Original Articles

Multiplexed and Microparticle-based Analyses: Quantitative Tools for the Large-Scale Analysis of Biological Systems

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While the term flow cytometry refers to the measurement of cells, the approach of making sensitive multiparameter optical measurements in a flowing sample stream is a very general analytical approach. The past few years have seen an explosion in the application of flow cytometry technology for molecular analysis and measurements using microparticles as solid supports. While microsphere-based molecular analyses using flow cytometry date back three decades, the need for highly parallel quantitative molecular measurements that has arisen from various genomic and proteomic advances has driven the development in particle encoding technology to enable highly multiplexed assays. Multiplexed particle-based immunoassays are now common place, and new assays to study genes, protein function, and molecular assembly. Numerous efforts are underway to extend the multiplexing capabilities of microparticle-based assays through new approaches to particle encoding and analyte reporting. The impact of these developments will be seen in the basic research and clinical laboratories, as well as in drug development. © 2006 International Society for Analytical Cytology

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A major goal for biomedical research in the 21st century will be to collect and integrate molecular information about genes, proteins, and numerous other biomolecules into the working models of cell and organism function from which predictions can be made. The rationale for pursuing such an ambitious goal stems from the very significant advances in molecular analysis that enable the sequencing of whole genomes, the highly parallel analysis of gene expression levels, and large scale identification of proteins in complex samples. These advances resulted from new molecular reagents and assay chemistries, new instrumentation with improved sensitivity and throughput, new computational tools, and a significant change in focus for experimental biology from one that focuses on individual molecules to one that considers the abundance and interactions of many different molecules as they function in networks of biochemical pathways in living systems.

However, just as these new technologies have enabled the rapid acceleration of data collection and interpretation, continued progress toward transforming this information into biological understanding is dependent on continued improvement in analytical technologies. In particular, it is critical to augment qualitative analysis methods that allow the identification of important molecules with quantitative measurements of their abundance and function. The ability to make quantitative measurements of the concentrations of many individual proteins, their interactions and the formation of macromolecular assemblies, and the measurement of these assemblies in live cells and organisms represent major challenges in understanding the systems of molecular networks and pathways that underlie physiology and disease. These challenges are being addressed through developments in microscopic imaging, optical spectroscopy, and mass spectrometry. In this article, we highlight how a new class of applications for flow cytometry, multiplexed molecular analyses using suspension array technology, is providing an important new set of tools for quantitative systems biology.

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EXPANDING THE APPLICATIONS OF FLOW CYTOMETRY: MULTIPLEXED MOLECULAR ANALYSIS

Flow cytometry is a term that aptly describes the major application of a very general analytical technique for the optical analysis of single particles in flowing sample streams. This approach to the study of cells has become so important that thousands of flow cytometers are present in universities, medical schools, and other academic and industrial research facilities throughout the world, and flow cytometric methods have impacted nearly every area of biomedical science. In the shadow of applications that focus on the analysis of whole cells, the general approach of making sensitive optical measurements in flowing sample streams has been exploited to make high resolution measurements of a variety of biological systems ranging from whole organisms(1,2) to single fluorescent molecules (3). For these measurements, the more general term of flow microfluorimetry might be used, but regardless of the terminology preferred, it is evident that the principles that underlie flow cytometry are useful for a great many more biological applications in addition to measuring cells.

One such application has its roots in the 1970s, when it was recognized that microparticles bearing antigen could serve as solid supports for the capture of antibodies from blood, and that by using differently-sized microspheres that could be distinguished by their light scatter properties several analytes may be detected simultaneously (4). In the 1980s and 1990s, this approach was extended to include antibodies (5), DNA (6), and lipids (7) displayed on the surface of microparticles. Today, this approach is gaining wide use; thanks to the ready availability of color-coded sets of multiplexing microspheres, assay kits, and instruments and softwares especially designed to facilitate bead-based multiplexed assays.

BEADS, DYES, INSTRUMENTS, AND ASSAYS

As described earlier, the use of microspheres as solid supports for molecular analysis using flow cytometry dates back to the 1970s, but a renewed interest in this approach, stimulated by a new set of biological questions that demand multiplexed measurements, has led to the development of reagents and instruments specifically designed to support multiplexed microparticle-based analyses. The key factor in the resurgence of microsphere-based assays was the development of fluorescence-encoded microspheres exhibiting discrete intensities of fluorescence from two different fluorescence dyes from Luminex Corporation. Ten intensities of two colors enable the encoding of 10^2 different microspheres that can be discriminated by a flow cytometer. These color codes can be thought of as forming a microarray, similar in function to the popular flat microarrays, but with single beads serving as array elements in a two dimensional fluorescence space replacing "spots" on a flat surface. The addition of ten intensities of one or two additional dyes would add dimensions to the encoding space and enable the development of microsphere sets with 10^3 or 10^4 discrete particle populations. While the analysis of such multi-color beads would be trivial for most flow cytometers, the preparation of such microspheres is apparently not, and the largest set of commercially available beads currently numbers ~100 encoded populations. Additional factors that limit the level of multiplexing are the reagents (antibodies, oligonucleotides, etc) and assay chemistries used (antibody sandwich assay, hybridization, PCR), which can exhibit significant cross reactivity and loss of quantitative response at high multiplex levels.

While the multiplexing levels on any platform can be limited by the features of the assay chemistry, there are many biological applications that are greatly enhanced by multiplexed analysis at the level of one hundred, or even a dozen, analytes. To address these more modest scale multiplex applications, microspheres encoded with different intensities of a single fluorophore in one or more sizes are now available from several commercial sources. These microsphere sets are designed to be used in conjunction with commonly available commercial bench top flow cytometers and enable anyone with access to a flow cytometry core facility to set up their own assays. The development of multiplexed assays requires some time and experience and, while resources are available to guide the researcher (8), many biologists will prefer to purchase assay kits that have been optimized and validated. At present, commercially available assay kits are available for the multiplexed measurement of cytokine and chemokine levels and for a handful of other immunology applications. In the following sections we highlight the major classes of multiplexed assays.

MULTIPLEXED ANALYSIS OF PROTEIN ABUNDANCE

Key measurements for understanding cell function are the amount and modification state of proteins. Mass spectrometry is presently the technique most closely identified with proteomic analysis. Coupled to a separation method, such as two dimensional gel electrophoresis, this tool is invaluable for identifying proteins in complex mixtures. While the excellent mass resolution of mass spectrometry is very useful for the qualitative identification of proteins, or more specifically peptide fragments thereof, variability in ionization efficiencies makes quantification of peptide/protein abundance difficult (9). Approaches using pre-analysis derivitization of samples with isotope-coded affinity tags have been developed to measure the relative abundance of proteins from an experimental and control sample (10) in a manner analogous to the two color fluorescence labeling method described earlier, but these provide limited information of the absolute concentrations of targets, owing to uncertainties in ionization efficiency for different molecules.

The standard for quantitative protein analysis is the enzyme-linked immunosorbant assay (ELISA). While the mode of reporting may vary (absorbance, chemiluminescence, fluorescence), the essence of the ELISA is the formation of a antibody–antigen "sandwich" complex involving an immobilized capture antibody, the target of interest captured from a sample, and a labeled reporter

antibody. The sensitivity of the sandwich immunoassay is generally determined by the affinity of the antibodies used, and can be in the pg/ml range with monoclonal antibodies with high affinity and specificity. The microwell plate is the most commonly used solid support for capture antibody determination, providing a format that is compatible with automation and the analysis of many samples. However, microplate-based ELISAs typically require ~100 μl of sample and measure only a single analyte.

Building on the approach of microarray-based gene expression analysis, several configurations of antibody arrays are being used for protein analysis. These arrays are comprised of antibodies spotted onto membranes, glass slides, or other solid supports and with detection being performed in a couple of different ways. The most quantitative approach employs the same sandwich assay format used for the ELISA (11), with purified protein standards being used for quantification. These approaches can give sensitivities approaching or even surpassing that of a conventional microwell plate-based single analyte ELISA, but require care to set up. Antibody cross reactivity can compromise both the sensitivity and specificity of the assay, and so antibodies must be screened and characterized in a multiplexed environment before use. Competitive assay formats can have a wider linear dynamic range (11), but generally have lower sensitivity and face the same limitations for multiplexing as the sandwich assays. A second class of assays involves the prelabeling of proteins prior to binding to immobilized antibodies (12). In this format, no second reporter antibody is required, making antibody cross-reactivity less of an issue and sample preparation times shorter. However, uncertainties regarding the variation of labeling among protein targets and the reproducibility between samples make this approach semi-quantitative at best. Purified protein standards are not typically used. A variation on this approach labels an experimental sample with one color of dye and a control sample with a second color of dye, and measures the ratio of the two colors after mixing and application to the antibody array (13). This two-color method allows for some normalization for sample to sample variation, but still measures only relative abundance rather than absolute concentrations.

While the spotted slide or membrane are the best known microarray platforms for highly parallel analysis, the advantages of encoded microparticles for multiplexed analysis are being increasingly appreciated. The most well-developed systems involve fluorescently-encoded microspheres analyzed via flow cytometry (14–16). The concept of using microspheres as solid supports for multiplexed assays by flow cytometry goes back nearly to three decades, but has recently been realized as a general use platform for the analysis of molecular interactions (14,17) and multiplexed analysis (18–21) through the availability of relatively low cost instruments with multiplexed assays as a primary appplication (Luminex LX100, Becton Dickenson FACSArray), commercial assay kits (Becton Dickenson, Bender Medsystems-BioRad, Biosource, OneLambda, R&D Systems, Upstate, and others), and encoded beads (Bang's Laboratories, Duke Scientific, Luminex, Spherotech). The microsphere-based platform has been used for analysis of receptor–ligand interactions, enzyme–substrate interactions, genetic analysis, and, most widely, immunoassays.

Bead-based immunoassays generally employ the sandwich assay design in which a microsphere-bound antibody captures an analyte and a fluorescence-labeled antibody is used as a reporter for measurement. By employing different capture antibodies on distinct microspheres and cocktails of reporter antibodies, it is possible to measure simultaneously the levels of multiple analytes. Standards relate the assessed fluorescence to concentration of analyte in the experimental sample. Early examples of these multiplexed assays featured "home-brewed" kits designed by individual researchers to detect multiple cytokines (18,22,23). Assay kits that detect as many as 22 different cytokines simultaneously based on this approach are currently available from a number of commercial sources. Smaller panels are available for phosphorylated signaling molecules, transcription factors, and matrix metalloproteases.

A second class of immunoassays, in which antigen is displayed on beads, aim to detect and characterize antibodies circulating in blood. In a typical assay configuration, purified antigen is immobilized on beads and used to capture antibodies present in blood or plasma, which is detected with a labeled class-or isotype-specific secondary antibody. This general approach has been used to detect exposure to infectious disease (5,24–27) and to monitor antigen-specific antibody responses to infection or vaccination (28,29).

In general, the bead-based systems provide sensitivity equal to or better than the conventional ELISA counterpart, require less sample processing time, and have the ability to measure multiple analytes simultaneously in samples as small as 20 μl or less of sample. Like the ELISA or any other immunoassay, the sensitivity and specificity of multiplexed microsphere assays depend on the use of high affinity, high specificity antibodies. Issues of specificity become even more critical in multiplexed assays and are major factors limiting high levels of multiplexing in immunoassays.

MICROSPHERE-BASED NUCLEIC ACID ANALYSIS

A second major area where optically encoded microsphere arrays are making an impact is in the area of nucleic acid analysis. For genetic and genomic analysis, the number of molecular features targeted for analysis can be quite large, tens of thousands in the case of gene expression analysis, hundreds of thousands in the case of single nucleotide polymorphisms. The flat DNA microarray, or DNA chip, developed from the need to make highly parallel molecular measurements from a single sample, and has emerged as a workhorse analysis platform (30). However, for studies involving large numbers of samples, the flat chip format is not especially well-suited to high throughput analysis. Optically encoded arrays of microspheres offer numerous advantages in terms of preparation and use over the conventional flat microarray.

Multiplexed nucleic acid applications can be grouped into two main categories: sequence detection and sequence analysis. Sequence detection applications include

gene expression analysis and the detection of PCR products. Sequence analysis is generally concerned with variations in nucleic acid sequence. The analysis of single nucleotide polymorphisms (or SNPs) has important implications for areas ranging from disease diagnostics and drug discovery to bacterial identification and forensic analysis, and the combination of a high level of multiplexing with the high serial throughput of flow cytometry make microsphere arrays an attractive format for these applications.

The primary step in the preparation of microsphere arrays for nucleic acid analysis is the functionalization of the microsphere surface with a nucleic acid. The nucleic acid is most often a synthetic oligonucleotide, but could also be an oligonucleotide analogue or natural DNA or RNA, depending on the application. As for immobilization of antibodies for immunoassays, nucleic acids may be immobilized on the microsphere surface using non-covalent physical adsorption, affinity binding methods, or covalent conjugation. The adsorption of DNA to a variety of surfaces, especially glass, is well known, and while glass beads have been used to display DNA for flow cytometric analysis, the relative lack of stability of the interaction as well as the fact that the current generation of commercial multiplexing microsphere reagents are composed of hydrophobic polymers make this approach of limited utility. More common are the use of affinity tags to tightly, but non-covalently, attach nucleic acid to a surface, especially biotin-modified nucleic acids to avidin- or streptavidin-functionalized surfaces. Such affinity interactions are generally tight, specific, and fairly stable, and have been used in a number of multiplexed applications. However, in terms of stability and specificity, the most widely used nucleic acid immobilization methods involve covalent attachment. The most popular approach is to couple amino-modified nucleic acids to carboxylated surfaces using carbodiimide chemistry. Synthetic oligonucleotides are readily obtained with an amino-modification at one or both ends, or at an internal position. Longer nucleic acids can be produced by enzymatic methods (i.e. PCR) by using amino-modified oligonucleotide primers or amino-modified nucleotide analogues. Sulthydral-modified nucleic acids can be prepared in similar manners for attachment via maleimide chemistry or for binding to gold surfaces. For both covalent and affinity-based non-covalent attachment, the inclusion of a multi-carbon linker to serve as a spacer seems to improve the efficiency of hybridization to a surface immobilized nucleic acid, though the degree of improvement can vary.

In general, nucleic acid analysis methods involve an assay chemistry and a readout, or detection step. Reverse transcriptase (for RNA targets), polymerase chain reaction, and other amplification strategies provide both amplification of the target sequence and opportunities to incorporate labeled nucleotide analogues for detection. For example, when PCR is performed using a labeled primer or nucleotide, the resulting PCR product is labeled. The PCR product can then be captured onto a microsphere by hybridization and detected. This approach has been used to analyze mRNA in gene expression studies (31,32) and leukemia detection (33,34) to measure microRNAs in human cancers (35), to detect nucleic acids from bacteria and viruses (36–40), and to discriminate single nucleotide polymorphisms(41,42). Variants of this method include the use of branched DNA as detection probes to amplify signals in mRNA analysis (43) and employing an immobilized allele-specific capture oligo, and a second allele-specific reporter oligo has been used to determine the haplotype of amplified DNA fragments (44).

Several approaches couple signal generation to the hybridization event for the detection of unlabeled nucleic acids in solution, and some of these have been adapted to microspheres. The Invader assay uses a structure-specific nuclease to cleave a quenched primer annealed to a target DNA (45,46) Performed in solution, this reaction exhibits isothermal amplification, in which a single target molecule can support the cleavage of many reporter molecules, and this approach has been demonstrated in the detection of a single nucleotide polymorphism on beads (47,48). Molecular beacons are hybridization probes with a hairpin structure that keeps a quencher in close proximity to a fluorophore (49). Upon hybridization to a target sequence, the hairpin structure is disrupted and the fluorophore. This approach has recently been adapted to microspheres for the single-plex detection of synthetic oligonucleotides and single stranded PCR products (50).

The direct capture of nucleic acid targets by oligonucleotides immobilized on microspheres is straightforward in concept, but in practice there are several challenges. First, capture of long nucleic acids by an immobilized oligonucleotide is inefficient, and the PCR product must be kept well under 1,000 nucleotides in length. Second, when double stranded DNA is used, the complementary strand competes with the immobilized oligonucleotide for the target strand, further reducing capture efficiency. These issues necessitate additional steps such as preparation of single stranded target prior to capture. For the resolution of single base differences in target DNA it is generally necessary to fine tune the hybridization probes and conditions to achieve the desired specificity, a task that is significantly more involved for multiplexed assays.

To circumvent these issues, assays have been configured to interrogate PCR products in solution rather than directly capturing the target onto a surface. One approach is to use a competitive binding assay in which the target competes with a bead-bound probe for a labeled oligonucleotide. In this case, the presence of target depletes the amount of free-labeled oligonucleotide available to bind to a bead, thus decreasing the signal on the bead (19).

A second approach is to use the target in solution as a template for the incorporation of a label into an oligonucleotide probe, which can then be detected when it is captured onto a bead. The incorporation of this label is generally achieved using enzymes, either a DNA polymerase to incorporate labeled nucleotides or a DNA ligase to attach a labeled oligonucleotide. A key component of the probe capture approach was the development of universal arrays for multiplexed nucleic acid analysis (51–53). Based on sets of oligonucleotides designed to exhibit specific

hybridization to a reverse complement, but negligible hybridization to any other oligonucleotide in the set (54,55), Microspheres bearing such oligonucleotide tags offer significant flexibility for assay design and development. By encoding soluble primers and probes with the reverse complement of the microsphere tags, specific detection or genotyping primers can be captured onto specific microspheres for detection. Modification of an existing assay or development of a new assay does not require the preparation of new beads, only the design of new primers with an appropriate capture tags to target the primer to the correct microsphere. Such universal arrays have been combined with a variety of enzyme-based genotyping methods for the analysis of single nucleotide polymorphisms. Polymerase-mediated extension of nucleotide analogues (51,52,56,57), or ligase-mediated coupling of oligonucleotides (53) have been shown to allow high levels of multiplexed SNP genotyping in a variety of systems. These methods take advantage of the requirement of these enzymes for correct nucleotide base pairing to provide exquisite specificity to single nucleotide sequence changes.

Another variant of the capture approach uses capture probes to bind to mRNA in solution followed by capture onto microspheres and detection using a branched DNA detection scheme (43). The use of branched DNA provides significant signal enhancement, allowing the detection of mRNA directly without the need for PCR or other amplification steps. As can be seen from these examples, the approaches to nucleic acid analysis are quite diverse, and new applications appear regularly.

BEYOND ANTIBODIES AND OLIGONUCLEOTIDES

The examples described earlier for immunoassays and nucleic acid analyses represent the best-developed uses of microsphere arrays for multiplexed molecular analysis. A range of different assays have been developed on the microsphere platform for use in screening, detection, structure-function studies, and other applications. Of particular significance are analyses of ligand-receptor, enzyme-substrate, and other interactions that are targets for drug discovery.

Ligand–receptor analysis is a very important area in both basic research, for understanding molecular mechanisms of signal transduction, and in drug development, for the screening and characterization of potential receptor agonists and antagonists. Flow cytometry has long been used to study receptors in their native environment of the cell, and in recent years microparticle-based methods have been adapted to study receptors under more controlled, in vitro conditions. In both cases, at the particle densities typically used in flow cytometry (1×10^5–1×10^6/ml), the receptor concentration is quite low (less than a nanometer), making it possible to titrate even high affinity ligands without significant ligand depletion (58). This feature facilitates the accurate measurement of binding affinity. The ability of the flow cytometer to resolve free from bound ligand enables real time measurement of ligand association and dissociation, and allows for higher resolution determination of kinetic rate constants than discontinuous methods that require a wash step. These features of flow cytometry, plus the ability to perform multiplex measurements, offer unique advantages for both understanding the mechanisms of ligand-receptor interactions and for identifying and characterizing compounds that can modulate them.

A distinguishing feature of cell surface molecular assemblies is the presence of the lipid bilayer, which determines the environment of receptor molecules and can control function. To facilitate the study of these interactions in controlled in vitro environments, several researchers have used artificial membrane bilayers supported on the surface of glass microspheres. Gilbert and colleagues have measured the binding of a number of plasma proteins including FactorVIII (7,59,60) and lactadherin (61,62) to supported lipid membranes. Nolan and colleagues have used microsphere-supported bilayers to measure the interactions of cholera toxin to its cell surface receptor ganglioside GM1 to measure binding rate constants and affinities, as well as the aggregation of receptor molecules within the membrane. In each of these cases, the supported bilayer membranes exhibited behavior consistent with a fluid membrane in which individual lipid molecules are free to diffuse within the plane of the membrane.

G protein-coupled receptors (GPCRs) are a particularly large and important class of receptors involved in numerous physiological processes. Microspheres have served as solid supports for GPCRs (63), G protein subunits (63–66), their ligands (65) and regulators (67) for use in characterizing various aspects of signaling mechanisms. Neubig and colleagues used immobilized Gα subunits on microspheres to measure the interactions with fluorescence-labeled β/γ subunit (66) and a regulator of G protein signaling (RGS) protein (67). Sklar and colleagues have developed methods for the display of detergent-solubilized GPCRs (68,69) and used these to characterize receptor-binding compounds. By measuring the binding of fluorescent receptor to immobilized ligand on beads, compounds that inhibit the ligand receptor binding were analyzed in a competitive assay format (63). Receptor agonists are identified by their ability to change the affinity of the fluorescent receptor for beads bearing immobilized G protein. Combined with mechanistic models of the ligand-receptor-G protein ternary complex (65), these approaches are providing insight into the molecular basis of full- and partial agonism.

Nuclear receptors are an important class of intracellular receptors and potential drug targets. They regulate gene transcription in coordination with coactivator or corepressor proteins that interact with a receptor's ligand binding domain. These interactions are attractive targets for drug development, and Iannone and colleagues have arrayed peptide domains corresponding to the different activators or repressors, and measured the effect of small molecule compounds on the binding of labeled nuclear receptors to these immobilized peptides (70–72).

A variety of enzyme–substrate interactions have also been analyzed using microsphere-based methods, including protease (73) (Saunders et al., this issue) and nuclease (74–78). In these applications, a fluorescently labeled sub-

strate is attached to the microsphere in such a way that enzymatic cleavage of the substrate results in release of the label and a decrease in microsphere fluorescence. Because the concentration of the immobilized fluorescent substrate is very low, below the K_D for the enzyme substrate–complex, the enzyme is generally provided in excess. In this concentration regime, the reaction can be measured under pre-steady state or single turnover conditions, which allow fundamental kinetic rate constants to be determined in a more straightforward manner than under more conventional steady state conditions. The use of encoded microspheres can allow such quantitative measurements to be made on several targets simultaneously, increasing the throughput of screening assays.

CONCLUSIONS AND PROSPECTS

The increased awareness of flow cytometry as a platform for high resolution in vitro molecular analysis as well as cellular analysis has stimulated a number of new developments in both the technology of flow cytometry as well as its applications.

One area of active research is in the development of novel encoding schemes. For example, semiconductor quantum dots have been employed as an alternative to organic fluorophores for fluorescence-based encoding (79–81). The concept of encoding has been carried beyond fluorescence to encompass Raman-encoded polymer beads (82,83), metallic nanometer rods (84), and light-powered radiofrequency transducers (85). These developments have the potential to lead to more robust encoded microparticles and higher levels of multiplexing than is possible with microspheres encoded with conventional small organic fluorophores.

A logical development in the area of assay design is the integration of cell-based assays and bead-based assays in a single tube. This approach has been used to simultaneously measure activated cells and released cytokines in whole blood (86) and more examples are likely to follow. Further blurring the distinction between cell-based assays and bead-based assays is the notion of using cells as microparticles for multiplexed analysis (87). In this configuration, fixed bacteria or yeast cells may encode information and display receptors or other molecules, but are not themselves the object of study.

The primary focus of this article has been approaches that employ microparticles as solid supports for molecular analysis, but these methods are related to an expanding set of applications that enable highly multiplexed analysis of particles or cells that are then selected by sorting for analysis. These approaches include yeast (88–90) and bacterial (91,92) display of antibody fragments and other proteins, bead-based display of peptide and other combinatorial chemical libraries (93–95), and microemulsion-based screening of single DNA or protein molecules (96–98). The widespread use of flow cytometry for quantitative cell analysis, for the maturation of microparticle-based multiplexing, and for the development of new molecular screening approaches makes it a key multiuse platform to address the emerging challenges of quantitative biology.

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