Designing Increasingly Complex Flow Cytometry Panels for High-Throughput Immunophenotyping

Offering simultaneous detection of an expanding array of cell markers, flow cytometry has become an indispensable high-throughput tool, used for the detection and quantification of diverse immune cell populations within heterogeneous samples. With a broad range of research and clinical applications, this analytical technique plays a pivotal role in understanding the immune system's role in disease progression, monitoring patient responses to treatment, and assessing the safety and efficacy of novel therapies and vaccines. Careful panel design is crucial to ensuring data accuracy, particularly when performing complex multiparameter assays.[1,2]

To ensure panel building success, a selection of best practice guidelines surrounding core aspects of immunophenotyping can be followed to assist with experiment design.

General Sample Preparation Tips

To ensure the success of your flow cytometry experiments, it's essential to prioritise meticulous sample preparation. Investing extra time in this phase can yield significantly improved results, saving you from the frustration of failed experiments and the need for multiple repetitions to achieve publication-quality data.[3]

Select Appropriate Tubes: Choosing the right tubes for your flow cytometry experiments is crucial. Some cytometer models allow flexibility in tube selection, like Bio-Rad's ZE5 Cell Analyzer, but others have restrictions. It's important to use tubes that are compatible with your cytometer model, considering factors such as round or flat bottoms and the type of plastics used. Additionally, be mindful of cell adhesion to different plastic materials. For instance, certain cell types, like monocytes, may adhere more strongly to polystyrene than polypropylene. Selecting the appropriate plastic can help minimise cell loss and the need for harsh detachment methods.

Handle Samples Gently: Maintaining the optimal condition of your cells is paramount. Avoid subjecting them to unnecessarily harsh conditions that can lead to cell death or generate artifacts, as this can significantly impact your results. When centrifuging samples, be cautious not to spin them at higher speeds than necessary, and avoid leaving samples in the centrifuge for extended periods, as prolonged pelleting can be detrimental. It's advisable to handle samples gently to prevent the formation of bubbles, which can harm cells. When aspirating media, ensure that you leave some supernatant behind to prevent cells from drying out in a pellet.

Prevent Clumping: Flow cytometry relies on analysing individual, single cells. Clumps of cells can obstruct the flow cytometer, causing clogs and inconvenience to other users. To reduce the risk of clumping, consider keeping wash and media buffers at a temperature of 4°C, particularly if your experiment permits this.

Dissociate Tissues Thoughtfully: Achieving single, intact cells suitable for flow cytometry is essential. When dissociating tissues, take care to minimise cell damage. Consider methods such as tissue mincing, mesh filtering, or enzymatic digestion based on the specific requirements of your cell type. If you are working with rare cell types, opt for gentler methods to preserve them. For example, neutral protease (dispase) is a milder treatment than trypsin and is commonly used for isolating iPSCs. Some cell types, like F4/80+ macrophages and follicular dendritic cells, may require enzymatic treatment for release, and the specific enzymes needed should be determined based on established protocols.

Key Considerations for Successful Panel Design

Immunophenotyping demands careful panel design as the inclusion of each additional fluorophore into the panel introduces complexities that can potentially compromise data accuracy. This is primarily due to the phenomenon of fluorescence spillover, where the emission from one fluorophore inadvertently spills over into the detection range of another, diminishing resolution and overall data quality (Figure 1).

Get to Know Your Flow Cytometer

Understanding the configuration of your flow cytometer is fundamental to effective panel design.[1] Most modern cytometers come equipped with three or more lasers, each

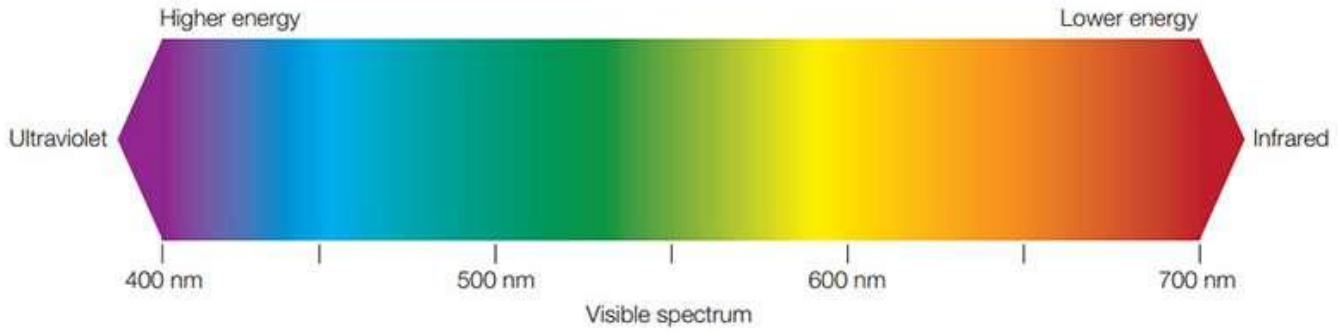


Figure 1: The electromagnetic spectrum

offering a distinct range of wavelengths. Additionally, the configuration of your cytometer hinges on the number and specifications of light filters and detectors. Together, these components determine the maximum number of fluorescent parameters that can be assessed concurrently, as well as which fluorophores can be effectively employed.

Taking a closer look at the detectors associated with each laser and the filters linked to each detector is imperative. These filters are typically labelled as XXX/YY, where XXX denotes the median wavelength of light passing through the filter (measured in nanometers), and YY signifies the wavelength range encompassed by the filter. For instance, a filter marked as 525/50 allows light with wavelengths between 500nm and 550nm to pass through. Alternatively, filters may be designated as XXX/LP or XXX/SP, with LP indicating "Long Pass", allowing longer wavelengths than XXX, and SP indicating "Short Pass", permitting shorter wavelengths. Careful selection of fluorophores relies on matching their optimal excitation and emission wavelengths to the available lasers and filters on your detector array. While exact matches may not always be feasible, employing a spectral viewer can help determine the most likely laser/filter/fluorophore combinations that will work effectively.

Considerations when Choosing the Right Fluorophore Combination

When choosing fluorophores, it is crucial to consider their individual properties to maximise data quality.[4] The compatibility of fluorophores depends on their excitation and emission profiles, as well as their relative brightness. These factors collectively determine the extent of spectral overlap between fluorophores which occurs when the emission spectra of two fluorophores intersect, allowing light from one to spillover into the detection range of the other. Addressing spectral overlap in multicolour panels is achieved through a process known as "fluorescence compensation" which can be determined using compensation controls, taking into consideration the unique fluorescent properties of each fluorophore. This method ensures that the fluorescence detected in a specific detector is attributed to the intended fluorophore (Figure 2).

Selecting fluorophores with narrow excitation and emission profiles minimises the potential for spillover. For single-laser excitation, spillover can be reduced by employing fluorophores with substantial differences in their Stokes shift, measuring the discrepancy between a dye's maximal excitation and emission wavelengths. In cases where multiple lasers are available, choosing fluorophores with significant differences in their excitation wavelengths – making them excitable by one laser but not another – significantly reduces spillover, particularly in instruments with spatially separated lasers.

To ensure reproducible results, it's imperative to use fluorophores conjugated to well-validated flow antibody clones and manufactured using robust methods to minimise lot-to-lot variation. Additionally, selecting photobleaching-resistant fluorophores that remain highly stable, even during fixation, is crucial for maintaining consistent staining. Opting for fluorophores that integrate seamlessly into common experimental protocols without the need for specialised staining buffers offers cost- and time-effective advantages.

Factoring in antigen density and marker expression profiles during fluorophore selection can further mitigate spillover. Bright fluorophores with high stain indices are ideal for detecting rare cell subsets or markers with low expression. Conversely, dimmer fluorophores are better suited for markers with abundant expression levels. Minimising the effects of

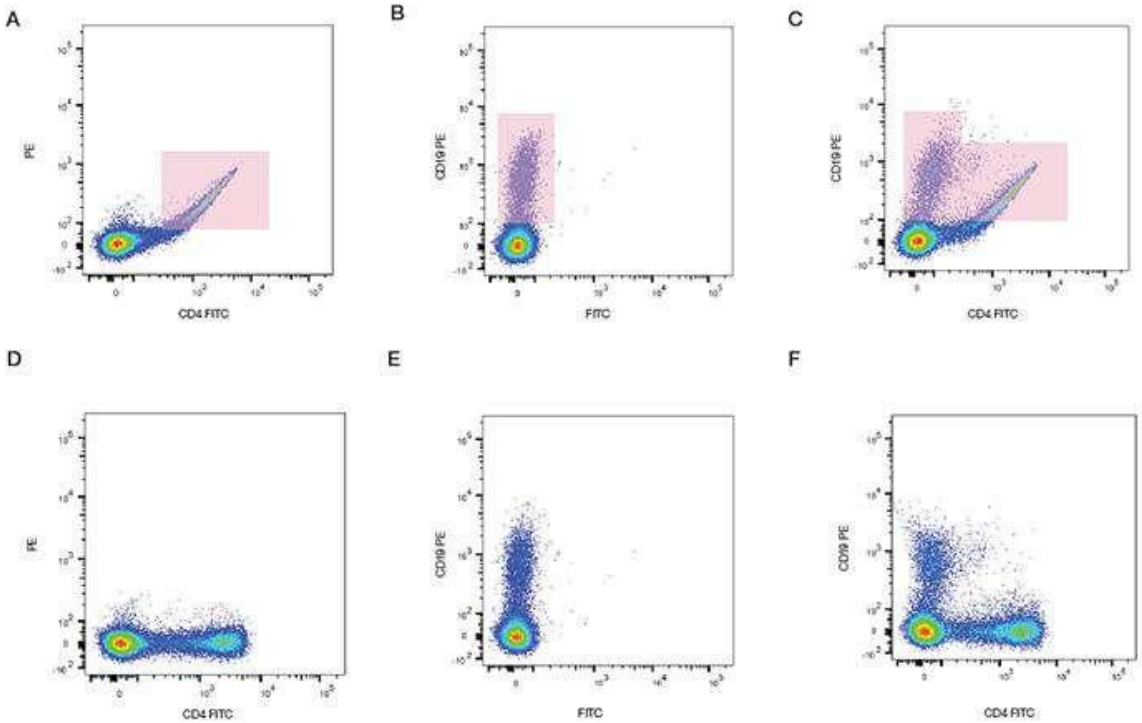


Figure 2: Fluorescence compensation corrects for spectral overlap. Peripheral blood was singly stained with CD4 FITC, CD19 PE, or both CD4 FITC and CD19 PE. When compensation was not applied, fluorescence spillover can be seen (top panel), which is removed after compensation (bottom panel).

spillover and spread can also be achieved by using closely overlapping fluorophores to detect mutually exclusive markers, such as CD3 and CD19. For cell subset markers found on a common cell type or markers exhibiting continuous expression patterns, selecting fluorophores with minimal or no overlap is crucial.

Select Appropriate Controls

Incorporating appropriate controls into flow cytometry experiments is vital for obtaining accurate, reliable data.[5,6] These controls can distinguish between specific signals and background noise, ensuring the quality and validity of your findings.

Unstained Controls: These controls involve setting up the instrument using unstained cells to visualise all cells on forward and side scatter plots, thereby adjusting photomultiplier tube (PMT) voltages to distinguish cells from electronic noise. This process helps determine background fluorescence or autofluorescence, enabling proper voltage settings for fluorescence channels. Using this approach can save time and sample during future experiments.

Single Staining and Compensation Controls: Compensation controls involve single-staining samples for each antibody in a multicolour experiment to determine spillover of fluorescence between detectors. Proper compensation settings ensure that only specific signals are used for analysis, improving data accuracy.

Fc Block Controls: The presence of Fc receptors on specific immune cells, such as monocytes, macrophages, dendritic cells, and B cells, introduces the potential for false positives and reduced resolution due to multiple antibodies binding to unintended targets. To combat this issue, Fc blocking reagents, can be added to the staining protocol (Figure 3). These reagents prevent nonspecific binding by ensuring that only antigen-specific interactions are observed. Alternatively, diluted serum from the sample's host species can be used to achieve a similar effect, e.g., using mouse serum for mouse cells.

Isotype Controls: Isotype controls play a critical role in surface staining experiments by verifying the specificity of antibody binding. These controls are raised against antigens not present on the cells under investigation, ensuring that the observed staining results from specific antibody interactions rather than artifacts. To maximise their effectiveness, isotype controls should match the host species, Ig subclass, concentration, and fluorophore of the primary antibody. Purchasing both the isotype control and primary antibody from the same supplier is advisable due to potential variations in fluorophore conjugation between suppliers. While the use of isotype controls can be a subject of debate among researchers, they can reveal issues such as inadequate Fc blocking.

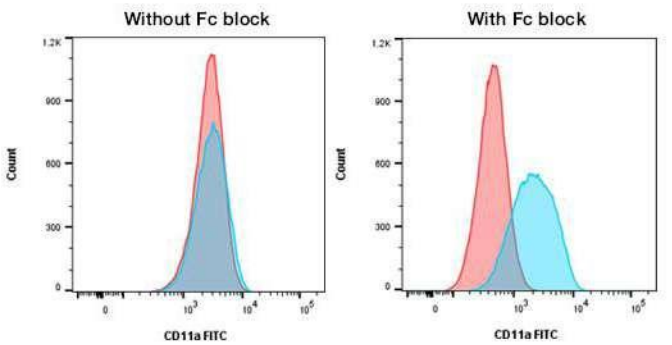


Figure 3: Staining of THP-1 cells with a Mouse Anti-Human CD11a antibody (blue histogram) or isotype control (red histogram) in the presence or absence of Fc block.

Fluorescence Minus One (FMO) Controls: FMO controls help identify fluorescence spread from neighbouring channels, helping set gating parameters. Each FMO control includes all fluorescently labelled antibodies except one, allowing researchers to assess the influence of each fluorophore on the panel and its potential spread into neighbouring channels. These controls are essential when working with multicolour panels, ensuring correct data interpretation.

Intracellular Staining Controls: Intracellular staining often presents challenges due to higher background levels caused by protein interactions. Isotype controls may not be suitable for this purpose, so other controls like a negative cell line or secondary antibody alone can be used to determine specific binding.

Biological Controls: Biological controls, encompassing known negative and positive samples, are essential for determining staining specificity and experiment limitations. These controls provide a reference point for interpreting results and assessing the dynamic range of fluorescence staining. Examples include cells with known antigen expression profiles, gene-edited cells, and stimulated/unstimulated controls for activation studies.

Perform Antibody Titration for Optimal Staining: Striking the right balance with antibody concentration is paramount. Excess antibodies can introduce background noise. The stain index, calculated as the difference between the median fluorescent intensity of the positive and negative populations divided by two times the standard deviation of the negative population, serves as a guiding metric (Figure 4).

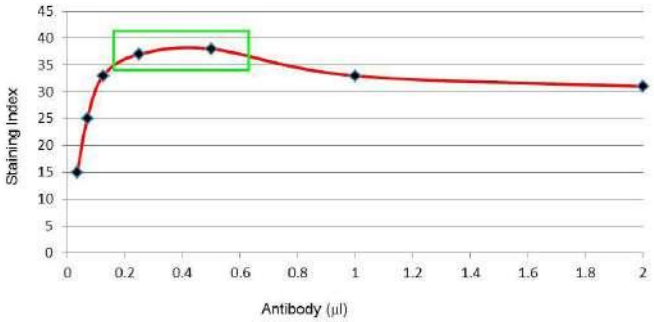
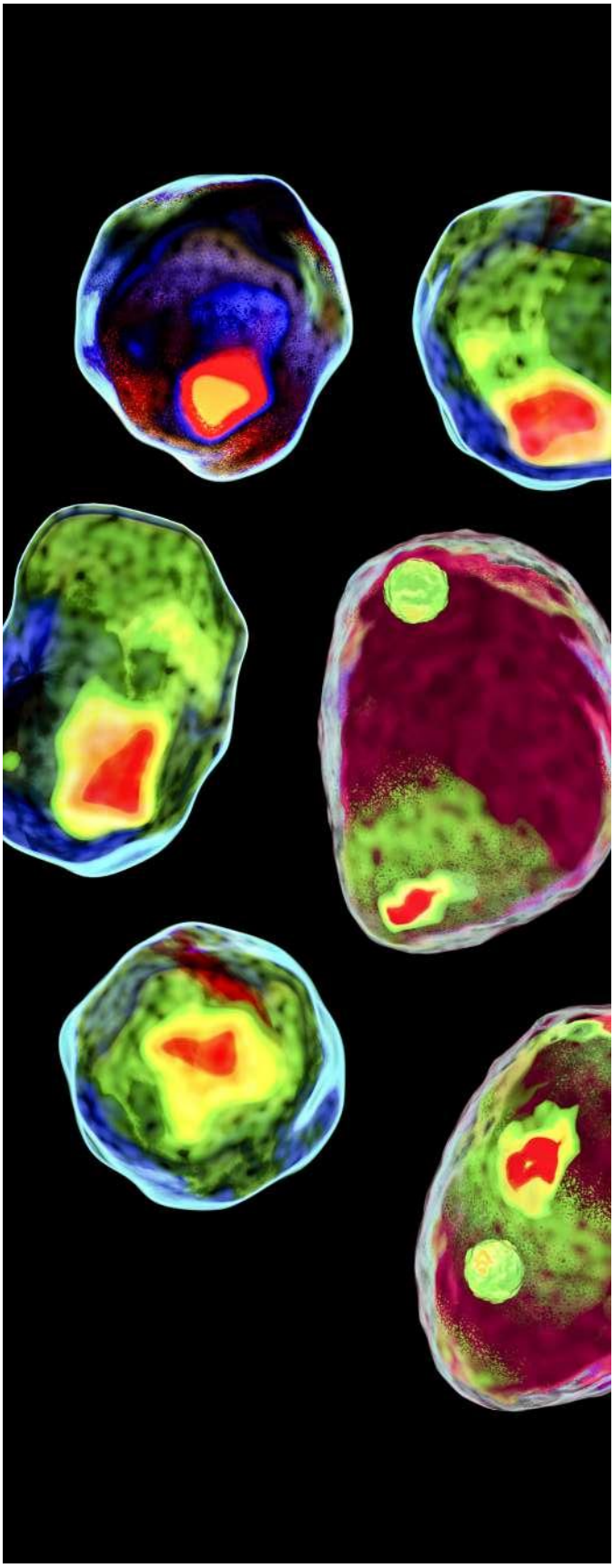


Figure 4: Calculating the stain index can help determine the ideal concentrations that will generate specific staining with the least amount of background (represented in the green box)

Create Dump Channels

When specific cell populations are irrelevant to the experimental design, creating a dump channel is essential. This approach proves particularly valuable when investigating rare populations, such as stem cells, or in contexts like vaccine development, where immune system cells of interest constitute a minute fraction of the total T cell population. By employing fluorophore-conjugated antibodies specific to cells you wish to exclude but not expressed on your target cells, you can



eliminate unwanted binding, fluorescence spillover, and signal contamination from your analysis. In scenarios where the detection of rare cells, such as hematopoietic stem cells, is paramount, employing a specific fluorophore to stain all other cells ensures they are disregarded. Additionally, a dump channel can exclude dead cells when using a viability stain, reducing signal noise caused by non-specific staining and enhancing data quality.

Incorporating Best Practices with Online Panel Building Tools

Leveraging online tools can simplify the complex panel design process and reduce the risk of selecting reagents that are incompatible with your flow cytometer. For example, dedicated panel building websites take instrument configuration and capabilities into account. Other online tools, such as spectral viewers, provide insights into spillover and excitation by each laser, highlighting compatibility issues between fluorophores. Similarly relative brightness tables assist in pairing fluorophores with specific targets, while marker expression data aids in understanding expression patterns before selecting a fluorophore.

Conclusion

Working with complex, multiparameter panels requires careful consideration of instrument capabilities, fluorophore properties, sample availability and antibody functionality. Moreover, leveraging online tools has also been underscored as a valuable resource to simplify the intricate process of panel design. While the process may involve multiple design and optimisation steps, adhering to best-practice guidelines and selecting appropriate controls can make the process more efficient and cost-effective which can ultimately lead to reproducible and reliable experimental outcomes.[7]

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