

## 1<sup>st</sup> – 2<sup>nd</sup> day

### Fixation (work in the hood)

- 4 % (v/v) PFA in PBS (1mL; 15 min; r.t.)
- wash with PBS (3 x 2 mL)
- -20°C MeOH (1mL; 20 min; -20°C - freezer);
- wash with PBS (3 x 2 mL)

### Permeabilization

- 0.2 % Triton X-100 in 1X PBS (1 mL; 10 min; r.t.)
- Note: permeabilization, if pre-extraction is skipped

### Blocking

- 3 % BSA in PBS (1mL; 20 min)

- ✿ prepare wet chamber
- ✿ dilute primary antibody



### Primary antibody

- dilute the antibodies in blocking solution  
anti-PCNA (PC10) mouse 1:500
- 50 µL/CV
- place CVs on parafilm into wet chamber; cells facing up? ? ?
- incubation (1 – 2 h; r.t.; wet chamber)
- wash with PBS (3 x 200 µL; 5min)

### Secondary antibody

- dilute the antibodies in blocking solution in the one eppendorf  
anti-mouse 1:500  
50 µL/CV
- incubation (1 h; r.t.; wet chamber - dark)
- wash with PBS (3 x 200 µL; 5min)

### DAPI

- stock 2 mg/mL
- dilute in PBS  
DAPI 1:1000
- incubation (5 min; r.t.; wet chamber - dark)
- wash with PBS (3 x 200 µL; 5min)
- dip to H<sub>2</sub>O
- dip to tissue to remove excess of liquid
- air dry (approx. 30 min; r.t.; in dark ? ? ?)
- mount with Fluoromont 4 – 4.5 µl/slide (cells facing down? ? ?)
- air dry ( r.t.; in dark ? ? ?)

## 3<sup>rd</sup> day

### Microscopy

- DM6000 (objective lenses: 63x, 1.42 NA, oil)

1 well ... 1 mL DMEM  
... 2 mL 1xPBS

PFA  
fixation via crosslinks

MeOH  
dehydration and denaturation  
for better access of PCNA epitope

3% BSA ... 1.5 g ... 50 mL

**option:** incubation of primary antibodies O/N

- place CVs on drop of diluted antibodies on parafilm into wet chamber; cells facing down? ? ?
- incubation (4°C; O/N; wet chamber)
- place CVs on parafilm into wet chamber; cells facing up? ? ?
- continue with wash etc. according to protocol ....

? ? ?

secondary antibody have  
to be kept in dark from on

### DAPI dilution

- Working in PBS

### Resources

- <https://www.thermofisher.com/ch/en/home/references/protocols/cell-and-tissue-analysis/protocols/dapi-imaging-protocol.html>
- <https://www.sigmaaldrich.com/deepweb/assets/sigmaaldrich/product/documents/164/535/10236276001.pdf>



### Wash during IF

- in wells: in each step put CVs back to well plate for wash, more solvent help to wash out previous reagents
- on parafilm: once you place CVs on parafilm just fill CVs with washing solution
- in both cases: can be done on shaking platform to improve wash

### Pre-extraction

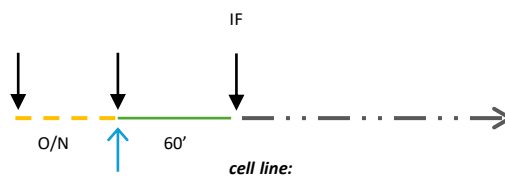
- wash away soluble, non-chromatin-associated proteins before fixing the cell sample
- specifically bound or structural proteins by reducing background noise and improving the signal-to-noise ratio
- particularly for nuclear proteins

### Note:

- <https://www.thermofisher.com/ch/en/home/life-science/antibodies/antibodies-learning-center/antibodies-resource-library/antibody-application-testing-protocols/immunofluorescence-protocol-adherent-suspension-application-testing.html>
- [https://andrslabmess.github.io/posts/IF\\_General-protocol/](https://andrslabmess.github.io/posts/IF_General-protocol/)
- <https://andrslabmess.github.io/posts/2.Monitoring-the-formation-of-R-loops/>

## 0 day

- seed cells 20 000 – 25 000 cells/cm<sup>2</sup>; O/N
- to be at approx. 70% confluence next day;
- treatment with dox



## 1<sup>st</sup> – 2<sup>nd</sup> day

1 well ... 1 mL DMEM  
... 2 mL 1xPBS

- ✿ pre-warm: DMEM, PBS
- ✿ cold PBS (in fridge or on ice; approx. 4°)
- ✿ cold 0.2 % Triton X-100 in PBS; ( approx. 4°)
- ✿ cold MeOH (-20°C)
- ✿ prepared all reagents and treatment

wash  
1. aspirate liquid  
2. add washing solution  
3. aspirate liquid  
..... repeat .....  
X. add desire liquid

			calculate	
treatment	c stock	c final	μL / 1 mL	

Cell culture room

- add 1 mL pre-warm DMEM to 6-well plate
- transfer CVs from plate to 6-well plate
- treatment
- incubate (..... min; 37°C; 6% CO<sub>2</sub>)
- wash cold PBS (2 mL) .... work in the lab on bench

Keep cells on ice

- Pre-extraction
- cold 0.2 % Triton X-100 in PBS (1mL; 10 min; on ice)
  - wash with cold PBS (2 x 2 mL)

- Fixation (work in the hood)
- 4 % (v/v) PFA in PBS (1mL; 15 min; r.t.)
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PFA  
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- Blocking
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3% BSA ... 1.5 g ... 50 mL

- ✿ prepare wet chamber
- ✿ dilute primary antibody

Wet chamber

- Primary antibody
- dilute the antibodies in blocking solution
  - anti-PCNA (PC10) mouse 1:500
  - 50 μL/CV
  - place CVs on parafilm into wet chamber; cells facing up? ? ?
  - incubation (1 – 2 h; r.t.; wet chamber)

- wash with PBS (3 x 200 μL; 5min)

- Secondary antibody
- dilute the antibodies in blocking solution in the one eppendorf
  - anti-mouse 1:500
  - 50 μL/CV

- incubation (1 h; r.t.; wet chamber - dark)
- wash with PBS (3 x 200 μL; 5min)

???  
secondary antibody have  
to be kept in dark from on

- DAPI
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  - DAPI 1:1000
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✿

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