Immunofluorescence and phalloidin labeling of mammalian cells

Contents

Materials for immunofluorescence and phalloidin labeling of mammalian cells

Forceps
For handling coverslips forceps with very fine tips are recommended e.g. Dumont forceps No 5 (straight) or Dumont forceps No 7 (bent) an (Fig. 1).

Fig.1 Dumont forceps

„humid chamber“
Different suppliers offer **humid chambers**, which are frequently rather expensive (>50 €). Alternatively large glass petri dishes may be used which need to be equipped with a tray for coverslips (e.g. a pipette tip holder form a pipette tip box) (Fig. 2).

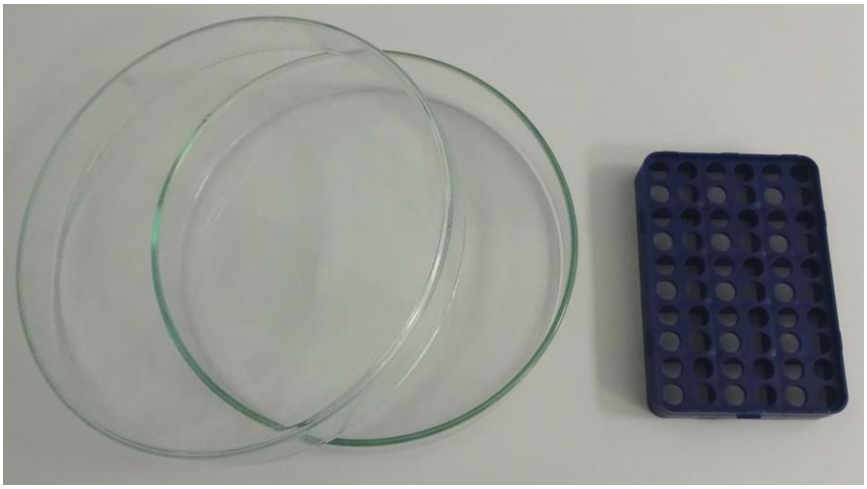
Fig. 2 **„humid chamber“**

Methanol, abs. ! TOXIC !
For fixation methanol should be chilled to -20 °C.

Formaldehyde solution ! TOXIC !
For fixation a freshly prepared formaldehyde solution which is buffered to a neutral pH is used. This solution should be prepared using para-formaldehyde. After preparation it should not be used longer than 1 - 2 weeks (when stored at +4 °C).
Alternatively, formaldehyde solutions may be frozen at -20 °C/ -80 °C direct after preparation. Then they might be stored and used for longer time periods.
The formaldehyde concentration and fixation time needs to be determined for each antigen/ primary antibody.

Primary Antibodies
A huge variety of primary antibodies is available.
For creation of test samples following commercial primary antibodies may be used.

Target	Source species	Antibody	Fixation
Nuclear Pore Complex	Monoclonal mouse antibody	Anti-Nup153 (Abcam, ab24700)	methanol or 2 - 4 % formaldehyde
Tubulin	Monoclonal mouse antibody	Anti-α-Tubulin (SIGMA, T6074)	methanol
Peroxisomes	Polyclonal rabbit antibody	Anti-PMP70 (Abcam, ab3421)	2 - 4 % formaldehyde

Extraction solution
Buffered solutions of different detergents as Tween20, Triton X 100, Saponin or SDS are used as extraction solution.
For many applications following solutions are used:
PBS + 0.1% – 0.5% Trition-X-100

Secondary Antibodies
Fluorophore coupled secondary antibodies, depending on the application.

Fluorophore labelled phalloidin ! TOXIC !
Typically phalloidin is shipped freeze-dried (i.e. in a solid form). Upon arrival it as to be dissolved in methanol (abs.).
Depending on the dye the solubility of fluorophore coupled phalloidin is approx.. 0.1 – 0.5 mg/ ml (methanol). For labelling of mammalian cells this stock solution needs to be diluted in aqueous buffer to a final concentration of ~20 - 50 µg/ ml.
(Depending on the application, cell line etc. phalloidin is used in concentrations between 0.1 and 100 µM in aqueous buffer.)

Phosphate buffered saline (PBS, pH 7 – 7,5)
137 mM NaCl; 2.7 mM KCl; 10 mM Na_2HPO_4; 2 mM KH_2PO_4

Blocking solution
For specific immunolabelling of cellular structures unspecific labeling needs to be avoided. To this end typically solutions containing different proteins which may not interfere with phalloidin or antibody reactions (e.g. bovine serum albumin, gelatin, yeast extract, milk (from milk powder)) are used.
For many applications following solution is used:
PBS + 1% – 5% bovine serum albumin

Embedding/ mounting media
A variety of different embedding media is available.
For STED microscopy we recommend following embedding media:
- Abberior Mount Liquid Antifade → for 3D STED
- Abberior Mount Solid Antifade → for 2D STED
- Mowiol/ DABCO → for 2D STED
- Prolong Antifade → for 2D STED

Coverslips
For the use of 100x oil immersion objective lenses, glass cover slips with a thickness of ~170 µm should be used i.e. No 1.5 or No 1.5H.
We do not recommend to use plastic cover clips or live cell chambers with plastic bottom because frequently only suboptimal results may be achieved.
Further no cover slips with grids gratings or similar should be used, because those structures might interfere with the imaging generation i.e. aberrations my occur.

Slides
The only limitation for slides is that they need to fit to the sample holder of the microscope. Super Frost slides or similar slides are not required.

Plastic or glass petri dishes
For fixation of the samples, washing of the samples…

Pipettes

Immunofluorescence-labelling on cultivated adherent mammalian cells

– Methanol Fixiation –

1. **Cultivation of cells**
   The cells are typically seeded on cover slips 12 – 36 h before labeling (Note 1, 2)

2. **Fixation and Blocking**
   Cover slips are fixed with cells facing upwards (Note 3; 4; 5; 6). Then the cover slips are washed with PBS (Note 6; 8; 9; 10). Finally unspecific binding sides are blocked with 2% BSA/ PBS for >5 min @ RT (Note 11) in a petri dish (2x).

3. **Incubation with primary antibodies**
   Cover slips are being taken out of the petri dish; excess BSA/ PBS is removed by placing the cover slip with the edge on a piece of tissue. Then cover slips are being put to a humid chamber. In turn the coverslips are overlaid with 25 µl (for 12 mm Coverslips) diluted antibody solution (in PBS/ BSA) (Note 12) and are incubated for 1 h at RT in a humid chamber.

4. **Washing**
   The cover slips are being taken out of the humid chamber. The antibody solutions are removed by pipetting of the liquid and then placing the cover slip with the edge on a piece of tissue. Then the cover slips are placed in a fresh petri dish containing PBS (Note 10; 13). Incubation > 5 min at RT (2x).

5. **Incubation with secondary antibodies**
   Cover slips are being taken out of the petri dish; excess PBS is removed by placing the cover slip with the edge on a piece of tissue. Then cover slips are being put to a humid chamber. In turn the coverslips are overlaid with 25 µl (for 12 mm Coverslips) diluted antibody solution (in PBS/ BSA) (Note 12) and are incubated for 1 h at RT in a humid chamber.

6. **Washing**
   The cover slips are being taken out of the humid chamber. The antibody solutions are removed by pipetting of the liquid and then placing the cover slip with the edge on a piece of tissue. Then the cover slips are placed in a fresh petri dish containing PBS (Note 10; 13, 14). Incubation > 5 min at RT (3x).

7. **Embedding, Storage, Stability**
   Finally the cover slips are being taken out of the petri dish; excess PBS is removed by placing the cover slip with the edge on a piece of tissue. Then the cover slips are mounted using the favored embedding Medium.
   In addition they may be fixed with nail polish completely or several small points (Note 15; 16).
   Ready-made samples should be stored at 4 °C (Note 18). Most samples are stable for a rather short time. For best resolution/ images with the best performance samples should be used not much longer than one week.

Immunofluorescence-labelling on cultivated adherent mammalian cells

– Formaldehyde Fixiation –

1. **Cultivation of cells**
   The cells are typically seeded on cover slips 12 – 36 h before labeling (Note 1, 2)

2. **Fixation, Extraction and Blocking**
   Cover slips are fixed with cells facing upwards (Note 3; 4; 5; 6, 7). Then the cover slips are washed with PBS (Note 6; 9; 10). Then cells are extracted using 0.1 – 0.5% Trition X 100 in PBS. Finally unspecific binding sides are blocked with 2% BSA/ PBS for >5 min @ RT (Note 11) in a petri dish (2x).

3. **Incubation with primary antibodies**
   Cover slips are being taken out of the petri dish; excess BSA/ PBS is removed by placing the cover slip with the edge on a piece of tissue. Then cover slips are being put to a humid chamber. In turn the coverslips are overlaid with 25 µl (for 12 mm Coverslips) diluted antibody solution (in PBS/ BSA) (Note 12) and are incubated for 1 h at RT in a humid chamber.

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5. **Incubation with secondary antibodies**
   Cover slips are being taken out of the petri dish; excess PBS is removed by placing the cover slip with the edge on a piece of tissue. Then cover slips are being put to a humid chamber. In turn the coverslips are overlaid with 25 µl (for 12 mm Coverslips) diluted antibody solution (in PBS/ BSA) (Note 12) and are incubated for 1 h at RT in a humid chamber.

6. **Washing**
   The cover slips are being taken out of the humid chamber. The antibody solutions are removed by pipetting of the liquid and then placing the cover slip with the edge on a piece of tissue. Then the cover slips are placed in a fresh petri dish containing PBS (Note 10; 13, 14). Incubation > 5 min at RT (3x).

7. **Embedding, Storage, Stability**
   Finally the cover slips are being taken out of the petri dish; excess PBS is removed by placing the cover slip with the edge on a piece of tissue. Then the cover slips are mounted using the favored embedding Medium.
   In addition they may be fixed with nail polish completely or several small points (Note 15; 16).

8. Ready-made samples should be stored at 4 °C (Note 18). Most samples are stable for a rather short time. For best resolution/ images with the best performance samples should be used not much longer than one week.

Phalloidin-labelling on cultivated adherent mammalian cells

– Formaldehyde Fixiation –

1. **Cultivation of cells**
   The cells are typically seeded on cover slips 12 – 36 h before labeling (Note 1, 2)

2. **Fixation, Extraction and Blocking**
   Cover slips are fixed with cells facing upwards (Note 3; 4; 5; 6, 7). Then the cover slips are washed with PBS (Note 6; 9; 10). Then cells are extracted using 0.1 – 0.5% Trition X 100 in PBS. Finally unspecific binding sides are blocked with 2% BSA/ PBS for >5 min @ RT (Note 11) in a petri dish (2x).

3. **Incubation with fluorophore labelled phalloidin**
   Cover slips are being taken out of the petri dish; excess BSA/ PBS is removed by placing the cover slip with the edge on a piece of tissue. Then cover slips are being put to a humid chamber. In turn the coverslips are overlaid with 25 µl (for 12 mm Coverslips) diluted antibody solution (in PBS/ BSA) (Note 12) and are incubated for 1 h at RT in a humid chamber.

4. **Washing**
   The cover slips are being taken out of the humid chamber. The antibody solutions are removed by pipetting of the liquid and then placing the cover slip with the edge on a piece of tissue. Then the cover slips are placed in a fresh petri dish containing PBS (Note 10; 13, 14). Incubation > 5 min at RT (2x).

5. **Embedding, Storage, Stability**
   Finally the cover slips are being taken out of the petri dish; excess PBS is removed by placing the cover slip with the edge on a piece of tissue. Then the cover slips are mounted using the favored embedding Medium.
   In addition they may be fixed with nail polish completely or several small points (Note 15; 16, 17).
   Ready-made samples should be stored at 4 °C (Note 18). Most samples are stable for a rather short time. For best resolution/ images with the best performance samples should be used not much longer than one week.

Notes

The most important rule for immuno-labeling is that specimens have to be prevented from drying out.

1. Seeding of cells may take place earlier if required. Depending on the doubling time, cultivation time, cell density and others cells may grow in micro-colonies.

2. If the cells are grown in very high densities, i.e. a confluent layer frequently a high background will become visible.

3. In contrast to other protocols, specimen/ cells are not washed with buffer or similar before fixation.

4. Cells are not subjected to a pre-extraction procedure before fixation.

5. The fixation is performed using an excess of fixative (> 1 - 2 mL/ coverslip).

6. For fixation the coverslips are placed into a fresh petri dish containing the fixative. Alternatively, the fixation may be performed as well in the petri dish the cells have been grown in.
   For fixation the growth medium is removed completely. Then the cells are submerged in fixative.

7. The fixation schould be done with freshly prepared or frozen fixative. Best use prewarmed fixative. Ready made 37% formaldehyde/ methanol should not be used.

8. Between fixation and blocking an extraction step may be required. For formaldehyde fixation it is crucial to extract the cells using detergents. For methanol fixed cells/ tissues, extraction is not required, however a short wash in PBS/ 0,1% Triton-X-100 may be advantageous for the labeling.

9. Cover slips with fixed cells may be stored several (1 - 2) days at 4 °C. However the Quality of the labeling may be affected by the storage of the samples.

10. Washing should be performed using excess PBS (> 1 - 2 mL/ cover slip).

11. Blocking should be performed using excess PBS/ BSA (> 1 - 2 mL/ cover slip).

12. For the antibody incubation cover clips should not be placed with cells facing downward on parafilm or similar films. Although this might reduce the amount of antibody needed for labeling cells may be affected while removing them from the parafilm.

13. After the antibody incubation cover slips should be washed in different petri dishes. Otherwise cross contaminations might occur.

14. If high background labeling of the specimen occurs, it can be further reduced by incubation with PBS/ Triton-X 100 or PBS/ BSA.

15. If embedding media should be used that are not hardening, cover slips have to be sealed using Twinsil or nail polish.

16. If TDE is used as embedding medium, twinsil may not be used since the TDE will hinder polymerization and hardening.

17. The use of TDE is not possible for phalloidin labelled cells.

18. Storage of samples at -20 °C should be avoided, because ice crystals may affect the quality of the samples.