# Understanding and Overcoming the Challenges of Flow Cytometry

**Flow cytometry is a powerful analytical tool that is widely used throughout drug development in various capacities. With an understanding of the range of applications flow cytometry offers, it is clear why it is heavily implemented in drug discovery and development. Studies have the ability to detect the expression of cell surface and/or intracellular molecules, determine the proportion of cell types within a heterologous cell population, and analyse cell volume and size.**

**However, the flexibility offered means goal-guided careful study design and a thorough technological understanding are required to define and inform panel selection, compensation, and gating in flow cytometry.**

**In this article, Director Scientific Engagement at Crown Bioscience, Pirouz Daftarian explores these requirements and highlights the need for drug developers to ensure that flow cytometry studies are purpose-driven in their design, to realise a world where patients get the right treatment at the right time.**

### The Wide Applications of Flow Cytometry

Flow cytometry offers the ability to analyse the chemical and physical characteristics of thousands of cells relatively quickly. Although flow cytometry can be used to distinguish between certain unlabelled cells, it is most often used to measure the fluorescence emission of fluorochrome-labelled reagents. These reagents are typically antibodies, cell tracers, cell trackers or conjugates that specifically bind to particular cell-associated antigens (markers). As different fluorochromes are excited at different wavelengths, many different fluorochromes can be used to specifically detect various markers within the same study. This allows flow cytometry to not only be used to study marker binding but also to differentiate and discriminate between subsets of cells by using markers known to bind to specific cell types.

Flow cytometry is inherently intricate. As well as careful selection of the best markers and fluorochromes for the study – and ensuring the instruments used can excite the fluorochromes – correctly analysing the data collected is also critical. Analysts must ensure the purpose of the study is defined, the use appropriate controls, define the validation parameters, consider signal-to-noise compensation, and have a robust gating strategy.

### Recognising the Challenges of Flow Cytometry

With so many considerations to be made throughout a flow cytometry study, it is imperative to pre-empt common challenges. This is particularly important when using flow cytometry to determine potential candidates for Investigational New Drug Applications (INDs), where understanding the mechanism of action (MoA) of a molecule is vital for effective clinical trial design.

### The Purpose of the Study is Fundamental to Study Design

Before starting any flow cytometry experiment, it is essential that the purpose of the study and the hypotheses it is based on are clearly defined. Setting the validation parameters, designing the reagent panel, choosing a gating strategy, and many other decisions made throughout will be dependent on understanding the purpose of the study.

If these decisions are not made with the purpose in mind, an abundance of data could be collected that does not answer the original hypothesis or could potentially lead to erroneous conclusions being made. As time is often critical in IND applications, it is vital to avoid poor study design which relies on a strong understanding of the principles of flow cytometry and its limitations.

### Designing the Reagent Panel Can be Complex

Panel design in flow cytometry encompasses the selection of a combination of reagents (usually fluorochrome-labelled antibodies) that recognise specific antigens on the surface of cells within the sample cell population. Commonly, panel selection will use a hierarchy approach to differentiate cells, with the reagent with the brightest fluorochrome having specificity to the smallest target cell population.

By selecting the right panels, misleading or non-specific binding of antibodies to the cells in the sample population can be avoided. However, this often relies on a strong understanding and familiarity with flow cytometry, as there are many factors that will impact panel selection. Depending on the study, these factors could include the size of the antibody, how the reagent binds to the marker, and whether binding could impact other cell interactions. Binding studies may be required prior to flow cytometry to elucidate these potential interactions.

### Validation Parameters will Depend on the Purpose

From the onset of the study, the validation parameters should be defined to ensure that the data are credible and reproducible within and between laboratories. These parameters will depend on the nature of the study; for example, if the study uses live cells, parameters should be set to ensure the viability of cells is maintained.

As well as the markers selected to achieve the goal of the study, another aspect that is important in validation is the choice of reagents that are specific to these markers. There are many commercially available antibodies of different sizes that offer various fluorochrome intensities and it is essential that those chosen are validated. This validation should ensure that the selected antibodies do not cross-react or bind non-specifically throughout the study.

### Collecting Events for Statistically Significant Results

When carrying out reagent panel design, the estimated proportion of target cells within a heterologous cell population

must be considered to ensure that statistically significant data is collected.

Low frequency of the target cell population is a common challenge in immunological studies. Occasionally, the proportion of target cells in a population may be so low it will not be detected by flow cytometry. This can be overcome in some circumstances using *in vitro* vaccination or *in vitro* culture to expand these low-frequency cells. However, using *in vitro* expansion methods could mean the results are no longer truly representative, and the decision to use these methods will be dependent on the purpose of the study.

**A Goal-guided Gating Strategy**

When designing the study, the gating strategy should be carefully considered with the purpose of the study in mind. Setting the gates incorrectly can mean the target cells that the study was developed to understand could be missed completely.

Fluorescence minus one (FMO) controls – where each control sample includes all fluorochromes except one – can allow for a highly accurate gate boundary to be set. Although FMO controls may not be needed in cases where well-defined positive and negative populations exist, these controls can be critical when there are rare cell populations or antigens with low expression. Additionally, FMO controls can be used to identify self-aggregation of antibodies. As setting up these controls is time-consuming it is essential that their need is determined during the early stages of study design.

**Selecting and Including the Right Controls**

There are five main types of controls that should be included in flow cytometry studies:

1. **Instrumental controls**
   These controls ensure the flow cytometer and its components are working as intended.

2. **Compensation controls**
   When two or more fluorochromes are used, compensation controls are needed to correct spill over of emissions into other channels.

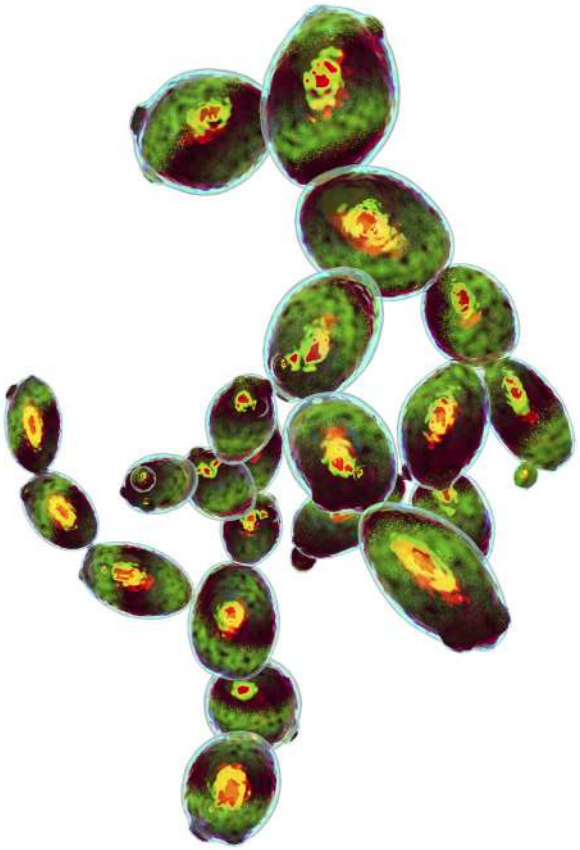
3. **Gated controls**
   These controls will help to set the gate (the area on the scatter plot or histogram produced to identify and define subsets of populations).

4. **Isotype controls**
   By using antibodies that are the same class and type as the primary antibody but lacking specificity to the target, isotype controls can be used to identify issues with the protocol.

5. **Experimental controls**
   Negative controls should identify false positives resulting from nonspecific antibody binding using cells known not to express the marker of interest. Positive controls with cells that express the target marker can be used to identify faulty antibodies causing false negatives.

**Key Lessons**

Flow cytometry is a powerful analytical tool that is instrumental in drug discovery and development. However, the wide variety of potential applications and many factors that must be considered throughout, including panel design, determining the necessary controls, and validation, lead to many potential challenges. It is therefore important to identify those with expertise and experience in flow cytometry who can help to overcome these hurdles and to ensure successful analysis and delivers to the purpose of the study.




## Pirouz Daftarian

Pirouz Daftarian completed his PhD training in immunology in the Faculty of Medicine of the University of Ottawa, in 1998. Since then, he has been leading studies on cancer immunology, vaccines and inflammation, in academia, and biotech. He joined Crown Bioscience / JSR Life Sciences in 2017 and led the developing applications for IO products and as a Director of Scientific Engagement for Inflammation and *In Vitro* IO. Of prior experience, pirouz was with NGM Biopharmaceutical, was a faculty at Miller School of Medicine of U of Miami and a Head Scientist, Cancer Biology, at IMV Inc. Pirouz has authored > 80 peer reviewed papers, has served as a reviewer of DOD, NIH, NCI, is a reviewer for several journals, and acted as a consultant to pharma and biotech companies.