Immunofluorescence (IF) protocol (adapted from Chaumeil J and Heard E)

1. Culture the cells on gelatine-coated coverslups for at least 24 hours
2. Change the medium at least 2 hours before (OPTIONAL)
3. Wash one with freshly made 1x PBS (2ml) (made on the day); BE ALWAYS SURE THAT CELLS ARE NEVER WITHOUT LIQUID THROUGHOUT THE PROTOCOL
4. FIX in freshly made (on the day) PFA 3% at RT for 10 min (2ml per well)

   Paraformaldehyde stocks are at 4% in the freezer (add 9 ml of PBS for a 50 ml eppendorf tube or 3ml for a 1ml eppendorf tube.

5. Wash 3x with freshly prepared 1xPBS, one wash immediately after the other (

6. Permeabilize with freshly 1x PBS, 0.5% Triton 100x (containing the RNAse inhibitor substance: 2mM Vanadyl Ribonucleaside complex-VRC in case of subsequent RNA-FISH) – On Ice for 3.5-5min (dependent on the cells); we use 4min for both PGK12.1 and LIF2.

   Permeabilization buffer:
   PBS 1x: 12ml
   Trinton 100x: 60 ul
   VRC 1%: 120 ul

7. Wash 3x with freshly made PBS 1x (one wash after the other)
8. Block in PBS1x/BSA 1%for at least 15 min (could be longer) at RT

   Preparation of the blocking solution:
   13 ml of PBS 1x + 2ml BSA (found in 2ml eppendorfs in the freezer)

9. Make a humid chamber (with dH2O) and put the slides with a correct identification

10. Prepare the primary antibody diluted in 40ul of 1x PBS/1%BSA (containing RNAseGuarde or analog – RNAse inhibitor: 23ul for 2ml, in case of subsequent RNA-FISH);
11. We put 39ul drop (not 40ul to avoid bubbles) on the slide (labelled accordingly) and with the use of tweezers/forceps (on the sides of the coverslips), we move the coverslips gently into the slide (first we remove : put cells facing down to be in contact with antibody solution; Incubate for 45min in the H2O humid chamber. Protect the coverslips from light for the rest of the protocol;
12. Put the coverslips back into the wells (cells facing up) and wash with freshly prepared 1x PBS for 5min at RT using a shaker (2ml per well)
13. Incubate with the secondary antibody; prepared and manipulated the same way as the primary antibody.
14. Wash 3x with freshly made PBS for 5 min at RT with shaking
15. If we do not do a RNA-FISH afterwards, we do now the DNA counterstaining for 2 min in 1xPBS containing 0.2mg/ml of DAPI

    Add 1 ul of DAPI to 10 ml of 1xPBS

16. Prepare the slide in 1 drop (15ul) of "milieu montege" and dry the slides with kimtech towels (pressing the paper 2x on the top); Seal with RIMEL and keep it in the fridge if we use the microscope in the same day; or keep it -80C if analysis on the microscope is to be done on a different day;

For RNA-FISH

1. After PBS washes, fix the samples in freshly made PFA 3%, 10 min at RT.
2. By the time, the probe is denaturating, wash 3x with PBS and 2x with 2X SSC
3. Prepare humid chamber (this time with used FA/SSC solution);
4. Add 12ul of probe solution (*check the preparation of FISH probe protocol) to the slide and move the coverslips with tweezers, take the 2xSSC excess with paper towel and put the cells face down into the slide (because the volume is low, the procedure should be done with care;
5. Incubate ON at 37C (incubator) in the FA/SSC humid chamber

Day 2:
1. Remove the coverslips by adding 2xSSC to float the coverslip
2. Wash 3x at 42C for 7 min in FA/SSC with agitation

   FA/SSC solution:
   FA(formamide): 50ml
   20X SSC: 10ml
   H2O: 40 ml

3. Wash 3x at 42C for 5 min in 2xSSC with agitation
4. DNA counterstaining in 2xSSC containing 0.2mg/ml of DAPI (10 ml of 2x SSC + 1ul of dapi) for 3 min at RT
5. Wash 2x with SSC2x (rapid)
6. Mount coverslips in 15ul of "milieu de montage" (glycerol-base mounting medium): Prepare the slide in 1 drop of "milieu montege" and dry the slides with kimtech towels (pressing the paper 2x on the top); Seal with RIMEL and keep it in the fridge if we use the microscope in the same day; or keep it -80C if analysis on the microscope is to be done on a different day

Preparation of the FISH probe:

- We normally use 3ul of the probe per coverslip;
- We prepare the probe adding 3ul of probe solution (made by nick translation) and add 1 ul of salmon sperm + 1/10 of 3M NaAc + 3x EtOH 100%
- Vortex
- Centrifuge at 30min with 12500rpm at 4C
- Remove supernatant
- Add 1 ml of EtOH 70% (cold – that is kept in the -20C).
- Centrifuge at 5min with 12500rpm at 4C
- Remove the supernatant
- Dry the pellet using a speed vac (for 2 min)
- Dilute the pellet in 6ul of formamide per coverslip (this dilution is quite difficult – TIP: Add just a bit of formamide, the rest saty in the tip; with the tip move pellet

around and try to dissolve it and then add the rest of the formamide; Put at 37C for 15min (could go longer) with agitation (1200rpm);

- Denature the probe at 75C for 7 min (with agitation)
- Put on the ice immediately
- Add equal amount (to the formamide volume) of hybridization buffer just before the preparation of the slides.