**Immunofluorescence protocol and potential useful suggestions to improve samples for imaging**

**Prepare cells**

1. Prepare coverslips by leaving them in 96 % ethanol for at least 24 hours. Then transfer them to a glass petri dish and dry autoclave them overnight.
2. Transfer a coverslip into each well.
3. **Coverslips for astrocytes is coated with approx. 300 ul Poly-L-Lysin (5ug/ml) for 30min-2 hrs at 37 °C (30ul(2mg/ml) to 12 ml H2O)**
4. Remove the coating and add 1 ml cellsuspension to each well with a coverslip.
5. Or leave a drop of cellsuspension on the coverslip and incubate for at least 4 hours and then add 1 ml media.
6. Incubate the plate at 37°C until the cells are ready to fixate.

**Fixation**

**!! MUST be carried out in a fume hood, as paraformaldehyde (PFA) is very toxic !!**

Thaw the PFA in 60°C water bath and let it cool down to RT.

**Coverslips**

1. 2 x quick wash in RT PBS pH 7.4
2. Fixate in 4 % PFA in 10 min (Some in EtOH)
3. 3 x 2 minutes wash in PBS pH 7.4

**Transwell inserts/membrans**

1. Transfer inserts to empty 12w plate
2. Wash the inserts 2x with PBS pH7.4 using 300 ul/well
3. Fixate in 4% PFA for 10-15 min (hood) in 300 ul/well
4. Wash 3 x 5 min with PBS pH 7.4
5. **If necessary** reduce high background by Incubating in 50mM ammonuim chloride in PBS pH 7.4 for 10 minutes

**Storage**

**Coverslips**

 Store in PBS pH 7.4 at 4°C (2-3 days)

 Store in 0,05% Natriumazid in PBS pH 7.4 at 4 °C (Longer period)

1. 3 x wash in PBS pH 7.4

**Inserts**

1. Add 800ul PBS pH 7.4 to the bottom of the well and 500 ul to the inserts
2. Store in 4°C up til 2 weeks

**Permabilization/blocking**

1. **Permabilize** the cells in fresh made 0.2 % triton in PBS pH 7.4 for minimum 10 minutes
2. **Block** in fresh made 2 % BSA in PBS pH 7.4 for minimum 20 minutes
3. 2 x wash in 0.05 % triton in PBS pH 7.4

**Immunomarking**

1. Dilute the **Primary antibody**:

 0.05 % triton in PBS pH 7.4 2% BSA + 0,05% triton in PBS pH 7.4

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Antibody | Number of coverslips/membranes | Dilution | Ul needed  | Ul antibody |
|  |  | 1: |  |  |
|  |  | 1: |  |  |
|  |  | 1: |  |  |

**Don’t use less than 0,5ul ab => 0,5ul x FF = ul needed**

**Coverslips**

1. Place a piece of parafilm on the table.
2. Place 30 µl droplets of diluted antibody onto the film and put the coverslips cell side down on top of the droplets. Be careful to turn the coverslips and avoid air bubbles.
3. Make a humid environment placing a damp cloth on the bottom of a dark lid. Place the lid over the coverslips.
4. Incubate in Primary antibody for 1 hour at RT Over night at 4°C
5. Transfer the coverslip back into the well, place them cell side up!
6. 3 x 5 minutes wash in 0.05 % triton in PBS pH 7.4

**Membranes**

1. Place a piece of parafilm on the table.
2. Place 20 µl droplets of diluted antibody onto the film
3. Cut the insert membrane in 4 pieces from the bottom and place the piece cell side down on top of the droplets.
4. Alternative place membrane pieces in a 48well plate with cell side up and add a drop of 30-40ul ab on top
5. Make a humid environment placing a damp cloth on the bottom of a dark lid. Place the lid over the membranes.
6. Incubate in Primary antibody for 1 hour at RT Over night at 4°C
7. Transfer the membranes back into the 48well plate, place them cell side up!
8. 3 x 5 minutes wash in 0.05 % triton in PBS pH 7.4
9. Dilute the **Secondary antibody** (Alexa normally 1:1000):

0.05 % triton in PBS pH 7.4 2% BSA + 0,05% triton in PBS pH 7.4

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Antibody | Number of coverslip/membranes | Dilution | Ul needed  | Ul antibody |
|  |  | 1: |  |  |
|  |  | 1: |  |  |
|  |  | 1: |  |  |

**Don’t use less than 0,5ul ab => 0,5ul x FF = ul needed**

**Coverslips**

1. Place a piece of parafilm on the table.
2. Place 30 µl droplets of diluted antibody onto the film and put the coverslips cell side down on top of the droplets. Be careful to turn the coverslips and avoid air bubbles.
3. Make a humid environment placing a damp cloth on the bottom of a dark lid. Place the lid over the coverslips.
4. Incubate in secondary antibody 1 hour in the dark at RT
5. Transfer the coverslip back into the well, place them cell side up!
6. 3 x 5 minutes wash in 0.05 % triton in PBS pH 7.4

**Membranes**

1. Place a piece of parafilm on the table.
2. Place 20 µl droplets of diluted antibody onto the film
3. Place the piece cell side down on top of the droplets.
4. Alternative place a drop of 30-40ul ab on top of the membrane
5. Make a humid environment placing a damp cloth on the bottom of a dark lid. Place the lid over the membranes.
6. Incubate in secondary antibody 1 hour in the dark at RT
7. Transfer the membranes back into the 48well plate, place them cell side up!
8. 3 x 5 minutes wash in 0.05 % triton in PBS pH 7.4

**Hoecst**

1. 2 x wash in ddH2O
2. Incubate in Hoecst (1 µl i 50 ml **ddH2O)** for 10-15 minutes in the dark at RT
3. 2 x 2 minuts wash in ddH2O
4. Quick wash in 70% ethanol and let the slides dry in the dark (15-30 minutes) **(not filters)**

**Mounting**

Note: we use Dako Fluorescence Mounting Medium
Link: [Mounting medium](https://www.agilent.com/en/product/immunohistochemistry/ancillaries-for-ihc/mounting-media/fluorescence-mounting-medium-76745)

1. Transfer 10 µl mounting media pr. Coverslip/membrane to a eppendorf tube (add a little extra)

**Coverslips**

1. Place each coverslip on top of 7-10 µl mounting media on an object glass – **cell side down**! Be careful – avoid air bubbles.

**Membranes**

1. Slide the membrane into 7 ul mounting media **cell side up** and place a coverslip\* on top. Be careful – avoid air bubbles.

\*wash in ETOH and dry

1. Dry overnight in the dark and seal with nail polish the next day. Store in fridge

**Mounting medie**

1. Transfer 10 µl mounting media pr. slide to a eppendorf tube (add a little extra)
2. Centrifuge for 3 minutes at maximum speed to get rid of bubbles
3. Use the degasser for 10 minutes if the results are very important.

**Immunofluorescens**

**Work Sheet**

**Coverslips Membrans**

**Date: Celletypes:**

**Fixation**

 Notes: methanol fixation can increase noise (especially in GFP). For GFP it is important to keep PH in all the solutions you use physiological, because the proton making the fluorophore is pH dependent

1. 2 x quick wash in RT PBS pH 7.4
2. Fixate in 4 % paraformaldehyd in 10 min (Some in EtOH)
3. 3 x 2 minutes wash in PBS pH 7.4

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**Storage**

1. Store in PBS pH 7.4 at 4°C

Store in 0,05% Azid in PBS pH 7.4 at 4 °C (Longer period)

C

1. 2 x wash in PBS pH 7.4

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 **Permabilization/blocking**

Incubate in 50mM ammonuim chloride in PBS pH 7.4 for 10 minutes

2 x wash in PBS pH 7.4

1. Incubate in fresh made 0.2 % triton in PBS pH 7.4 for minimum 10 minutes **(permabilization)**
2. Block in fresh made 2 % BSA in PBS pH 7.4 for minimum 20 minutes

C

1. 2 x wash in 0.05 % triton in PBS pH 7.4

**Immuno marking**

Notes:

Primary antibodies: you need to find suitable antibodies at companies like Thermo Fisher, Abcam, SantaCruz, Nordic BioSite, BD Biosciences, Sigma, Dako, Invitrogen.

Secondary antibodies. Use Alexa fluor dyes, links:

[405](https://www.thermofisher.com/dk/en/home/life-science/cell-analysis/fluorophores/alexa-fluor-405.html)

[488](https://www.thermofisher.com/dk/en/home/life-science/cell-analysis/fluorophores/alexa-fluor-488.html)

[568](https://www.thermofisher.com/dk/en/home/life-science/cell-analysis/fluorophores/alexa-fluor-568.html)

[647](https://www.thermofisher.com/dk/en/home/life-science/cell-analysis/fluorophores/alexa-fluor-647.html)

1. Dilute the **Primary antibody:**

0.05 % triton in PBS pH 7.4 2% BSA + 0,05% triton in PBS pH 7.4

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Antibody | Number of coverslips/membranes | Dilution | Ul needed (30ul/coverslip) | Ul antibody |
|  |  | 1: |  |  |
|  |  | 1: |  |  |
|  |  | 1: |  |  |

**Don’t use less than 0,5ul ab => 0,5ul x FF = ul needed**

1. Incubate in Primary antibody for 1 hour at RT Over night at 4°C
2. 3 x 5 minutes wash in 0.05 % triton in PBS pH 7.4
3. Dilute the **Secondary antibody** (Alexa normally 1:1000):

0.05 % triton in PBS pH 7.4 2% BSA + 0,05% triton in PBS pH 7.4

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Antibody | Number of Coverslips/membranes | Dilution | Ul needed (30ul/coverslip) | Ul antibody |
|  |  | 1: |  |  |
|  |  | 1: |  |  |
|  |  | 1: |  |  |

**Don’t use less than 0,5ul ab => 0,5ul x FF = ul needed**

1. Incubate in secondary antibody 1 hour in the dark at RT
2. 3 x 5 minutes wash in 0.05 % triton in PBS pH 7.4
3. 2 x wash in H2O
4. Start preparing the mounting media
5. Incubate in Hoecst (1 µl i 50 ml H2O) for 10-15 minutes in the dark at RT
6. 2 x 2 minuts wash in H2O
7. Quick wash in 70% ethanol and let the slides dry in the dark (15-30 minutes) (not filters)
8. Mount in 7-10 µl mounting media
9. Dry overnight in the dark and seal with nail polish the next day. Store in fridge

|  |  |
| --- | --- |
| 1 |  |
| 2 |  |
| 3 |  |
| 4 |  |

**Remember to make a rapport containing information about:**

1. **The cells.**
2. **The antibodies and the concentrations they were used in.**
3. **The position of the different slides on the object glass.**
4. **Date and name.**