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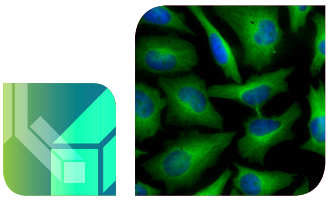
10 Tips for selecting and using Fluorophores in IF experiments



1 Familarize yourself with your microscope set up

Applies especially to emission/excitation/dichroic filters and in the case of confocal microscopes the laser lines. Getting to know your set-up ensures that you can select fluorophores that can be optimally excited and detected.

2 Consult a spectrum viewer or a fluorophore reference chart to assess fluorophore/microscope compatibility

These references will help you determine the maximum excitation and emission wavelengths of the fluorophore conjugated antibodies available in your lab. Compatibility evaluation can be straight forward for certain filters, such as FITC/TRITC filters, as these have been named after the fluorophores they are meant to be used with. As members of dye families, such as Alexa Fluor® 488, have been named/numbered according to their approximate excitation maxima (in nm), these numbers also provide a rough guidance for laser and filter selection.

3 Select fluorophores with high extinction coefficients (ε)

One defining factor of a fluorophore's brightness is its extinction coefficient (a measurement of the probability of absorbing a photon of light); the higher the value of the extinction coefficient the brighter the fluorophore. For example the rather dim DyLight® Fluor 350 has an extinction coefficient of only 15,000 while the bright DyLight Fluor 650 dye has an extinction coefficient of 250,000 (Thermo Fisher Scientific Inc 2012).

4 Choose fluorophores with high quantum yields (Φ)

The quantum yield is a read-out of the efficiency of the fluorescence process (Φ is calculated by dividing the number of emitted photons by the number of absorbed photons). A 100% efficient fluorescence process would have a quantum yield of 1 (the maximum quantum yield possible). The commonly used and very bright Alexa Fluor 488 fluorophore for example has a high quantum yield of 0.92 (Molecular Probes® 2010).

5 Avoid fluorophores with a high susceptibility to photobleaching

Photobleaching is a photochemical destruction process that reduces the intensity of the fluorescence signal; for example FITC and R-Phycoerythrin are known to photobleach quickly. Where possible we recommend you use photostable fluorophores, such as Alexa Fluor or DyLight Fluor dyes. Alternatively, you can also reduce the intensity/exposure time to the excitation light or use mounting media containing antifade reagents. For more information about mounting media, please refer to the "Mounting coverslip section" of the Bio-Rad IHC-P tips and tricks ebook (bio-rad-antibodies.com/ihc-ebook).

6 Use new generation dyes that stay fluorescent over a broad pH range

Many conventional fluorophores, such as FITC, are not recommended for staining protocols using acidic buffers as the fluorescence intensity signal is highly sensitive to an acidic environment (Cancer Institute, The University of Mississippi Medical Centre 2015).

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7 Ensure your fluorophore staining is spectrally differentiable from your counterstain

Counterstains provide background contrast and put the observed staining into perspective (e.g. by visualizing nuclei). It is important that your conterstain and fluorophore staining can be easily distinguished. For example, DRAQ5™ should be used as a nuclear counterstain rather than DAPI when using antibodies conjugated to blue emitting fluorophores such as Alexa Fluor 405 or DyLight Fluor 405.

8 Use fluorophores with narrow emission spectra in multi-color IF experiments to avoid spectral overlap

Spectral overlap is also known as bleed-through and describes the detection of one fluorophore in another fluorophore's filter set. Bleed-through makes it difficult to observe discrete fluorescence signals and complicates the evaluation of co-localization experiments. Therefore when selecting fluorophores the emission and excitation spectra should be checked for spectral overlap with the help of a spectrum viewer. Ideally, there should be no spectral overlap between the fluorophores. Quantum dot conjugates, which have broad absorption and very narrow emission spectra, are therefore perfectly suited for multi-color experiments.

9 Decide what antigen to detect with which fluorophore in multi-color experiments

The brightest fluorophore should be reserved for the detection of the antigen with the least abundant expression level. The dimmest fluorophore should be used for detecting the most abundant antigen.

10 Include appropriate controls

In order to verify that the observed fluorescence is a result of staining rather than an unspecific artifact we recommend you consider inlcuding these three controls:

- Autofluorescence/cell background staining control. As cells (especially those that have been damaged or have started to undergo apoptosis (Hibbs 2004)) can display natural fluorescence it is crucial to observe IF samples microscopically before every staining experiment. Additionally, a label/fluorophore control should be included by performing the complete staining protocol without the addition of fluorophore conjugated antibodies.
- Positive and negative controls. Include cell lines in which your protein of interest is either over-expressed or absent (e.g. a knock-out cell line). If you do not see staining in the positive control something has gone wrong with the staining protocol. Alternatively, if you see staining in the negative control you know that the staining/observed fluorescence is nonspecific.
- If secondary antibodies rather than directly fluorophore conjugated primary antibodies are to be used, a secondary antibody only control should be performed following the same staining protocol without the addition of a primary antibody. This type of control is used to verify that the secondary antibody does not nonspecifically bind to certain cellular compartments. For multi-color immunofluorescence experiments we recommend the use of cross-adsorbed/pre-adsorbed secondary antibodies as those minimize the risk of the secondary antibody reacting with endogenous immunoglobulins or an undesired primary antibody.

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