Immunofluorescence

The reactivity of antibodies can vary widely. Also monoclonal and polyclonal antibodies do not necessarily react with the same antigenic form of a component. Because of the low structural resolution of light microscopy it will be more important to preserve the antigenicity of an component. Different fixation procedures can be tried to optimize the immunostaining. The choice of fixatives will also depend on the subcellular localization of the antigen (soluble, membrane bound, cytoskeleton associated). Detergents should be used with caution for membrane bound antigens, while they will be helpful to lower the background of cytoskeletal components. If immunoelectron microscopy has to be carried out, the structural preservation of the cells must be very high. Therefore, glutaraldehyde followed by the proper quenching of the reactive groups should be used.

Cell Culture:

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Preparation of cover glasses for immunofluorescence microscopy

Most cultured cell lines need an adhesive surface to grow on. Cells growing in normal culture dishes can be used for testing an antibody; however, the surface is too uneven for optimal microscopy of single cells (cells are quite often tilted to one side resulting in an uneven focussing).

- Incubate cover glasses in 50% H_2SO_4 for 1 hour using a porcelain rack (Thomas Scientific).
- Wash cover glasses for 30 min in running tap water.
- Rinse with dH_20.
- Incubate cover glasses in 40 µg/ml poly-L-lysine (MW ~70-90kD) for 1 hour at room temperature.
- Wash cover glasses for 1 hour in running tap water.
- Rinse cover glasses 3 times 5 min each in dH_2O.
- Dry cover glasses on filter paper in a dust-free area.
- Sterilize cover glasses inside the laminar flow chamber under UV light for at least 4 hours.

Cell culture

- Detach cell from the plastic surface by incubating them in trypsin solution.
- Resuspend detached cells in culture medium and transfer them to culture dishes with the cover glasses.

- Culture cells up to semi-confluency.

Fixation Protocols:

Depending on the stability or accessibility of the antigen various fixation protocol can be used.
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(1) Methanol fixation (for cytoskeletal components): The methanol fixation is an easy method; however, it frequently solubilizes and removes membrane bound antigens. By a simple precipitation of the protein, methanol only provides low structural preservation.

- Rinse the cover glass with PBS.
- Fix cells by incubating the cells in pre-cooled 100% methanol at -20 oC for 10 min.
- Wash cells with PBS.

(2) Formaldehyde fixation (for membrane associated components):
FA stock solution: Dissolve 16 g paraformaldehyde in about 80 ml dH_2O by stirring at 70 oC (in fume cupboard). Add a few drops of 1 N NaOH to depolymerize the paraformaldeyde. Adjust the pH to about 7.0 and check with pH paper). Cool down to room temperature and bring up to 100 ml. Filter through an 0.45 µm Millipore filter and mix with an equal amount of double strength buffer. Divide into convenient aliquots and store frozen at -20^{o}C. Discard after thawing.

- Rinse cells with PBS at room temperature.
- Fix in 3-4 % paraformaldehyde in PBS for 15 min at room temperature.
- Wash 3-times 5 min each with PBS containing 100 mM glycine.
- Permeabilize cells with 0.1% Triton X-100 in PBS for 1 to 4 min.
- Rinsed with PBS.

(3) Paraformaldehyde/glutaraldehyde fixation (method of choice for double labeling of membrane bound and cytoskeletal antigens):
Rinse cells with PBS at room temperature.

- Fix in 3 % paraformaldehyde, 0.02% glutaraldehyde in PBS for 15 min at room temperature.
- Permeabilize by dipping cells for 10 seconds in 100 % methanol (-20^{o}C).
- Incubate cells for 3-times 10 min in 0.5 mg/ml $NaBH_4$ in PBS, pH 8.0 to reduce aldehyde groups and then rinsed with PBS.

  Note: Handle $NaBH_4$ with caution. $NaBH_4$ should be dissolved just before use. Bubbles will form (release of H_2).

(4) EGS (ethyleneglycol-bis-succinimidyl-succinate) fixation (method to preserve microtubules and membrane bound antigens):

- EGS stock solution: dissolve 45 mg in 1 ml DMSO (75 mM). Note that EGS is highly unstable in water.
- Rinse cells with pre-warm (37^{o}C)PBS.
- Dilute EGS stock in PBS to 10 mM.
- Drain off excess PBC from cell and immediately transfer them into EGS

  Note: less than 1 min should be elapse between adding EGS stock and transferring the cover slips in to the solution.

- Incubate (covered) at 37°C for 10 min.
- Rinse 3-times with PBS (include 100 mM glycine in 2nd wash).
- Permeabilize with 0.1 % Triton X-100 for 4 min at room temperature.
- Rinse with PBS.

Note that EGS is rather expensive and should be used in small amounts.

(5) Fixation of the cytoskeleton: If components of the cytoskeleton are of interest, cell can be extracted prior fixation.

- Rinse cells with PBS
- Incubate cells in extraction buffer (100 mM Pipes-KOH, 2 mM EGTA, 1 mM $MgCl_2$, 0.5% Triton X-100, pH 6.8) for 1 to 2 min at room temperature.
- Fix in methanol or formaldehyde/glutaraldehyde (see above).
- Rinse with PBS.

Staining Protocol:

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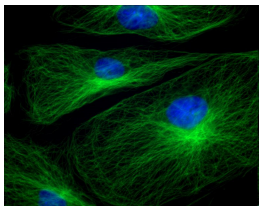


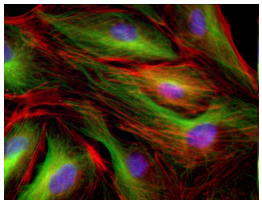
- Drain off culture medium and rinse cover slips with PBS.
- Drain off PBS with any of the above mentioned fixation methods.
- Wash in PBS 3-times 5 min.
- Permeabilize with 0.01% Triton X-100 in PBS for 30 sec (if needed).
- Wash in PBS 3-times 5 min
- Incubate in 1% BSA, PBS pH 7.5 for 30 min to block unspecific binding of the antibodies.
- (alternative blocking solutions are: 1 % gelatine, 1 % bovine or horse serum)
- Incubate with primary antibody in 1% BSA, PBS pH 7.5 for 60 min (or over night at r.t. depending on antibody concentration and the accessibility of the antigen).
- Wash with PBS pH 7.5, 3-times 10 min.
- Incubate 2nd antibody in 1% BSA, PBS pH 7.5, 60 min at r.t.; e.g., goat anti-rabbit Texas Red (Accurate), 1:80
- Wash with PBS pH 7.5, 3-times 10 min.

Optional:

- Incubate in 0.1-1 mg/ml Hoechst or DAPI (DNA stain) for 1 min.
- Rinse with PBS.
- Mount in PPD-mounting medium (90% glycerol).
- Seal with nail polish.

Final Notes:

Double Immunofluorescence:



Double Immunofluorescence is carried out just as single labeling. Antibodies derived from different animal can be mixed and incubated as a cocktail (example: rabbit anti-A and mouse anti-B). The same is valid for secondary antibodies (example: goat anti-rabbit Texas Red conjugated and goat anti-mouse fluorescein conjugated). If secondary antibodies cross-react, they should be pre absorbed against each other or for example

with liver acetone powder of the appropriate animal. One might, however, experience a reduction or loss of signal with one of the antibodies, if the antibody binding sites of the primary antibodies are in close proximity.

Protein A - Protein G:

Protein A is a cell wall component produced by several strains of Staphylococcus aureus. Protein G is a bacterial cell wall component isolated from group G streptococci. Protein A and protein G bind to the Fc region of most mammalian immunoglobulins (**see Table**). Protein G has an additional binding site for albumin which can be avoided by using recombinant protein G. Fluorophor-tagged protein A and G provide a very useful detection system for antibodies derived from different animals systems.

Avidin - Biotin:

Avidin is a tetrameric protein originally isolated from chicken egg white with a MW of about 67K and an isoelectric point of about 10. One mole Avidin will bind four mole biotin. The high pI of Avidin can cause binding to acidic structures such as DNA. Biotin is a naturally occurring vitamin with a MW of 244.31 and an isoelectric point of 3.5. The Avidin-Biotin interaction is the strongest known non covalent, biological interaction (Ka=1015 M-1). The bond formation is rapid and is unaffected over wide range of pH. Streptavidin is a biotin binding isolated from cultures of Streptomyces avidinii. It has Avidin-like binding characteristics; however, it has a MW of about 16K and an isoelectric point of 5-6.

References:

Beesley, J.E. (1989) Colloidal gold: A new perspective for cytochemical marking. Royal Microscopy Handbook #17. Oxford Univ. Press. pp 48.

Blose, S.H. & Feramisco, J.R. (1983) Fluorescent methods in the analysis of cell structure. Cold Spring Harbour Laboratory.

Fujiwara, K. & Pollard, T.D. (1976) Fluorescent antibody localization of myosin in the cytoplasm, cleavage furrow, and mitotic spindle of human cells. J. Cell Biol. 71, 848-875.

McBeath, E. & Fujiwara, K. (1984) Improved fixation for immunofluorescence microscopy using light-activated 1,3,5-triazido-2,4,6-trinitrobenzene (TTB). J. Cell Biol. 99, 2061-2073.

Richman, D.D., Cleveland, P.H., Oxman, M.N., & Johnson, K.M. (1982) The binding of Staphylococcal protein A by the sera of different animal species. J. Immunol. 128, 2300-2305.

Savage, M.D., Mattson, G., Desai, S., Nielander, G.W., Morgensen, S., & Conklin, E.J. (1992) Avidin-Biotin Chemistry: A handbook. Pierce Chemical Company. pp 467.

Wang, K., Feramisco, J., & Ash, J. (1982) Fluorescent localization of contractile proteins in tissue culture cells. In: Methods in Enzymology 85, 514-562.

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