

# Guideline for Designing an Experiment in Multicolor Flow Cytometry

The need for multidimensional analysis using complex stain combinations has been expanding considerably. Especially to distinguish, classify or isolate rare cells populations, it is necessary to measure several independent properties. For example, it is common to use many markers to define and characterize a phenotype in rare subpopulation like stem cells compartments or rare progenitors cells. The more parameters needed, the more users will face [compensation](#) issues, technical limits, or compatibility between the fluorochromes chosen. Thus, it is critical to understand and follow some basic rules when you design a new panel or stain for a flow cytometry experiment.

## **1. Consider the Brightness of your fluorochromes:**

When designing your panel, you need to choose the brightest fluorochromes that can be used on the instrument you are planning on using (FACSCalibur, LSRII, FACS Canto or FACS AriaII). You can find a [Fluorochromes Brightness Chart](#) on this website, note that this chart gives an idea of the fluorochrome [brightness](#). If you use only one color the typical choice will be PE or APC as those colors are among the brightest ones. Remember that the brightness of each fluorochrome depends on the buffers used, the instrument configuration (filters, PMT, etc...), the kind of instrument you use, the ratio Fluorochrome/Protein, the antibody clones etc...

## **2. Choose the right fluorochrome for your markers:**

When it comes to assign a fluorochrome to a marker, always keep in mind to use the brightest fluorochromes for the least expressed proteins and the dimmest fluorochromes for the most highly expressed proteins. It is really important to know or at least have an estimation of the level of expression for each of your markers as it determines which fluorochrome to choose. It will greatly optimize the results of the experiment. If you don't have any idea of the level of expression, try to assign the brightest color to the most important marker in your panel.

## **3. Limit spectral overlap between the fluorochromes:**

Choosing the fluorochromes which have the least [spectral overlap](#) will enhance the quality of your results. Thus, you need to choose fluorochromes with non-overlapping emission spectra. You can find a chart showing the theoretical amount of overspilling for

each fluorochrome combination on the website (link). Generally, the further apart the maximal emission, the less spectral overlap will occur. Some fluorochrome pairs ([proximity pair](#)) will generate more overspilling than others and you will need to compensate in order to have valid results. Typically, association such as APC/APC Cy7; PE Cy5/APC; FITC/PE are some choice to avoid if possible as they generate a large amount of over spilling. Moreover, this property of fluorochrome is intrinsic for each fluorochrome. This rule can be in conflict with the first rule, which was to choose the brightest fluorochromes. In another words, you may have to sacrifice a certain amount of brightness in one detector to avoid spillover in another.

#### **4. Know the Fluorochromes properties that you choose:**

Each fluorochrome has specific properties. Large choice of [tandems dyes](#) are available, it is important to know that tandems might be subject to uncoupling, sensitive to [photobleaching](#) and may need an increased incubation time, so they usually constitute a second choice after a non-tandem dye. Monitoring the uncoupling during the staining by running a simple color that is due to the emission of the excitation partner is a good way to remove [noise background](#). For example, FITC is sensitive to acidic conditions, thus if your protocol includes acidic buffers FITC won't be a good choice to stain your cells. Cychrome or PE Cy5 is the primary choice for intracellular staining as it's capable of conserving a decent [signal](#) for intracellular staining even after the harsh washes with methanol. PE Cy5 is a good choice of color to stain your cells for a "Dump channel" such as lineage dump or viability check. 7AAD being detected in the same channel as PE Cy5, it is useful to dump the subpopulation of cells you don't need within this channel. Go to: [fluorochromes description](#).

#### **5. Maximise the usage of your lasers:**

Many cytometers include several lasers. Try to choose a color in each laser excitation range before to think having a second color for the same laser. This considerably reduces the amount of compensation you need to perform between the different colors. For example, a three color panel could be chosen as follows: APC for the red laser, PE for the blue laser and Pacific Blue for the violet laser. Though, in this case, Pacific Blue being a dim color, could be substituted for a second choice in the blue laser since this laser can have up to five colors.