Sample Preparation
Guidelines for optical imaging

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General sample preparation

a. Fixation

Several fixation methods that are suitable for IF are accessible in literature. The goal of all is to keep the cell structure as close as possible to the natural state. In the reality, always fixation and further steps as permeabilizing will unfortunately affect the cell morphology, the localization of proteins and the availability of the antigens of interest. Therefore, you should always try different reagents, times and concentrations to optimize these for your special experiment.

Aldehyde fixative (Paraformaldehyde, Formadehyde and Formalin) cross-link proteins and maintains cellular morphology relatively well. They act somewhat slower than organic solvents, especially on thick samples.

- 4% formaldehyde for 10 min is a good starting point for mammalian cells.

i.e. Sources of supply:

Formaldehyde solution (Formalin) contains 10-15% methanol as stabilizer, 37 wt. % in H2O.

Paraformaldehyde (powder or prilled), source of methanol free formaldehyde.

Organic solvents (**methanol/ acetone)** precipitate proteins; the protein "shell" of the cell is maintained. These detergents have a similar fixation effect as the use of aldehyde fixatives, but one will lose all small molecules throughout the rest of the protocol. In addition, because they denature proteins they should not be used with fluorescent proteins in your sample such as GFP.

However in case that you are interested in naturally buried antigens, which makes it difficult to bind to antibodies, these antigens might be better exposed after methanol fixation and therefore accessible.

If you lose your immunolabelling in 100% methanol, you should try 10 min in a combination of methanol /acetone at -20°C, because methanol is best for structure fixation but acetone is less damaging.

i.e. Sources of supply:

Methanol (MeOH)
Acetone

b. Permeabilization

Permeabilization facilitates the access of an antibody into the fixed cell. Many different substances are available, including Triton X-100, Tween-20 and Saponin. How long you should perform this step is depending on your target of interest. If it is a membrane protein, you should not remove the whole membrane lipids by a long Permeabilization step, otherwise you will lose also the membrane bound proteins. Therefore, it usually makes sense to try different detergents, different concentrations and incubation times in order to optimize them for the experiment.

i.e. Sources of supply:

Triton™ X-100
TWEEN® 20

c. Blocking

A dramatic but positive effect on the result has a good blocking step. Here you can test different substances, BSA, fish skin gelatin milk or serum. Importantly, do not use serum of the same species as the one in which the primary antibody was raised! Often the procedure has worked well using the serum in which the secondary antibody was created! Good starting point is blocking for 30min at RT with 5% serum / 0.05% Tween-20 in PBS.
The primary antibody should be diluted in this blocking solution, dilution range from 1:10 to 1:1000. Incubation should last for one hour at room temperature, or overnight at 4 °C and 50µl antibody solution are sufficient for a standard cover glass in a humid chamber.

Wash the sample several times (staying on the sample for at least 3x5min RT) with PBS+0.05%Tween after antibody incubation to reduce the background.

i.e. Sources of supply:

Bovine Serum Albumin
Gelatin from cold water fish skin
Donkey serum
Sheep Serum

d. Labelling

Most microscopes are very well equipped so that they can image a variety of fluorophores. However, you should always check before the labelling whether the desired setup (laser / filter) is present at the microscope and whether the fluorophores are suitable as a combination to each other.

You can easily check this using the Fluorescence SpectraViewer of ThermoFischer. In addition, the fluorophore conjugates -coupled to secondary antibody – must be bright and stable and cross-adsorbed for good species specificity.

Dilute your fluorophore-conjugated secondary antibody with 5%serum/0.05%Tween/PBS (dilution range between 1: 200 to 1: 1000) and incubate for 1 hour at RT or overnight at 4°.

Coverslip thickness

Most objectives are exact set to a cover glass thickness of #1.5. A small deviation of this thickness has a drastic effect to the quality of recording particularly with high-numeric aperture lenses. See for more details:

www.miap.eu

"The importance of #1.5 thickness coverslips for Microscopy" (pdf)

Mounting

There are many good commercial embedding media available with different properties (i.e FluoromountG, Prolong Gold, Prolong Diamond, Prolong Glas, Vectashield, Ibidi Mounting). However, it is also possible to use self-produced Movio l-, Agarose - or Glycerol - mixtures for embedding.

All mounting media make the sample more robust and serve to adapt the refractive index to the required RI of the objective to give the best performance. In addition, the mounting solution often also reduce photobleaching.

Select a mounting medium that does not contain DAPI or any other dye. If you have a dye in your mounting medium, you will not be able to obtain the high signal-to-noise ratio required for quality images. The presence of dye in the mounting medium will dramatically increase the background signal in your sample.

Further, be aware that in hardening mounting media, the samples compress as they cure and shrink in z. direction In contrast, when using a soft mountant, compression does not occur and sample morphology is better preserved.

Rinse briefly the coverslip by dipping it in distilled water a couple of times to wash out the salt. Otherwise the coverslip will show salt stain when the salt is dried. Always seal the slides after letting them dry.

i.e. Sources of supply:

Fluoromount™ Aqueous Mounting Medium
VECTASHIELD® Antifade Mounting Medium
Prolong (Diamond, Glass, Gold)
Ibidi Mounting
Agarose, low gelling temperature

Imaging conditions - acquisition of physiological data (live cell imaging)

Especially during the acquisition of physiological data, it is important to achieve an optimal result with the least possible amount of laser light. Therefore, consider the following topics:

- Use much less excitation light than you would use for fixed cell imaging.

- Avoid too many acquisition channel and use long wavelengths that cause less cellular damage.

- If the question can also be answered using non-fluorescent techniques such as bright-field, differential interference contrast (DIC), or phase contrast imaging, use this as it does little or no harm to the cells

- Do a few z slices and minimize number of time point accordingly to the dynamics of your cell events.

Further Tips and Pitfalls

For a more extensive information on these subjects, we recommend the following article:

Seeing is believing? A beginners' guide to practical pitfalls in image acquisition

Everybody, who wants really good and amazing microscopic pictures for publication, should be ready to spend more deeply into the matter. It is definitely worth.

Sources:

https://microscopy.duke.edu/guides/intro-sample-prep-immunofluorescence
http://microscopy.arizona.edu/sites/default/files/sites/default/files/upload/sample_prep_sr-sim_v2.pdf
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