**Protocol for Immunoflourescent Imaging (IF)**

**IMPORTANT:**

\*After use open lid to evaporate moisture

Cytospin centrifuge

Belongs to Cami's team

• must ask before use!

• Turn off after use!

Cassette

Cassettes components can be found either in 37'C incubator

Clean filter paper can also be found in box in cupboard above microinjector bench

To set up:

• Must Align the hole!

• Must use the correct surface of glass slide

**Lab's protocol**

**Prepare cells**

Prepare 50,000 cells (cell line/mouse cells) in 200ul PBS/HBSS- but minimum cell number needed is 2000 cells

(*Centrifuge the cell suspension at 1,200 rpm for 5 min, discard supernatant*.)

1. After collecting cells, **wash twice** with 90%PBS/2%FBS or HBSS

(Prepare 90%PBS/2%FBS- Take 9mL PBS+200uL FBS +800uL ddH20) \*for 10ml soln

\*(**Prepare** ***1xPBS Solution -Dissolve 10 tablets PBS into 1litre ddH2O***)

1. Resuspend cell pellet in **200-300ul** 90%PBS only or HBSS (Don't add FBS at this step!)

**Cytospin**

1. Label glass slides with PENCIL.

2. Set up the cassette: slide, filter, funnel for cells (make sure holes are aligned)

3. Turn on centrifuge, after putting in the cassettes

4. Pipette the cells suspension into the funnel **(250uL-300uL)**

5. close the lid (must push down the middle part)

6. Spin 300-500rpm 5 mins room temp (medium acceleration) (cell line- **400rpm 5 mins**)

**Note:** the speed is cell dependent, e.g. if fragile cells use 300rpm. Alex used 400rpm for cell line. Also *larger cells requires slower speed*  
rpm too low = cells may clumps together-> double layers/clumps  
rpm too high = cells too disperse

7. Unpack the cassettes, observe the slide under microscope  
***Good outcome = single cell layer in tight confluency***Poor outcome = several layers of cells/too little cells

8. Leave the glass slides to air-dry in room temperature (prevent moisture!)  
Air-dried slide can be kept for 1 year

9. Rinse the filter, funnel for cells with tap water, then place in 37'C incubator to dry

**Fixation**:

1. **Draw circle with PAP pe**n around the cells for hydrophobic barriers, then Add **4% PFA** **50ul** (get from 4C fridge) for **15 mins at room temp**.

2. **Soak in PBS to stop reaction** for 10 mins (*slides can keep at this step for 1 week, at 4'C*)

3. Tap off excess PBS before store in 4C room in the black cassette. (only if not enough time to proceed permeabilization, otherwise continue next step)

**Permeabilization**

1. If need to stain Ab, need to treat with **0.5% Triton-X 100** for cell membrane permeabilization for at least 5-10 mins at **room temp (use 10 mins)**

**(Prepare 0.5% Triton- 50uL Triton-X 100 + top up with PBS to 10mL)**

1. **Drop onto** the fixed sample on the slide (**use 50-100uL**) and **leave it 10 mins at RT.**
2. Remove Triton-X-100 and wash the cells three times with 1X PBS**. (*soak in).***
3. Prepare the paper soak with water and put into the cassette to prevent slide drying while waiting washing.
4. Dry the edges with kimwhite.

**Blocking** (to prevent non-specific binding)

1. Blocking (PBS + 0.5% Triton + 5% lamb serum) for **1-hour RT** inside the cassette.

(Prepare 5% lamb serum- take 500uL of aliquot 100% lamb serum in Eppendorf + top up to 10mL PBS/0.5% Triton-X*) (meanwhile prepare primary antibody)*

1. Wash with PBS /0.5% Triton (PBST) for **three times each 5 mins. (**Soak in and shaker)

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Primary Antibody** | **Host** | **Dilution** | **2nd Antibody** | **Dilution** |
| **Centrin-2** | Rabbit IgG | 1:100  (1:200) | Goat anti Rabbit **555**  488 | 1:500  1:200  (1:300) |
| **Alpha-tubulin** | Mouse IgG | 1:500  (1:500) | Goat anti Mouse  **488**  555 | 1:500  1:200  (1:300) |
| **Pericentrin**  **(do separately)** | Rabbit IgG | 0.1-0.5ug/mL  (0.8ug/ml) | Goat anti Rabbit **555**  488  **647** | 1:500  1:200  (1:300) |
| **ˠH2AX** | Mouse IgG | 1:250 | Goat anti Mouse  555  647 | (1: 300) |
| **PLK4** | Rabbit polyclonal | 1:500 | Goat anti rabbit  488 | (1:300) |

**Immunostaining**

1. **Dilute primary Ab with blocking buffer (PBS/0.5% Triton/5% lamb serum-** Nelson**)** @ in commercial Ab diluent (green colour in 4C fridge- Bowie) -ratio follow table attached.
2. Add 50-80uL primary Ab (depending on circle size) onto the slide and keep/incubate at 4C room overnight.
3. Decant the solution and **wash the cells three times in PBS, 5 min** each wash.
4. **Dilute secondary antibody with blocking buffer (PBS/0.5% Triton/5% lamb serum)**
5. Incubate cells with the both **secondary antibodies for 1-2 hr at room temperature** in the dark.
6. Decant the secondary antibodies solution and **wash three times with PBS** for **5 min** each in the dark.
7. Dry with kimwhite.

**Counterstaining and Mounting**

1. Put **a drop** of the antifade prolong Mounting medium with DAPI onto the sample on slide.
2. Put on the coverslip and make sure no bubble trap inside the sample (put slanting the coverslip).
3. **Airdry the slide and seal with nail polish** to prevent movement of slide.
4. If desired, visualize the target antigen by fluorescence microscopy. Check under fluorescent microscope.
5. Keep in dark at 4C room/fridge or -20C if longer time before confocal imaging.