Flow Cytometry - FMO FAQ

By Andrew Kinman, PhD., Immunology and Flow Cytometry Scientist at Xeno Diagnostics

What is an FMO?

FMO stands for fluorescence minus one. Think of it as total fluorescence: the combined emission signal from all fluorophores that are being used, minus a single fluorophore. For example, if 5 fluorophores are being used, an FMO sample would be comprised of 4 of the 5 fluorophores; thus, there would be a total of 5 FMO samples each comprised of 4 fluorophores, omitting a different fluorophore for each sample.

Why do I need FMOs?

An FMO helps identify the true baseline negative cell populations. Studies should also have unstained controls to help verify negative populations, but FMOs serve as unique negative control for multi-color experiments. In multicolor experiments, there is the possibility of fluorescence spillover; where the emitted light from a different fluorophore in the sample may overlap slightly with the target fluorophore of interest causing increased signal for negative populations.

Do I need an FMO if I expect my sample to be negative for one of my stains?

Yes, still perform the FMO normally just in case there is any non-specific binding that may add to potential spillover.

What happens if I do not use an FMO?

Without an FMO, an investigator may over or underestimate where the cutoff (gate) is between positive and negative populations. For example: in some cases, it may be possible to see that stained samples have two distinct populations. We would expect that these two populations are a positive and negative population. However, both populations may be positive, one population bright (high expression) and while the other is dim (low expression; but still positive). Without an FMO it can be difficult to tell the difference between the two possibilities.

There may be additional confounding factors that make gating difficult, such as changes in cell source: primary vs immortalized, fresh vs cultured, stimulated vs resting, and specimen (donor); as well as various treatments and culturing conditions which may increase variability. Having an FMO removes one more source of ambiguity and provides confidence when interpreting the data.

What cells should I use for an FMO?

The best practice is to use cells that match the test samples. Ideally samples that contain both a positive and negative population should be included. This is because, depending on the complexity of the staining panel, it may be necessary to gate on a series of positive cell populations to identify the cell subset of interest before adjusting the negative gate for the FMO.

Can I use compensation beads for an FMO?

No, compensation beads are not a great substitute for cells. Firstly, compensation beads do not have the same level of autofluorescence that cells do. Secondly, the beads will have a different binding profile from cells (e.g., beads will bind all the antibodies, whereas a cell will bind the antibodies in different proportions) which would affect the fluorescence spillover. Lastly, there are certain stains that beads will not bind: membrane dyes, DNA dyes, viability dyes, etc.

What FMO gates should I set first?

Set gates in order of gating strategy for samples. Example: Tregs with CD3+CD4+CD25+FoxP3+. First gate on main population of lymphocytes (FSC x SSC), then gate on singlets (FSC-H x FSC-A). Next, set FMOs on live cells (if using viability dye); then set FMO for CD3, then CD4, and

proceed until the final gated cells are the most "narrow" cell subset.

What if I am testing different cells from multiple different sources in the same experiment?

It depends. If testing multiple different cells as a mixture within the same well, it should be acceptable to use the same mixture as an FMO. However, if the cells are kept separate, and will be analyzed separately; it will be likely that separate FMOs will need to be prepared for each cell type. If the cells are expected to be similar (e.g., testing primary vs immortalized cells of the same lineage) it may be possible to use a single FMO. Although it may be a good idea to conduct a pilot study to verify that there is no difference between their individual FMOs.

What cells should I use for an FMO when I'm testing for activation markers with varying levels of stimulated cells?

The preference at Xeno Dx is to use a mixture of unstimulated and that are maximally stimulated cells matching highest level of stimulation of test samples. Having the positive population (upregulated activation marker) present when performing an FMO ensures that the negative FMO population is accurate (e.g., if non-specific binding/spillover from other fluorophores is affected by activation marker).

If concern remains about whether a maximally stimulated FMO is accurate for a minimally stimulated sample, then it is recommended to run a pilot study verifying FMOs at low, medium, and high stimulation levels.

What is the difference between an FMO and an isotype control?

The purpose of an isotype control is to control for potential non-specific binding that may naturally occur in cell samples. After all, cells and proteins can be "sticky" (ionic, hydrophobic, and Van der Waals forces). Hence, there may be some additional fluorescence in a sample caused by non-specific adsorption that appears as increased staining when compared to unstained control, even if the sample is negative for that specific cell population of interest.

An isotype control does not always perform in a uniform manner, and can vary between antibody controls of the same isotype. Isotype controls cannot control for spillover from other dyes or replicate other stains that may be used such as nuclear stains, viability stains, membrane dyes, etc. FMOs have been replacing the use of isotype controls.

What if I am using a dump channel with different antibodies using the same fluorophore?

Treat that group of antibodies as a single stain. Remember, FMO's are more about the fluorophore, adjusting for potential fluorophore spillover to verify negative cell populations.

How do I use an FMO if I am performing viability, surface staining, intracellular staining, and phosphorylated /kinase staining?

FMOs are still needed with these types of experiments. It is important to match the staining treatments and timing to that of your normal test samples. This can involve doing multiple rounds of staining (fixable viability dyes, followed by surface, followed by intracellular and phospho-kinase); or it may be necessary to perform phospho-kinase stains separately to arrest phosphorylation as soon as possible. Either way, follow the same protocol established for the test samples.
