A comprehensive resource for state-of-the-art flow cytometry methods

Guidelines for the use of flow cytometry and cell sorting in immunological studies.

Cossarizza A, Chang HD, Radbruch A et al. (2017) *Eur J Immunol* 47:1584–1797.

With the advent of monoclonal antibodies conjugated to fluorophores, flow cytometry has become an essential tool for immunological studies. While the flow cytometer is relatively easy to operate, it can be challenging to master the technical aspects—instrument setup, sample preparation, data acquisition, and analysis—to get the most out of flow cytometry experiments. Under the leadership of Andreas Radbruch and Andrea Cossarizza, numerous authorities in the field were invited to share knowledge in their particular area of expertise. As a result of this community effort, the article in the *European Journal of Immunology* aggregates currently accepted methods along with a comprehensive collection of information, protocols, tips, and advice, with the goal of establishing guidelines for the use of flow cytometry and cell sorting in immunological studies.

This article is organized into eight chapters representing key areas of interest, with multiple sections within each chapter. It begins with an overview of flow cytometry equipment, followed by a description of the principles of spectral, imaging, and mass cytometry. Cell sorting, instrument setup, and quality control are discussed, including details on correctly compensating for fluorescence spillover. Fundamentals of experimental design, reagent selection, sample preparation, data acquisition, and cell sorting are each presented, with recommendations for data analysis and handling. The chapter on cytometric parameters includes an extended discussion of 17 different techniques and areas of interest. Perhaps the most interesting to the immunologist is the chapter depicting cytometric phenotypes of 10 cell and tissue types. Finally, a comprehensive list of nearly 1,000 references is included. Here we highlight a few key concepts.

Compensation

Fluorescence spillover, which occurs when a fluorophore's emission is detected in multiple channels, remains a source of frustration for scientists. Correctly compensating for spillover is crucial for accurate population identification, but when performed incorrectly it can lead to poor and even invalid data. A spillover value (SOV) can be calculated using single-color control samples. Compensation is the mathematical process by which the SOVs are used to create a matrix that can be employed to subtract or correct background due to fluorescence spillover.

Four principles for performing compensation are presented: 1) the fluorescence spectrum of a single-color compensation control should be identical to that used in the experiment; 2) autofluorescence of positive and negative populations should be equal; 3) fluorescence of the positive population should be as bright as possible; and 4) at least 5,000 events should be collected in order to calculate an accurate SOV. The use of antibody capture beads, such as Invitrogen™ UltraComp eBeads™ Compensation Beads and Invitrogen™ AbC™ Total Compensation Beads, for single-color compensation controls provides an accurate method for determining fluorescence spillover; moreover, these beads are simple to use and require no biological material, so sample can be conserved.

Sample preparation

Before starting any flow cytometry experiment, reagent and sample preparation should be considered. It is good practice to optimize and validate all reagents used. Just as important is how the cell sample is prepared. A single-cell suspension is required, and therefore techniques for disaggregation (typically mechanical and enzymatic procedures) into single cells are needed when using cells from tissue or adherent cell culture.

When compared with measuring cell-surface targets, the analysis of intracellular targets presents additional challenges and requires fixation and permeabilization procedures. Cell fixation is generally performed using either crosslinking fixatives such as formaldehyde or dehydrating alcohols such as methanol and ethanol. Formaldehyde has the advantage of generally preserving the overall conformation of native proteins, as well as fixing posttranslational modifications and inhibiting the degradation of these targets. Formaldehyde-based reagents such as those found in the Invitrogen™ eBioscience™ Intracellular Fixation and Permeabilization Buffer Set and Invitrogen™ FIX & PERM™ Cell Permeabilization Kit are readily available. →

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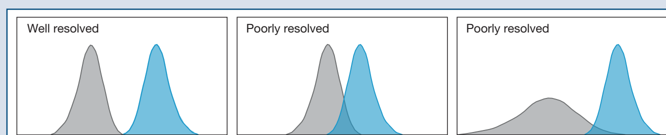
Mastering the technical aspects of flow cytometry*

Some key takeaways on controls and experiment design.

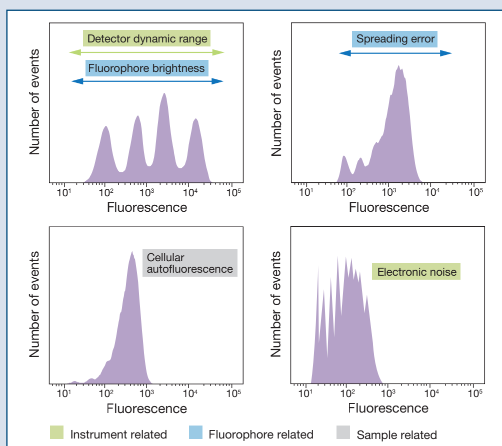
OBJECTIVE of a flow cytometry experiment: To obtain well-resolved cell populations.

WHAT determines resolution? Resolution is a function of signal separation and spread.



HOW can resolution be enhanced? Fluorophore, sample, and instrument factors affect resolution.

Separating positive and negative signal: Factors that determine separation



Separating positive and negative signal is critical to accurate detection and interpretation of results. There are multiple sources of fluorescent signal that need to be accounted for, including those from the fluorophore, sample, and instrument.

Determining dye compatibility in multicolor experiments: A spillover-spreading matrix (SSM)

LIVE/DEAD Fixable Violet
LIVE/DEAD Fixable Green
LIVE/DEAD Fixable Yellow
LIVE/DEAD Fixable Red
LIVE/DEAD Fixable Far Red
LIVE/DEAD Fixable Near-IR

Alexa Fluor 405 — CCR6
Alexa Fluor 488 — CD294
PE — CD161
Alexa Fluor 647–PE — CD127
PE–eFluor 610 — LAG
APC — CCR5
Alexa Fluor 680–APC — CD8
Alexa Fluor 750–APC — CD19

Relative degree of overlap: 6.5, 3, 1, 0.1, 0

In this hypothetical SSM, emission overlap of several LIVE/DEAD stains is matched to a range of antibody conjugates. The higher the degree of overlap between the stain and antibody conjugate, the greater the spillover.

WHY are these factors important? ↑ Separation + ↓ Spillover = ↑ Resolution

Top 5 compensation mistakes

5. Multiple color controls are analyzed in the same tube instead of individually.
4. An insufficient number of events are collected to obtain an accurate spillover value (SOV); >5,000 events are required.
3. Autofluorescence of the positive and negative controls is not the same.
2. The fluorescence of the positive control is not at least as bright as the biological sample.

And the number 1 compensation mistake is...

1. Use of positive color controls that are spectrally similar—but not identical—to the dyes used in the actual experiment.

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Panel design

Advances in hardware and reagent development allow for the detection of up to 30 fluorescence parameters simultaneously, revealing complex biological mechanisms and cell populations. However, they also require the use of a carefully chosen, highly multiplexed antibody panel. Because these panels typically utilize fluorophores that overlap, the principles of single-color compensation controls apply, with the phenomenon known as spreading error (SE) accounted for. SE is an intrinsic characteristic that arises from the counting error associated with low photon numbers. SE is not caused by compensation but it is revealed in compensated data, as the effects of counting error are observed at the low end of a log-scale fluorescence plot. If the SE is very high in a particular detector, a dim marker may not be resolved sufficiently from background signal. Creating a spillover spread matrix (SSM) is useful for characterizing dye and instrument performance and choosing optimal antibody–fluorophore combinations.

Viability assessment

It is essential that the viability of the cell population under examination be known, regardless of the sample preparation method used, and several approaches are discussed. For example, viability assays based on the use of impermeant DNA-binding dyes assess the integrity of the plasma membrane. A healthy intact cell membrane keeps the dye out of the cell, whereas a compromised cell membrane allows the dye to enter the cell, where it binds to the DNA and becomes brightly fluorescent, thus identifying the cell as dead. Impermeant DNA-binding dyes such as propidium iodide (PI), 7-AAD, TO-PRO-3, and the Invitrogen™ SYTOX™ dyes are generally added as the last step in a workflow, and not washed out before data acquisition.

It is important to note that DNA-binding dyes cannot be used on fixed or permeabilized cells. In contrast, protein-binding dyes such as the Invitrogen™ LIVE/DEAD™ and eFluor™ Fixable Dead Cell Stains can be used to assess viability in workflows that require fixation or permeabilization. In cells with compromised membranes, these fixable dyes covalently react with proteins both in the cell interior and on the cell surface, whereas in live cells they only react with cell-surface proteins. The difference in fluorescence intensity is typically on the order of 50-fold, allowing easy discrimination between dead and live cells, and the staining pattern is preserved after fixation. When using protein-binding dyes, it is critical that labeling protocols be performed in the absence of proteins in the staining buffer.

Minimal sample perturbation in ROS evaluation

Reactive oxygen species (ROS) are associated with oxidative stress and include hydrogen peroxide (H_2O_2), superoxide anion ($\cdot O^{2-}$), and hydroxyl radical (·OH), which all have relatively short half-lives. Ideally, functional studies for measuring ROS should be performed in whole blood, with minimal sample manipulation, in order to mimic physiological conditions as closely as possible. A no-lyse, no-wash sample preparation method is described that involves simply staining the whole blood sample, diluting it, and then acquiring the sample on the cytometer. Often a fluorescence threshold is set by gating with a CD45 antibody or a vital DNA-binding dye so that only cells of interest are examined. A second approach is discussed that combines the analysis of the blue (488 nm) and violet (405 nm) side scatter laser light; this differential side scatter method allows for detection of red blood cell (RBC), white blood cell (WBC), and platelet populations in the sample because the hemoglobin in RBCs is absorbed by the violet light.

mRNA detection

The immune system comprises heterogeneous cell populations and, upon stimulation, cell-specific responses that trigger production of specialized proteins can be detected at the mRNA level. To fully understand the contribution of cellular heterogeneity to biological function, a single-cell approach is required. Flow cytometry is the gold standard for the study of heterogeneous cell populations. With the novel Invitrogen™ PrimeFlow™ RNA Assay, researchers can detect the dynamics of RNA and protein expression simultaneously using flow cytometry; see "Flow cytometry assay for simultaneous detection of HIV RNA and Gag protein" on page 16. The PrimeFlow RNA assay employs fluorescence *in situ* hybridization (FISH) and branched DNA (bDNA) signal amplification for the detection of up to 4 RNA targets per cell using a standard flow cytometer, and this RNA detection can be combined with immunophenotyping using intracellular and cell-surface antibodies.

Access these guidelines

This publication contains useful information for anyone using flow cytometry as a tool in their research, and it is of particular interest to immunologists. Download this special issue (volume 47, issue 10) of the *European Journal of Immunology,* an open-access publication from the Wiley Online Library, at onlinelibrary.wiley.com/doi/10.1002/eji.201646632/full. ■

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