Immunofluorescence Protocol

Experiment Outline

1.	Remove cell culture medium	10 min.
2.	Fix cells	15 min.
3.	Permeabilise cells	15 min.
4.	Block unspecific antibody binding	60 min.
5.	Primary antibody incubation	60 min.
6.	Wash	10 min.
7.	Secondary antibody incubation	45 min.
8.	Wash	10 min.
9.	DAPI incubation	10 min.
10.	Wash	10 min.
11.	Microscopy	> 30 min.

Wash and fixation

Background: We want to maintain the structure of the cells from the start of the experiment until we visualise them under the microscope. For that reason, we fix the cells – as a form of snapshot of how the cells look at the time of interest. Cells are washed to remove the growth medium because it contains a range of proteins that we do not want to be fixed with the cells. We wash the cells in phosphate buffered saline (PBS) to maintain the osmolality (isotonic) on both sides of the cell membrane, and to prevent cell lysis, which would happen if we use sterile water (hypotonic). After the wash step, we fix the cells using 4% paraformaldehyde (PFA). PFA establishes covalent chemical bindings between amine-groups on macromolecules and fixes them to each other, which prevents further movement.

Step:

1. Wash cells carefully with PBS, taking care to avoid that the cells detach from the cell culture dish.
2. Fix cells for 10 minutes with 4% PFA at room temperature (RT).
3. Remove the PFA and wash carefully twice with PBS.

Permeabilisation

Background: The purpose of permeabilisation is to breach the integrity of the cell membrane, i.e. make small holes, to create access for the antibodies to all of the cellular compartments. In our case, we wish to create access to the cell nucleus, thus we need to make holes in two lipid membranes. We use Triton X-100 as a destabilising agent (surfactant) because this molecule dissolve lipids in a hydrophilic solution, practically removing them, to leave behind a permeable membrane.

Step:

1. Aspirate PBS.

2. Add PBS + 0,5% Triton X-100.
3. Incubate for 10 minutes.
4. Wash once with PBS.

Blocking

Background: We want to block unspecific binding of our antibodies to other proteins than their specific antigen. Antibodies have a very high affinity for their specific antigen, however, protein-protein interactions such as electrostatic or hydrophobic attraction can permit unspecific binding to other proteins. To avoid this, we block this type of binding using a protein-rich solution that binds to these "sticky patches" instead of the antibodies. When we add the antibody after blocking, the antibodies can displace the blocking protein and bind to their antigen, while the blocking proteins prevent low-affinity binding.

Step:

1. Aspirate PBS.
2. Add PBS + 1% Bovine Serum Albumin (BSA)
3. Incubate 1 hour at RT

Primary and secondary antibody incubation

Background: We use antibodies to detect a specified target protein. Antibodies are proteins with a very high affinity for other proteins or their fragments (epitopes). Antibodies can be both polyclonal or monoclonal. Polyclonal antibodies are prepared by injecting a host animal with the desired epitope, e.g. rabbit or goat. In this way, the host animal's B-cells produce many different antibodies against the foreign epitope, which can be isolated and purified for use in the laboratory. In our case, we wish to detect the protein y-H2AX using a method known as indirect immunofluorescence. This means that we use two antibodies. The first (primary) antibody will bind to y-H2AX, whereas the second antibody is conjugated to a fluorescent molecule and binds to the primary antibody. In this experiment, we only wish to detect one protein so the species of the host animals doesn't matter. However, it would have been important if we wanted to visualise more proteins with different colours. Phalloidin is a toxin from the mushroom *Amanita phalloides* (Death Cap) that binds to F-actin in the cytoskeleton, which enables us to visualise the cell structure simultaneously.

Step:

1. Aspirate blocking buffer.
2. Wash with PBS.
3. Add primary antibody.
4. Incubate 1 hour at RT.
5. Wash 3x3 minutes with PBS
6. Add secondary antibody and phalloidin-TRITC
7. Incubate 45 minutes at RT. Cover samples with tin foil during incubation.
8. Wash 3x3 minutes with PBS

Visualisation of the nucleus and mounting on coverslips

Background: To visualise the cell nucleus in the microscope, we label them with DAPI. DAPI is a fluorescent molecule that binds to AT-rich areas of the DNA.

Step:

1. Aspirate PBS.
2. Add PBS + 1µg/mL DAPI.
3. Incubate 10 minutes at RT. Cover samples with tin foil during incubation.
4. Wash with PBS
5. Remove PBS, but leave approximately 200µL in the well
6. Ready for microscopy.

Often people would mount the coverslips as shown on the image, but since we are using chamber slides, the cells are already growing directly on the coverslip and we can omit this step.