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Review Article

Flow Cytometry: A versatile and Powerful Tool for Drug Discovery and Development

Basak Aru[1], Gulderen Yanikkaya Demirel[1]

[1]Immunology Department, Faculty of Medicine, Yeditepe University, Istanbul, Türkiye

Corresponding Author: Gülderen Yanikkaya Demirel (E-mail: gulderen.ydemirel@yeditepe.edu.tr)

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Abstract

Flow cytometry, a pivotal tool in clinical and research labs since the discovery of cell markers in the mid-1970s, plays a crucial role across all phases of drug discovery. Modern flow cytometers can detect rare cell types relevant to disease pathogenesis, measure numerous parameters simultaneously, thus, offer versatility in drug screening. In drug discovery studies, flow cytometry contributes to the assessment of drug pharmacokinetics, pharmacodynamics and safety in animal models and clinical trials. It can also be used to monitor drug efficacy and identify biomarkers for diagnosis and prognosis.

In essence, flow cytometry is a versatile, instrumental technique that supports drug discovery from target identification through to clinical development, limited only by the creativity of the researcher and the availability of fluorescent labels or specific size/scatter related findings. This review article focuses on the use of flow cytometry in drug discovery and drug development studies, summarizing not only conventional assays such as immunophenotyping, measurement of programmed cell death pathways and cell division to provide insights into drug effects and patient responses, but also novel approaches including mass cytometry, spectral cytometry, and droplet cytometry.

Keywords: Flow cytometry, drug discovery, drug development, mass cytometry, spectral cytometry

1. Introduction

Flow cytometry has been an indispensable tool for clinical and research laboratories for many decades now. After the discovery of cell markers in mid-1970's, use of flow cytometry has become an important part for all phases of drug discovery. It is known that only approximately 0.1% of new drug candidates can survive from preclinical research to marketing approval stage (1). The need for better assays and tools for evaluation and characterization of these drug candidates is still present and flow cytometry is one of the essential instruments in drug screening studies. Flow cytometry can be applied to various stages and aspects of drug discovery and development such as target identification, screening, mechanisms of action, biomarker discovery, toxicity testing and clinical trials (2–5).

It has some advantages over other methods by being multiparameter, high throughput availability and single cell analysis.

Modern flow cytometers can measure from 10 to >40 colors, providing the ability to measure many parameters simultaneously on each cell. Using bead technologies, cytometers can also measure soluble proteins such as cytokines, chemokines, enzymes, and all types of proteins in soluble format (6). Recent advances in imaging flow systems, mass spectrometry cytometers, spectral cytometers and the development of high throughput screening cytometry systems have increased the capabilities of single cell profiling for systems level approaches to drug discovery.

Target identification is the first step in the drug discovery process, where a disease is elucidated and potential targets for intervention are selected. Flow cytometry allows the analysis of cell populations and the detection of rare cell types that may be involved in disease pathogenesis. Examples of the use of flow cytometry include the identification and isolation of specific cells such as stem cells, cancer cells, different subtypes of immune cells that express specific markers or receptors that can be targeted by drugs. Cytometry systems can be used to screen libraries of small molecules or antibodies for their binding affinity and specificity to the target cells, using fluorescent tags or labels to identify novel ligands or modulators of the target molecules.

Target validation is the next step in the drug discovery process where the selected targets are confirmed to be relevant and essential for the disease. Flow cytometry is a useful tool for the functional characterization of the target molecules and their role in the disease mechanism. It can be used to measure the activation, signaling, proliferation, differentiation, various cell death modalities such as apoptosis, or migration of target cells in response to modulation of the target molecules by drugs or other agents (7). Cytometry can also be used to assess the effects of gene knockdown or knockout of target molecules on target cells using techniques such as RNA interference or CRISPR-Cas9. Once a target is identified and validated, the drug candidates must be optimized to achieve the desired outcome with high specificity and low toxicity. At this stage, cytometry can be used for optimization of the drug properties and the selection of best candidates for further development. With various assays, potency, efficacy, selectivity, and kinetics of the drug candidates can be determined by cytometry.

Flow cytometry can be used to assess the pharmacokinetics, pharmacodynamics, and safety of drug candidates in animal models for biodistribution, pharmacological imaging and toxicity testing. It can also be used in the clinical development phase of drugs being tested for efficacy and safety in human trials. Monitoring the effects of drugs and identifying biomarkers for diagnosis and prognosis are important areas of application for

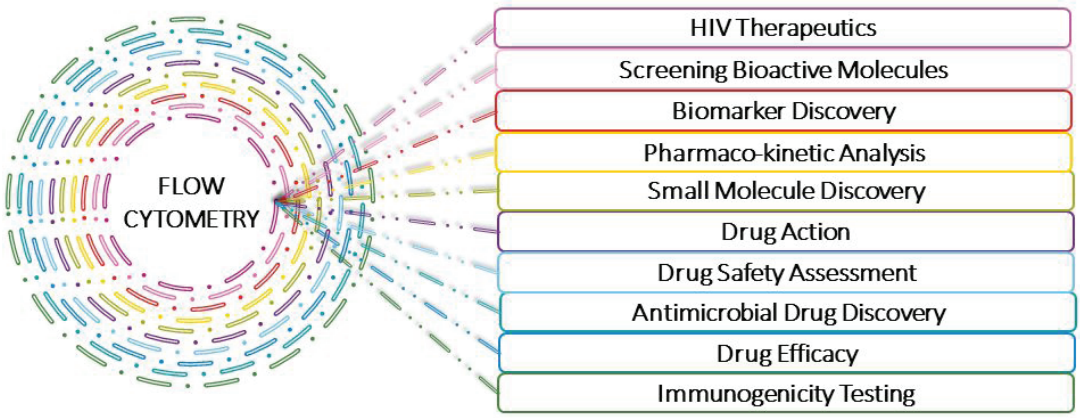

Figure 1. Use of flow cytometry at different stages of drug discovery and development

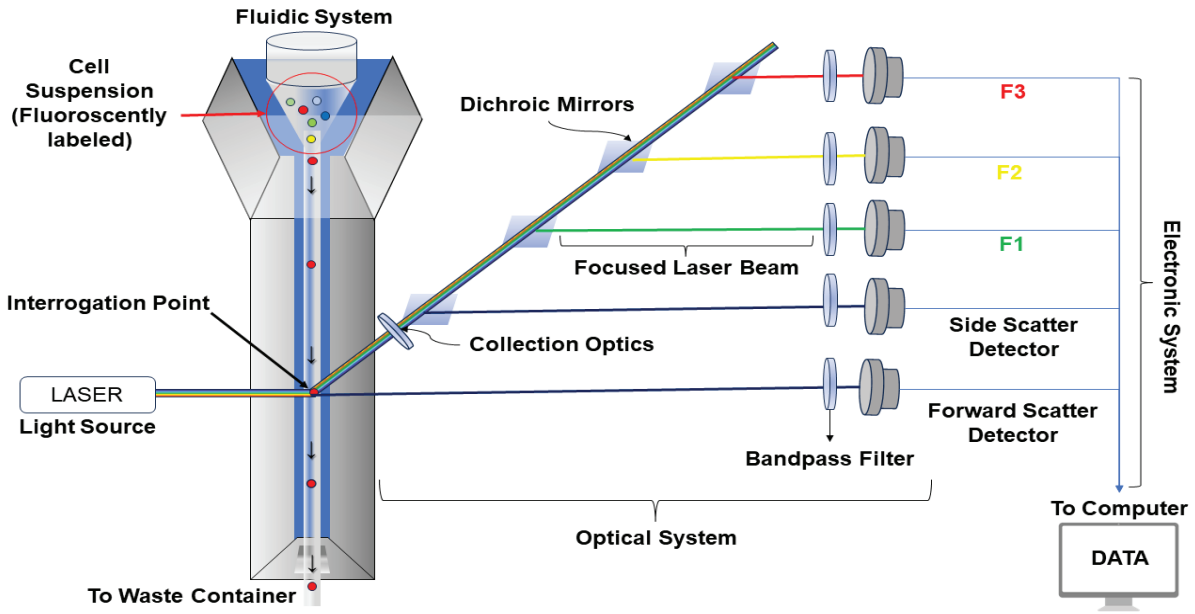

Figure 2. Schematic drawing of conventional flow cytometry (10,11). Illustration is adapted by Furkan AYDIN.

flow cytometry. It can be used to measure changes in target cells or other cells in blood or body fluids by measuring parameters such as blood cell count, immunophenotype, function, DNA cell content and response to drugs.

Hence, flow cytometry is a versatile and powerful tool that supports the drug discovery process from target identification through to clinical development. The parameters measured are only limited by the imagination of the researcher or clinician if there is a fluorescent label or size/scatter related specific finding about the event being evaluated (**Fig 1**). It is an essential technique in the discovery and development of new drugs for various diseases.

It should be noted that flow cytometry is used in different analytical areas, including research, preclinical applications, and routine methods provided as a medical laboratory service, all of which should follow standardization and validation procedures (8). However, method validation in flow cytometry is challenging due to the complexity of the technology and is expected to become even more complex in the future as high-parameter research methods such as clustering become routine applications (9).

2. Flow cytometry methods in drug discovery

2.1. Immunophenotyping

Immunophenotyping is an important technique in immunology that characterizes and identifies cell populations based on the expression of surface markers or antigens. This method uses monoclonal antibodies specific for certain cell surface proteins, allowing the discrimination and classification of different cell types within a sample. Immunophenotyping plays a fundamental role in understanding the complexity of the immune system, aiding in the diagnosis of disease, monitoring treatment response and elucidating immune-related disorders (12). This technique has been extensively used in flow cytometry, allowing the simultaneous analysis of multiple markers on individual cells, providing detailed insights into the composition and functionality of immune cells (13). In addition, recent advances in high-dimensional technologies, such as mass cytometry, have further improved the precision and depth of immunophenotyping analyses, facilitating comprehensive immune profiling and advancing our understanding of immune system dynamics (14–16).

In drug discovery studies, immunophenotyping is often used to assess the impact of potential therapeutic agents on the immune system and to identify drug candidates that modulate immune

cell responses (14,17). In addition, the combination of immunophenotyping with advanced high-dimensional approaches such as RNA sequencing has the potential to revolutionize the drug discovery landscape by providing unparalleled insights into the intricate workings of the immune system and its modulation by pharmacological agents (18,19). Single-cell resolution analyses have revealed heterogeneity within immune cell populations, shedding light on previously unexplored cellular subsets and their differential responses to therapeutic interventions (20).

2.2. Evaluation of proliferation

The measurement of cell proliferation by flow cytometry encompasses a range of methods designed to comprehensively analyze and quantify cell division dynamics within heterogeneous cell populations (21–23). Furthermore, the incorporation of multiple fluorochromes into flow cytometry assays allows the simultaneous assessment of proliferation markers alongside other cellular characteristics, providing a multi-faceted understanding of cell behavior (24–26).

A widely used technique involves the incorporation of nucleotide analogues such as bromodeoxyuridine (BrdU) or 5-ethynyl-2'-deoxyuridine (EdU) during the DNA synthesis phase of the cell cycle (27,28). In this method, cells are incubated with these analogs, which are incorporated into newly synthesized DNA. After incubation, the cells are fixed, permeabilized and exposed to specific antibodies conjugated to fluorescent markers, allowing the identification and quantification of cells actively synthesizing DNA. This BrdU/EdU incorporation assay is critical in determining the proportion of cells in S phase, providing a snapshot of the actively dividing cell population within a sample.

Another widely used technique for assessing cell proliferation by flow cytometry is DNA content analysis, which relies on staining cellular DNA with fluorescent dyes such as propidium iodide (PI) or Hoechst (21). After fixation, permeabilization and nuclear staining, different phases of the cell cycle are distinguished according to their DNA content, allowing researchers to study the distribution of the cell cycle between the G0/G1, S and G2/M phases, providing crucial information about cell cycle distribution and proliferation rates within a population.

Carboxyfluorescein succinimidyl ester (CFSE) is a fluorescent dye widely used in biomedical applications to track and quantify cell division (29). Upon cellular uptake, CFSE is cleaved into its fluorescent form by intracellular esterases, resulting in its covalent binding to intracellular molecules (30). The fluorescence of CFSE halves with each cell division, allowing the precise determination of the number of divisions a population of cells has undergone. The dye's ability to stain different cell types without affecting cell viability has made it a valuable tool in the study of lymphocyte proliferation, immune responses, and cell kinetics. Today, CFSE-based assays in flow cytometry are widely used to characterize the dynamics of cell proliferation and differentiation in both in vitro and in vivo experimental settings. There are also other dyes on the market such as CellTrace™ Violet Cell Proliferation Kit (Thermo Fisher Scientific), Tag-it Violet™ Proliferation and Cell Tracking Dye (BioLegend Inc.) and Violet Proliferation Dye 450 (VPD450) (Beckton Dickinson Biosciences), all of which work on a similar principle to CFSE staining. Besides, cell proliferation can also be assessed using flow cytometry by intracellular staining to evaluate proliferation markers such as Ki-67 or proliferating cell nuclear antigen (PCNA), which will be discussed in more detail (31,32).

2.3. Toxicity measurements

Over the past decade, the mechanisms underlying cell death, which include a wide range of signaling cascades that regulate the initiation, execution and post-mortem disposal of cells have become a focus of interest for many researchers in drug discovery studies (33). Here, we aimed to summarize the flow cytometric applications aiming to measure different modes of programmed cell death pathways. It should also be noted that in addition to programmed cell death pathways, cell death and viability can also be measured with flow cytometry by labelling dead cells with fluorescent dyes that are excluded from viable cells due to membrane integrity such as 7-Aminoactinomycin (7-AAD), ethidium homodimer-III or 4′,6-diamidino-2-phenylindole (DAPI); these intercalating dyes can also be combined with cell-permeable viability indicators

to differentiate between healthy and dead cell populations, although these stains do not provide information on the mode of cell death.

2.3.1. Apoptosis

Relying on the simultaneous measurement of membrane permeability and morphological changes observed during the course of apoptosis, Annexin V/Propidium Iodide (PI) staining is a widely used method in flow cytometry for evaluating this pathway (34,35). Briefly, this method is based on the principle that during apoptosis, phosphatidylserines located at the inner leaflet of the plasma membrane are translocated to the outer leaflet, where they can be detected by Annexin V. At the same time, PI is used to stain necrotic cells with increased membrane permeability, allowing to distinguish between apoptotic and necrotic cells. Since PI is excluded from live or early apoptotic cells due to the presence of an intact plasma membrane, but can enter late apoptotic and necrotic cells due to the loss of membrane integrity, Annexin V/PI staining can detect early apoptotic, late apoptotic, necrotic along with viable cells (36). Currently, there are several protocols available for Annexin V/PI staining in flow cytometry to detect apoptosis. The most common procedure involves staining the cells with Annexin V and PI, followed by flow cytometry analysis to differentiate between healthy, apoptotic, and necrotic cells (37,38). A modified version of the protocol involves RNase treatment steps to limit the number of false-positive staining events (36). When running Annexin V/PI analysis, it should be kept in mind that the presence of calcium in the cell suspension is critical for Annexin V binding, thus, avoiding buffers containing calcium chelators and maintaining the calcium concentration is important to eliminate the risk of false results; in addition, ideal calcium concentration may be determined for different cell types for optimum results (39)

Activation of endonucleases that specifically cleave chromosomal DNA at internucleosomal regions is a hallmark of apoptosis. Based on fluorochrome-labeling of 3′-OH termini of DNA strand breaks *in situ* with exogenous terminal deoxynucleotidyl transferase (TdT) is named as Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling (TUNEL) assay, and considered as the gold standard for detecting apoptosis (40,41). Moreover, by combining a DNA stains such as PI or DAPI followed by multi-parametric analysis of cells by flow cytometry enables evaluating the correlation between apoptosis and cell division (40).

Additionally, apoptosis can also be assessed by measuring intracellular proteins known to be involved in the apoptotic process: this can be achieved either by measuring active forms of the proteins (42,43), evaluating caspase activity with cell-permeable fluorogenic caspase substrates (44), or fluorochrome-linked caspase inhibitors (45). These approaches can be combined with Annexin V/PI staining or TUNEL assay to provide a better insight into the apoptotic process.

2.3.2. Autophagy

Autophagy is an evolutionarily conserved pathway for clearing cytosolic aggregated proteins, damaged organelles, invading microorganisms as well as maintaining metabolic balance (46). Modulation of autophagy has been shown to hold great potential in the treatment of various diseases, thus is receiving interest in drug discovery studies (46,47).

Being located in the autophagosomes of cells undergoing the autophagic process, microtubule-associated protein 1A/1B-light chain 3 (LC3-II) is the most prominent biological marker for autophagy detection. Currently there are various studies reporting flow cytometry based LC3-II measurements to evaluate autophagy, which is either performed by labelling the protein with an antibody, or using transduced cells that express green fluorescence protein-tagged LC3 (48–50). Along with LC3-II, other proteins that play a role in autophagy such as Beclin-1 can also be investigated simultaneously (51,52). Alternatively, autophagy can also be measured by investigating the levels of selective autophagy substrates, which are usually recognized by autophagic proteins, and their degradation can be measured using lysosomal inhibitors (53). A well-known example for such substrates is SQSTM1/p62, though it should be noted that its expression is regulated at the transcriptional level, thus, its mRNA levels should also be evaluated to eliminate

the possibility of the involvement of transcriptional induction.

While autophagic markers can provide a general insight to autophagy, assessment of autophagic cargo flux is needed for specific and accurate detection of autophagic activity. For investigating monitoring bulk and selective autophagy, employing the fluorescent coral protein Keima is another approach (54). This protein is excited at 440 nm and 586 nm under neutral and acidic conditions, respectively, allowing to monitor its delivery from the cytosol to lysosomes (53). However, this probe is not suitable to be used in fixed samples and can only be combined with LC3-II staining if transduced cells are used.

In addition to the methods mentioned above, there are commercial kits such as CYTO-ID® Autophagy detection kit (Enzo Life Sciences), FlowCellect™ Autophagy LC3 Antibody-based Assay Kit (Merck Millipore) and Autophagy Assay Kit (Abcam) where the cells can be subsequently evaluated with flow cytometry.

2.3.3. Ferroptosis

First proposed in 2012, ferroptosis is a form of regulated cell death characterized by iron-dependent lipid peroxidation, leading to the accumulation of reactive oxygen species (ROS) and subsequent cell demise. This process involves the dysregulation of cellular redox balance and antioxidant defense mechanisms, ultimately resulting in oxidative damage to cell membranes (55,56). Ferroptotic pathway is distinguished by the loss of lipid peroxide repair capacity by the phospholipid hydroperoxidase GPX4, the presence of redox-active iron, and the oxidation of polyunsaturated fatty acid (PUFA)-containing phospholipids (57). Ferroptosis has been shown to overcome drug resistance in cancer (55) in addition to holding importance in neurodegeneration, stroke, traumatic brain injury, ischemia-reperfusion injury, cardiomyopathy, and kidney degeneration (58). However, a significant challenge in assessing the therapeutic possibilities of ferroptosis lies in accurately identifying under pathological conditions.

Since ferroptosis was identified relatively recently, assays regarding its evaluation are not as well established as apoptosis. In flow cytometry, BODIPY™ 581/591 C11 probe is widely used to detect ferroptosis by determining the amount of lipid peroxides in cellular membranes (59,60). Another probe is Liperfluo which directly reacts with lipid hydroperoxides to yield fluorescent Liperfluo-OX which is excited and emitted at 524 and 535 nm wavelengths, respectively (61). Certain proteins including long-chain-fatty-acid—CoA ligase 4 (ACSL4), Transferrin receptor protein 1 (TFRC, CD71), Cyclooxygenase 2 (PTGS2), or glutathione specific gamma-glutamylcyclotransferase 1 (CHAC1) protein expressions have been shown to be enhanced; autophagy also promotes ferroptosis, and evaluating LC3-II may reflect the degree of ferroptotic damage, which can all be measured via flow cytometry (62).

2.3.4. Pyroptosis

Pyroptosis is a type of inflammatory programmed cell death which is induced by caspase cleavage and gasdermins and results in the secretion of inflammatory mediators interleukin 1β and interleukin 18 (63–65). This cell death pathway has been reported to be involved in carcinogenesis as well as autoimmune, neurodegenerative or allergic diseases (66–69). Therefore, pyroptosis is considered as an attractive target for treating a wide array of diseases (70,71).

In flow cytometry, activation of pyroptotic pathway can be detected via evaluating gasdermin D and active caspase-1 levels, or secretion of IL-1β and IL-18. For caspase-1, intracellular staining can be performed by fixing and permeabilizing the cell membrane, followed by labelling an appropriate antibody of the target, or with a commercial kit that includes a fluorescent caspase-1 substrate such as FAM-FLICA™ Caspase-1 Assay Kit (72,73). For detecting IL-1β and IL-18, inflammatory mediators released as a result of pyroptosis, commercial bead-based flow cytometric arrays which are suitable for blood and plasma samples in addition to cell culture media (74).

2.4. Flow-fluorescence *in situ* hybridization (Flow-FISH)

High-throughput analyses evaluating gene expression are widely used in early drug discovery to identify disease-associated genes. To further characterize the expression of selected genes, in situ hybridization (ISH) using RNA probes (riboprobes) is a powerful tool to localize mRNA expression at the cellular level, which is especially beneficial for

investigating novel drug targets (75). Flow-FISH (Fluorescence In Situ Hybridization) is a technique that combines the principles of fluorescence in situ hybridization with flow cytometry, allowing for the simultaneous detection and quantification of specific nucleic acid sequences within individual cells within a heterogeneous population (76). In this method, cells are first treated with fluorescently labeled nucleic acid probes that target complementary sequences of interest (77). These probes hybridize to their specific targets within the cells. Subsequently, flow cytometry is employed to analyze the labeled cells, measuring the fluorescence signals emitted by the hybridized probes. Moreover, by combining flow-FISH with fluorescence-activated cell sorting (FACS), the target cell populations can be obtained and investigated at the single stage (78).

Flow-FISH enables the examination of genetic or RNA content at a single-cell resolution within a mixed population, providing insights into cellular heterogeneity, gene expression patterns, and telomere length (76,79). Taking typically 1-2 days, flow-FISH is faster compared to conventional methods which involve culturing cells (80). In addition, in terms of evaluating telomere length, flow-FISH was reported to be more accurate, reproducible, sensitive, and specific in comparison with quantitative PCR (qPCR) (81). Likewise, even though sensitivity of monochrome multiplex–quantitative polymerase chain reaction (MM–qPCR) and flow-FISH was found to be similar, specificity of MM–qPCR was reported to be significantly lower compared with flow-FISH (82).

2.5. Receptor-ligand binding assays

Ligand binding assays in flow cytometry encompass methodologies to investigate molecular interactions between ligands and their receptors expressed on the cell surface or within the cellular milieu (83). These assays utilize fluorescently labeled ligands or antibodies to detect and quantify specific binding events. The integration of flow cytometry with ligand binding assays offers a powerful means to examine receptor-ligand interactions at a single-cell level with high sensitivity and multiparametric analysis capabilities. Moreover, they can be combined with other cellular markers to determine specific events in a heterogenous cell suspension (84).

Flow cytometry-based binding assays serve as a cornerstone in characterizing product activity by probing its interaction with a specific cell surface receptor: in the case of monoclonal antibodies (mAbs) aimed at obstructing ligand-receptor binding, employing *in vitro* binding assays emerges as a potent surrogate for assessing the therapeutic mAbs' potency to illuminate its efficacy by gauging its ability to impede the crucial interaction between the ligand and its cellular receptor (85). Summarizing, these assays play a pivotal role in the realm of drug development, offering a crucial avenue for assessing candidate therapeutic agents that specifically target receptors or ligands; in addition to evaluating their specificity, this process can significantly contribute to refining and perfecting the design of pharmaceutical interventions, charting a course towards more precise and effective treatments.

2.6. Receptor occupancy assays

Implementing the precision medicine framework in drug development requires the integration of a range of information to facilitate more informed decision-making regarding target selection and a better understanding of the pharmacological aspects of the drug, including bioavailability, pharmacodynamics, and pharmacokinetics(86). For developing biologically-based therapeutics, measuring the binding of the biotherapeutic to its cellular target, Receptor Occupancy (RO), can aid in determining the Minimal Biological Effect Level (MABEL) as well as revealing optimal dosing and administration schedules(86,87). In addition, data generated in RO assays can be used in safety assessment as long-term maximum RO can be a hallmark of overdosing or long-term binding, which can lead to serious side effects and even toxicity. RO assays measure unbound or free receptors, total available receptors and/or the fraction of bound receptors (**Fig 3**). These assays are largely applied it evaluating therapies directed at immune conditions as well as targeted oncology therapy, making flow cytometry an ideal instrument for performing RO assays. Moreover, with flow cytometry, target engagement on specific cell subsets within heterogeneous mixtures can be achieved (4).

While flow cytometry stands as a valuable tool in evaluating receptor density and occupancy during both preclinical and clinical pharmacodynamic studies, it's crucial to perform optimization, standardization and validation studies as it is the

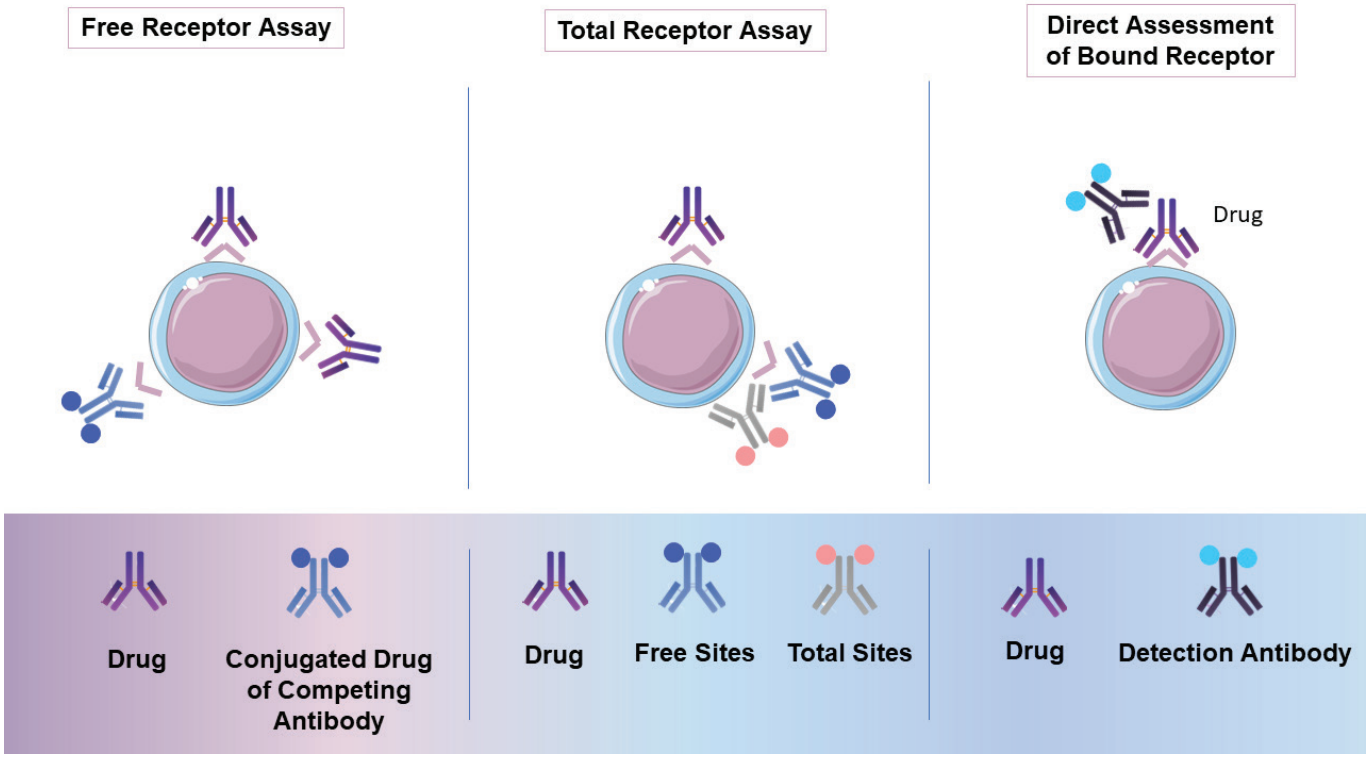

Figure 3. Three different formats of Receptor Occupancy (RO) assays (88). Free receptor assays quantify the unbound fraction of receptors in the presence of a drug, which is achieved after incubating with a fluorochrome-conjugated antibody, especially designed to compete with the drug for occupancy at the identical epitope. These assays are commonly employed to ascertain dosage levels. Total receptor assays, on the other hand, evaluate the combined presence of both unbound and bound receptors on cells These assays employ a dual-antibody approach: one antibody competes with the drug for binding, while another targets a distinct epitope on the receptor. This method is employed when anticipating the drug's potential impact on the up- or down-regulation of the receptor when administered at specific concentrations, and successful execution of this method relies upon the availability of a non-competing antibody. Finally, direct assessment of bound receptor assays are preferred in scenarios where receptor expression remains low or the target cells are scarce. Within this modality, the antibody directly engages with the drug, amplifying the detection of drug molecules bound to the receptor and thereby significantly enhancing assay sensitivity.

initial step to ensure consistency and reliability in the assessment process (4,9,87). Methods regarding flow cytometric RO assays remain challenging to develop due to various obstacles including low antigen expression, rare frequency of the target population, presence of soluble targets, and stability of reagents in addition to samples.

2.7. Enzyme activity assays

Enzyme activity assays serve as a cornerstone in understanding biological processes, providing insights into enzymatic reactions' kinetics and mechanisms. Traditionally, these assays rely on bulk measurements, providing averaged values for a population of cells while to directly explore how certain enzymes relate to different cell types and their functions, it's essential to use single-cell enzyme activity assays which enable investigating enzymes within individual cells, providing insights into their roles across various cell types and functions (89,90). Within this context, flow cytometry allows the assessment of enzymatic activity present in individual cells, uncovering heterogeneity and variations that might be obscured in bulk measurements. This single-cell resolution offers valuable insights into cellular heterogeneity and the diverse roles enzymes play within different cellular subpopulations (91). Furthermore, cell sorting enables isolation of cells based on their enzymatic activity levels, and by coupling flow cytometry with cell sorting capabilities, researchers can isolate subpopulations exhibiting specific enzymatic activities for further downstream analyses (92).

When measuring enzymatic activity by flow cytometry, fluorochrome-labelled antibodies can be used to measure enzyme concentration as an indirect indicator of enzyme activity, or the generation of a fluorescent product instead of solely relying on antibody-based assessments can be employed.

There are also studies where fluorogenic substrates originally developed for imaging and fluorimetry have been adapted to flow cytometry format (93).

2.8. Intracellular protein staining

Intracellular protein staining using flow cytometry represents a pivotal technique in modern cellular biology, enabling the examination and quantification of specific proteins within individual cells (94–96). However, according to the nature of the protein-of-interest, the protocol that should be applied should be chosen carefully. Measurement of secreted proteins such as cytokines and chemokines consist of four steps: (1) *in vitro* activation, (2) fixation, (3) permeabilization, and (4) immunostaining. For analyzing a specific cell subset in a heterogeneous cell population, cells surface markers are also stained simultaneously with intracellular cytokines. For stimulating cytokine secretion, *in vitro* cellular activation is generally achieved by phorbol-12-myristate-13-acetate (PMA) and ionomycin, or via antigens while inhibitors of cytokine secretion, Brefeldin A or monensin are used to increase intracellular cytokine concentration in order to increase Signal-to-Noise ratio (97,98). Nevertheless, both inhibitors exert cytotoxic effects and thus, should not be incubated more than 12 hours though cytokine accumulation generally reaches to its maximum within 2 to 4 hours after treatment with these inhibitors, but ideal incubation durations should be determined according to the cytokines that will be evaluated. For measuring cytokines with dim and low-frequency such as IL-4, IL-5, and IL-13, fluorochromes with high signal-to-noise ratios such as phycoerythrin (PE) and allophycocyanin (APC) are recommended.

For fixation and permeabilization, aldehyde-based fixatives such as paraformaldehyde (PFA) and saponin are commonly preferred. However, when evaluating nuclear antigens along with cytokines, saponin will not allow nuclear staining, and permeabilization buffers may not enable cytokine staining as the PFA/saponin system (98). In addition, number of cells that will be acquired to obtain statistically significant numbers of cytokine-positive events should be determined (99). When using PMA and ionomycin for cell activation, around 15% of CD4+ cells exhibit IFN-γ positivity, and about 4% show IL-13 positivity in healthy donors, thus, lower number of PBMC (e.g., 20,000 total events) can provide an ample count of cytokine-positive events. Nevertheless, with antigen-specific activation, these frequencies usually range much lower, from 0.001% to 0.10%. Hence, researchers must collect a sufficient number of cells during flow cytometry to achieve statistically significant counts of cytokine-positive events(98).

Besides secreted proteins, flow cytometry can also be used for evaluating cytoplasmic and nuclear proteins. This method is especially feasible for investigating heterogeneous cell populations such as peripheral blood mononuclear cells (PBMC), murine splenocytes, bone marrow cells as these samples have mainly remained outside the scope of biochemical analysis. Similar to evaluating cytokines, intracellular proteins require a fixation step which is followed by permeabilization to provide access to the cytoplasm. To date, extensive studies have been carried out to determine the ideal fixation and permeabilization reagents (100–102), and many epitopes have been reported to be successfully stained including enzymes (103), native and phosphorylated proteins (104–106), viral particles (107), and immunoglobulins (108). In this context, 'phosphoflow', the staining of phosphorylated proteins, has been studied for more than two decades and has outlined essential requirements for the effective detection of intracellular phosphoproteins. Firstly, rapid fixation of cells is essential to preserve the authentic phosphorylation state of the proteins of interest. Second, efficient cell permeabilization is necessary to allow specific antibodies to access the targeted epitopes within the cells. Thirdly, the antibodies used for staining both cell surface proteins to distinguish subsets and phosphorylated components within intracellular proteins should accurately identify and securely bind to epitopes altered by the fixation process (109). Aiming to develop a general protocol for phosphoflow, the first effective and reproducible approach for intracellular phosphoprotein detection using flow cytometry was reported in 2003, where the authors have investigated the ideal stimulation, fixation and permeabilization conditions(109). Over the past decade, collaborative efforts between academic and industrial researchers have led to the development of refined fixation and permeabilization methods specifically tailored to the detection of various phosphoproteins,

which some of them now commercially available. Moreover, small molecule fluorescent dyes such as fluorescein isothiocyanate (FITC), Alexa Fluor 488® and Alexa Fluor 647®, have proven to be superior for phosphoflow applications thanks to their small size that facilitates antibody entry into cells in addition to their minimal interference with the ability of antibodies to bind to their target proteins (110). In summary, it can be concluded that advances in both reagents and assay techniques will significantly expand the application of phosphoflow beyond preclinical research as this approach holds great promise for monitoring immune responses and assessing immune status following vaccination and immunomodulatory interventions. Additional investigations, including adherence to Good Laboratory Practice (GLP) standards, are essential for the incorporation of phosphoflow cytometry into clinical applications (111). Another critical area for further exploration is to assess the potential of phosphoflow as a sensitive tool for tracking antigen-specific T-cell responses, particularly in the development of vaccines and immunotherapies for diseases such as HIV and cancer. There's also a need to understand how immunomodulators and immune response suppressors affect intracellular signaling pathways downstream of T cell and cytokine receptors in people with cancer, chronic viral infections and autoimmune diseases (112).

2.9. Analysis of extracellular vesicles (EVs)

Extracellular vesicles (EVs) are small lipid bilayer compartments released by cells that contain nucleic acids, proteins and lipids. Long thought to be as insignificant as platelet dust, recent studies have shown that EVs play a role in cell-to-cell signaling and can serve as drug delivery vectors and disease biomarkers (113,114). Moreover, their ability to target specific cells also positions them as potential drug delivery vehicles (115). EVs of different origins have the ability to influence both normal physiological and disease-related processes. Despite their significant potential, the current methods used in EV research are somewhat limited. Currently, EVs are predominantly studied using nanoparticle tracking analysis and bulk molecular techniques such as Western blot. However, these methods fall short of fully dissecting the observed heterogeneity of EVs as visualized by electron microscopy (EM). While EM is instrumental in revealing the diversity within EV samples, its limitations hinder the performance of more complex and quantitative EV analyses.

Studying EVs using flow cytometry presents challenges due to their sizes being too small to detect (116). A common strategy for analyzing EVs on conventional flow cytometers is to attach these structures to beads of a size that ensures accurate detection on the flow cytometer. In one of the first studies to use this method, the researchers have isolated and characterized exosomes between 30 and 100 nm in cell culture supernatants by binding them to latex beads (117). However, advances in flow cytometry have made it possible to isolate EVs without beads: in a recent study, researchers removed plasma proteins to isolate EVs by size exclusion chromatography followed by flow cytometric analysis, and 95% of the samples were positive for the exosome marker CD63 and contained neural cell adhesion molecule (NCAM) (118).

EV analysis is a challenging area of cytometry, but there are ongoing efforts to develop new stains and diagnostic tools for the identification of EVs and their drug cargo. Guidelines for the identification of these small vesicles have been developed by interested research groups (119).

2.10. Microbial applications

Flow cytometry is a good tool for evaluation of the effect of anti-microbial drugs. Effective antifungal drugs can inhibit the fungal growth or may change the fungal cytological features. In addition to phenotypic profiling studies, utilization of imaging flow cytometers (IFC) have the potential of improving and enhancing cytological profiling. IFC combines the abilities of flow cytometry and fluorescent microscopy with advanced data processing algorithms. With this method, measurement of different components of the cell, size, shape, texture, nuclear DNA morphology, integrity of cell wall, membrane permeability, surface and intra cellular markers are also possible (120–123). There are studies for development of highly sensitive nano level detection systems for high throughput systems but due to low amount of sample and limitations in cell numbers they have not yet reached to daily use stage (124).

3. Recent technological developments

What we have discussed so far has focused on classical cytometry systems. Recent developments in cytometry systems including high-throughput flow cytometry, Mass-TOF, droplet flow cytometry, and spectral cytometry, will enhance the use of cytometric methods.

3.1. High-throughput screening of compound libraries

While flow cytometry is a widely validated technique used in preclinical development from target selection and validation to mechanism of action identification, its applicability to drug screening has been very limited due to the lower number of assays that can be performed per patient sample and the lack of automation (125). High throughput screening systems can assist in drug screening by reducing reagent costs, compound usage and cell consumption through the ability to sample small volumes with negligible waste. With these systems, many compounds can be tested in a short time in 96, 384 or even larger capacity plates, and various high-throughput flow cytometry (HTFC) systems with fast autosampler devices tailored to traditional flow cytometers have been reported by different research groups. HTFC is an advanced method that uses automated sample processing and offers a promising way to screen large compound libraries. Recently, a novel system called HyperCyt has emerged that streamlines automated HTFC analysis by rapidly aspirating cell samples from microplate wells and transporting them to the flow cytometer (126,127). Since its commercial launch in 2006, the HyperCyt sampling technology has been attracting attention, particularly from companies favoring flow cytometry for a range of research objectives, including SiRNA-based functional genomics screens, hybridoma screens for therapeutic antibodies and immune cell analysis from primary tissues.

High-throughput flow cytometry platforms are likely to benefit from two recent trends in drug discovery. Firstly, there's a growing focus on moving away from target-centric screening towards target-agnostic or mechanism-informed phenotypic screening approaches. Second, large pharmaceutical companies are increasingly investing in the development of biologic drugs, particularly novel antibodies. These trends have likely contributed to the increased utility and interest in high-throughput flow cytometry systems. The appeal of HyperCyt lay not only in its faster sample processing, but also in its ability to handle smaller sample volumes while mitigating cell loss, a common problem with conventional flow cytometry due to dead volume during sample backflushing (127).

3.2. Mass cytometry

Mass cytometry is a modern variation of flow cytometry diverging from the traditional approach by replacing fluorescent labels with heavy metal ion tags on antibodies. This technique employs heavy metal isotopes not found in biological systems, solving the issue of overlapping fluorescence spectra and allowing for a greater number of parameters to be analyzed at once in comparison to conventional cytometry methods. In mass cytometry, cells are treated with a mixture of probes and antibodies, each labeled with a distinct non-radioactive heavy metal isotope.

For single-cell analysis, cell suspensions are aerosolized so that each droplet contains only one cell. These individual cells are then sent through argon (Ar) plasma, which atomizes and ionizes the sample, transforming each cell into an ion cloud of the elements present it contains. A quadrupole, acting as a high-pass filter eliminates lower-mass ions of biological origin, primarily of biological origin, leaving a cloud of ions tied to the isotope-conjugated probes.

Within the Time of Flight (TOF) chamber, ions are sorted by their mass-to-charge ratio. When these ions hit the detector, their counts are amplified and converted into electrical signals. Practical limitations, such as the availability of isotopes with sufficient purity and antibody conjugation chemistries, restrict applications to approximately 60 parameters per mass cytometry panel, as opposed to the theoretical claim of 120 parameters.

An alternative single-cell technology with even greater dimensionality is single-cell RNA sequencing (scRNAseq), providing a quantitative measure of gene expression levels per cell. While scRNAseq is a potent genomic tool for dissecting cell populations, it is constrained by higher costs and can only be run on a limited number of single

cells. In contrast, mass cytometry experiments can acquire data on a much larger scale, ranging from 10^6 to 10^7 cells. Despite offering more reliable results, the cost of disposables remains a hindrance to the widespread adoption of these systems (15). Mass cytometry cannot provide information about the cell size, internal complexity and autofluorescence profile which can be counted as a drawback depending on what the researchers are looking for (128).

Mass cytometry data can be analyzed by using SPADE Cluster Analysis, Principal Component Analysis and Boolean Gating (129,130).

3.3. Spectral cytometry

Spectral cytometry, a technique developed based on conventional flow cytometry, employs a spectrograph and a multichannel detector system, such as CCD. In contrast to conventional flow cytometry optics, which observe photons based on their wavelength, spectral flow cytometers disperse photons according to their wavelength. Utilizing a prism to disperse emitted light, this technique offers the advantage of using a more extensive array of colors for a sample, yielding accurate results with reduced reliance on color compensation (131). Just like conventional cytometers, careful panel selection is crucial for optimal performance in spectral cytometers. Another beneficial feature is the automated subtraction of cellular autofluorescence. In studies comparing mass cytometry and spectral cytometry results, researchers underscore the significance of selecting an efficient panel (128,132).

3.4. Droplet cytometry

Cytometry experts have extensively investigated this method, using traditional cytometers for its application. The technique involves droplet microfluidics to encase individual cells in tiny, picoliter-scale microdroplets. Among the droplet types suitable for cytometry in aqueous environments are double emulsion droplets (water-in-oil-in-water) and hydrogel-based droplets. Integrating flow cytometry with these droplets enhances cell characterization beyond traditional markers, facilitating simultaneous analysis of genomes, epigenomes, or transcriptomes of single cells encapsulated in droplets. This approach has led to significant breakthroughs, including enzyme and protein evolution, analysis of cell variance under drug exposure, detection of rare cells in microbial populations, pinpointing antibiotic resistance genes, and discovering new biomarkers (133).

4. Conclusion

In summary, we wanted to highlight the importance of the described techniques throughout different phases of drug development. To ensure dependable outcomes, it is imperative to have proficient and experienced users who adhere to the fundamental principles of flow cytometry and possess the expertise to select appropriate reagents. The possibilities that can be explored through cytometry are only constrained by one's imagination.

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Conflict of Interest

Authors have no conflict of interest to declare.

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