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Flow Cytometry for Drug Discovery, Receptor Pharmacology and High Throughput Screening

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Summary

While flow cytometry is viewed as a mature technology, there have been dramatic advances in analysis capabilities, sorting, sample handling and sensitivity in the last decade. These advances contribute to its application for biological and chemical diversity, sample throughput, high content, and complex systems biology. This article will evaluate the new opportunities for flow cytometry relating to receptor assembly and pharmacology, as well as a range of screening applications.

Introduction

The modern flow cytometer analyzes and sorts cells or particles at rates up to 50,000 per second. Particles suspended in a liquid are passed through a focused laser beam and the optical signals are processed in real-time. While historically, single cells have been the particles of interest, flow cytometry is equally adept at examining microspheres. A broad range of flow cytometric applications for biotechnology includes applications in diagnostics and vaccine development, genomics, proteomics and protein engineering, drug discovery, reproductive biology, plant and marine biology, toxicology, and single molecule detection (1). The technology has been recently reviewed (2–4).

The modern flow cytometer measures multiple parameters (fluorescence intensity as well as light scatter) for each particle and discriminates cell or particle populations based on size, morphology, and other properties. Thus flow cytometry combines the potential for high content analysis, the ability to measure multiple parameters on a single class of particles, and with multiplexing, the ability to measure multiple classes of particles simultaneously. These features can be combined so that high content along with multiplexing is also possible.

It is less well-appreciated that flow cytometers can perform homogeneous analysis of molecular assemblies or ligand binding by discriminating the fluorescence of molecules associated with the particles in an environment of fluorescing molecules that are not associated with the particles. This discrimination may be observed at concentrations up to several hundred nanomolar concentration of the unbound species when the nonspecific binding to other assay components, which is reagent dependent, is limited. Commercial flow cytometers can detect 100’s to 1000’s of fluorescing molecules per particle depending upon the fluorescence properties of the molecule. Particles sizes detected range from submicron to tens of microns, with specialized instruments allowing detection and sorting to hundreds of microns.

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Detection of fluorescence or other optical properties of cells in flow cytometers can distinguish a range of cell characteristics including surface markers, protein expression, intracellular ions and other biochemical species, DNA content, redox state, and cell functions such as apoptosis, cell cycle, and phosphorylation status. The ability to examine particles at the rate of thousands per second has made flow cytometry clinically useful for diagnosing immune status or tumor prognosis, in basic research for evaluating signaling pathways through phosphoprotein analysis, and in industry to examine cell function ex vivo in animals or patients treated with drugs. There are thousands of flow cytometry assays in the literature (5).

Cell sorters can produce highly purified, viable cell populations for further analysis or research. Particularly exciting molecular technologies use combinatorial protein expression to create libraries of cells in which each cell is a separate assay for the expression of a single protein or peptide that produces a unique cell phenotype or in strategies for cell-based two hybrid analysis. Microspheres are used as solid supports for immunoassays of proteins, sequence analysis of nucleotides, and molecular assemblies of transcription factors or molecular assemblies involving receptors, signaling partners, toxins, and libraries of chemical compounds.

Given these capabilities, flow cytometry has only recently been used in screening of soluble compound libraries even though it has played an accessory role in other phases of drug discovery (6). In recent years, flow cytometers have been integrated with plate based sampling, with each well in the sample plate treated as an individual sample. Sampling rates, up to 96 wells in 30 minutes are practical for secondary screens, and pathway or biomarker analysis. Alternatively, the contents of entire plates can be delivered continuously, and treated as a single data file, permitting flow cytometry to be used in primary screening.

Diversity of biological targets

Flow cytometry is compatible with large numbers of biological assays and targets. Flow cytometry is used clinically with antibodies directed at specific antigens to enumerate leukocyte subsets. These cell surface receptors are classified in a Clusters of Differentiation (CD) system with specific CD patterns in specific cell populations. With multiple antibodies, subsets of blood cells can be identified, yielding the patient's immunophenotype. Human Immunodeficiency Virus (HIV) is monitored as a decrease of CD4-positive T cells, which decreases the ability for the patient to mount an immune response.

Probes for flow cytometry extend beyond antibodies (7). Fluorescent molecules include vital dyes, probes for DNA content that show a marked enhancement in fluorescence upon binding DNA, dyes that change fluorescence to measure intracellular calcium and other ions, probes for enzymatic activity, and probes for protein expression that involve fusion proteins with fluorescent proteins. Cell-based functional assays include stains and markers for surface, cytoplasmic, and nuclear components. Flow cytometry excels at phenotypic assays, such as antibody staining and light scatter (size and shape), to discriminate complex cell populations in hematology, immunology, cancer biology, reproductive biology, marine biology, microbiology, pharmacology, and toxicology, Methods compendia for flow cytometry include Methods in Cell Biology Volumes 33, 63, and 64 and Current Protocols in Flow Cytometry (5).

Flow cytometry has been extended to *ex vivo* analysis of *in vivo* efficacy of therapeutic agents (8). Cell-based assays evaluated the effects of the novel protein kinase C (PKC) inhibitor enzastaurin on intracellular phosphoprotein signaling (8) in blood obtained from patients before and after receiving daily oral doses of enzastaurin. These samples were stimulated *ex vivo* with phorbol ester and were then assayed for PKC activity. Multiparametric analysis of peripheral blood mononuclear cells showed that monocytes in cancer patients had decreased PKC activity following enzastaurin administration.

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Molecular Assembly

Flow cytometry allows homogeneous detection of molecular assembly, analysis of binding affinity, subsecond resolution of binding kinetics, often with femtomole sensitivity (9–13). While the best known binding applications in flow cytometry involve antibodies or other cell surface ligands, the particles detected by flow cytometry do not have to be cells or cellular constituents. Microspheres or beads of varying composition (latex, glass, and dextran) have been used to capture analytes or to study molecular binding interactions. Applications have included proteins, peptides, small molecules, oligonucloetides, toxins, and phospholipids with incorporated receptors. Attachment schemes range from non-specific absorption to epitope tagging schemes such as peptide epitopes for antibody capture, his-tagged proteins with Ni^{2+}-chelates, avidinbiotin, and glutathione to capture glutathione transferase fusion proteins. Targets include GPCR, channels and transporters, transcription factors, hybridization, kinases and phosphoproteins, proteases and nucleases. The possibility of detecting multiple colors means that assemblies with multiple components can be observed.

The molecular assembly assays are comparable to surface plasmon resonance (SPR) and scintillation proximity assays (SPA). In SPR, one component is displayed on a surface and the second component, using fluorescence rather than mass, is captured and assembled on the surface. In SPA, radiolabled assemblies cause particles to scintillate. Flow cytometry measurements are performed as static end point assays or with advanced sample handling as real-time assays with subsecond resolution.

Because flow cytometers measure a signal pulse for each particle above a background signal, it is possible to resolve bound assemblies at free concentrations to 100 nM and higher in a homogeneous format without a wash step (10). Moreover, assays can be performed quantitatively. Epitope tagging schemes with particles can be used to isolate, capture and display epitope tagged proteins from cell lysates, often without purification of the components. Recently, flow cytometry has been used in a screening mode to identify small molecules that inhibited the interaction of the signaling Gα subunit and the RGS protein (14). Bead assemblies have also been applied to analysis of protease-substrate interactions (15). Opportunities for analysis of molecular assembly in drug discovery are described in more detail below in the context of HTS flow cytometry.

G protein-coupled receptor molecular assemblies (16–18) have been investigated with solubilized a fusion protein consisting of the β2-adrenergic receptor and green fluorescent protein (β2-AR GFP). In one case, the β2-AR GFP was bound to beads displaying a conjugated ligand, dihydroalprenolol. This format permits analysis of the K_d for binding of the fusion protein to the ligand, and, in competition with other β2-adrenergic receptor ligands, provides K_d values for agonists and antagonists. The other format involved beads displaying chelated Ni^{2+} which bound purified his-tagged G protein heterotrimers. These beads bound the binary complex of agonists with β2-AR GFP. When the ligand concentration is varied, ternary complex formation between ligand, receptor, and G protein yield dose-response curves. The extent of the high affinity ternary complex formation plateaues at a constant high level for ligands previously classified as full agonists and at reduced levels for ligands previously classified as partial agonists. These bead assays showed that GTPγS induced dissociation rates of the ternary complex were the same for full and partial agonists. Taken together, these results suggested that the association rather than the dissociation of the signaling complex was what differentiated partial agonists from full agonists. By linking different assembly components onto the beads, it was possible to show that the fastest disassembly steps were not consistent with separation of the Gα and Gβγ subunits.

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When performed simultaneously with color coded beads, the two assemblies (ligand beads and G protein beads) discriminated between agonist, antagonist or inactive molecule in a manner appropriate for high throughput, small volume drug discovery. The ligand beads were sensitive to the presence of all ligands, while the G protein beads were sensitive only to agonists, and discriminated full and partial agonists. From a quantitative perspective, these studies show applications of flow cytometry for measurements of numbers of binding interactions per particle, the binding constants, and the rate constants, all in a homogeneous format.

High Content

Flow cytometry provides opportunities for multiplexing (19) and high content analysis that includes pixel by pixel imaging (3,20). The expansion of multiparameter flow cytometry has been driven by the need to understand the complexity in biological systems. High content flow cytometry is analogous to microscopy with multiple fluorescence signatures arising from multiple excitation sources and multiple emission wavelengths with imaging. A detailed review of cellular imaging in drug discovery has recently appeared (21).

Multiple fluorescence colors are also used to measure multiple analytes simultaneously with suspension arrays. Whereas in planar arrays the address system is spatial, affinity reactions occurring at defined locations on the array, in suspension arrays, the address is encoded into the particle as an optical signature. Optical signatures currently include fluorescent dyes as well as particle size and light scatter. With only two dyes, each encoding ten distinct levels of fluorescence, 100-plex arrays can be produced. Unique spectroscopic signatures, associated with Raman spectra, could potentially be encoded into larger arrays (http://www.ljbi.org/fundingj.htm). Multiplexed particle-based immunoassays to study genes, protein function, and molecular assembly are becoming routine. Applications include cytokine quantification, single nucleotide polymorphism genotyping, phosphorylated protein detection, and characterization of the molecular interactions of nuclear receptors. Multiplexed drug discovery screens are described below.

For cell suspensions, array technologies are also evolving. In receptor pharmacology, for example, cyanine-labeled neuropeptide Y has been used as a universal Y(1), Y(2), and Y(5) receptor agonist. Calcium mobilization was measured in different channels with the aid of fluo-4 and fura red. A combination of dyes allowed the simultaneous determination of Y(1), Y(2), and Y(5) receptor selectivity and receptor-mediated response at the same time (22).

Krutzik and Nolan (19) developed fluorescent cell barcoding to enhance the throughput of flow cytometry. For barcoding, each cell sample was labeled with a different signature, or barcode, of fluorescence intensity and emission wavelengths, and mixed with other samples before antibody staining and analysis by flow cytometry. Using three fluorophores, the authors were able to barcode and combine entire 96-well plates. Antibody consumption was reduced 100-fold and acquisition time was reduced to 5–15 minutes per plate. Using barcoding and phospho-specific flow cytometry, the authors screened a small-molecule library for inhibitors of T cell-receptor and cytokine signaling, simultaneously determining compound efficacy and selectivity. They also analyzed IFN-gamma signaling in cells from primary mouse splenocytes, revealing differences in sensitivity and kinetics between T cell and B cell subsets.

Highly multiplexed applications are needed in immunology where immune responses depend upon a network of cells expressing unique combinations of cell surface proteins. Multiparameter flow cytometry for clinical diagnostics has historically been limited by the available probes and instrumentation to about six colors. New multiple laser flow cytometers and new probes, including semiconductor nanocrystals (quantum dots), have recently extended the capabilities of flow cytometry to resolve 17 fluorescence emissions (23,24). Phenotyping multiple antigen-specific T-cell populations have promise in vaccine development as well as

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the understanding of adaptive and innate cellular immune mechanisms. These approaches are both multiplexed since multiple populations are resolved and high content since each population is characterized on the basis of multiple markers.

High Content Analysis of Phosphoprotein Networks

Phosphoprotein profiling is a mainstay of bead-based multiplexing where phosphoproteins from cell lysates are first captured by an antibody on a bead and then quantified by a second antibody. When performed with intact cells, the measurement of phosphoproteins in cells uses the same types of fluorescently labeled, phosphospecific antibodies (26,27). However, in contrast to detecting phosphoproteins as analytes, the information about the distribution of the phosphoproteins, on a cell by cell basis is retained. By exposing cancer-cell signaling networks to potentiated inputs, rather than relying upon the basal levels of protein phosphorylation, investigators have been able to identify unique profiles correlated with genetics and disease outcome (28). In addition to measuring and correlating individual parameters, high content technologies may contribute to a systems understanding of physiology and disease at the cell level. It has been possible to discriminate disease-modulating agents and drugs by analyzing a complex network of cell types, treatments and readouts by machine-learning algorithms (29). Multiparameter flow cytometry has recently been used to monitor eleven intracellular proteins and phospholipids in human peripheral blood cells subjected to nine treatments (30). The data were processed with machine-learning algorithms to identify known signaling pathways and to predict new ones to be verified experimentally.

Image Flow Cytometry

High content analysis has been extended to an image analysis flow cytometer that combines CCD technologies and an optical architecture for high sensitivity and multispectral imaging of cells (3,20,31). The system has fluorescence sensitivity comparable to a PMT-based flow cytometer with imaging in six channels and 0.5 micron pixel resolution. Biological applications include analysis of viral loads, and pathway components contributing to nuclear translocation and apoptosis (32). Coupling of morphometric with photometric measures made it possible to distinguish live cells from cells in the early phases of apoptosis, as well as late apoptotic cells from necrotic cells (33). The scale of the image data acquisition, on a cell by cell basis, limits throughput compared to intensity based measurements.

Sorting Speed and Particle Size

Flow cytometry sorts cell populations with the desired phenotype. Because sorting rates approach 50,000 cells per second (or 4.32 billion cells per day), practical applications range from sorting rare cell populations in complex mixtures to selecting transfected cells expressing specific targets for drug discovery. For these reasons, it is likely that cell sorting will play an important role in stem cell research. More recently, sorting in flow cytometry has been coupled with combinatorial techniques which enable primary screens to explore both biological and chemical diversity.

For biological diversity, cell systems are configured to express libraries of peptides and proteins for both protein engineering and drug discovery. Bacterial expression systems have become associated with protein engineering (34–37) with enzyme libraries displayed on the surface of microbial cells or microbeads. These libraries, with 10^9 different variants can be screened with fluorogenic substrates. The methods are applied to the identification of novel biocatalysts or the directed evolution of substrate specificity to investigate structure/function relationships. The approach has been relatively more successful in altering affinity and specificity as compared to enhancing catalytic activity (37). Protein engineering has potential applications in the synthesis of novel drugs *in situ* correction of genetic errors (36).

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As gene sequences are revelaed, efficient methodologies to functionally characterize these genes *in vivo* are needed. A novel drug discovery approach has used retroviral vectors in which combinatorial oligonucleotide inserts create intracellularly expressed peptides. With one vector per cell, each cell becomes an assay for the peptide encoded by that insert (38). Sorting cells with the desired phenotype, and sequencing the insert, can reveal novel regulatory pathways. When the library involves genomic inserts, the assays for the insert can reflect novel pathways of protein-protein interaction (39).

The sorting capabilities of flow cytometry have been extended from the 1–10 micron size range into the millimeter size range (http://www.unionbio.com/). Sorting rates for millimeter sized particles are typically reduced two orders of magnitude from the speeds achieved for micron sized particles. These new capabilities are adding to the utility of flow cytometry for analysis of chemical and biological diversity.

For chemical diversity, combinatorial libraries of small molecules can be displayed on microspheres (40) of sufficient size that the active molecules can be identified by mass spectrometry. These particles, in a one-bead one-compound (OBOC) format, can be used in conjunction with binding of fluorescence ligands, or receptors, as well as in cell-based applications to detect chemical species which interact with the desired target. Recently (41), combinatorial chemistry identified high affinity peptidomimetics against α4β1 integrin from both diverse and highly focused OBOC combinatorial peptidomimetic libraries. The molecule identified was used to image α4β1-expressing lymphomas in a mouse xenograft model when conjugated to a near infrared fluorescent dye.

In biological diversity, the new sorting technology may enable the analysis and sorting of small multicellular animals such as C. elegans, D. melanogaster, Daphnia, Medaka, Mosquito, Sea Urchin, Xenopus, Zebrafish, and Zooplankton as well as large cells or cell clusters such as adipocytes, duct cells, hepatocytes, pancreatic cells, and stem cells. These capabilities would amplify the power of flow cytometry for the secondary screening of more complex biological systems.

Sample Handling and Automation

Flow cytometry has traditionally been used for the analysis of individual samples. However, flow cytometers have now been coupled to a variety of input systems. For example, automation of delivery systems of flow cytometers is leading to their use with bioreactors that can be monitored continually (42,43), with multwell plates (see below), with subsecond reaction kinetics (10), and with devices that produce shear forces on cells or cell aggregates to model the environment of flowing blood (44,45).

Until recently, the throughput of flow cytometry for automated analysis of multiple discrete samples of cells has been limited, with commercial systems capable of processing ~2 samples per minute. This bottleneck precludes the screening of large compound collections. We have described successive generations of sample handling technology to address this issue. The first used a reciprocating multiport flow injection valve to deliver 10 endpoint assays per min, 4 on-line mixing experiments per min and, in secondary screens, a 15-point concentration gradient of soluble compound in ~2 min (11,46). The second uses a peristaltic pump in combination with an autosampler to boost assay throughput (47,48). As the sampling probe of the autosampler moves from well to well, a peristaltic pump sequentially aspirates particle suspensions from each well. Between wells, the running pump draws a bubble of air into the sample line resulting in the devliery of a series of bubble-separated samples. This system has been validated for cell-based high throughput endpoint assays for ligand binding, surface antigen expression, and immunophenotyping. Quantitative measurements have been demonstrated at rates to 40–100 samples per minute over a 4-decade range of fluorescence

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intensity with input cell concentrations of 1–20 million cells per ml and source well volumes of 5–15 μl. Currently, 384 well plates are routinely sampled in 10 minutes with sample volumes of 1–2 μl. The particle counting ability of flow cytometry can be adapted for high throughput analysis of compound solubility. The data from all wells of a microplate is collected in a single data file. The time-resolved data, with periodic gaps corresponding to the passage of the sample-separating air bubbles, are analyzed by software. Flow cytometry has been successfully used for small molecule discovery for GPCR (49–51) with fluorescent ligand and cell based approaches. Multiplex data sets are beginning to be generated.

Screening for the NIH Roadmap and Future Discovery

High throughput flow cytometry is now part of the NIH Roadmap Molecular Libraries Initiative (MLI). The NIH Roadmap aims to accelerate biomedical research and create new tools for discovery. The MLI is focused on chemical biology and consists of initiatives for individual investigators and centers to discover small molecules useful as biological probes, imaging agents, and potentially as leads for drug discovery. The New Mexico Molecular Libraries Screening Center (NM MLSC) is the home of HT Flow Cytometry and one of ten centers performing screens on research targets submitted by the international community. The centers identify active molecules and chemically optimize those molecules for biological activity. Information about NMMLSC may be found at http://screening.health.unm.edu/.

The NM MLSC impacts HT flow cytometry in several ways. First, through outreach, we are collaboratively developing biological targets for screening (See Table 2), allowing the technology to be shared with the discovery community. Second, we are exploring unique features of flow cytometry for high content and multiplex screening intended to permit the determination of small molecule selectivity and specificity in a single step. Once the screens are completed against the NIH Small Molecule Repository, the data will be available on PubChem. The grant number in Table 2 can be used to track the screening progress and to locate descriptions of the target proposal and the assay.

Automation of the HT flow cytometric platform is in process the NM MLSC with a goal of delivering five 384 well plates to a flow cytometer every hour, with 10 parameter or 20-plex assays. We project that the HT platform, with modifications to the fluidics system, is compatible with sampling in 1536 well plates at the same rate as for 384 well plates. We also view the application of flow cytometry to SiRNA libraries as a significant future opportunity.

Summary

Flow cytometry provides a unique set of capabilities for the analysis of cells and particles with a wide range of applications that include molecular assembly and receptor pharmacology, high content analysis of signaling pathways and images, multiplexing of biological targets, sorting of large particles and small organisms, and plate based analysis for screening and discovery.

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Table 1

Flow Cytometric Capabilities

Capability	Feature	Utility	Application
Analysis	Homogeneous detection	Real-time discrimination of particle bound and unbound molecules	Ligand Binding, Molecular assembly or binding interactions
	Multiparameter	10 or more colors and scatter signals	High Content, e.g, Phosphoprotein Signal Analysis
	Multiplexing and barcoding	2 or more colors and scatter signals	Suspension arrays for genotyping, detection of organisms and analytes
	Rate via digital signal processing	50,000 events per second	Thousands of flow cytometric assays
	Size Discrimination	Particles sizes ranging from submicron to 10's of microns	Cells, particles, insoluble precipitate screening of compound libraries
Imaging	Multiparameter image capture for each cell or particle	1,000 events per second	Cell morphology and topography
Sample Handling	Bioreactor	Programmed flow	Process Control
	Multiwell	1–2 per minute	Commercially Integrated plate loaders
	Mutliwell	10 wells per minute	Macro samples segmented by air bubbles delivered continuously
	Multiwell	40–100 wells per minute	Micro Samples segmented by air bubbles delivered continuously
	Multiwell, real-time mixing	Gradient formation, 4 per minute	Cell response pharmacology
	Real-time Mixing	Second time resolution	Cell Response, Ligand Binding
		Subsecond time resolution	Ligand Binding
	On line shear	Cone and plate, parallel plate	Cell adhesion under shear
	On line shear	Syringe	Cell activation by shear
	On line shear	Couette	Cell adhesion and activation under shear
Sensitivity of Detection	Conventional	100's of dye molecules	Full range of cytometric assays
		10's of phycobiliproteins	Immunophenotypic assays
		10's of nanoparticles	
		Single molecule detection	
Sorting cells and particles	High Speed Sorting	50,000 events per second	Each cell is an assay
	Large Particle	100–1000 events per second	One bead one compound screening; multicellular organism, large cell or cell cluster screening

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Table 2

High Throughput Flow Cytometry for the NIH Roadmap

	Assay	NIH Status and tracking
1	Cytotoxicity/384 well plate miniaiturized	
2	FPR/FPRL1 duplex	1 R03 MH076381-01
3	Allosteric Ligands for the VLA-4 Integrin affinity activation and inhibition	1 X01 MH077638-01
4	Ligands of GRP30 and Classical Estrogen Receptors	1 X01 MH077627-01
5	ATP Hydrolysis-dependent disassembly of the 26S proteosome	1 X01 MH077613-01
6	Androgen Mediated LnCAP Differentiation	1 X01 MH078937-01
7	Quorom Sensing for Bacterial Virulence	1 X01 MH078952-01
8	Arrestin binding to GPCR tail	1 X01 MH077637-01
8	Bcl-2 Family Protein Multiplex	1 X01 MH079850-01
9	GTPase Multiplex	1R03 MH081231-01
10	Multiplexed flow Cytometry Screens for RGS inhibitors	1R21 NS057014-01
11	Assays for compounds that block or stimulate cell death	1R21 NS057023-01
12	Efflux Pump Inhibition for Leukemia Therapy	In Development