Immunofluorescence protocol for cytoskeletal preparations

1. Autoclave coverslips and pair of tweezers, which you are going to use to place the coverslips into the culture dishes.
2. Place up to three coverslips in one tissue culture dish, always work steril under the hood !!!.
3. Add 10 ml of culture media to each dish.
4. After splitting your cells, add appropriate amount of cells to each dish. Note: cells need some time to adhere on coverslips, therefore you should give the cells at least 24 hr. after the passage to do so and before you procede with the protocol. Amount of the cells passaged per dish depends on the individual cell growth rate. However note, at the timepoint of fixation cells should be ~ 40-50% confluent. To be able to get good images under the microscope you need single isolated cells.

For further processing:

1. Draw off culture media
2. Rinse the dish 2x with warm PBS. Note: This is important !! otherwise you will have a high background later on.
3. Transfer coverslips to 35 mm dish (6 well culture dishes). Note: It is easier for the further treatment of the coverslips , if you lined out the bottom of the wells with parafilm before hand.

Extraction of soluble proteins

Extraction of soluble proteins

1. Wash coverslips 2x with cytoskeletal stabilizing buffer (CSB).
2. Incubate cells in CSB + 0.2% Triton X100 for 5`@ 24oC. This step permeabilizes the cells and extracts soluble cell contents.
3. Rinse 2x with PBS

Fixation

1. Add 30æl of 4% Paraformaldehyde for 30` @ room-temperature (RT).
2. Wash 1x with PBS for 5`.

Note: During washing period always place 6 well dishes on orbital shaker.

Washes

1. Wash 1x with PBS/ 0.02% Tween 20 for 5`
2. Wash 1x with PBS/0.02% Tween 20/ 1% BSA for 5`

Primary antibody

Prepare 1o-antibodies in PBS + 3% BSA, dilute according to manufacturers recommendations, or see above protocol.

1. Add 30æl of 1o antiboby in PBS/3% BSA
2. Incubate in a humidified chamber for 451 @ 37oC. (We normally used a waterbath).

Washes

1. Wash with PBS/0.02% Tween 20/1% BSA for 5`

Secondary antibody

Prepare 2o antibodies in PBS/3% BSA, dilute according to manufacturers recommendations

1. Add 30æl of each antibody per well
2. Incubate in humidified chamber for 45` @ 37oC

Note: If you have got more then two antibodies repeat antibody and wash steps as often as needed. After adding your last antibody proceed as following:

Washes

1. Wash with PBS/ 0.02% Tween 20 for 5`
2. Wash with PBS for 5`

Dry

1. Dry @ 37oC in oven (we normally use bacterial incubator) for ~ 45`

Mounting

Mounting media: mix PBS, 50-60% glycerol, 2.5% 1,4 diazobicyclo(2,2,2)-octane = Dabco Quencher

1. Use 10-15æl
2. Apply mountant on slide and add coverslip with cells facing mountant on top.
3. Seal coverslides with Nail Polish - don`t forget to label slides.

CSB (cystoskeleton stabilizing buffer)

4M Glycerol
25mM PIPES pH 6.9
1mM EGTA
1mM MgCl2