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Protocol Tips for Immunofluorescence.docx

Tips for Immunofluorescence

When implementing protocols for new antibody-antigen combinations and for intracellular staining in particular, do consider the following points and be prepared to try more than one protocol before you reach conclusions or give up.

Fixation

Fixation may damage or mask antigenic sites. Some cell-cell adhesion antigens can be functionally affected by antibody binding.
Aldehyde fixatives (e.g., 2-4% paraformaldehyde for 10-20’, formaldehyde, glutaraldehyde) – possible method of choice for double labeling of membrane bound proteins. Allows access to cells and subcellular compartments. Aldehyde usage may require “quenching” to reduce autofluorescence. Incubation time may have to be optimized.
Organic solvents (methanol, ethanol, acetone) do not alter target proteins covalently but nuclear and mitochondrial penetration may be compromised and lipid-linked proteins may be removed. Not suitable for membrane proteins.
Combination: Formalin - 37% formaldehyde, 10-15% methanol.

Permeabilization

Aldehyde-fixed samples might require a permeabilization step either using detergents (from mild to harsh: saponin/digitonin, Tween 20, 0.1-0.5% Triton X100, NP40) or methanol or acetone.

Blocking

You may start with 10% normal serum (generally matching secondary antibodies in the experiment) 10’-1h for sections or 1-5% BSA for 30’ for cells in culture.

Buffers

While PBS may be best for cell surface staining, for intracellular antigens you may want to try PHEM buffer (60mM Pipes, 25mM Hepes, 10mM EGTA, 2mM $MgCl_2$ pH6.9) or other buffer more similar to the intracellular environment.

Antibodies

Monoclonal antibodies may have lower affinity than polyclonal antibodies. Polyclonal antibodies may have higher background.
Polyclonal antibodies raised against the whole antigen may still exhibit selectivity to a subset of the whole antigen molecule.
Primary (direct) immunofluorescence using a single antibody linked to a fluorophore avoids issues of antibody cross-reactivity but is less sensitive than indirect, where using a secondary antibody may amplify the signal.
Titrate primary antibody and check long (slow but targeted) incubation overnight at 4°C.
Antibody optimization should be carried out separately before combination in double/triple staining.
Run secondary control without primary antibody.
Consider using sodium azide as a preservative and BSA as a stabilization in the antibody diluent.
Make sure that the slides are kept in a humid environment.

Bleaching

Always use anti-fluorescence-quenching mounting medium.
Consider employing more modern fluorophores (e.g., Alexa or DyLight Fluors) which are less prone to bleaching and older generation dyes (e.g., FITC)

From:
http://blog.ptglab.com/index.php/immunostaining-cultured-cells/
http://www.immunohistochemistry.us/immunohistochemistry-staining/immunohistochemistry-staining-tips.html
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