levels of one analyte as a time. Proteins are a part of a complex system and clinical diagnosis requires the determination of multiple factors to interpret the pathophysiology of a single health problem. Thus, these requirements increase the cost, due to the number of assays, with the subsequent economic pressures for the Health Systems. In addition, the time spend in their performance is increased and this must be optimized in a diagnostic setting. Besides, the determination of several analytes included into an endocrine system may lead to different error degrees in each individual determination that may avoid

a desirable interpretation of the illness. In addition, there is a minimal risk of radiation and the problems of stability of radioisotopes may interfere in the quality of radioimmunoassay.

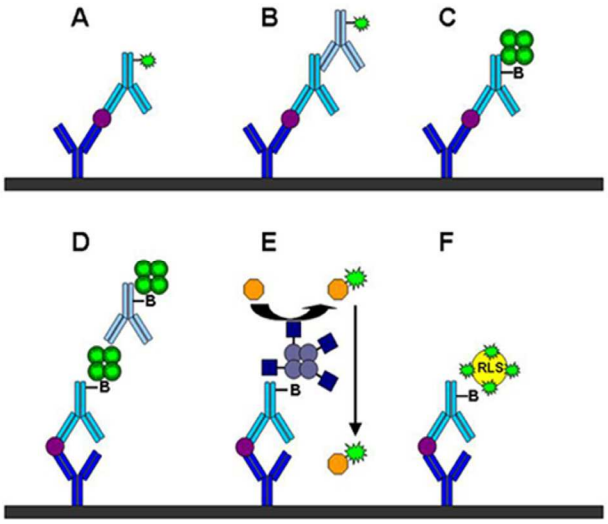
All these concerns have been minimized in the past decades. As previously reported, one of the greatest advances has been the use of monoclonal antibodies, which represents a continuous source of identical molecules with high affinity and specificity (Hoogenboom, 2005). Also, the development of software for data processing and the incorporation of non-isotopic reporters, such as enzymes that generate chromogenic, fluorescent and chemiluminescent products have improved these classical assays. The development of amplification methods (Figure 1) together with the use of above mentioned products has improved the sensitivity of these assays, allowing for the progressive use of smaller sample size, making it possible their application to pediatric samples (Barrios et al., 2007).

2.2 Basic principles

The discovery of new fluorescent compounds has improved the sensitivity of classical immunoassays. In recent years, protein microarrays have emerged as a powerful tool to provide quantitative data of proteins in biological samples. In traditional protein microarrays, capture reagents are immobilized in defined locations and in the presence of substrates, microspots are generated. After that, the deconvolution of the multiplexed measurements, allows for the identification and quantification of the set of selected proteins (Mirzabekov & Kolchinsky, 2002).

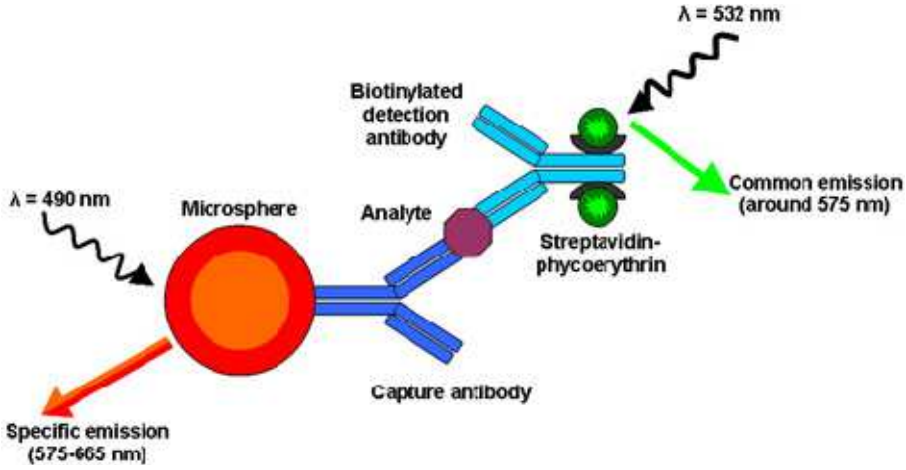
A key advance in multiplexed measurements has been the spatial segregation of the mentioned immobilized protein, encoded beads yield so-called MBIA. Multiplexed bead immunoassay kits contain microspheres, that are highly uniform with the same size (around 3-6 μm, depending of the manufacturer) and are polystyrene beads cross-linked during polymerization for physical and thermal stability. These microspheres are grouped into sets; each one is subsequently embedded with specific quantities of red and orange fluorescent dyes. The different proportion of these dyes gives each specific set of microspheres a unique spectral signature. Each specific set of microspheres is used as the solid support for the conjugation with a distinct reactant for each particular analyte. Among the reactants, there are enzyme substrates, antigens, receptors and antibodies.

In the MBIA, the capture antibody of each immunosorbent assay is coupled to one of 100 different microsphere bead sets, a 10 x 10 matrix with capacity and the combination of red and orange florescent dyes. After the capture of the analyte, the complexes are washed applying vacuum separation. The vacuum is applied to a 96-well plate, in order to separate the liquid, whereas the microspheres are retained in the filter. Afterwards, the addition of a solution with a second biotinylated antibody followed by resuspension allows for the creation of a "sandwich". After a new washing, a complex of streptavidin conjugated to phycoerythrin is added. The amount of the analyte binded to the beads is then quantified through the use of phycoerythrin, a green-fluorochrome reported dye, excited by the array reader at a wavelength of 532 nm, whereas the emission is detected around 575 nm (Figure 2). Thus, in each immunosorbent assay, the intensity of this green fluorochrome measured by the reader is directly proportional to the amount of analyte bound to the surface of each microsphere (Thrailkill et al., 2005; Van der Heyde & Gramaglia, 2011).



A, fluorescent bound detection antibodies. B, fluorescent labeled compounds-tertiary antibodies. C, biotinylated detection antibodies with streptavidin-phycoerythrin conjugate. D, streptavidin-phycoerythrin conjugate staining amplified with biotinylated anti-streptavidin-phycoerythrin antibodies. E, streptavidin-linked horseradish peroxisade linked to a species-specific tertiary antibody activates chemiluminiscent substrates or generates different flourophores. F, resonance light-scattering colloid gold particles coated with an antibiotin antibody. Modified from Nielsen & Geierstanger. J Immunol Methods 2004; 290: 107-120.

Fig. 1. Signal generation and amplification methods.



The capture monoclonal antibody is coupled to the bead (microsphere). After binding of the analyte a second biotinylated antibody is added. After addition of a streptavidin-phycoerythrin conjugate, dyes embedded in the beads and phycoerythrin are excited (wavy lines) and both compounds give two types of light emission. Modified from Barrios et al., Rev Esp Pediatr 2007; 63: 157-161.

Fig. 2. Schematic representation of an isolated reaction of a MBIA with the reagents used in this technique.

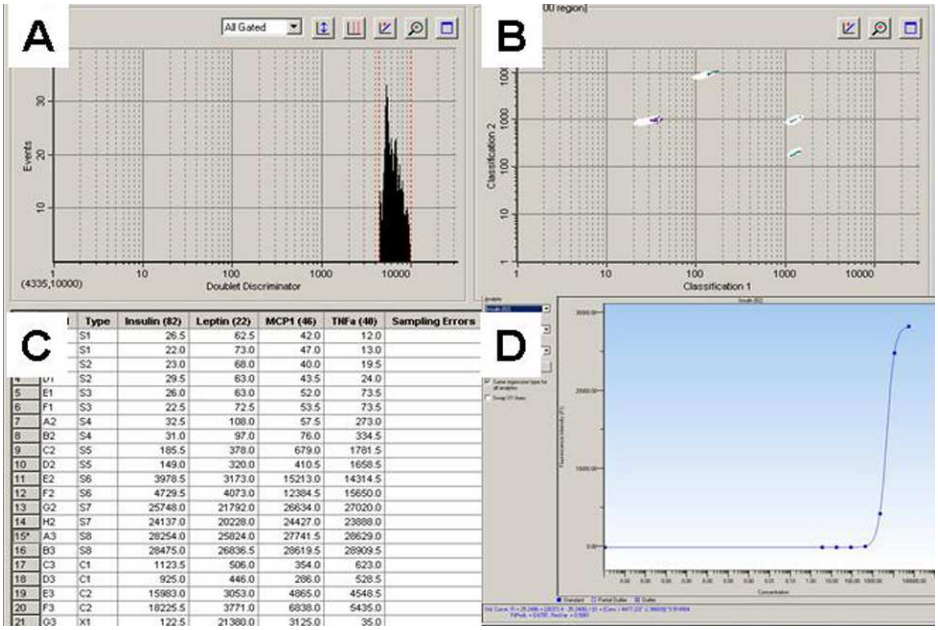
There are two lasers in the adapter array reader for MBIA, the first laser classifies each microsphere and its bound analyte and the second quantifies the amount of analyte bound to each microsphere. The first laser, known as red laser, excites the dyes of each microsphere and the fluorescent signal of each microsphere is separated with selective filters and converted in intensity units by the combination of fluorescent detectors and a digital processor, being the microsphere arranged. The second laser, named green laser, stimulates the fluorochromes bound to the analytes (in our case, phycoerythrin) and the signal is distinguished with emission filters and translated to intensity units by specific detectors and a signal processor, and the amount of analyte is measured. This data are acquired by different adapted flow cytometers attached to computers with special software (Figure 3).

Fig. 3. Suspension array system with high throughout put fluidics system.

This special cytometer is a dual-laser, flow-based microplate reader system. The content of each well is drawn up into the reader. The laser and associated optics detect the fluorescence of the individual beads and the fluorescence signal on the bead surface. This identifies each assay and reports the levels of target protein in the well. The detected intensity of fluorescence on the beads indicates the quantity of analytes. Thus, the system calculates the green fluorescence and the combination of red and orange fluorescence of each microsphere by using three detectors. The software separates the pool of microspheres using the orange and red fluorescence data and integrates these data with the average amount of green fluorescence for each bead set (Figure 4). A high-speed digital processor manages the data output, analyzed as fluorescence intensity on the software.

2.3 Assay guidelines and technical considerations

One of the main focuses of this review is to analyze the assay procedure in order to obtain reliable results with this technique. The current literature contains many examples of user and manufacturer product evaluations; however, some recommendations that will improve the performance of MBIA must be taken into account.



Each set of microbeads is quantitated in the histogram (A) and separated by the Luminex© system in a two-dimensional bead map (B), permitting the simultaneous quantitation of different analytes in the same sample. Results are given as fluorescence mean intensity (C) and the concentration of the analyte in the problem sample is obtained by extrapolation from a standard curve (D).

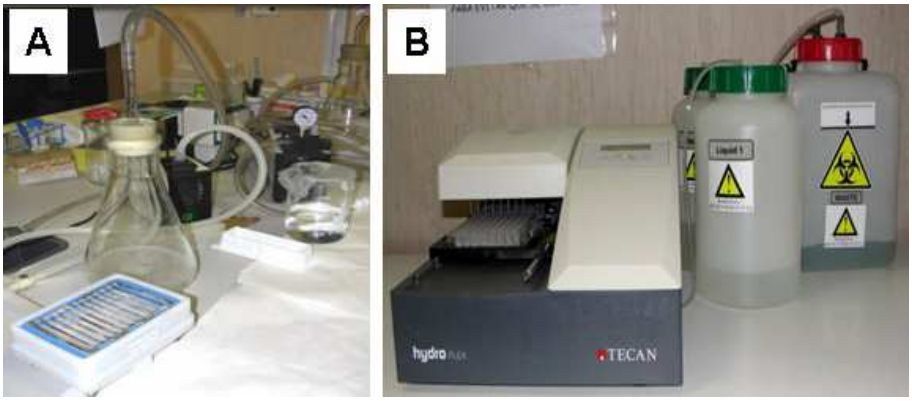
Fig. 4. Separation and quantification of the analyte concentrations by the suspension array reader system.

As the microspheres have different fluorescent dyes that are light sensitive, we must protect the beads from direct light by covering the tubes with aluminium foil. Sometimes, this procedure is not necessary, because the manufacturer provides the MBIA kits with dark tubes, thus avoiding light exposition. We also must keep the microspheres at 4ºC, avoiding freezing.

Another problem is the eventual aggregation of the microspheres. Sonication during 15-60 seconds keeping the tubes on ice is a procedure to separate aggregated microspheres, but it may cause hurt in the microsphere suspension. We usually used gentle vortexing during 5-10 minutes to obtain a homogeneous mixture. Thus, we may avoid not only a potential harmful effect on the sample, but also the possible loss of a part of the liquid phase during sonication. Another common alternative process is the use of a bath sonicator, by placing the probe sonicator tip in a bath of water and inserting the tube with microspheres near the tip, avoiding touching it.

Multiplexed bead immunoassays require washing during the assay period to separate the analyte of second antibody bound to the microsphere surface from the unbound reactants. The most common procedure is the vacuum separation. The reactions are performed in microtiter filter-bottom 96-well plates and vacuum is applied to the plate allowing the liquid to filter through while retaining the microbeads on the filter (Figure 5A). A new procedure

is the use of magnetic microbeads and conventional 96 well plates. In this situation, a microplate washer with a magnetic platform is used to separate these microspheres of unbound fraction (Figure 5B).



A, vacuum manifold system. The microtiter filter-bottom 96-plate is on a support and vacuum is applied with a conventional pump. Liquid is collected into a manifold. B, Microplate washer with magnetic platform. Conventional ELISA 96-plate and magnetic microbeads are used to separate liquid of microbeads.

Fig. 5. Separation of microbeads and liquid containing unbound fraction.

We may also develop new MBIAs, coupling the capture antibodies to the microspheres. First, we must choose a set of microbeads with significant diverse proportion of dyes fluorescence compounds (red and orange) in order to maintain sufficiently separated the reading areas obtained in the two-dimensional diagram by the suspension array reader system (see figure 5B). In this way, we will avoid potential overlapping among different bead readings.

Microspheres with fluorescent dyes are supplied at standard concentrations, but during the coupling process may diminish due to loss during washing. This loss is not uniform and it may change for multiple causes, such as pressure of vacuum manifold, and reactant employed in coupling, among others (Bio-Plex Manager Software, User Guide). It is difficult the optimization of the assay because we must take into consideration the different proportions of reactants (antibodies, analytes, fluorophore conjugates, assay buffers, etc.), temperatures and times of incubation and perhaps the most critical variable, the total surface area (total number of microbeads). To obtain reliable results, it is necessary to know the number of microspheres by counting them in a hemocytometer. These are also crucial aspects in the new MBIA using small quantity of sample, the microfluidic bead-based immunoassay that it was be developed to perform a multiplexed assay in a capillary, requiring only 1 μl assay volume (Yu et al., 2010).

Monoclonal capture antibodies must be bound to a specific each set of microspheres. There are some kits to perform this procedure; however is difficult to establish not only the adequate number of beads and concentration of the capture antibody, but also the quantity of secondary antibody to obtain a good assay performance (Djoba-Siawaya et al., 2008). Assay performance of new developed MBIAs must be compared with classical methods, such as radioimmunoassay o ultra-sensitive enzyme-linked immunosorbent assay,

considered as "gold standards" in the laboratory (Krouwer et al., 2002; Elshal & McCoy, 2006). The development of new panels using MBIA requires testing the effect of the biological matrix. Serum or plasma specimens are complex samples that are frequently diluted before addition in the well. The diluent has to imitate the sample matrix in order to obtain the same fluorescent background. There are many commercially available diluents and it is also necessary to analyze the effect of dilution and its addition to interpolate unknown concentrations (Pfleger et al., 2008).

Another aspect is the optimization of the parameters of the acquisition of data and analysis of results in the reader, by using the associated software. We must start by adjusting the needle according the manual instructions. The height of the array reader sample needle must be adjusted when the style of microtiter plate has been modified, to optimize the sample acquisition. The next step is the calibration of the array reader, necessary for optimal performance and reproducibility of results (Bio-Plex Manager Software, User Guide). Commercial manufacturers provide some kits containing calibration microspheres with stable fluorescent intensities in the emission wavelength ranges of the classification channels. The calibration process employs these microbeads to regulate voltage settings for optimal microsphere classification and reporter readings over time.

After preparation of the protocol, we need to define additional characteristics, as the number of bead counts, sample size and bead map selection to obtain an adequate histogram and bead map (Figures 4A & 4B). These graphs are updated during the reading (Figure 4C). Results are extrapolated from each standard curve for each analyte (Figure 4D). At the end of the reading process, the software generates a results file, containing the data, protocol parameters used and analysis tools for interpreting the data. Thus, we can reanalyze raw data by testing the effect of dilution or the regression method used. We are also able to change the double discriminator gate range and to recalculate the data based in the new range. During the reading, this discriminator of the array reader determines the amount of light scatter of the beads detected by the red laser. Particle size is proportional to light scatter and an internal discriminator gate identifies particles smaller o larger than single microbeads, including aggregates that may interfere in the results.

In addition to these studies, development of a new MBIA or the inclusion of a new analyte to a pre-existent multiplexed panel also requires the analysis of classical assay characteristics, as sensitivity, specificity, precision, recovery and linearity, among others (Krouwer et al., 2002; Liu et al., 2005; Dossus et al., 2008; Martos-Moreno et al., 2010).

3. Interest of MBIA in pediatrics

The study of growth hormone axis, pituitary hormone panel or cytokine expression profiling, among other examples, have become as established guidelines for the identification and characterization of several diseases. However, the determination of multiple parameters by classical immunoassays is a laborious process requiring a big amount sample volume, a problematic aspect for patients, especially when these subjects are newborns or children.

Multiplexed immunoassays based on protein microarray platforms have been used in the detection and confirmation of biomarkers associated with several diseases (Hsu et al., 2008; Sauer et al., 2008; Paczesny et al., 2009). Nevertheless, most of these studies have been

performed in adults and it is necessary to carry out these determinations in children. Among studies in children, most of them have been conducted to analyze serum cytokine profiling (Pranzatelli et al., 2011). In addition, most of the parameters determined in boys and girls during pubertal development show variations in their concentrations, due to anthropometric and biochemical changes influencing some of these variables. Thus, MBIA is a good method, because it allows for the simultaneous determination of multiple parameters in a single assay, avoiding inter-assay variations. This property offers reliable data and could help in the diagnosis and or follow up of pediatric patients. Thus, different studies, clinical trials or monitoring of pediatric patients during disease treatment require the determination of multiple parameters during extended periods of time (Martos-Moreno et al., 2011a). As an example, in the Table 1, we show the effect of weight loss on several adipokines in obese children after dietary intervention.

This technique also facilitates in obtaining a "pool" of reference values during childhood and puberty and may improve the knowledge of the physiology of any given endocrine axis. As it is mentioned above, the determination of several analytes at the same time, avoid the variability among them and allows a better interpretation of the physiology of the studied axis. In addition, the obtaining of reference data in children involves the recruitment of boys and girls in different Tanner stages, due to the effect of sex and biochemical changes through pubertal development. MBIA could be a choice method, as not only integrates all parameters in one determination, but also allows analyzing longitudinal changes in all of them. Here we show reference data of different adipokines in boys and girls through childhood and adolescence (Table 2).

Group	Adiponectin	Leptin	Resistin	TNF-α	IL-6
Control	15.8 ± 6.3	4.3 ± 3.1	13.8 ± 6.2	4.6 ± 2.2	2.2 ± 2.0
Obese B	16.3 ± 7.4	36.9 ± 13.6**	14.7 ± 6.5	6.1 ± 2.0	2.5 ± 2.0
Obese -1	19.1 ± 7.2	16.4 ± 13.3**##	12.2 ± 5.5	5.4 ± 3.2	2.1 ± 1.5
Obese -2	25.7 ± 11.4*#	16.1 ± 11.3**##	15.7 ± 8.4	4.8 ± 2.4	2.0 ± 1.3

Adiponectin, leptin, resistin, tumoral necrosis factor-α (TNF-α) and interleukin-6 (IL-6) levels were measured by MBIA in healthy children (control) and prupubertal obese subjects at baseline (Obese B) and after reduction of their body mass index by 1 SDS (Obese -1) and 2 SDS (Obese -2). Data expressed as mean ± standard deviation. *p<0.01, **p<0.001 vs. control; #p<0.01, ##p<0.001 vs. obese B. Modified from Martos-Moreno et al. Clin Chem Lab Med 2010; 48: 1439-1446.

Table 1. Serum concentrations of adipokines in control and obese children

Another aspect of special interest in Pediatrics is the sensitivity of the MBIAs. Serum samples of children and newborns have low levels of some analytes and sensitivity is crucial to determine them. The fluorescent readout if MBIA is more sensitive than the colorimetric signal of ELISA, where it is required a step of enzyme amplification. Moreover, the sensitivity can be augmented by reducing the number of bead in each assay, increasing in this manner the ratio of analyte to capture antibody without reducing the number of capture antibodies per bead. In addition, MBIA may be more reproducible than ELISA. Thus, the replicates usually show little variation in fluorescence, whereas ELISA significant variations between experiments and between plates within assays (Leng et al., 2008). Multiplexed bead

assays are more accurate because the data are calculated from the mean of 50-100 beads, each of which functions as an individual replicate. This is an additional advantage in longitudinal studies, very frequents in pediatric population.

The MBIAs may be adapted to carry out immunoassays for identifying antibodies. It is necessary to introduce variations in the assay design (Morgan et al., 2004). Here, purified antigens are conjugated to the beads and incubated with any sample of interest, followed with species-specific anti-immunoglobulin reagent, labelled with a fluorochrome. The mean fluorescence intensity is directly proportional to the amount of antibody bound to the antigen on the bead. These adapted assays have been employed to detect antibodies in diagnosis of celiac disease (Yiannaki et al., 2004), autoimmune thyroid disease (Tozzoli et al., 2006) and meningitis (Shoma et al., 2011) during childhood.

Multiplex applications are not restricted to the detection of proteins and antibodies, as this technique has also been used during a decade for the simultaneous detection of different DNA sequences. Among the specific applications include genotyping of single nucleotide polymorphisms, screening of genetic diseases, genotyping of the major histocompatibility complex and molecular analysis of infectious organisms (Ye et al.., 2001; Cesbron-Gautier et al., 2004; Dunbar et al., 2003). The main use of MBIA in this field has been the detection of mutations associated with disease; so, one of the first applications of genotyping was the analysis of multiple variants of β-globin in capillary blood of neonates (Colinas et al., 2000). Also this technology has been used for genotyping in samples from patients with a predisposition to thrombophilia (Musher et al., 2002) and detection of fusion transcripts of chromosomal translocations in children with acute lymphoblastic leukemia (Wallace et al., 2003).

Tanner stage	Adiponectin	Leptin	Resistin	TNF-α	IL-6
I					
Female	15.6 ± 4.2	4.8 ± 3.6	13.5 ± 5.0	4.2 ± 1.6	2.6 ± 2.2
Male	16.2 ± 5.6	3.9 ± 2.7	12.6 ± 6.3	4.2 ± 2.0	1.9 ± 1.8
II					
Female	16.7 ± 12.2	5.7 ± 2.7	15.9 ± 4.3	4.0 ± 1.6	2.6 ± 2.2
Male	17.0 ± 5.0	4.1 ± 2.8	16.9 ± 7.1	4.0 ± 1.3	2.4 ± 1.8
III + IV					
Female	18.0 ± 6.2	11.4 ± 4.2	16.5 ± 3.8	4.6 ± 1.7	1.3 ± 0.4
Male	13.4 ± 6.8	6.4 ± 2.7	16.8 ± 7.0	4.4 ± 1.3	1.5 ± 0.6
V					
Female	12.1 ± 4.9	12.1 ± 4.3	29.4 ± 8.9	4.9 ± 1.1	1.5 ± 1.2
Male	18.3 ± 5.2	6.2 ± 3.1	22.8 ± 8.5	4.6 ± 1.6	1.0 ± 0.5

Adiponectin, leptin, resistin, tumoral necrosis factor-α (TNF-α) and interleukin-6 (IL-6) levels were measured by MBIA in healthy girls and boys in the different pubertal stages (Tanner stage). Data expressed as mean ± standard deviation. Modified from Martos-Moreno et al. An Pediatr (Barc.) 2011b; 74: 356-362.

Table 2. Reference values of adipokines in children throughout development

4. Limitations

In spite of the advantages of this technology, it is important to keep in mind the scarce development of assays in the field of pediatric disorders. Therefore, the development of new multiplexed panels is needed, especially in those pediatric pathologies related to endocrinology of growth and pubertal disorders.

Special attention should be paid when reporting absolute values with MBIA. The acceptance of this technique depends on the acquisition of comparable results to those achieved by using classical techniques, accepted as the "gold standards" in the laboratory. To date, we and others have compared these methodologies for some hormones (Martos-Moreno et al., 2010; Liu et al., 2005), but further comparisons are needed, especially for analytes that are present at very low concentrations in pediatric samples, since the concentrations obtained with MBIA and classical ultra-sensitive immunoassays are distinct for some analytes and also show that the differences between the methods are composed of both constant and proportional components, preventing a direct comparability between both assays.

A limitation in some MBIAs is the interpretation and reporting of analyte values at extremely low concentrations. One of the possible advantages of this assay is that the dynamic range seems to be much broader than classical ELISAs. However, many sensitivity issues in the very low range of concentrations remain unresolved (Liu et al., 2005; Leng et al., 2008), especially for several cytokines.

Therefore, the establishment of reference values for the pediatric population and the improvement in the detection of determined parameters that are present at low levels in serum is a challenge for the investigators and the MBIA manufacturers, respectively.

5. Future research

These methods have an excellent accuracy and reliability, together with an excellent sensitivity for most analytes. However, some analytes are currently determined by ultra-sensitive immunoassays. A challenge of this technology is to improve the sensitivity of some analytes present at very low concentrations in biological fluids, as well as in tissues, in order to its applicability in new areas of routine diagnosis.

Another important aspect is the improvement of specificity for some parameters, which is essentially limited by the quality of antibodies employed in the MBIA (Vignali, 2000). Thus, the use of some monoclonal antibodies in classical immunoassays, such as enzimoimmunoassay or radioimmunoassay, does not affect in a significant manner the results, but it may be a big issue for MBIA. The near future will show whether MBIA exhibit a greater applicability in diagnostics in Pediatrics.

Another objective is the development of commercial kits that allow the simultaneous analysis of factors that require extractive procedures or alternative processes for assessment, such as growth factors, together with other molecules in which it is not necessary this preliminary stage. It is also required the improvement of MBIAs to evaluate the molecular heterogeneity of certain hormones that have several isoforms (Popii & Baumann, 2004). In

this sense, the development of immunology allows characterizing new monoclonal antibodies against different epitopes, solving some problems of inadequate sensitivity or cross-reactivity with classical antibodies (Feldhaus et al., 2003).

These assays will improve the throughput by greatly enhancing the quantity of information achieved from a single experiment. Moreover, the development of new microfluidic bead-based immunoassays will allow diminishing the expenditures of reagents and reducing the requirements of biological sample, very important in pediatric patients. Based upon their capabilities, MBIA will be proposed for use in disease screening and probably will open up new possibilities in the follow up of patients during therapy.

6. Conclusion

Multiplexed bead immunoassays could result more cost-effective for the measurements of selected analytes, diminishing inter-assay variations and reducing the volume of sample needed, what would be particularly interesting in Pediatrics, especially when limited amounts of samples are available, which is usually the case of young children. The current advantages in time, ability to simultaneously measure analyte concentrations in the same conditions and high performance of multiplex analysis will allow MBIA to be a more useful tool in evaluating the pediatric diseases.

7. Acknowledgments

The authors wish to thank Dr. Julie Ann Chowen for English revision and critical review of the manuscript. This work was supported by grants of Fondo de Investigación Sanitaria (CD07/00256 to E.B.R.), CIBERobn and Fundación Endocrinología y Nutrición.

8. References

Barrios, V.; Martos-Moreno, G.A. & Argente, J. (2007). Aspectos metodológicos y utilidad diagnóstica de los inmunoensayos múltiples en suspensión en Pediatría. *Revista Española de Pediatría*, Vol.63, No.2, pp. 157-161, ISSN 0034-947X

Bio-Plex Manager Software 4.1. User Guide. (2005) Bio-Rad Laboratories, Inc. 2000 Alfred Nobel Drive. Hercules, CA, USA, P/N 10003414.

Bomert, M.; Köllisch, G.; Roponen, M.; Lauener, R.; Renz, H.; Pfefferle, P.I. & Al-Fakhri, N. (2011) Analytical performance of a multiplexed, bead-based cytokine detection system in small volume samples. *Clinical Chemistry and Laboratory Medicine,* (DOI: 10.1515/CCLM.2011.631), ISSN 1434-6621

Cesbron-Gautier, A.; Simon, P.; Achard, L.; Cury, S.; Follea, G. & Bignon J.D. (2004). Luminex technology for HLA typing by PCR-SSO and identification of HLA antibody specificities. *Annales de Biologie Clinique (Paris),* Vol.62, No.1, pp. 93-98, ISSN 0003-3898

Chandra, H.; Reddy, P.J. & Srivastava S. (2011). Protein microarrays and novel detection platforms. *Expert Review of Proteomics,* Vol.8, pp. 61-79, ISSN 1478-9450

Colinas, R.J.; Bellisario, R. & Pass, K.A. (2000). Multiplexed genotyping of beta-globin variants from PCR-amplified newborn blood spot DNA by hybridization with allele-specific oligodeoxynucleotides coupled to an array of fluorescent microspheres. *Clinical Chemistry,* Vol.46, No.7, pp. 996-998, ISSN 0009-9147

Djoba Siawaya JF, Roberts T, Babb C, Black G, Golakai HJ, Stanley K, Bapela NB, Hoal E, Parida, S.; van Helden, P. & Walzl G. (2008). An evaluation of commercial fluorescent bead-based luminex cytokine assays. *PLoS One,* Vol.3:e2535, ISSN 1932-6203

Dossus, L.; Becker, S.; Achaintre, D.; Kaaks, R. & Rinaldi, S. (2009) Validity of multiplex-based assays for cytokine measurements in serum and plasma from "non-diseased" subjects: comparison with ELISA. *Journal of Immunological Methods,* Vol. 350, pp. 125-132, ISSN 0022-1759

Dunbar, S.A.; Vander Zee, C.A.; Oliver, K.G.; Karem, K.L. & Jacobson, J.W. (2003). Quantitative, multiplexed detection of bacterial pathogens: DNA and protein applications of the Luminex LabMAP system. *Journal of Microbiological Methods,* Vol.53, No.2, pp. 245-252, ISSN 0167-7012

Elsshal, M.F. & McCoy, J.P. (2006). Multiplex bead array assays: performance evaluation and comparison of sensitivity to ELISA. *Methods*, Vol.38, No.4, pp. 317-323, ISSN 1046-2023

Feldhaus, M.J.; Siegel, R.W.; Opresko, L.K; Coleman, J.R.; Feldhaus, J.M.; Yeung, Y.A.; Cochran, J.R.; Heinzelman, P.; Colby, D.; Swers, J.; Graff, C.; Wiley, H.S. & Wittrup. KD. (2003). Flow-cytometric isolation of human antibodies from a nonimmune Saccharomyces cerevisiae surface display library. *Nature Biotechnology,* Vol.21, No.2, pp. 163-170, ISSN 1087-0156

Hoogenboom, H.R. (2005). Selecting and screening recombinant antibody libraries. *Nature Biotechnology,* Vol.23, pp. 1105-1116, ISSN 1087-0156

Hsu, H.Y.; Wittemann, S.; Schneider, E.M.; Weiss, M. & Joos T.O. (2008) Suspension microarrays for the identification of the response paterns in hyperinflammatory diseases. *Medical Engineering & Physics,* Vol.30, No.8 , pp. 976-983, ISSN 1350-4533

Krouwer, J.S.; Tholen, D.W.; Garber, C.C.; Goldschmidt, H.M.J.; Harris-Kroll, M.; Linnet, K.; Meier, K.; Robinowitz, M. & Kennedy, J.W. (2002) Method comparison and bias estimation using patient samples; approved guideline. 2nd edition (EP9-A2). *Clinical and Laboratory Standards Institute*; Vol.22, No.19, pp. 1-53, ISSN 0273-3099.

Lee SA; Tsai HT; Ou HC; Han CP; Tee YT; Chen YC; Wu, M.T.; Chou, M.C.; Wang, P.H. & Yang, S.F. (2008). Plasma interleukin-1beta, -6, -8 and tumor necrosis factor-alpha as highly informative markers of pelvic inflammatory disease. *Clinical Chemistry and Laboratory Medicine,* Vol.46, No.7, pp. 997-1003, ISSN 1434-6621

Leng, S.X.; McElhaney, J.E.; Walston, J.D.; Xie, D.; Fedarko, N.S. & Kuchel, G.A. (2008) ELISA and multiplex technologies for cytokine measurement in inflammation and aging research. J*ournal of Gerontology Series A Biological Sciences and Medical Sciences,* Vol.63, No.8, pp. 879-884, ISSN 1079-5006

Liu, M.Y.; Xydakis, A.M.; Hoogeveen, R.C.; Jones, P.H.; Smith, E.O.; Nelson, K.W. & Ballantyne, C.M. (2005). Multiplexed analysis of biomarkers related to obesity and the metabolic syndrome in human plasma, using the Luminex-100 system. *Clinical Chemistry,* Vol.51, No.7, pp. 1102-1109, ISSN 0009-9147

Martos-Moreno, G.A.; Burgos-Ramos, E.; Canelles, S.; Argente, J. & Barrios, V. (2010) Evaluation of a multiplex assay for adipokine concentrations in obese children. *Clinical Chemistry and Laboratory Medicine,* Vol.48, No.10, pp. 1439-1446, ISSN 1434-6621

Martos-Moreno, G.A.; Kratzsch, J.; Körner, A.; Barrios, V.; Hawkins, F.; Kiess, W. & Argente J. (2011a) Serum visfatin and vaspin levels in prepubertal children: effect of obesity and weight loss after behavior modifications on their secretion and relationship with glucose metabolism. *International Journal of Obesity (London),* (DOI: 10.1038/ijo.2010.280), ISSN 0307-0565

Martos-Moreno, G.A.; Burgos-Ramos, E.; Canelles, S.; Argente, J. & Barrios, V. (2011b). Analysis of insulin and cytokines during development using a multiplexed immunoassay: implications in pediatrics. *Anales de Pediatría (Barcelona),* Vol.74, No.6, pp. 356-362, ISSN 1695- 4033

Mirzabekov, A. & Kolchinsky, A. (2002). Emerging array-based technologies in proteomics. *Current Opinion in Chemical Biology,* Vol.6, No.1, pp. 70-75, ISSN 1367-5931

Morgan, E.; Varro, R.; Sepulveda, H.; Ember, J.A.; Apgar, J.; Wilson, J.; Lowe, L.; Chen, R.; Shivraj, L.; Agadir, A.; Campos, R.; Ernst, D. & Gaur, A. (2004). Cytometric bead array: a multiplexed assay platform with applications in various areas of biology. *Clinical Immunology,* Vol.110, No.3, pp. 252-266, ISSN 1521-6616

Musher, D.; Goldsmith, E.; Dunbar, S.; Tilney, G.; Darouiche, R.; Yu Q.; López, J.A. & Dong, J.F. (2002). Association of hypercoagulable states and increased platelet adhesion and aggregation with bacterial colonization of intravenous catheters. *The Journal of Infectious Diseases,* Vol.186, No.6, pp. 769-773, ISSN 0022-1899

Nielsen, U.B. & Geierstanger, B.H. (2004) Multiplexed sandwich assays in microarray format. *Journal of Immunological Methods,* Vol.290, pp.107-120, ISSN 0022-1759

Paczesny, S.; Krijanovski, OI; Braun, TM; Choi, SW; Clouthier, SG; Kuick, R; Misek, DE; Cooke, KR; Kitko, CL; Weyand, A; Bickley, D; Jones, D.; Whitfield, J.; Reddy, P.; Levine, J.E.; Hanash S.M. & Ferrara J.L. (2009). A biomarker panel for acute graft-versus-host disease. *Blood,* Vol.113, No. 2, pp. 273-278, ISSN 0006-4971

Pfleger, C.; Schloot, N. & ter Veld, F. (2008). Effect of serum content and diluent selection on assay sensitivity and signal intensity in multiplex bead-based immunoassays. *Journal of Immunological Methods,* Vol.329, No.1-2, pp. 214-218, ISSN 0022-1759

Popii, V. & Baumann, G. Laboratory measurement of growth hormone. *Clinica Chimica Acta,* 2004; Vol.350, No.1-2, pp. 1-16, ISSN 0009-8981

Pranzatelli, M.R.; Tate, E.D.; Travelstead, A.L. & Verhulst, S.J. (2011) Chemokine/cytokine profiling after rituximab: reciprocal expression of BCA-1/CXCL13 and BAFF in childhood OMS. *Cytokine,* Vol.53, No.3, pp. 384-389, ISSN 1043-4666

Sauer, G.; Schneiderhan-Marra, N.; Kazmaier, C.; Hutzel, K.; Koretz, K.; Muche, R.; Kreienberg, R.; Joos, T. & Deissler, H. (2008). Prediction of nodal involvement in breast cancer based on multiparametric protein analyses from preoperative core needle biopsies of the primary lesion. *Clinical Cancer Research,* Vol.14, No.11, pp. 3345-3353, ISSN 1078-0432

Shoma, S.; Verkaik, N.J.; de Vogel, C.P.; Hermans, P.W.; van Selm, S.; Mitchell, T.J.; van Roosmalen, M.; Hossain, S.; Rahman, M.; Endtz, H.P.; van Wamel, W.J. & van Belkum, A. (2011). Develpoment of a multiplexed bead-based immunoassay for the simultaneous detection of antibodies to 17 pneumococcal proteins. *European Journal of Clinical Microbiology & Infectious Diseases,* Vol.30, No.4, pp. 521-526, ISSN 0934-9723

Tanner, J.M. (1962). Growth and Adolescence, 2nd edition. Oxford: Blackwell Scientific Publications.

Tetin, S.Y. & Stroupe, S.D. (2004). Antibodies in diagnostic applications. *Current Pharmaceutical Biotechnology,* Vol.5, pp. 9-16, ISSN 1389-2010

Thrailkill, K.M.; Moreau, C.S.; Cockrell, G.; Simpson, P.; Goel, R.; North, P.; Fowlkes, J.L. & Bunn, R.C. (2005). Physiological matrix metalloproteinase concentrations in serum during childhood and adolescence, using Luminex Multiplex technology. *Clinical Chemistry and Laboratory Medicine,* Vol.43, No.1, pp. 1392-1399, ISSN 1434-6621

Tozzoli, R.; Villalta, D.; Kodermaz, G.; Bagnasco, M.; Tonutti, E. & Bizzaro, N. (2006). Autoantibody profiling of patients with autoimmune thyroid disease using a new multiplexed immunoassay method. *Clinical Chemistry and Laboratory Medicine,* Vol.44, No.7, pp. 837-842, ISSN 1434-6621

Van der Heyde, H.C. & Gramaglia, I. (2011) Bead-based multiplexed analysis of analytes by flow cytometry. *Methods in Molecular Biology,* Vol.699, pp. 85-95, ISSN 1064-3745

Vignali, DAA. (2000). Multiplexed particle-based flow cytometric assays. *Journal of Immunological Methods,* Vol. 243, pp. 243-255, ISSN 0022-1759

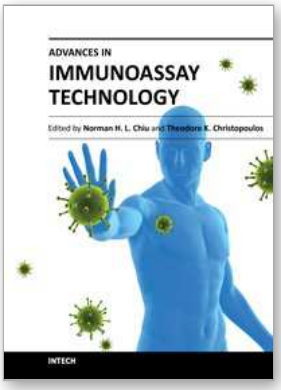
Wallace, J.; Zhou, Y.; Usmani, G.N.; Reardon, M.; Newburger, P.; Woda, B. & Pihan, G. (2003). BARCODE-ALL: accelerated and cost-effective genetic risk stratification in acute leukemia using spectrally addressable liquid bead microarrays. *Leukemia,* Vol.17, No.7, pp. 1404-1410, ISSN 0887-6924

Yalow, R.S & Berson, S.A. (1959). Assay of plasma insulin in human subjects by immunological methods. *Nature,* Vol. 184, pp. 1648-1649, ISSN 0028-0836

Ye, F.; Li, M.S.; Taylor, J.D.; Nguyen, Q.; Colton, H.M.; Casey, W.M.; Wagner, M.; Weiner, M.P. & Chen, J. Fluorescent microsphere-based readout technology for multiplexed human single nucleotide polymorphism analysis and bacterial identification. *Human Mutation,* 2001; Vol.17, No.4, pp. 305-316, ISSN 1059-7794

Yiannaki, E.E.; Zintzaras, E.; Analatos, A.; Theodoridou, C.; Dalekos, G.N. & Germenis, A.E. (2004). Evaluation of a microsphere-based flow cytometric assay for diagnosis of celiac disease. *Journal of Immunoassay and Immunochemistry,* Vol.25, No.4, pp. 345-357, ISSN 1532-1819

Yu, X.; Hartmann, M.; Wang, Q.; Poetz, O.; Schneiderhan-Marra, N.; Stoll, D.; Kazmaier, C. & Joos, T.O. (2011) µFBI: a microfluidic bead-based immunoassay for multiplexed detection of proteins from a µl sample volume. *PLos one*, Vol.5, No.10, e13125, ISSN 1932-6203



Advances in Immunoassay Technology

Edited by Dr. Norman H. L. Chiu

ISBN 978-953-51-0440-7
Hard cover, 180 pages
Publisher InTech
Published online 23, March, 2012
Published in print edition March, 2012

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.

INTECH
open science | open minds

InTech Europe
University Campus STeP Ri
Slavka Krautzeka 83/A
51000 Rijeka, Croatia
Phone: +385 (51) 770 447
Fax: +385 (51) 686 166
www.intechopen.com

InTech China
Unit 405, Office Block, Hotel Equatorial Shanghai
No.65, Yan An Road (West), Shanghai, 200040, China
中国上海市延安西路65号上海国际贵都大饭店办公楼405单元
Phone: +86-21-62489820
Fax: +86-21-62489821