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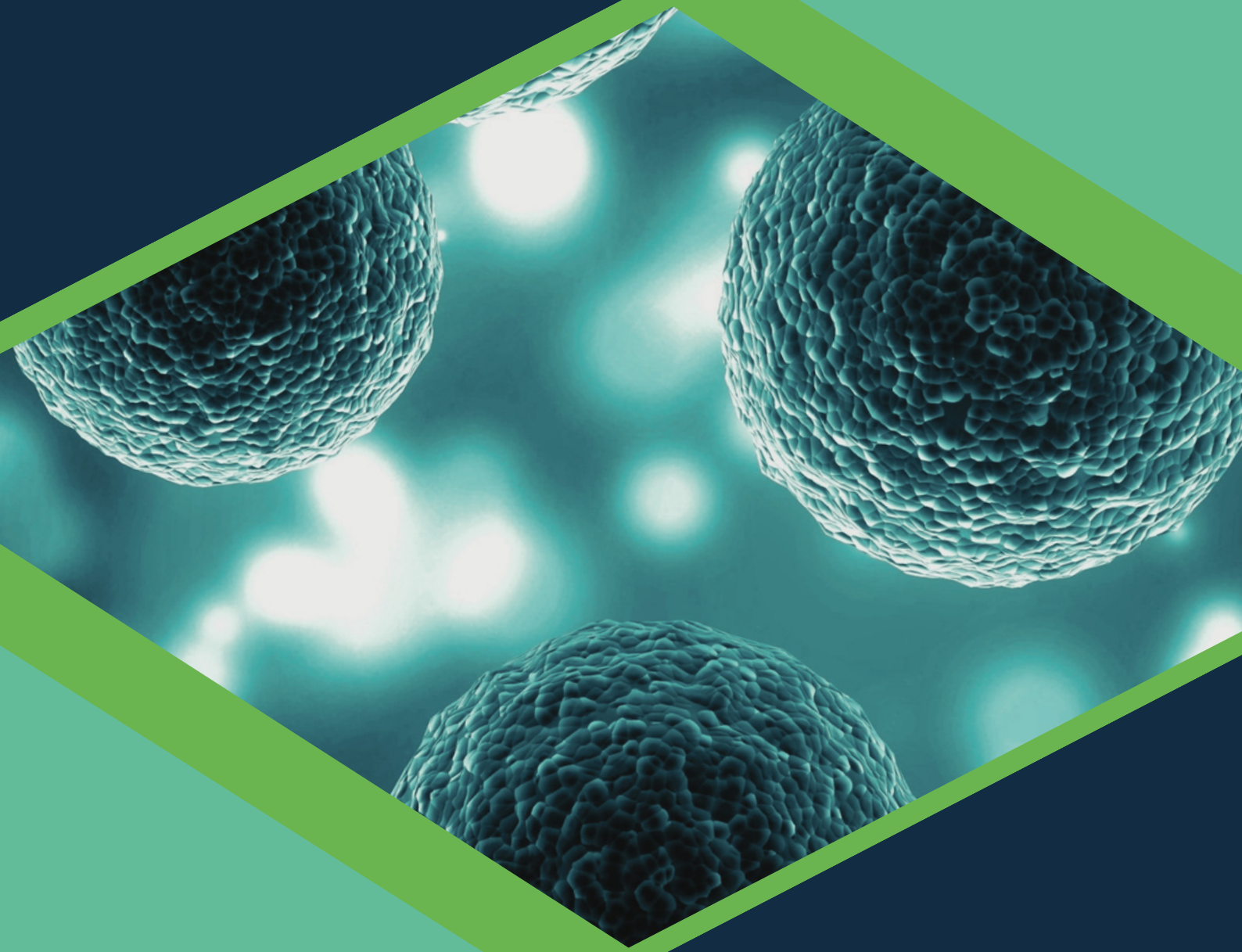

Flow cytometry in cancer therapy development

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Contents

Foreword

Flow cytometry can measure the physical and chemical characteristics of samples such as blood, bone marrow or lymph tissue. It allows researchers to measure various parameters simultaneously including the specific expression of tagged markers present on a cell's surface therefore making it ideal for cancer diagnosis.

Its utility in the clinical setting in terms of cancer diagnosis and monitoring is widely recognized. It can be used to analyze DNA content, detect cancer cell phenotypic markers and detect rare events such as circulating tumor cells.

Flow cytometry is also utilized within the drug development process, from target identification to lead development, as it can facilitate investigations into the efficacy and toxicity of the drug. This is significant for immuno-oncology research.

In this eBook, we will explore the recent advances in flow cytometry, its application in drug discovery and how to overcome the challenge of sample stability in flow cytometry analysis. Also, we have the latest insights into flow cytometry and oncology research in our interview with Richard Cuthbert (Bio-Rad, UK).

We hope you enjoy this eBook!

Amy White
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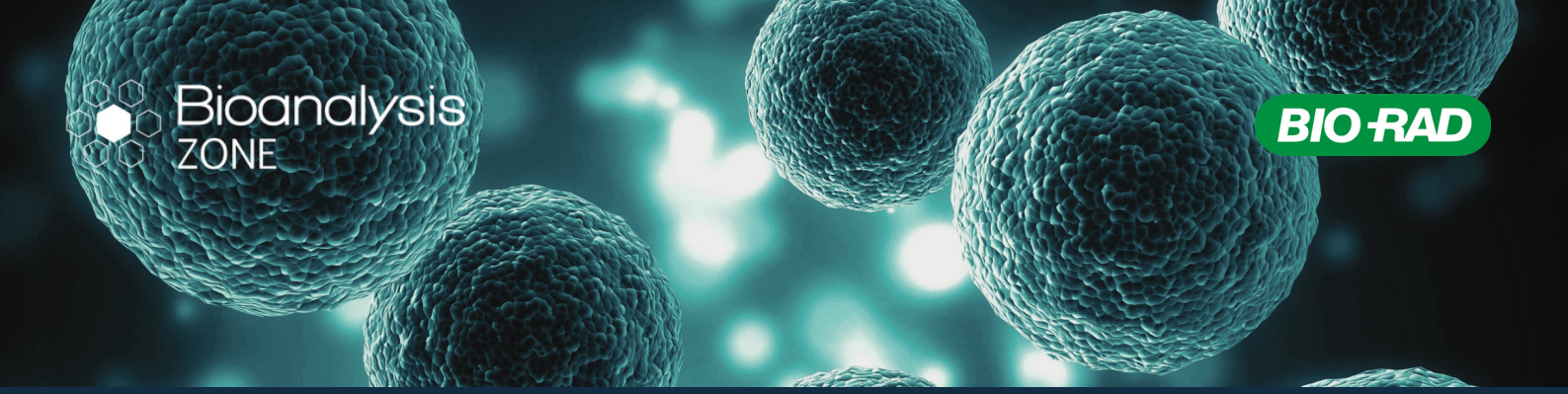

60 seconds with Richard Cuthbert: flow cytometry

Richard Cuthbert holds an honors degree in Biomedical Chemistry and a PhD in the field of Regenerative Medicine. He has over 10 years of experience in biomedical research at the University of Leeds (UK) where his main research interests were stem cell biology, immunology and colon cancer. Richard joined Bio-Rad (UK) in 2019 as a Flow Cytometry Specialist and is now a Flow Cytometer Product Manager.

What attracted you to the field and do you have any advice for others starting out in flow cytometry?

I started out using flow cytometry because I was studying mesenchymal stem cells (MSCs). I needed to measure the number of MSCs in a bone marrow aspirate. The most common method of doing that is to plate out your cells in appropriate media and perform a colony forming assay. The problem with that is its relatively labor intensive, but more importantly takes 2 weeks to complete. Flow cytometry offers a rapid method of identifying and quantifying cell populations in a heterogeneous mixture of cells. Meaning that once we had a validated method of quantifying MSCs using flow cytometry I could get my results in less than 2 hours. It's that ability to get data at speed, and the sheer volume of data that can be collected using flow cytometry, that attracted me and that is also what makes it an ideal screening platform.

I would admit that compared to some other techniques, flow cytometry does require a greater amount of validation and optimization and there is definitely a learning curve, which can be steep and long. But, I would suggest that the return on that investment makes it well worthwhile. The amount and detail of information that can be acquired, and also the versatility of flow cytometry makes it an ideal technology that can be used to validate and strengthen conclusions drawn from data acquired using other techniques.

My advice to anyone starting out now, is to take advantage of all the learning resources that are available these days that can help you climb that learning curve. At Bio-Rad we have developed a range of resources that are aimed at beginners to give them a really solid understanding of flow cytometry from first principles. They include guides, a workbook, which leads the reader though practical exercises that they can perform in their own lab, and tools to help set up experiments. They have been designed to be helpful regardless of the manufacturer of your instrument or the reagents you are using and are completely free to use and download from our website.

What are the challenges associated with using flow cytometry as a screening platform?

As I have already mentioned, the volume of data and the speed that data can be generated is one of the key advantages of flow cytometry and that's exactly what you need for screening. However, there is more than one way of defining speed. Going back to my MSC experiment, what I needed was to collect lots of data points from a single sample, MSCs are very rare, even in bone marrow, so I was collecting at least half a million events per sample to measure a reasonable number of MSCs. In order to do that quickly you need an instrument that is able to collect data at a high event rate.

Another way to define speed is how quickly you can process individual samples, for example from a 96-or 384-well plate. Here, many instruments have a problem because they are designed to process samples individually. Typically, a sample is drawn into the sample line until it reaches the flow cell and data starts to be collected. Once you have enough data, collection stops and the sample line is purged ready for the next sample, but all this takes time. To address this limitation, instead of drawing samples individually, some instruments can be set to only partially fill the sample line with a small volume of each sample consecutively. These samples are separated by air and water gaps in the sample line. The instrument can recognize the boundaries of each sample and so there is a continuous one-way flow and that means multiple samples can be processed much more efficiently.

Finally, automation. Screening experiments that require a very large number of samples really benefit from automating the process of data acquisition. Some instruments are just not suited to that and in other cases users may be limited in terms of the automation solutions they can access due to the flexibility of the instrument's application programming interface, or API. Of course, for the ideal solution you would have an instrument that has a high event rate, uses smart sample handling and has a flexible API and that is what Bio-Rad developed with the ZE5 Cell Analyzer.

Moving forward to preclinical trials, could you explain 21 CFR Part 11 compliance and is there any support out there for this software?

Put simply, 21 CFR Part 11 is the portion of the code of federal regulations that regulates electronic records, and it applies to any laboratory under any federal records mandate. Obviously, this is a US regulation, but many other countries follow broadly similar rules. It becomes very important once research progresses past the discovery phase and although technically it's possible to use any instrument in a compliant manner, in practice, it's much more practical to use instruments that have software that supports compliance.

For the ZE5 Cell Analyzer we chose a collaborative approach. Rather than integrating compliance features into the instrument software itself, we partnered with Xybion Digital (NJ, USA) who have a really nice piece of software called Compliance Builder. The great thing about that is it doesn't interfere with how you use the instrument while still offering comprehensive compliance support. Another huge advantage is that it can be used on pretty much any instrument, not just flow cytometers. This means that labs can harmonize their record keeping with a single system and generate audit reports much faster than if they we having to collate reports from multiple separate sources.

Can you comment on any recent research that has been significant in terms of utilizing flow cytometry for cancer research?

One recent piece of research that really stands out for me was a paper published in *Nature Medicine* in April this year. It was a report on a Phase 3 trail of a drug called motixafortide, which is a CXCR4 inhibitor. The hope is that this drug can be used to improve outcomes for patients suffering from multiple myeloma. One strategy for treatment is to harvest stem cells from the blood following mobilization of those cells from the bone marrow. The patient is then treated with chemotherapy that kills off a lot of the immune system including the cancer cells. The immune system can then be reconstituted by autologous transplantation of the stem cells that were harvested previously. The problem with the current standard of treatment is that quite often the number of stem cells harvested is insufficient and that's what this new drug hoped to improve, by stimulating more efficient mobilization of stem cells into the blood.

We knew from speaking to our customers in the past that measuring stem cells in the blood is something that the ZE5 Cell Analyzer is really good at because of its ability to measure rare events quickly.

We hosted a webinar last year following the publication of a paper in *Blood* by one of our customers on that subject. But whereas that study was very much at the beginning of the road to drug development, the paper published in *Nature Medicine* is at the other end of that journey. As an instrument manufacturer its always great to see your product used extensively in a high impact publication, but when you know that work is going to be saving lives it is extra special.

CAR-T therapy is often hailed as a huge breakthrough for the treatment of cancer. How important is flow cytometry in the oncology field?

Absolutely crucial, I doubt that it could exist without it. Flow cytometry is useful at every stage of CAR-T development. Aside from the huge role it has played in understanding the immune system it has been used throughout the development process. It can be used, for example, to help optimize the transgene dose, confirm specificity of the transgene to the target molecule, assess the activation and exhaustion of CAR-Ts *in vivo*, confirm the viability of the transgenic cell product and monitor the composition and potency of the finished product and this is by no means an exhaustive list as I mentioned earlier it's a hugely flexible technology.

We published a White Paper recently that demonstrated some of the workflow for CAR-T development in which we use the ZE5 Cell Analyzer as well as other Bio-Rad technologies to perform several elements of CAR-T development. It was a really nice piece of work that demonstrated how all these technologies fit together in the context of CAR-T cells. I also know there have been many publications using the ZE5 Cell Analyzer in this context over the last couple of years.

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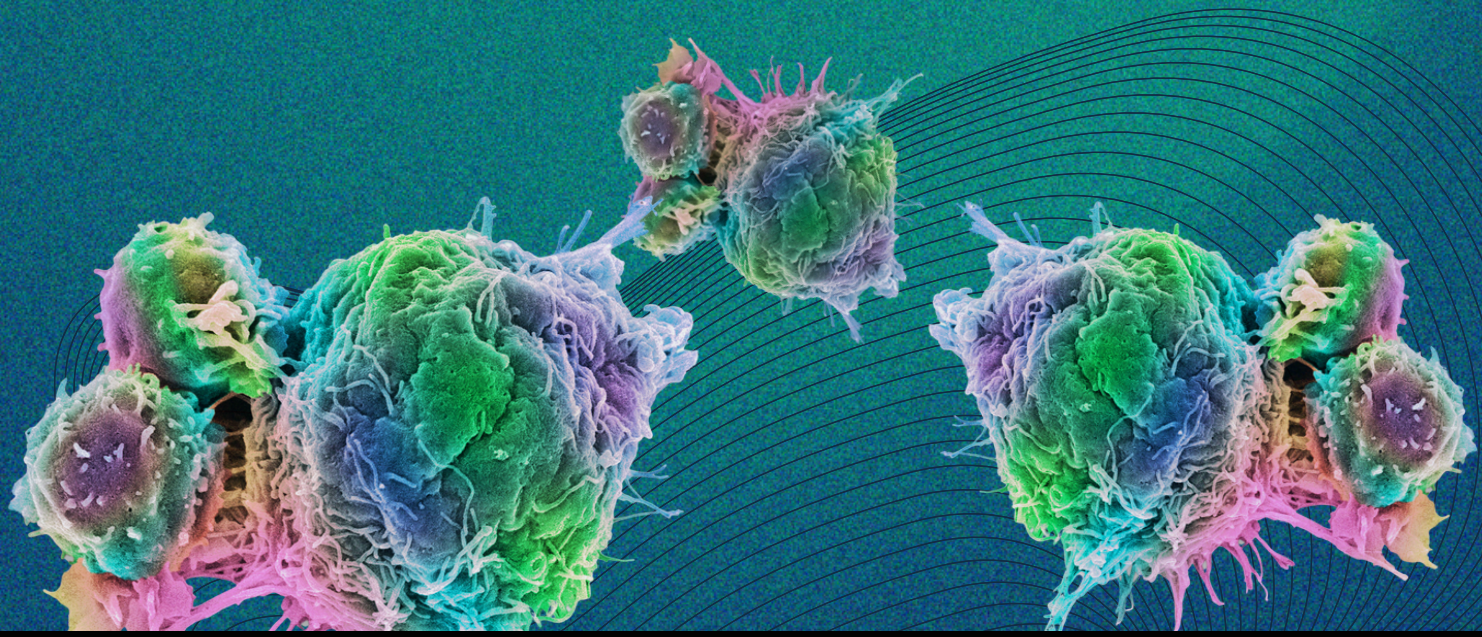
Selected cancer publications synopsis'

Flow cytometry is an invaluable tool that can help build our understanding of cancer and aid in developing new treatments. The ZE5 Cell Analyzer is perfectly suited for many aspects of cancer research. Here we provide a brief summary of some recent, high impact publications utilizing this innovative flow cytometer, highlighting how it may be used at important stages of drug discovery.



EPHB4 and EPHRINB2 act in opposition in the head and neck tumor microenvironment

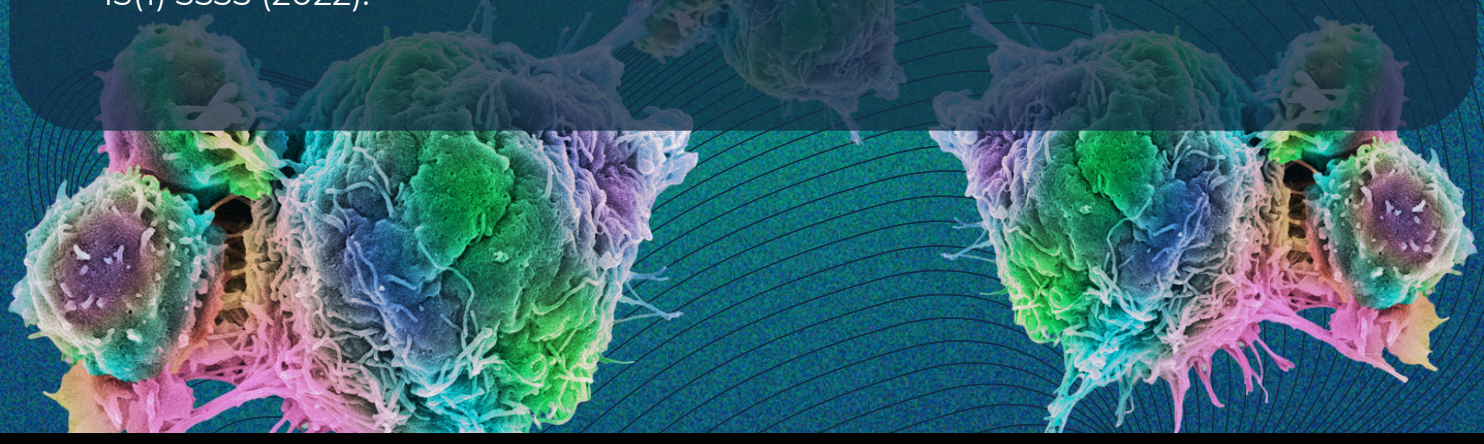
Brief Summary

Bhatia *et al.* used genetically engineered mice, recombinant constructs, pharmacologic agonists and antagonists to examine the effects of targeting ephrin type-B receptor 4 (EphB4) and ephrinB2 in head and neck squamous cell carcinoma. They report that perturbation of EphB4 signaling enhances angiogenesis, leads to an increase in the proportion of regulatory T-cells (Tregs) and promotes tumor growth and progression. Whereas loss of ephrinB2 inhibits tumor growth, leading the authors to identify ephrinB2 as a tumor promotor and its receptor, EphB4 as a tumor suppressor and also suggest opportunities for rational drug design.

The ZE5 Cell Analyzer was used to assess the influx of regulatory and cytotoxic T-cell populations in tumors. Their cytotoxicity and activation status were also confirmed using intracellular flow cytometry, which was also used to confirm the deletion of CD4+ Foxp3+ Tregs as well as to examine the resultant effect on dendritic cell influx, proliferation, and activation status.

Basic Science/Target identification

Bhatia S, Nguyen D, Darragh L.B., *et al*. EphB4 and ephrinB2 act in opposition in the head and neck tumor microenvironment. *Nat. Commun.* 13(1) 3535 (2022).



Albumin nanoparticle containing a pi3Kγ inhibitor and paclitaxel in combination with α-PD1 induces tumor remission of breast cancer in mice

Brief Summary

Since therapeutic strategies aimed at immunomodulation of the immunosuppressive microenvironment of tumors have shown limited success, the authors of this publication investigated immunomodulation of lymph nodes as an alternative treatment strategy. Using an immunomodulator contained within an albumin nanoparticle in combination with α-PD1 they achieved long-term remission and elimination of lung metastases in mouse models. This was associated with the remodeling of the immune microenvironment, the polarization of M2 to M1 macrophages, an increase in T-cells, B-cells and dendritic cells, and a decrease in regulatory T-cells.

The ZE5 Cell Analyzer was used throughout this publication to measure the proportions of M1 and M2 macrophages, both in tumors and lymph nodes of mice. It was also used to show the drug-dependent changes in the proportion of M1 and M2 macrophages, as well as an increase in the proportion of dendritic cells and activated dendritic cells following drug treatment. The authors also used a novel fluorescent labeling technique to show drug localization to tumor-associated macrophages and tumor cells within tumors and lymph nodes.

Animal Models/Pre-Clinical Research

Song Y, Bugada L, Li R, *et al.* Albumin nanoparticle containing a PI3Kγ inhibitor and paclitaxel in combination with α-PD1 induces tumor remission of breast cancer in mice. *Sci. Transl. Med.* 14(643) (2022).

Motixafortide and G-Csf to mobilize hematopoietic stem cells for autologous transplantation in multiple myeloma: a randomized phase 3 trial

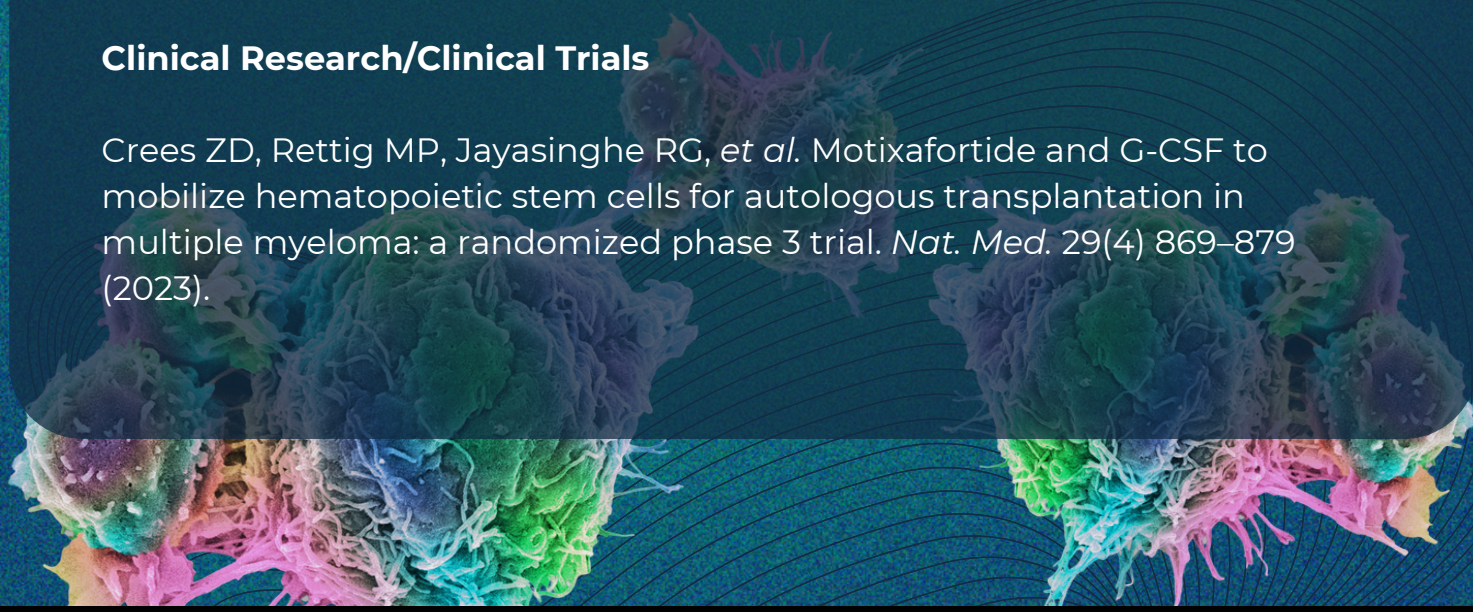
Brief Summary

Autologous hematopoietic stem cell transplantation improves survival in multiple myeloma. Using granulocyte colony-stimulating factor (G-CSF) alone to stimulate hematopoietic stem and progenitor cells (HSPCs) mobilization is suboptimal in many cases. This report of a prospective phase 3 trial, focused on the effectiveness of a novel CXCR4 inhibitor motixafortide, in combination with G-CSF, to mobilize HSPCs. The primary endpoint was achieved in 92.5% of individuals and 88.8% met the secondary endpoint, both with a high level of significance over the G-CDF/placebo control.

The ZE5 Cell Analyzer was used to quantify the numbers and proportions of nine individual CD34+ HSPC subsets in 48 patients following HSPC mobilization. The authors used t-distributed stochastic neighbor-embedding (t-SNE) projection to display the merged flow cytometry data. Flow cytometry was also used to examine CXCR4 expression on CD34+ HSCP subsets as measured by two monoclonal antibodies recognizing CXCR4 in day 1 apheresis products from treatment groups.

Clinical Research/Clinical Trials

Crees ZD, Rettig MP, Jayasinghe RG, *et al.* Motixafortide and G-CSF to mobilize hematopoietic stem cells for autologous transplantation in multiple myeloma: a randomized phase 3 trial. *Nat. Med.* 29(4) 869–879 (2023).



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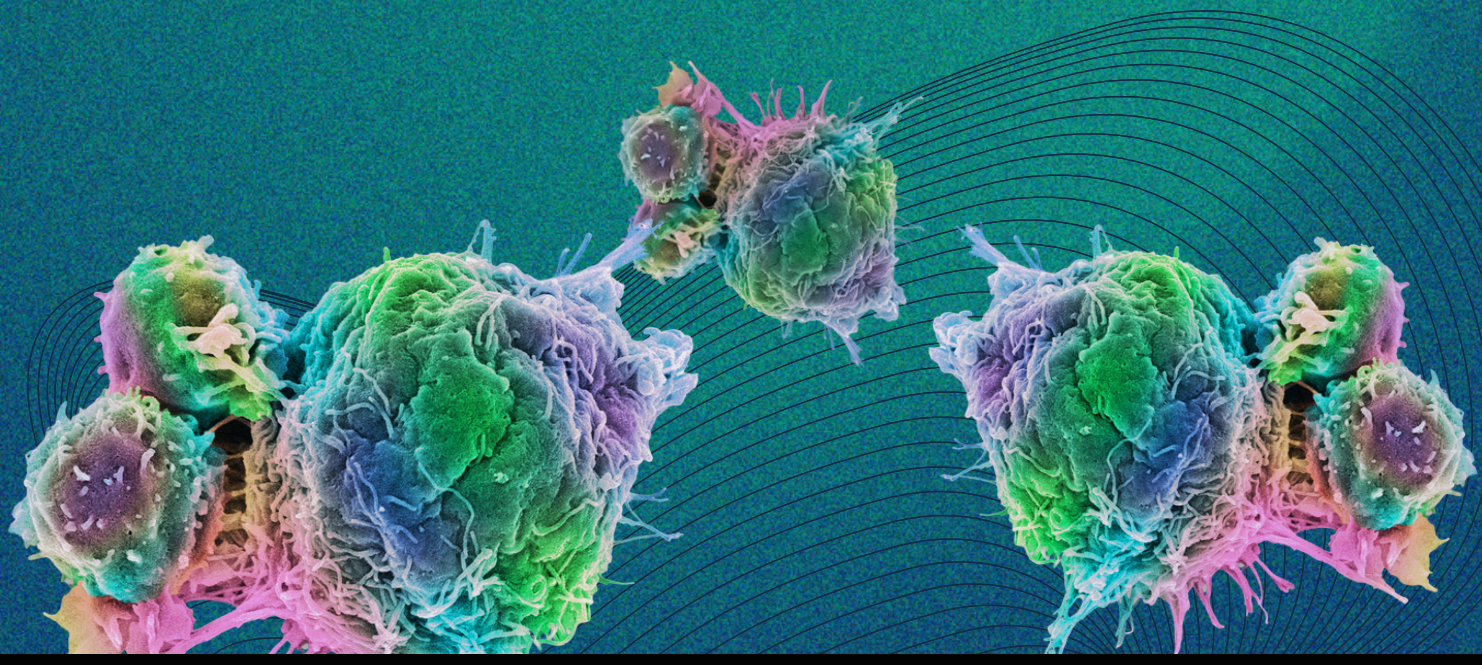
The ZE5 Cell Analyzer in Cancer Research

With a universal plate loader, fast sample acquisition, up to 5 lasers and 30 parameters, the ZE5 Cell Analyzer can significantly enhance throughput while remaining versatile enough for a wide range of applications relevant to cancer research. Its open platform and agnostic API allows integration with any robotic workcell making it ideal for screening for potential therapeutic compounds. With support for 21 CFR Part 11 compliance from Xybion's Compliance Builder software, it is possibly the only flow cytometer you will ever need.

For more information on how the ZE5 Cell Analyzer can benefit your research visit bio-rad.com/ZE5Cancer



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High-Impact Publications in Cancer Research, January 2022–July 2023

ZE5 Cell Analyzer Publications

Aikins ME et al. (2022).
Cancer stem cell antigen nanodisc cocktail elicits anti-tumor immune responses in melanoma.
J Control Release 351, 872–882.

Apavaloaei A et al. (2022).
Induced pluripotent stem cells display a distinct set of MHC I-associated peptides shared by human cancers.
Cell Rep 40, 111241.

Bhatia S et al. (2022).
EphB4 and ephrinB2 act in opposition in the head and neck tumor microenvironment.
Nat Commun 13, 3535.

Blokon-Kogan D et al. (2022).
Membrane anchored IL-18 linked to constitutively active TLR4 and CD40 improves human T cell antitumor capacities for adoptive cell therapy.
J Immunother Cancer 10, e001544.

Cordo' V et al. (2022).
Phosphoproteomic profiling of T cell acute lymphoblastic leukemia reveals targetable kinases and combination treatment strategies.
Nat Commun 13, 1048.

Crees ZD et al. (2023).
Motixafortide and G-CSF to mobilize hematopoietic stem cells for autologous transplantation in multiple myeloma: A randomized phase 3 trial.
Nat Med 29, 869–879.

Du W et al. (2023).
WNT signaling in the tumor microenvironment promotes immunosuppression in murine pancreatic cancer.
J Exp Med 220, e20220503.

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Evgin L et al. (2022).
Oncolytic virus-mediated expansion of dual-specific CAR T cells improves efficacy against solid tumors in mice.
Sci Transl Med 14, eabn2231.

Hattori T et al. (2023).
Creating MHC-restricted neoantigens with covalent inhibitors that can be targeted by immune therapy.
Cancer Discov 13, 132–145.

Jang HJ et al. (2023).
Dual receptor T cells mediate effective antitumor immune responses via increased recognition of tumor antigens.
J Immunother Cancer 11, e006472.

Kim MY et al. (2022).
A long-acting interleukin-7, rhIL-7-hyFc, enhances CAR T cell expansion, persistence, and anti-tumor activity.
Nat Commun 13, 3296.

Lidström T et al. (2023).
Extracellular galectin 4 drives immune evasion and promotes T-cell apoptosis in pancreatic cancer.
Cancer Immunol Res 11, 72–92.

Maniar R et al. (2023).
Self-renewing CD8+ T-cell abundance in blood associates with response to immunotherapy.
Cancer Immunol Res 11, 164–170.

Mao C et al. (2022).
In situ vaccination with cowpea mosaic virus elicits systemic antitumor immunity and potentiates immune checkpoint blockade.
J Immunother Cancer 10, e005834.

Merlotti A et al. (2023).
Noncanonical splicing junctions between exons and transposable elements represent a source of immunogenic recurrent neo-antigens in patients with lung cancer.
Sci Immunol 8, eabm6359.

Ng KW et al. (2023).
Antibodies against endogenous retroviruses promote lung cancer immunotherapy.
Nature 616, 563–573.

O'Neal J et al. (2022).
CS1 CAR-T targeting the distal domain of CS1 (SLAMF7) shows efficacy in high tumor burden myeloma model despite fratricide of CD8+CS1 expressing CAR-T cells.
Leukemia 36, 1,625–1,634.

Song Y et al. (2022).
Albumin nanoparticle containing a PI3Kγ inhibitor and paclitaxel in combination with α-PD1 induces tumor remission of breast cancer in mice.
Sci Transl Med 14, eabl3649.

Velez-Delgado A et al. (2022).
Extrinsic KRAS signaling shapes the pancreatic microenvironment through fibroblast reprogramming.
Cell Mol Gastroenterol Hepatol 13, 1,673–1,699.

Yao L et al. (2023).
Single-cell discovery and multiomic characterization of therapeutic targets in multiple myeloma.
Cancer Res 83, 1,214–1,233.

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Review

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Flow cytometry: principles, applications and recent advances

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Flow cytometry (FCM) is a sophisticated technique that works on the principle of light scattering and fluorescence emission by the specific fluorescent probe-labeled cells as they pass through a laser beam. It offers several unique advantages as it allows fast, relatively quantitative, multiparametric analysis of cell populations at the single cell level. In addition, it also enables physical sorting of the cells to separate the subpopulations based on different parameters. In this constantly evolving field, innovative technologies such as imaging FCM, mass cytometry and Raman FCM are being developed in order to address limitations of traditional FCM. This review explains the general principles, main applications and recent advances in the field of FCM.

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Keywords: apoptosis • cell cycle analysis • cell sorting • flow cytometer • fluorescence • intracellular staining

Introduction

Flow cytometry (FCM) is a technique which enables rapid analysis of statistically significant number of cells at single cell level. The main principle of this technique is based on scattering of light and emission of fluorescence which occur when a laser beam hits the cells moving in a directed fluid stream. Light scattering by these cells and cellular components occurs due to their structural and morphological properties. Fluorescence is emitted from a fluorescent probe and its intensity is proportional to the amount of fluorescent probe bound to a cell or a subcellular component. Fluorescent probes used in FCM may be fluorochromes (explained in the 'Fluorochromes' section within the 'Fluorescence and compensation' section) linked to antibody molecules or fluorescent dyes that either bind specifically to cellular components or exhibit emission variation depending on some microenvironmental characteristics. Interactions between the cells and the laser beam, in other words, light scattering, and/or fluorescence emission can be observed for each individual cell using specific detectors. Data obtained can then be correlated with characteristics of different cells and their components [1].

The term parameter refers to the physical or chemical characteristics of a cell (i.e., size, granularity or fluorescence features derived from either antibodies or dyes) that is measurable in cytometry. FCM allows multiparametric detection. In addition, it also provides single parameter distributions for a given population. Conventional instruments offer simultaneous detection 15–20 parameters [2,3]. However, most advanced instruments like MoFlo Astrios EQ offer detection of 50 parameters simultaneously [4]. Some of the commonly measured cellular parameters include cell size, membrane integrity and relative abundance of biomolecules. Besides eukaryotic cells, these characteristics can also be measured in microorganisms, nuclei and particles. Due to the enormous information that FCM generates, it is commonly applied for cell function analysis, disease diagnosis, therapy monitoring, detection of rare cells etc. [5].

Flow cytometers are of two types: Analyzers can perform only detection of light scattering and fluorescence emission whereas Sorters (e.g., BD FACSAriaIII™) can analyze as well as sort cells or particles based on various parameters such as size, fluorescence etc. [6]. In the next section, we describe major components of flow cytometers.

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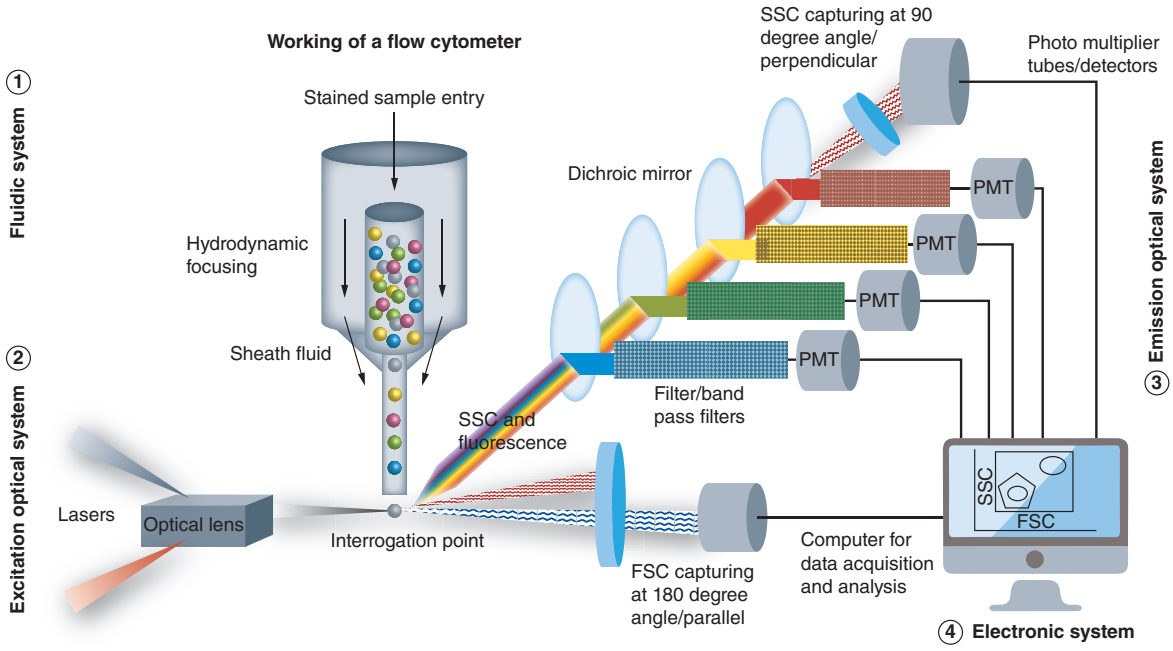

Figure 1. Principle of a flow cytometer. When single cells in a hydrodynamically focused cell suspension interact with the laser beam, the light scattered and fluorescence signals emitted by the cells are collected by the detectors which convert these signals into an electrical current. Electronics convert these analog current signals into digital signals and software represents the data in the form of two parameter dot plots or one parameter histograms. FSC: Forward scatter; PMT: Photomultiplier tube; SSC: Side scatter.

Components

A typical flow cytometer comprises of:

1. Fluidics: transport the cells to the laser interrogation point.
2. Optics: excite and collect signals generated by light scatter and fluorescence emission.
3. Electronics: convert optical signals into digital signals;
4. which are then processed by a computer.

Basic principle of a typical flow cytometer is depicted in Figure 1.

Fluidics

As cell suspension is injected into the flow cell of the flow cytometer, cells move in a single file across a laser beam which is focused perpendicular to the flow. Sample stream is hydrodynamically focused using an isotonic buffer (sheath fluid) that focuses sample stream into the central core inside the flow cell along the axis. Since the sample stream pressure is higher than the sheath fluid stream pressure, sample stream gets focused into a narrow stream. Using this technology, it is possible to detect up to 70,000 cells per second. Modern instrument such as MoFlo XDP cell sorter is capable of analyzing >1,00,000 cells per second [7]. The flow cytometer operator can adjust the rate at which cells are injected into the flow cell depending on the type of experiment. To perform qualitative measurements such as immunophenotyping, flow rates are set at a higher value. For analyzing DNA content, a higher resolution is a prerequisite which can be achieved using slower flow rates wherein sample stream becomes narrower thus increasing the precision of illumination. Proper operation of fluidic components is crucial for precisely intercepting cells with the laser beam. Fluidics system should always be without any air bubbles and/or debris and should be maintained at an optimum pressure [3,8].

Optics

Optics of a flow cytometer comprise of lasers, lenses and collection systems (filters and mirrors) at fixed positions. The laser beam is focused using crossed cylindrical lenses. Laser light is coherent and monochromatic which is

desired for uniform illumination of the cells with light of a specific wavelength. The laser excitation lines in conventional instruments include ultraviolet (350 nm), violet (405 nm), blue (488 nm) and red (640 nm) [9]. Yet, nowadays, yellow-green (561 nm) laser is being more commonly used than ultraviolet laser [10].

When laser beam hits a cell, the excitation light is scattered in the forward as well as side directions. Forward-scattered light (FSC) indicates the cell size whereas parameters, including granularity and cell morphology have an effect on the side-scattered light (SSC). Fluorescence is emitted from the fluorescent probe used to stain cells or cellular components. Collection optics consists of lenses that collect fluorescence emitted from the cell. A set of mirrors and suitable filters separate specific wavelengths and focus them towards the appropriate optical detectors. Suitable filters (such as long pass, short pass or band pass filters) are positioned so that an optical detector can detect a specific fluorochrome. Band pass filters transmit only a specific range of wavelengths, short pass filters allow wavelengths equal to or shorter than a specific wavelength to pass through and long pass filters transmit wavelengths equal to or longer than a specific wavelength to reach the detector [3,11]. Modern instruments such as CytoFLEX use repositionable band pass filters and non-standard filters. In these instruments, Wavelength Division Multiplexer (WDM) deconstructs and measures multiple wavelengths of light using fiber optics and band pass filters to separate the light wavelengths [12].

Photodetectors convert the signals generated by light scattering or fluorescence into electrical signals. Most commonly used detectors are photodiodes and photomultiplier tubes (PMTs). PMTs have a greater sensitivity as compared with photodiodes. FSC signals are stronger, which can be detected using photodiodes whereas the signals with weaker intensities (like SSC and fluorescence) can be recorded by PMTs [3]. Nowadays, avalanche photodiodes (APDs) are also used which have better sensitivity and resolution in the long wavelength regions [13]. The resulting signals are processed by electronics and data is visualized using the software.

Electronics

PMTs and photodiodes generate an electric current on the basis of the signals captured by them. Maximum amount of signal is detected when the cell passes through the middle of the laser beam. After generation of the electrical current by detectors, the obtained analog signal is amplified by a pre-amplifier and then converted into digital signal by analog to digital converters (ADCs) for further computations. This digital signal is then amplified by either linear or logarithmic amplifier. The amplified digital signals are assigned with channel numbers and are represented on two parameter dot plots or single parameter histograms illustrating the intensity of various parameters (such as light scatter or fluorescence) [2,3]. Depending on the amplifier used, distributions look different. Mode of amplification used is decided based on the dynamic range required [14,15].

Today cytometers are often used to detect things other than cells. 'Event' is a common term used to denote a single cell or a single particle detected by the instrument. All events are individually correlated to all the parameters collected. The signals can be combined in different ways to allow visualization of all subpopulations [2]. Data storage is done in Flow Cytometry Standard (FCS) format that is a list mode data file (raw file) that lists the FCM data for all parameters [16].

The instrument should be set so as to respond to signals derived only from an event and not from the debris, or spikes arising from electronic noise. For this purpose, a threshold is set on FSC or SSC or both so that an event is only detected when the signal goes beyond this threshold level [17].

Sorting

A cell sorter can physically sort the cells according to their properties. A cell suspension is injected into the sheath fluid stream of a sorter. Droplet sorting is the most traditional method, wherein the hydrodynamically focused cells are enclosed into droplets that arise from vibrating sheath stream generated by a nozzle. In a cell sorter, cells pass through the laser beam and their light scatter and fluorescence signals are detected in the same manner as that in a non-sorting instrument. Cells then continue to flow downstream and as the stream sonically breaks up into drops, the cells get enclosed in those drops. Electrical charge is imparted to these droplets for their further deflection [5]. This is followed by collection of cells on the basis of desired parameters such as cell size, fluorescence etc. (Figure 2). Advanced cytometers that are used today are able to charge the droplets in six different ways so as to separate a population into six subpopulations in the appropriately positioned collection tubes or a 96-well plate [4]. Since unwanted cells end up in uncharged droplets, they do not get deflected and pass down the center into the waste container [11,17]. Live cells sorted in a sterile environment can be subsequently cultured. Sorted cells can be used for further assays. Nozzle-dependent sorting can be performed at a speed of up to 25,000–30,000 cells per

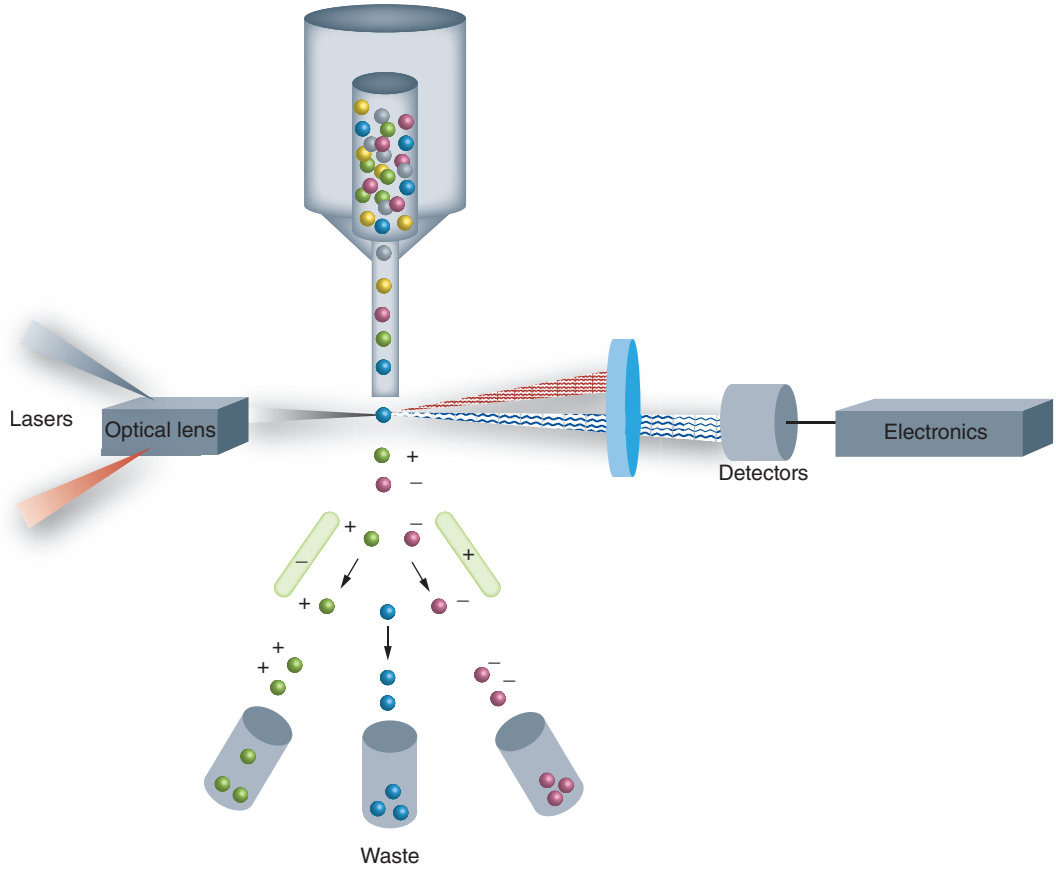

Figure 2. Cell sorting. A cell sorter imparts charge to the cells enclosed in droplets based on desired cellular parameters. Charged cells are subsequently deflected based on their charge toward the positive or negative deflection plates and are thereby sorted.

second, giving high purity and yield in the range of 80–90% depending on the target cell frequencies. Rare cell populations can be efficiently isolated using sorters with high recovery rates [18]. Also used nowadays is microchip cell sorting which offers several advantages over traditional methods such as equipment size reduction, elimination of use of biohazardous aerosols and simple protocols but yet suffers from limitations such as lower throughput and speed thus making processing of large number of samples unfeasible [19].

Fluorescence & compensation

Fluorochromes

Fluorescence is the emission of photons by certain molecules after their illumination with light of a specific wavelength. These molecules are called fluorochromes. Upon light absorption, electrons of a fluorochrome move from a resting state to an excited state emitting light of longer wavelength than that of the excitation light. This effect is called as the Stokes shift. Each fluorochrome absorbs light optimally at a maximum excitation wavelength although it can also be excited by a range of wavelengths suboptimally which is known as excitation spectrum of the fluorochrome. Based on this information, fluorochromes can be selected so that they can be excited using lasers in the available instrument. Similarly, each fluorochrome emits light optimally at a specific emission wavelength, but it can also emit over its whole emission spectrum. For maximum collection of the emitted fluorescence, a specific optical filter is selected. The spectral information is also useful to determine which fluorochromes can be used together [8,20].

Fluorescence measurements captured across multiple wavelengths can provide relatively quantitative information about fluorescent probe-labeled cells or cellular components. Flow cytometers use separate fluorescence channels (FL) to detect emitted fluorescence. An argon laser (488 nm) is the typical light source in the single laser instruments. It can excite various commonly used fluorochromes viz. FITC, PE and Alexa fluor 488 [17,20].

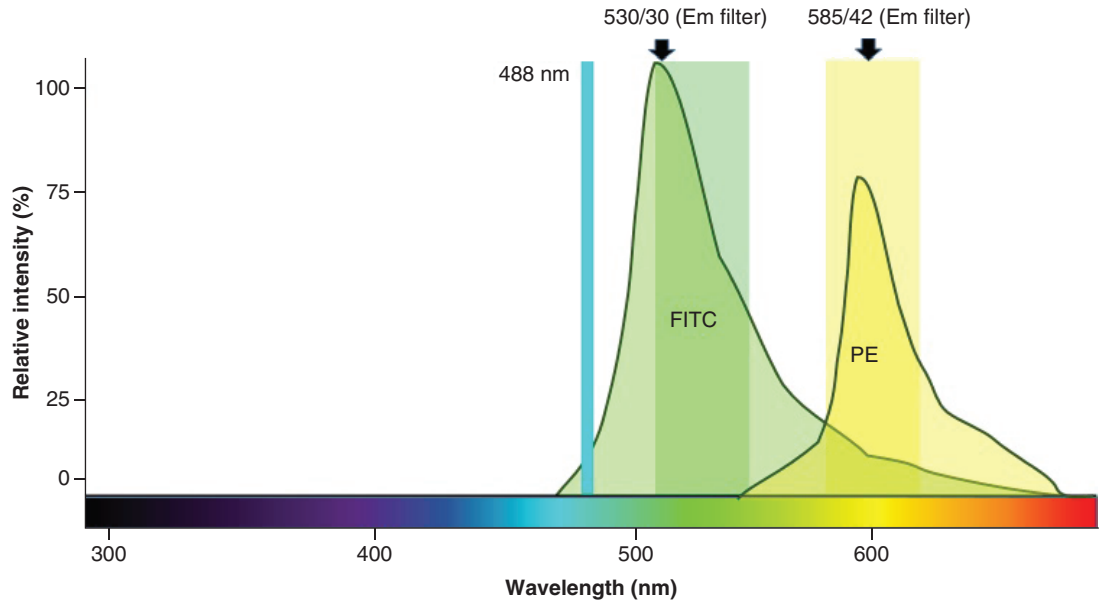

Figure 3. Emission spectra of FITC and PE that illustrate spectral overlap. Both FITC and PE fluorochromes can be excited by a 488 nm laser line. Although FITC and PE have clearly separate peak emission wavelengths which can be transmitted towards specific detectors by suitable emission filters (Em filters), there is an overlap between spectra of emission wavelengths of these two fluorochromes.

Overlapping emission spectra of various fluorochromes limits the maximum number of detectable markers in traditional flow cytometers; since in such multicolor experiments, each fluorescence channel is directly correlated to a biomarker. For overcoming this limitation, fluorochromes with differing excitation spectra are used. Presently, up to seven lasers offering excitation wavelengths between 325 nm and 808 nm are used which make the selection of fluorochromes highly flexible [21].

Tandem dyes are fluorescent molecules that comprise of two closely attached fluorochromes. In case of such dyes, the emission spectrum of one fluorochrome (donor) and the excitation spectrum of the other fluorochrome (acceptor) are overlapping. Thus, energy absorbed by donor is transmitted to the acceptor, which further emits it in the form of fluorescence. This energy transfer occurs only when the fluorochromes are in a close proximity.

Quantum dots are fluorescent nanocrystals; those used in FCM are 10–20 nm in size. They exhibit a wide excitation spectrum and a very narrow emission spectrum as compared with that of the conventional fluorochromes [3]. Therefore, appropriate combinations of quantum dots and fluorochromes can be used together [16]. However, the fact that they can be excited by all laser lines limits their use in combination with available fluorochromes. Also, they exhibit a 'blinking' behavior in which fluorescence emission is interrupted by dark periods of no emission [22].

Compensation

Fluorochromes show broad emission spectra. For example, although FITC fluoresces mainly in green, it emits over a spectrum of emission wavelengths which contain a range of colors from green to red. For many commonly used fluorochromes, though the peak emission wavelength is distinct for every fluorochrome, the emission spectra often overlap between them. Figure 3 shows the spectra for FITC and PE with two stylized band pass filters superimposed on them. A part of the fluorescence emitted by FITC is transmitted through the filter for PE and detected in the PE channel. This is called the spectral overlap. Spillover of FITC signal into PE channel detector results in appearance of a PE-positive population. The spectral overlap is compensated by subtracting a fraction of the FITC signal from total PE signal [20]. For setting the compensation, PMT voltages on all the fluorescence channels in use are set in order to attain a clear separation between negative and positive cell populations. Then cells labeled with single fluorochrome are analyzed and the compensation is applied by subtracting the fraction of one fluorescence signal from another. (There is no PE in the FITC only tube; hence the apparent fluorescence in the PE channel is only caused by FITC spillover.) For two or three fluorochromes, such a manual method of compensation is suitable; with

four or more colors an automated method should be used. In modern machines, softwares are used for calculating the compensation which is applied after the samples are analyzed [23].

Quality control

Calibration

An instrument is standardized, calibrated and monitored on a regular basis for quality control using particles (commercially available synthetic or biological). Commercially available synthetic reference particles (commonly known as reference beads) are often matched with the spectra of the fluorochromes employed in the experiment. However, at times, rainbow beads are used which are able to fluoresce in the complete desired spectrum. In addition, a routine check of the linearity of PMT using calibration beads helps to detect improper functioning of the machine [24]. All of these measures ensure that the signals are detected within a desired range with only a minimal deviation.

However, reference beads have two limitations: First, they may have different light scattering properties as compared with cells. Second, fluorescence from a bead and that from a cell for a given fluorochrome, though can be similar, can never exactly match [25].

Molecules of equivalent soluble fluorochrome (MESF) is a standard unit for fluorescence measurement. Commercially, calibration beads are available which are conjugated with a known number of fluorochrome molecules. This quantity is reported in terms of MESF units. In such cases, absolute quantities of fluorescence signals for samples can be calculated in MESF units by analyzing the beads and samples at the same PMT voltage at the time of analysis. Using such calibration, results can also be compared across different instruments and/or across experiments carried out on different days [26,27].

Voltage gain

Electronics are used for voltage control of the PMTs which determines the sensitivity of PMTs to light signals. An optimal range of voltages (gain) can be determined for experiments on a daily basis using reference beads. The gain is applied in such a way that every fluorochrome being used displays a maximum signal in the PMT assigned to that fluorochrome-specific channel and a lower signal in all other channels [28]. Further, for every particular experiment, gain is set using unstained cells such that these cells appear in the lower range of the logarithmic scale of the specific fluorescence channel. However, these applied gains are only for reference. Voltage adjustments are required on a daily basis for different experiments based on different parameters and/or sample types under study.

Gating

The most crucial and laborious step while manually analyzing the FCM data is the identification of homogeneous cell populations in a given sample and a comparison of such subpopulations across samples. This is achieved by a process called as gating. It involves visually comparing two or three dimensional plots followed by selection of the desired regions for subsequent analysis [2].

Sequential gating is employed for analyzing multidimensional datasets. This method uses software for applying sequential manual gates so as to select regions in two dimensional plots (Figure 4). During the gating step, boundaries are determined by the operator and are not always guided by rules. However, manual analysis of high-dimensional datasets is tedious. In order to overcome this constraint, lots of efforts have been put into developing automated gating strategies which use both supervised and unsupervised statistical methods [29]. These methods directly identify cell subpopulations from the high dimensional FCM data, thus overcoming limitations of manual gating [30–32].

At this point, gating controls can be employed to set up the gate boundaries for classifying cells on the dot plots. These controls help to distinguish specific signals from the non-specific ones. Gating controls are critical when there is no clear separation between fluorochrome-positive and negative cells. This is commonly observed in case of some surface markers such as CD25. A negative control (e.g., unstimulated cells) or a fluorescence-minus one (FMO) control can be used as gating controls [23].

Visualization of data on linear, logarithmic & bi-exponential scales

Data acquired by FCM can be expressed on a linear, logarithmic or bi-exponential scale which is selected on the basis of type of an experiment. For example, linear amplification is used during DNA analysis which involves only a limited difference (two-fold) in the DNA content. However, when immunofluorescence is performed for protein

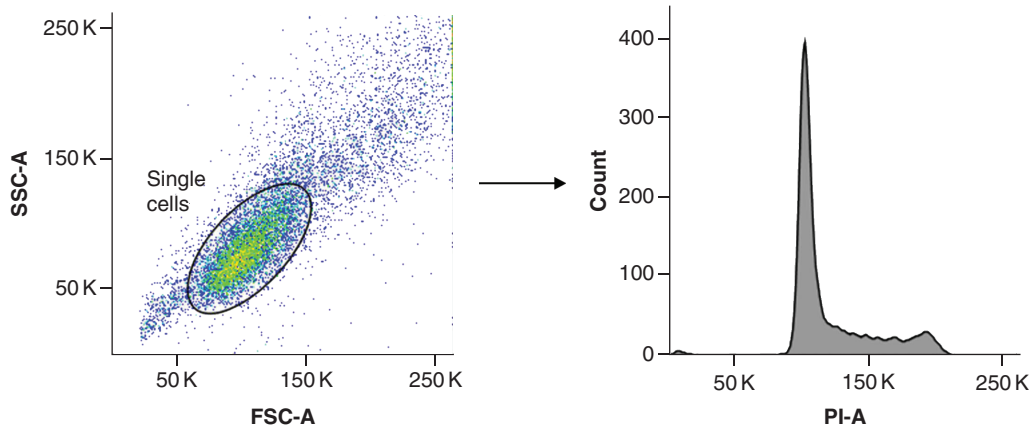

Figure 4. Manual gating based on scatter properties to plot single parameter histogram. For cell cycle analysis, healthy single cells are gated based on FSC-A vs SSC-A dot plot. A distribution of DNA fluorescence intensities (resulting from propidium iodide staining) can be plotted for gated cells as single parameter histogram on a linear scale.
FSC-A: Forward scatter area; PI-A: Propidium iodide fluorescence area; SSC-A: Side scatter area.

detection, a wider range (100- to 10,000-times) of fluorescence intensities is roofed, because of the immense cell-to-cell heterogeneity. Thus, a logarithmic amplifier is used to cover the complete spectrum of fluorescence signals [8].

Yet, the logarithmic scale does not correctly represent cells with fluorescence levels below zero that result from subtracting background and fluorescence compensation. To correct this, bi-exponential scale is used which approximates the normal logarithmic scale at the higher range and at the same time allows visualization of the data below zero on the linear scale [33].

Data analysis: modern approaches

Recent advents in the modern FCM enable researchers to measure increasingly high number of parameters at single cell level thus generating large number of multidimensional datasets. The complexity of these datasets makes their manual analysis using classical approaches challenging (due to several disadvantages of manual analysis such as low reproducibility, time-consuming, subjective and biased), therefore demanding the development and use of novel computational and statistical strategies for the same. Nowadays, different computational methods are being used at several stages during data analysis. These include storage of well-annotated FCM data in repositories, data cleaning and pre-processing (for dimensionality reduction) and for different types of analyses such as data visualization, population identification, and biomarker detection using several clustering and automated gating techniques [21,34]. Compensation on multicolor systems requires use of softwares such as WinList and FlowJo although it has limitations due to inaccurate conversion from linear to logarithmic signals by analog circuitry of the flow cytometer [35]. Therefore, computational approaches cannot completely replace manual analysis and rather should be used as complementary tools. Additional manual analysis is still a good practice for quality control.

Sample preparation

The sample to be analyzed by FCM needs to be a single cell suspension. So, sample preparation for FCM-based analysis is easier for suspension cell types (e.g., suspension cell lines or primary blood cells). However, solid tissues or adherent cell culture samples need to be treated to disintegrate the tissues or cells into a single cell suspension and only then they can be used for FCM-based analysis. Two main methods for disintegration of the tissues or adherent cells in culture are mechanical or enzymatic dissociation. Mechanical dissociation of the sample is performed by applying a mechanical force that breaks down the tissues in the growth medium thus releasing the cells. The solution is further filtered by centrifugation to remove large particles and the pellet is resuspended in growth medium to obtain a single cell suspension. In the enzymatic digestion, cells are treated with enzymes such as trypsin, EDTA, etc. [36–38]. After obtaining the cells in suspension, sample must be centrifuged to remove such enzymes. Cells that have undergone mechanical or enzymatic disintegration should preferably be checked for their viability. Besides

these methods, recently, use of temperature-sensitive plates for cell detachment has been demonstrated successfully without significant loss of cell viability/function [39].

Applications

Major applications of FCM are summarized in Supplementary Table 1 and some of these important applications are discussed in details below.

Cell cycle

Cell cycle analysis has been the basic application of FCM. DNA amount in cells is commonly the only parameter measured during such kind of analysis. Exponentially growing cells in culture go through a series of changes during cell cycle progression. DNA measurements allow identification of these changes, by compartmentalizing the cell population into three basic cell cycle phases: G0/G1, S and G2 + M. Normal non-dividing (G0), or quiescent (G1) cells contain diploid (2n) DNA content in somatic cells. During the S (synthesis) phase, duplication of the DNA occurs. Thus, during G2 and M phases, DNA content of the cells is double as compared with that in the cells in the G0/G1 phase [40]. Cell populations having less than G0/G1, in other words, sub G1 DNA content are identified as apoptotic cells since extensive degradation of DNA is a hallmark of apoptosis [41]. Cell cycle analyses are performed using fluorescent dyes that bind stoichiometrically to DNA. Some dyes exhibit intercalative binding (e.g., propidium iodide (PI)) whilst others show an affinity for DNA A-T rich regions (e.g., Hoechst 33342 and DAPI) or G-C rich regions (e.g., mithramycin) [42]. Since such dyes have affinity for both DNA and RNA, RNAse treatment is required for DNA quantification during cell cycle analysis. Cells have to be fixed prior to DNA staining. Formaldehyde, when used as a fixative, cross-links the chromatin and reduces the dye binding capacity. Therefore, dehydrating fixative such as ethanol is commonly used which also provides stability during long term storage. After fixation and staining, when sample is analyzed by FCM, the total fluorescence emission from the cell is considered as equivalent to the cellular DNA content. Since the data acquired does not indicate the absolute quantity of cellular DNA content, reference cells/nuclei with varied DNA content should be employed for comparative positioning of the cells under investigation. Commonly used reference nuclei are chick erythrocyte nuclei (CEN) and calf thymocyte nuclei (CTN) [43]. For cell cycle analysis, samples should be analyzed at a slower flow rate (<1000 cells per second) for achieving a good discrimination between singlets and doublets. Such slow flow rates provide higher resolution which is desired for DNA content analysis since they reduce the size of the sample stream and increase the uniformity and precision of the illumination [3]. Simultaneous detection of DNA (using Hoechst 33342) and RNA (using Pyronin Y) can also be done by following the same protocol without using RNAse [44].

Cell proliferation

Since the late 1980s, two different methods for cell cycle and proliferation analysis are commonly used which are based on the detection of 5-bromo-deoxyuridine (BrdU – a thymidine analog) that gets incorporated into the DNA during replication. In the first method, measurement of cell proliferation along with cell cycle analysis is carried out using PI staining and BrdU labeling. Incorporation of BrdU into the cellular DNA of S phase cells can be detected by FCM using FITC-tagged monoclonal antibody against BrdU which gives a direct measure of cell proliferation along with cell cycle analysis by PI staining [45].

Another technique is based on the property of BrdU, that when it is incorporated in replicating DNA of cells in S phase, it reduces fluorescence intensity of cells stained with Hoechst 33258. So, after a continuous incubation with BrdU and DNA staining with Hoechst 33258, one can differentiate the cells according to the number of divisions they have undergone and can recognize the proliferative cellular fraction [46,47].

Apoptosis

Understanding the mode of cell death (necrosis vs. apoptosis) is very important, as it can cause pro- or anti-inflammatory responses. Apoptosis can be distinguished from necrosis using the biochemical and morphological changes associated with it. The change in light scattering properties of apoptotic cells is commonly used as a marker for identifying them. During early apoptosis, the cell exhibits noticeably reduced intensity of forward light scatter and an enhanced side scatter [48]. In addition, the fragmented DNA leaks out from fixed apoptotic cells during rinsing and staining procedures of cell cycle analysis. Such events with fractional DNA content detected by

flow cytometer are represented as the 'sub G1' peaks on the DNA histograms and are considered to be apoptotic cells [49,50].

Phosphatidylserine which is located on the inner surface the lipid bilayer of live cells flips during apoptosis and is exposed on the outer leaflet of the membrane. It can then be bound by annexin V. Thus, fluorochrome-labeled annexin V proves to be an important marker for the detection of early apoptotic cells by FCM. Besides, loss or presence of the cell membrane implies the nature of cell death, in other words, necrosis or apoptosis. Therefore, annexin V labeling method is used in conjunction with a vital DNA staining dye such as PI to identify mode of cell death, in other words, apoptosis or necrosis [51].

Yet, this protocol is more suitable for cells in suspension since in case of adherent cells, results may vary depending on cell harvesting methods used as well as cell type/cell line being employed [52,53]. Although this assay is usually carried out using live cells, Mariotti *et al.* [54] have proposed a method that allows multiparametric analysis of fixed and permeabilized cells for identification of apoptotic/necrotic populations using annexin V/PI staining along with simultaneous detection of surface markers and intracellular cytokines.

Apoptotic cells can also be identified using FCM by detecting breaks in the DNA strands. This is achieved by labeling their 3′-OH termini with fluorochrome-conjugated nucleotides using exogenous terminal deoxynucleotidyl transferase (TdT). This methodology is termed as TdT dUTP nick end labeling (TUNEL) assay [55–57].

Surface molecule staining

Surface molecule staining is another most commonly used application of FCM which allows the characterization and identification of cellular subpopulations. It also permits identification of the degree of cell differentiation and/or specific cellular functions based on the relative quantification of associated surface proteins [58]. Surface molecule staining does not require any special preparation step such as fixation and permeabilization. In fact, such staining should be done using live cells to circumvent any possible denaturation that could arise during the pre-treatment steps. Yet, simultaneous surface molecule staining and intracellular staining has been reported by several groups [59–61]. Adherent cell lines or primary cells derived from tissues require pretreatment using enzymes to dissociate the tissue for obtaining cells in suspension for FCM-based analysis. However, during tissue dissociation, antigenicity of surface molecules may get affected. Therefore, conditions for dissociation need to be optimized for surface molecule staining of different tissue/cell types [62,63].

Intracellular protein staining

Intracellular protein detection by FCM requires fixation and permeabilization of cells which make epitopes of intracellular proteins accessible to fluorochrome-tagged antibodies against them. Fixatives are dehydrating agents such as ethanol, methanol or acetone which coagulate proteins and also permeabilize the cells by creating holes in the lipid membrane. Fixation is performed commonly using formaldehyde in the concentration range of 1% to 4%. It fixes the protein structure in place by crosslinking lysine residues. Two-step method of fixation with formldehyde and permeabilization with methanol has been identified as the best combination for intracellular staining of cell signaling proteins [24,39,64,65].

Various non-ionic detergents are available such as Triton X-100, Tween-20, and saponin which can be selected for permeabilization based on localization of the protein of interest [66]. Transcription factors are usually nuclear and therefore their staining requires the use of stronger detergent such as Triton X-100 which permeabilizes both the plasma membrane and the nuclear membrane. Time and conditions of fixation and permeabilization need to be optimized carefully for intracellular staining of specific protein/s of interest. Depending on the experimental need, fluorochromes need to be selected, for example, transcription factors are rare hence bright fluorochromes should be used for staining them while cytokines can be stained with less bright fluorochromes as they are known to accumulate in higher numbers within the cell when their secretion is blocked [67]. Simultaneous quantification of multiple cytokines is routinely carried out using multiplex microbead assays [68]. Intracellular phospho-proteins are detected by a technique termed 'phospho-flow' [64,69]. Different methods of fixation and permeabilization for multiparameter surface marker and intracellular staining of kinase cascades in suspension cells have been extensively optimized and discussed by Perez *et al.* [70].

Classical cell detachment methods are known to damage cellular components. Therefore, intracellular protein abundance detection in adherent cells using classical FCM is challenging since it uses such methods for cell detachment [39]. Yet, it has been successfully demonstrated using carefully optimized methods of cell detachment such as cold trypsinization, non-enzymatic temperature-sensitive method and using alternatives to trypsin in some

primary cell types and adherent cell line models [37,39,71]. Microsphere-based FCM approach has been employed to study effects of extracellular matrix components on cell signaling in adherent cells [72].

The abundance of surface or intracellular proteins is compared using overlaid histograms of fluorescence intensities of different samples. Change in the fluorescence intensity is typically quantified using mean/median fluorescence intensity (MFI) and/or the percentage of cells which exhibit fluorescence signal above that of the unstimulated cells. In some cases, percentage of positive cells is identified by applying a threshold so that all cells with fluorescence intensities higher than the threshold are considered to be positive [17].

Fluorescent cell barcoding

As the scale of FCM experiment expands, researchers often encounter several limitations such as the antibody cost and longer time for sample acquisition. Also, due to large number of samples, the assay may suffer from staining variability among the samples. To overcome this problem, a multiplexing approach known as fluorescent cell barcoding (FCB) has been developed which saves the antibody consumption, reduces the sample acquisition time by improving the throughput of FCM experiments and eliminates staining variability among samples [65]. Individual cell samples are labeled or barcoded with specific quantities of amine reactive N-hydroxysuccinimide (NHS)-derivatives of fluorochromes (commonly known as FCB dyes) which impart unique signatures to the samples. Further, multiple samples can be mixed, stained, and analyzed as a single sample. The samples can be deconvoluted during software analysis on the basis of their fluorescence intensities in the barcoding channel. Prior to antibody staining, samples are mixed together which significantly reduces the antibody consumption. In addition, FCB increases the data robustness and throughput, minimizes pipetting error and staining variation, and enhances the speed of acquisition. FCB can be used for multiplexing during intracellular or surface marker staining not only in suspension cells but also for adherent cell lines [37,39,73–76]. Many FCB dyes are available, which allow multiparameter barcoding. Another technique named fluorescent genetic barcoding using retroviral transduction enables generation of stable cell lines with unique fluorescent signatures [77,78]. Retrogenic color barcoding has been used for mapping and tracking natural killer (NK) cells upon murine cytomegalovirus (MCMV) infection [79].

Other intracellular parameters measurable by FCM include mitochondrial membrane potential (using specific fluorescent probes such as JC-1, Mitotracker green, rhodamine 123) [21,80–82]; Ca^{2+} concentration (using Indo-1 acetoxymethyl ester, Fluo-3, Fluo-4, Fura red) [83–85]; and reactive oxygen species (ROS) (using 2′–7′ dichlorofluorescein diacetate, i.e., DCFH) [86,87].

Rare cells

Cell types that are present in low abundance in an organism are categorized as rare cells. Rare cell populations are of significant value in diverse areas such as basic research, translational medicine and diagnostics. In several disease conditions, these cells provide critical information about status of the disease, for example, circulating tumor cells (CTCs) in the bloodstream, cancer stem cells etc. Rare populations of stem cells in normal adults have important therapeutic applications. Optimal methodologies are required for detection of these cell types because of their low frequency of ~0.01%. Based on the expected frequency of rare cell type being studied, the amount of biological material required for obtaining a sufficient number of those cells needs to be calculated. On the basis of experimental need, enrichment of the rare cell population may be required, for which specific markers that enable unambiguous identification of that particular rare cell population need to be defined. During sample analysis, gate boundaries should be laid so as to exclude unwanted cell types, doublets and debris [88–92].

Extracellular vesicles

Cells release extracellular vesicles (EVs) of 0.1–1 μm into the extracellular environment in response to activation or apoptosis or viral infection. EVs may function as vehicles for intercellular communication. Their role both as markers and pathogenic effectors in various disease conditions such as cancer has increased interest in their detection in clinical practice [93]. Specific biomolecules are selectively incorporated into the EVs before their release into the extracellular environment [21,94,95]. To measure EVs by FCM, two strategies are used. In the first strategy, vesicles are selected according to their size (using FSC-based threshold) and then fluorescence intensities are analyzed only for those preselected populations. In the other strategy, vesicles are preselected on the basis of fluorescence-based threshold and then the specific populations are identified by scatter-based gating. EVs can be isolated from conditioned media of cultured cells or from body fluids such as blood, urine etc. Methods employed for EV isolation from blood significantly affect EV count and function [96]. EV populations are detectable after incubation

of such conditioned media/body fluids with fluorochrome-labeled monoclonal antibodies against specific antigens expressed by those EVs (which depend upon cell type from which those EVs are derived) [97].

Microbiology

FCM is a rapid and inexpensive method for characterizing and analyzing microbial communities [98]. Dual parameter analysis can be applied to identify different bacteria in a given sample. Direct staining of cellular components using fluorochromes along with cellular size detection is the easiest way for FCM-based identification of microorganisms. Another common application of FCM in microbiology is testing the viability of bacteria using vital dyes such as erythrocin B, fluorescein diacetate etc. FCM has also been applied in the probiotic count assessment, allowing identification of viable and non-viable cells, and also for providing additional information on the physiological state of the cells using specific fluorescent probes [99]. Antimicrobial effects and susceptibility testing to antibiotics has also been carried out by FCM in bacteria using fluorescent probes [100].

Nanoparticles

Nowadays, nanoparticles (NPs) are commonly used for targeted drug delivery. Quantifying the interactions of NPs with cells is crucial for designing efficient drug delivery systems which is possible using FCM. The uptake of NPs is routinely assessed by changes in side scatter of cells [101,102]. The association of NPs with cells can also be analyzed with a histogram overlay of treated and untreated samples [103]. Also, antibody-conjugated fluorescent silica NPs have been used for the detection of pathogens [104].

Limitations

Although traditional flow cytometers offer unique advantages over other techniques, they do suffer from some limitations. As an example, for FCM-based analysis, it is essential that cells are in a single cell suspension. While recent improvements in cell dissociation techniques have broadened the possibility of using FCM for analysis of samples from tissues and solid tumors, investigators often encounter complications such as reduced cell viability and functional alteration following disruption of tissue structures. Also, traditional FCM provides little information about subcellular localization of components, although recently some groups have tried to address this problem [105–107].

Flow cytometers are very expensive and sophisticated instruments. They are prone to problems such as blockages of the fluidic systems and also require warm-up, laser calibration and cleaning after every use [25]. Therefore, maintenance of these instruments requires trained personnel and extensive routine quality control protocols that need to be followed. With continuous development of the FCM technology, enormous amount of data is being generated that warrants complex analyses and specialized training for the same [2].

Recent advances

Latest innovations in lasers, fluorochromes, quantum dots and computer processing software have developed the current state-of-the-art in multicolor FCM. However, multicolor FCM has to overcome intrinsic shortcomings which arise due limited number of spectrally resolvable fluorochromes that are currently available [20]. In order to overcome this limitation, a new platform has been developed that combines FCM and mass spectrometry, called mass cytometry which uses stable element isotope-conjugated antibodies. Recently, Fluidigm has developed Helios™ CyTOF® which utilizes monoclonal antibodies labeled with heavy metal tags and offers single cell analysis with simultaneous measurement of up to 100 parameters at one time. Mass cytometry has been employed for understanding functional complexity of hematopoiesis using simultaneous measurement of 34 parameters and signaling states in hematopoietic cellular subsets at single cell level [108]. Imaging mass cytometry has also been applied to solid tumors e.g. in human breast cancer samples, for identifying cell subpopulations, cell-to-cell interactions and cellular heterogeneity within the tumor [109]. Mass cytometry has also been used for mapping cellular reprogramming of mouse embryonic fibroblasts into induced pluripotent stem cells since it allows direct access to post-translational modifications and cell signaling states in single cells [110]. It has also been used to demonstrate functional diversity of human $CD8^+$ T cells by analyzing large number of proteins [111]. Recently introduced Acoustic-based flow cytometers use acoustic focusing principle in order to achieve a narrow core stream and uniform laser illumination [15].

In order to limit the difficulties of spectral overlaps and interferences in conventional flow cytometers, a recently developed technology called Spectral Analyzer creates a spectral fingerprint by measuring the entire fluorescent

emission spectra for each fluorochrome in a multicolor sample. During analysis, each spectrum is separated to get a precise signal for each fluorochrome. Spectral analyses are being routinely used for detection in high-dimensional FCM [112,113].

A great limitation of traditional FCM data is that it does not provide an image. Single cell detection is only on the basis of light scatter. These cells are represented by dots on two parameter plots. Unwanted particles like debris, aggregates or air bubbles are also detected in the same manner leading to ambiguity. This shortcoming has been resolved by imaging flow cytometers which measure cellular signals and plot them in the same manner as in the classical FCM. In addition, these cytometers provide a microscopic visual image (bright field and fluorescence) for each recorded event [18]. This allows rapid simultaneous analysis of a sample for cell morphology and multiparameter fluorescence at single cell as well as population level. Thus, imaging cytometers have the ability to track protein levels within individual cells (like that in a fluorescence microscope) and can also process large number of cells just like a flow cytometer. They are especially useful in any application that requires coordinated information about the cellular location along with the fluorescence expression in large cell populations [112]. ImageStream® 100 Multispectral Imaging Cytometer has been successfully employed for discriminating live, necrotic, early and late apoptotic subpopulations of Jurkat cells using combinations of fluorescence intensity and morphometry [114]. Recently, virtual-freezing fluorescence imaging flow cytometry (VFFIF) technique has been developed that allows longer exposure time for microscopy grade fluorescence image acquisition and thus enables high-throughput imaging FCM of single cells at >10,000 cells per second without sacrificing sensitivity and spatial resolution [115]. Cell Search Engine (iCSE), which integrates high-throughput cell imaging, cell focusing, and cell sorting enables deep learning assisted image activated sorting which has been demonstrated using microalgal cells and blood cells [116]. Nawaz *et al.* (2020) have recently designed a novel approach for cell sorting using real-time fluorescence and cell deformability cytometry (RT-FDC), standing surface acoustic wave (SSAW) sorting and deep neural networks. It was demonstrated for label-free sorting of neutrophils from the whole blood [117]. Nitta *et al.* have developed an intelligent image activated cell sorting platform (IACS) and have shown its utility for sorting of cells based on protein localization and cell-to-cell interactions [118].

Recent innovations allow high-content, high-throughput screening using FCM, achieving up to thirty-times faster analysis of samples using these technologies. There have been revolutions in terms of sample handling, for example, in plug FCM, a reciprocating multiport flow injection valve is used. Another advanced technology, HyperCyt, makes use of a peristaltic pump along with an autosampler for improving the endpoint assay performance [119].

Recently, Li *et al.* have reported design, fabrication and characterization of microfluidics-based cytometry which is capable of cell size characterization and can also detect intracellular proteins [120]. In these types of cytometers, cells conjugated with fluorochrome-labeled antibodies are subjected to aspiration through a microchannel in which fluorescence intensity is measured.

FCM has also been used for detecting viral sequences by multiplexed analysis of PCR products using microspheres and analysis of single nucleotide polymorphisms [121]. Flow cytometers can be used for the analysis and sorting of human sperms based on their sex chromosome content so as to minimize the probability of an embryo getting affected by a sex-linked disease during *in vitro* fertilization [122]. RNA FCM can detect multiple RNA transcripts (including the low abundance transcripts) from single cells with high sensitivity in heterogeneous biological specimens [123]. Recently developed techniques such as Branched DNA Technique and PLAYR enable simultaneous quantification of multiple mRNA transcripts and protein expression at the single cell level [124,125].

Sample preparation for traditional FCM involves invasive cell extraction from a live organism that can change cellular properties and limit the long-term cellular studies in their natural bio-environment. Additional limitations of *ex vivo* FCM include its low sensitivity while detecting rare cells such as CTCs, time-consuming sample processing procedures and discontinuity of sampling at limited time points, hindering early stage disease diagnosis which eventually could delay the therapy [126]. Recently, Tan *et al.* reported the use of diffuse *in vivo* FCM for detecting extremely rare fluorescently-labeled CTCs directly in the bloodstream of mice [127]. This technique enables detection of cells in the bloodstream without the need for drawing blood samples with the help of specially designed, dual fiber optic probes that can be placed on the skin surface above the major blood vessels. Signal processing algorithm is then used not only to count individual cells moving in arterial or venous directions, but also to measure their depth and speed.

Recently, use of optical methods other than fluorescence has been shown to be a promising approach for obtaining information about cellular structure and molecular-level signatures of subcellular components at single

cell level. Carvalho *et al.* successfully demonstrated optical biopsy using Raman spectroscopy technique for early stage cancer diagnosis using human primary oral mucosa and oral cancer cell line models [128]. Hiramatsu *et al.* have developed a high-throughput, highly sensitive Raman flow cytometer capable of chemically probing single live cells on the basis of scattering of incident photons resulting from intracellular molecular vibrations without the need of labeling [129]. An automated optofluidic-based Raman-activated cell sorter (RACS) was employed for sorting of stable-isotope-probe-labeled microbial live cells for their further single-cell genomic and metagenomic studies or subsequent culture [130]. Raman image-activated cell sorter enables stimulated-Raman-scattering image-based sorting of fluorescent label-free single live cells which was successfully demonstrated in stem cells, adipocytes, protists and algae [116]. The challenges faced during high-throughput single cell analysis were addressed by Merola *et al.* by coupling digital holography with tomographic FCM on diatom algal cells and erythrocytes [131]. Thus, such optical methods have proven to be a useful approach to further enhance capabilities of FCM.

Conclusion & future perspective

FCM technique that originated in 1960s is an indispensable and widely used technique for several applications in biological research and clinical diagnostics even today. Its abilities to rapidly detect multiple parameters in single cells and to sort them have revolutionized the study of heterogeneous as well as rare cell populations. Advanced FCM technologies and applications continue to be developed in order to address the limitations of the traditional FCM technique and together these will continue to foster our knowledge of basic as well as clinical aspects of molecular cell biology.

Executive summary

- Flow cytometry (FCM) is a powerful tool for single cell analysis.
- It works on the principle of light scattering and fluorescence emission that occurs when a cell/particle passes through a laser beam.
- Main components of a flow cytometer include fluidics, optics and electronics.
- Although earlier used mainly for cell cycle and cell proliferation analysis, FCM is now routinely used to study many other cellular parameters as well such as intracellular protein abundance, mitochondrial membrane potential etc.
- FCM is not restricted to the cell analysis but can also be used for analyzing nanoparticles, extracellular vesicles etc.
- It offers unique advantage of physically sorting the cells with high purity which can be further used for different assays.
- Automated gating strategies have made handling and analysis of multidimensional FCM datasets easier.
- Recent advances in the instrumentation (such as spectral cytometry, imaging FCM, *in vivo* FCM etc.) offer solutions to the limitations of traditional FCM technique.

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Review

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Bioanalysis

Critical reagents in flow cytometry, instrumentation and application in drug discovery development

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Flow cytometer is a powerful cellular analysis tool consists of three main components; fluidics, optics and electronics. Flow cytometry methods have been used in all stages of drug development as like ligand binding assays (LBA). Both LBA and flow cytometry methods require specific interaction between the critical reagents and the analytes. Antibodies and their conjugates, viable dyes and permeabilizing buffer are the main critical reagents in flow cytometry methods. Similarly, antibodies, engineered proteins and their conjugates are the main critical reagents in LBA. The main difference between the two methods is the lack of true reference standards for flow cytometry cellular analysis.

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Keywords: critical reagents • flow cytometry • LBA

Flow cytometry and ligand-binding assays (LBA) have been used in all stages of drug development, from early discovery to preclinical and clinical studies. Flow cytometry is a powerful tool to analyze multiple characteristics of a single cell/particle simultaneously by optical means using laser light and the multiparametric analysis is performed on individual cells at a very high rate. Multiple cell populations can be characterized using a combination of reagents against cell surface and intracellular markers in any single cell suspensions. LBA is a method for quantification of proteins, antibodies, nucleic acids and other macromolecules by comparing immunoreactivity of calibrators of known concentrations to the samples of unknown concentration. Critical reagents are the essential components of an assay whose characteristics are pivotal to assay performance. Antibodies and their conjugate are the main critical reagents used in both LBA and flow cytometry methods. Therefore, the characterization of the critical reagents is essential to the assay performance. This article is a conference report on type of critical reagents used in flow cytometry presented in a training session at the 13th WRIB conference held in New Orleans in April 2019.

Flow cytometry: instrumentation key components

A typical flow cytometer consists of three systems: fluidics, optics and electronics. First, the fluidics system is designed to move the cells one by one in the flow cell by injecting the cells into the center of the sheath fluid stream at a slightly higher pressure and hydrodynamic focusing allows single cells analysis at high speed, thousands of events per second. Recent advanced cytometers use microfluidics flow chips or microcapillaries.

The second, the optical system includes light source usually lasers with excitation optics and detectors (photomultiplier tubes). Multiple dichroic mirrors move the lights and the bandpass filters allow the specific band of the optical spectrum to reach the respective detectors. Clinical flow cytometers with two to three lasers are common, however, there are research instruments with five or more lasers which can detect up to 50 parameters. Next generation of flow cytometers are mass cytometers and spectral analyzers. Mass cytometry is a combination of traditional flow cytometry with TOF MS where the antibodies are labeled with heavy metal tags. Traditional fluorescent dye labeled antibodies are used in spectral analyzers. Spectral analyzers capture the full emission spectrum by a set of detectors. Current advanced spectral flow cytometer has 186 detectors with seven lasers.

The third system, electronics consists of photodetectors (photomultiplier tubes or photodiode) which converts the emission light into photocurrent that can be processed by the computer for data analysis. Newer cytometers use

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digital signal processing compared with older generation instruments, which used analog pulse processing. Flow cytometry data are displayed in one-dimension (histogram) or two-dimensional plots.

Three types of measurements are taken when the cells pass through the laser light at the interrogation point: the discrimination of cells by size is determined by measuring forward angle light scatter, the granularity or intracellular complexity is measured by side light scatter and the fluorescence emission. The number of fluorescence signals measured depends on the number of lasers, available fluorophores and the detectors. Due to broader emission spectrum of fluorescence dye, the emission signals are detected not only in primary detector but also in other detectors and it is called spectral overlap. The spill over signal is adjusted by compensation table created using single color stained cells or beads with each fluorochrome. Flow cytometers equipped with sorters are used to separate the cells for further analysis.

Main applications in drug discovery & development

Flow cytometry methods are used in early drug discovery for target validation, compound screening and mechanism of action studies. Similarly, in preclinical and clinical studies, flow cytometry methods are used for measuring safety and toxicity, efficacy, pharmacokinetics (PK), immunogenicity and pharmacodynamics (PD) biomarker evaluation [1].

Cellular characterization & functional analysis

High-throughput platforms are available for the characterization and functional analysis in early drug discovery for target identification [2]. Cell cycle analysis determines the frequency of cells in each phase of the cell cycle using fluorescent intercalating DNA dyes, such as propidium iodide. Cell proliferation is measured *in vitro* by labeling cells with fluorescent dye and for each cell proliferation cycle the intensity of the dye is reduced by 50% [3]. The frequency of cells undergoing programed cell death is measured by dye conjugated annexin-V which binds to phosphatylserine in the plasma membrane which is exposed during apoptosis [4]. In cell signaling studies, the phosphorylation of protein kinases is measured to study of the mechanism of action of the compounds [5]. High-throughput platforms are also used in screening thousands of hybridoma secreting clones rapidly.

Multiparameter analysis & quantitation of different cellular components

Flow cytometry methods measure not only percentage of cell populations but also absolute cell counts indirectly either using internal reference counting beads in a single-platform cytometer or using total leukocyte counts from complete blood count or directly in volumetric cytometers. In the peripheral blood, the various subsets of cell populations are determined based on the expression of cell surface markers identified by immunophenotyping. The measurement of both percentage and absolute counts of major cell populations (TBNK Panel: total T cells, CD4 T cells, CD8 T cells, B cells and natural killer cells) has been widely used not only in diagnostic laboratories but also as safety biomarker during drug development [6]. The most common cellular populations determined by flow cytometry in the human peripheral blood are presented in Table 1.

Analysis of intracellular signaling

The optimization of fixation and permeabilization of the cell membrane enabled the intracellular analysis of signaling and functional markers inside the cells by flow cytometry [5]. The intracellular molecules included cytokines, FoxP3, Bcl-2, estrogen receptors and various phospho-proteins (Stat1, Stat5, Stat6, Akt, S6, ERK, MEK, cJun and p38 and others). The intracellular phospho-protein kinase assays are aiding to discover novel therapeutic compounds for activation or inhibition of kinase signaling cascades, such as PI3-kinese inhibitors of Akt, S6, Stat1 and others. Intracellular cytokine staining (ICS) by flow cytometry has been widely used for studying cytokine production at the single-cell level. ICS assays are employed to study the antigen-specific responses to vaccines and also to characterize different T-helper subsets based on the cytokine production (IFN-γ for Th1 cells; IL-4, IL-5 and IL-13 for Th2 cells; IL-9 for Th9 cells; IL-17A and IL-22 for Th17 cells; IL-13 and IL-22 for Th22 cells) [7].

Receptor occupancy determination of drug-receptor occupancy

Advances in biotechnology aided the invention of biologics as therapeutic agents and the binding of the biologics to their cellular target is measured using receptor occupancy assays by flow cytometry. Receptor occupancy assays are used to determine the optimum dosing for efficacy, safety and administration schedules of biologicals [8].

Table 1. Identification of common cellular populations in the human peripheral blood.

Cell population	Leukocyte marker	Primary marker(s)	Secondary marker(s)
Leukocytes	CD45+		
T cells	CD45+	CD3+	
T-helper cells	CD45+	CD3+CD4+	
Naïve	CD45+	CD3+CD4+	CD45RA+CD197+
Central memory	CD45+	CD3+CD4+	CD45RA-CD197+
Effector memory	CD45+	CD3+CD4+	CD45RA-CD197-
RA+ effector	CD45+	CD3+CD4+	CD45RA+CD197-
T-regulatory cells	CD45+	CD3+CD4+	CD25+FoxP3+
Th1 cells	CD45+	CD3+CD4+	CD183+
Th2 cells	CD45+	CD3+CD4+	CD194+CD196-
Th17 cells	CD45+	CD3+CD4+	CD194+CD196+
T-cytotoxic cells	CD45+	CD3+CD8+	
Naïve	CD45+	CD3+CD8+	CD45RA+CD197+
Central memory	CD45+	CD3+CD8+	CD45RA-CD197+
Effector memory	CD45+	CD3+CD8+	CD45RA-CD197-
RA+ effector	CD45+	CD3+CD8+	CD45RA+CD197-
Double-positive T cells	CD45+	CD3+CD4+CD8+	
B cells	CD45+	CD19+	
Naïve B cells	CD45+	CD19+	CD27-IgD+
Memory B cells	CD45+	CD19+	CD27+IgD-
Transitional B cells	CD45+	CD19+	CD24+CD38+
Plasmablasts	CD45+	CD19+	CD24-CD38+
NK cells	CD45+	CD16+CD56+	
Monocytes	CD45+	CD14+	
Granulocytes	CD45+	CD11b+CD15+	
Neutrophils	CD45+	CD11b+CD15+	CD66b+
Basophils	CD45+	CD11b+CD15+	CD123+
Eosinophils	CD45+	CD11b+CD15+	CD125+CD193+
Platelets		CD41+CD61+	
Red blood cells		CD235a+	

Free receptor occupancy assays measure the receptors not bound by biotherapeutic agent, with fluorescent labeled competing antibody or biotherapeutic agent. Total receptors are measured with fluorescent labeled non-competing antibody to the same receptor target. Bound receptor levels are measured with fluorescent labeled anti-drug antibodies. The receptor expression levels in the receptor occupancy assays are measured by the fluorescence intensity or molecules of equivalent soluble fluorochrome [9].

Cell & gene therapy

In gene therapy, the expression of transgene product levels is monitored by flow cytometry methods. In cell-based therapy, flow cytometry has been employed to purify specific cell populations. For example, CD34+ hematopoietic stem and progenitor cells are purified for effective reconstitution in patients undergoing chemotherapy [10]. ICS assays are used to measure the cellular immune response against capsid and transgene products in gene and cell therapies [11]. Recent advances in gene editing tools enabled the successful generation of T cells against tumor cells with insertion, deletion or modification of genes within T cells, such as cloned T-cell receptor or chimeric antigen receptor (CAR) T cells. Two autologous anti-CD19 CAR T-cell therapies had been approved by US FDA for the treatment of acute lymphoblastic leukemia and diffuse large B-cell lymphoma [12]. Flow cytometry methods play a key role in adoptive cell therapies to monitor infused CAR-T cells (cellular kinetic) and immune profiling of various subsets of cellular components. The monitoring of patient CAR-T cellular kinetics and persistence is useful for understanding adoptive T-cell therapies [13].

Critical reagents in flow cytometry

Critical reagents are the essential components whose characteristics influence assay performance. The antibodies and their conjugates are the main critical reagents for flow cytometry methods [14]. The human cluster differentiation molecule (HCDM) plays a vital role in characterizing the structure, function and distribution of leucocyte surface and other molecules. Antibody reagents against human CD markers are well characterized in a series of the human leukocyte differentiation antigens workshops by HCDM. These workshops involve the testing of multiple monoclonal antibody clones against the same molecule and the confirmation of their specific reactively. So far ten human leukocyte differentiation antigen workshops have been conducted and characterized the leukocyte molecules from CD1–CD371 [15]. The reagents against CD markers are available from commercial vendors; mostly for research use only and some as analyte specific reagent. It is recommended to use analyte specific reagents in flow cytometry methods, if available [16]. Other critical reagents in flow cytometry includes viability dyes and permeabilizing buffers used in intracellular staining. All critical reagents should be characterized during the development of the method [14].

Similarities & differences between LBA & flow cytometry critical reagents

Critical reagents used in LBA and flow cytometry methods include antibodies (monoclonal and polyclonal), engineered proteins and their conjugates [17]. Majority of the reagents are procured from commercial vendors. Certificate of analysis is available for commercially purchased reagents. But in some cases, antibodies against rare antigen/molecule need to be produced, for example, reagents against each CAR molecule needs to be developed in CAR T-cell methods. The characterization of the critical reagents include determination of identity, purity, concentration, binding affinity, antibody isotype, specificity and stability [18]. The extend of the characterization is based on the context of use of the reagent and the assay. The titration of the reagents is also critical to establish best signal to noise ratio. When using multiple lots of reagents in a study, lot-to-lot bridging should be performed and documented. The documentation in the form of standard operating procedure is required for critical reagent and it should include the reagent preparation, qualification, labeling (if required) and storage temperature. Critical reagent life cycle management system includes the reagent characterization, supply chain, maintenance, long-term storage, inventory and knowledge database. This is critical in support of analytical methods in longitudinal studies [16,17,19].

The main difference between LBA and flow cytometry critical reagents is the lack of true reference standards for flow cytometry cellular analysis. In the absence of reference standards and the standard curve, data generated from flow cytometry analysis is considered as quasi-quantitative for cellular analysis [20]. In LBA, calibrators and quality controls are made from reference standard materials. Bead array analysis in flow cytometer is similar to LBA assay where calibrators are used to create standard curve for various analytes.

Conclusion

Flow cytometry is a laser-based powerful cellular analysis tool which measures multiple parameters, such as size, shape, cell surface and intracellular molecules on single cells simultaneously at a high rate. The instrumentation includes three key components; fluidics for transporting cells in a stream, optics for detection and electronics for informatics. The number of parameters detected depends on the number of lasers and detectors; clinical cytometers usually have two–three lasers which can detect 6–12 parameters while research cytometers have more than three lasers which can detect up to 50 parameters. The flow cytometry methods are used in every stage of drug development, from preclinical to patient stratification and selection. The flow cytometry methods in bioanalytical and biomarker laboratories are validated as fit-for-purpose to evaluate the safety and PD biomarkers, including target engagement and receptor occupancy. The critical reagents for flow cytometry methods include antibody reagents against cellular markers, viable dyes and permeabilizing buffers. Even though, the antibody reagents against CD markers are well characterized, the staining reagents should be evaluated with proper controls during development of the method. The characterization and documentation of critical reagents in flow cytometry methods are similar to LBA reagents. Calibrators and quality controls are prepared from reference standard materials in LBA and no true reference standards are available for cellular markers in flow cytometry.

Future perspective

Flow cytometry is a multiplex cellular analysis platform used in PK and PD assays. Flow cytometry assays are validated as fit-for-purpose driven by the context of use. In cellular therapy, like CAR T-cell therapy, flow cytometry plays a critical role in the bioanalysis of cellular kinetics of infused CAR-T cells. Mostly commercial reagents are

used in flow cytometry assays and the characterization of critical reagents is important, especially when used in the clinical studies involving multiple clinical sites. A minimum characterization of reagents includes the assessment of specificity, F/P ratio and titration in the appropriate reaction volume. The performance of the critical reagents should be continuously monitored for long-term stability. It is recommended to use assay specific reagents when available and a partial validation should be performed when changing a reagent in the middle of a study. Bioanalytical community is working to establish the best practices for critical reagent characterization and assay performance monitoring.

Summary points

- Flow cytometry is a laser-based cellular analytical tool and a typical flow cytometry instrumentation includes three systems: fluidics, optics and electronics.
- Flow cytometry and ligand binding assays (LBA) are used in bioanalytical laboratory to support all stages of drug development.
- Both LBA and flow cytometry methods require specific interaction of the critical reagents and the analytes/receptors.
- The main critical reagents for LBA and flow cytometry assays are antibodies, their conjugates and the buffers. The other reagents, such as viable dyes in flow cytometry assays and the engineered proteins in LBA are also critical for the performance of the assays.
- Critical reagents have a direct impact on the bioanalytical data generated. The characterization of the critical reagents include identity, purity, concentration, binding affinity, antibody isotype, specificity and stability.
- In LBA, calibrators and quality controls are made from reference standard materials, but there are no true reference standards for cellular markers in flow cytometry assay.

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Editorial

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Bioanalysis

Approaches to overcome the challenge of sample stability for flow cytometry analysis in clinical trials

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"there are many considerations that have to be made when establishing a flow cytometry assay to ensure that the cells and markers of interest are preserved and high quality analytical results are obtained."

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Keywords: biomarkers • cell fixation • clinical trials • flow cytometry • sample stability

Flow cytometry is a technology that has been increasingly valuable in support of biomarker analysis for clinical trials. It has many clinical applications including the ability to provide data to support safety or exploratory studies by utilizing basic or complex immunophenotyping, pharmacokinetic, receptor occupancy, target engagement, cell activation, functional and intracellular expression assays. The assays on this platform often provide important data in support of clinical trials, and thus it is important to ensure the data generated is of good quality. Flow cytometry is inherently complex in nature due to the inherent biological variability of cells. The technology itself also has its challenges in that the cytometer setup requirements are complex. The intricacy of data output and interpretation of results adds another layer of complexity. Aside from these obvious complexities of the technology itself, sample stability is a big challenge for flow cytometry given the fact that there is a time window in which cells begin to deteriorate once removed from the body [1]. The challenge of limited stability has logistical and operational consequences for using assays to support multicenter clinical studies and often leads to high study costs due to the need to perform multiple sample shipments and run samples across multiple analytical batches. The increase in the number of analytical runs can also contribute to data variability. In this regard, there are many considerations that have to be made when establishing a flow cytometry assay to ensure that the cells and markers of interest are preserved and high quality analytical results are obtained. In general, cell subset markers are only stable for a few days and in some cases, specifically for activation markers or intracellular assays, may require analysis within 24 h. To overcome this short analysis window, several approaches can be taken to extend sample stability in flow cytometry each with its own feasibility and degree of complication.

Method development considerations

At the beginning of the assay development activities, it is imperative to understand the context of use including but not limited to the trial design, patient characteristics and how the data will be utilized, but also trial logistics, such as number and location of sites and their proximity to the bioanalytical lab. One of the key parameters to evaluate before starting assay development is the stability of the cells and markers of interest and ensuring that the stability window is within the time it would take for the study samples to be analyzed. Matrix selection is one of the first considerations for preservation of cells during transport to the bioanalytical lab prior to acquisition on the cytometer. Often, merely the type of anticoagulant and storage temperature (e.g., refrigerated vs ambient) in certain assays can lead to longer sample stability of cells and their markers [2]. A widely used approach for extending sample stability in flow cytometry is as simple as sample fixation. There are various ready to use fixative solutions that are available commercially and even blood collection tubes that already contain reagents to fix cells immediately upon blood draw. However, cell fixation methods are not always applicable since they can result in loss of the target of

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interest and may not be suitable for certain sample processing requirements like assays that require cell stimulation beforehand.

Cell fixation & freezing

Taking fixation a step further and freezing fixed whole blood is another method for extending sample stability. In this method, blood is typically collected in a blood collection tube that already contains a commercial fixative. The fixed blood is then transferred to a polypropylene tube and frozen at -70°C or below up to a specified amount of time. The main advantage of this method is extending the stability of the sample by freezing beyond simply fixing the sample. The additional freezing step with this approach adds time and sample handling with the need to transfer the fixated whole blood to a secondary tube prior to freezing, as well as the need for freezer space to store these samples. In some situations the additional burden of adding these processing steps, although complicating logistics and requiring frozen storage, is worth the effort for better quality data.

Cell lysing & fixation

A third approach for extending sample stability includes lysing, fixing and freezing whole blood samples at the clinical sites. In this approach, a sample is lysed with lysing solution, fixed with a commercial fixative and then stored in the freezer at -70°C for a specified amount of time that is determined in the method validation. This method has even demonstrated sample stability with rare cell populations such as Tregs [3]. A drawback of this method includes the need to train clinical sites on how to properly perform sample processing as improper sample handling at each step can easily affect the sample and data integrity in the study.

Isolating peripheral blood mononuclear cells

Finally, another highly used approach is isolation of peripheral blood mononuclear cells (PBMCs) from whole blood and subsequent storage in vapor phase liquid nitrogen for extended sample stability. Its major advantage is the long duration of stability that can be established. However, given the complexity of the isolating PBMCs, the quality of data is highly dependent on the efficiency of cell isolation and the viability of the cells prefreeze and postfreeze. Thus, also with this method, training of sites that are collecting and processing the samples on proper PBMC isolation techniques is of utmost importance.

The four approaches mentioned are a few examples that can be used to develop flow cytometry assays that allow for extended samples stability in support of clinical studies. Each approach is dependent on the properties of cells and markers of interest and has to be evaluated early on in method development to determine if it is feasible to work for that assay type, marker and cell subset [4]. Like with any biomarker assay, flow cytometry assays have to be approached with an understanding of the context of use in order to design an assay that will provide meaningful data [5]. With this in mind, it is important to understand the time frame for shipment of samples from the clinical specimen collection site to the testing laboratory at an early stage to ensure integrity of the data acquired from the flow cytometer. Regardless of how close the lab is to the clinical site, one still has to validate sample stability to cover time of draw to time of analysis and this has to be proven in the method validation. Defining a proper validation approach to assess sample stability that will cover all aspects of the sample transit postdraw and prior to analysis is crucial. Having extended sample stability does have many benefits including lowering costs and improved data quality by batching samples in fewer analytical runs. It also allows for buffer time to cover delayed sample shipments. Finally, it provides flexibility for sample reanalysis if needed. As technology and methods continue to evolve, we will continue to find new and better ways to preserve the integrity of cells to overcome the challenge of limited sample stability.

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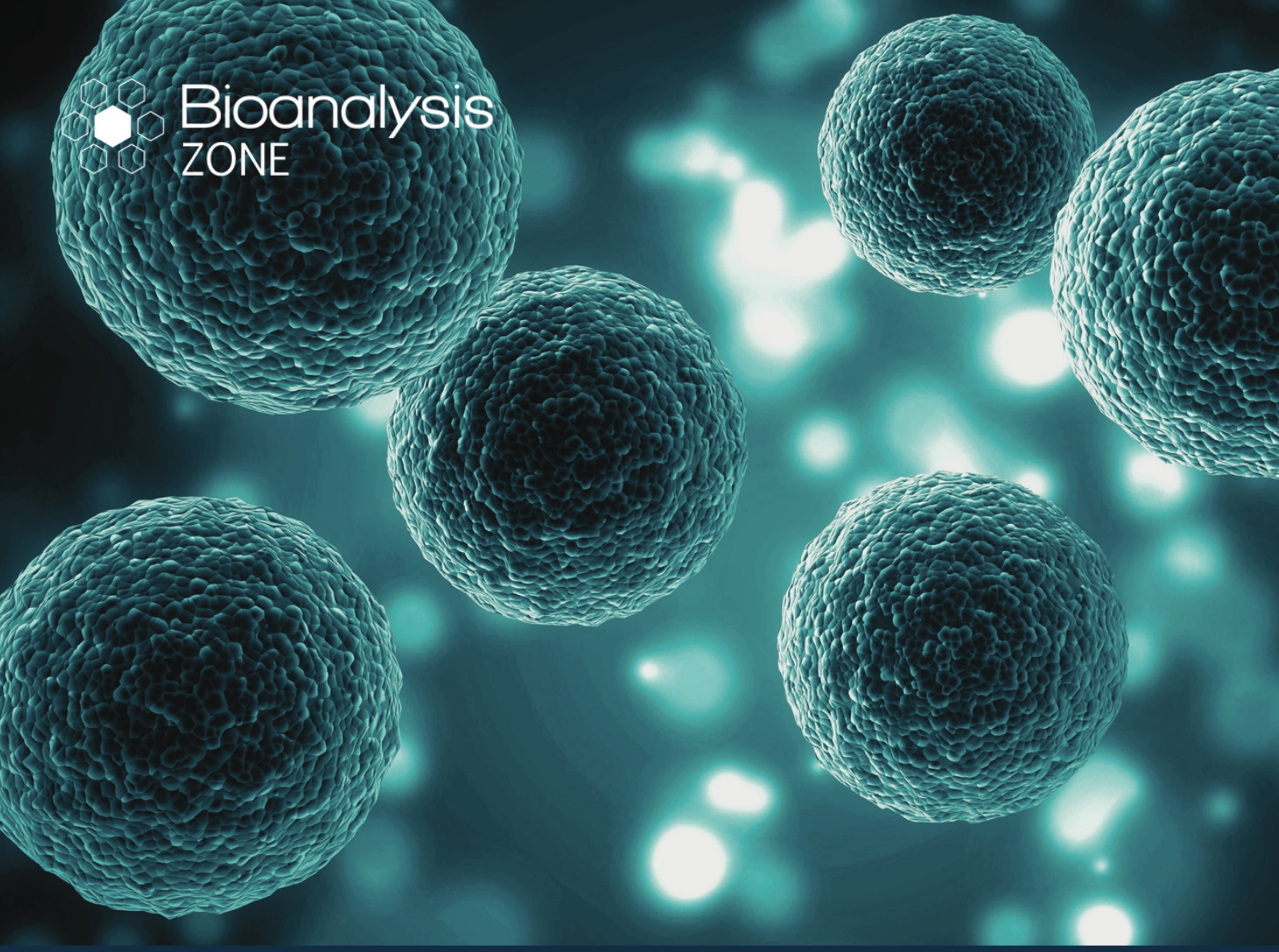

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