Sample preparation for RNA FISH

Considerations:

- From the fixation step on everything has to be done in an RNAse-free environment.
- Remember to make tweezers RNAse-free using the RNAse ZAP, followed by a rinse in RNA-free water.
- If possible, place the tissue section on #1.5 coverglasses; otherwise on a microscope slide.
- The attachment of the tissue might need optimization depending on the source of the material.

Options:

1. Adherent cells for RNA FISH
    - 1.1 Purpose: preserve the 3D morphology of the genome
    - 1.2 Purpose: no need to preserve the 3D morphology of the genome
        - 1.2.1 Need for methanol/acetic acid treatment
        - 1.2.2 No need for methanol/acetic acid treatment
2. Suspension cells for RNA FISH:
    - 2.1 Purpose: preserve the 3D morphology of the genome
3. Fresh Frozen (FF) samples for RNA FISH

1. Adherent cells for RNA FISH

1.1 Purpose: preserve the 3D morphology of the genome

PFA fixation followed by Triton X-100:

1. Rinse coverslips quickly in 1 x PBS (+Ca, +Mg) (pre-warmed to 37°C) at RT, repeat once.
2. Exchange the PBS to 4% PFA/ 1x PBS (w/o Ca and Mg) at RT (freshly prepared using the 16% stock from EMS Diasum or an equivalent).
    a. Note! Make sure you have a generous amount of the PFA solution (~2 ml per well of a 6-well plate, add a higher volume than the volume of the 1 x PBS in step 1.
3. Incubate at RT for 10 min.
4. Wash the PFA away with 1 x PBS (w/o Ca and Mg), 3 x 5 min. at RT while shaking (make sure this PBS is RNAse-free (Ambion)
5. OPTIONAL STEP if high background fluorescence is seen one can quench the reaction of crosslinking using glycine:
    a. Wash the PFA away with 1 x PBS/125 mM Glycine (w/o Ca and Mg), 2 x 5 min. at RT while shaking (make sure this PBS and Glycine are RNAse-free) and wash 2 x with 1 x PBS (RNAse-free, w/o Ca and Mg).
6. Exchange the PBS with 1 x PBS/0.5% Triton X-100 and incubate at RT for 10 min. (make sure the Triton solution is RNAse-free!).
7. Wash with 1 x PBS, 2 x 5 min. at RT.
8. If the samples are not used immediately for hybridization, store them in 1x PBS/RVC (ribonucleoside vanadyl complexes) at 4°C (but this is very risky as most likely you will keep on losing RNA gradually upon storage).

1.2 Purpose: no need to preserve the 3D morphology of the genome

1.2.1 Need for methanol/acetic acid treatment

Methanol/acetic acid fixation of cells (for cells grown on coverslips):

1. Rinse quickly in 1 x PBS (+Ca, +Mg) (pre-warmed to 37°C) at RT, repeat once.
2. Add methanol/acetic acid (3:1 vol/vol) solution. Add it quickly so that the coverslips get covered by it immediately, instead of floating on top of the solution as may happen if you pour slowly.
3. Exchange to a fresh portion of the methanol/acetic acid solution and incubate at RT for 15 min.
4. Store at 4°C until needed (wrap the dishes with parafilm). Alternatively, store in 70% ETOH at 4°C for up to a year.

1.2.2 No need for methanol/acetic acid treatment

PFA fixation followed by Ethanol (for cells grown on coverslips)

1. Rinse coverslips quickly in 1xPBS (+Ca, +Mg) (pre-warmed to 37°C) at RT, repeat once.
2. Exchange the PBS to 4% PFA/1 x PBS (w/o Ca and Mg) at RT (freshly prepare PFA using the 16% stock).
    a. Note! Make sure you have a generous amount of the PFA solution (~2 ml per well of a 6-well plate, add a higher volume than the volume of the 1 x PBS in step 1.
3. Incubate at RT for 10 min.
4. Wash the PFA away with 1 x PBS (w/o Ca and Mg), 3 x 5 min. at RT, while shaking (make sure this PBS is RNAse-free (Ambion)).

5. OPTIONAL STEP if high background fluorescence is seen, one can quench the crosslinking reaction using glycine:
    a. Wash the PFA away with 125 mM Glycine/1 x PBS (w/o Ca and Mg), 2 x 5 min. at RT while shaking (make sure this PBS and Glycine are RNAse-free) and wash 2 x with 1 x PBS (RNAse-free, w/o Ca and Mg).
6. Exchange the PBS to ice-cold 70% Ethanol.
7. Exchange the 70% Ethanol with fresh and ice-cold 70% Ethanol (also ice-cold) and store the cells in it at 4°C (can be stored for up to a year, seal the dishes with parafilm).

2. Suspension cells for RNA FISH

2.1 Purpose: preserve the 3D morphology of the genome

Fixing cells in suspension with PFA:

1. Spin the cells at 100-300 x g for 5 min. at RT depending on the cell type (100 x g for relatively large cells like fibroblasts, 300 x g for small suspension cells like KBM7).
    - Always monitor the appearance of the pellet after centrifugation as well as how transparent the medium becomes in order to judge the centrifugation: to optimize this step monitor also the amount of cells remaining in the supernatant by pipetting a small droplet onto a microscope slide and observing it under a microscope.
    - If clumping occurs one may wash the cells in 1 x PBS containing 0.5 mM EDTA.
    - Depending on the cell concentration/amount needed do this centrifugation in a 50 ml, 15 ml or a protein low-bind 1.5 ml tube.
2. For a 50 ml falcon tube format, resuspend the cell pellet in 10 ml of 1 x PBS (+Ca, +Mg).
3. Add 10 ml of 8% PFA/1 x PBS (w/o Ca and Mg) to the 10 ml of the cell suspension at RT (freshly prepared using the 16% stock from EMS Diasum or an equivalent).
4. Incubate at RT for 10 min. while rotating.
5. Stop the PFA by adding 20 ml of 500 mM Glycine/1 x PBS solution (RNAse-free, w/o Ca and Mg) to a final concentration of 250 mM Glycine, and immediately proceed to the next step.
6. Centrifuge for 5 min. at 300–1000 x g and exchange the PFA solution to 10 ml of RNAse-free 1 x PBS (w/o Ca and Mg), pipette up and down.
7. Centrifuge for 5 min. at 300–500 x g, remove the supernatant and resuspend the pellet in 10 ml of 1 x PBS (w/o Ca and Mg)/0.5% Triton X-100 (made RNAse-free) and incubate for 5 min. at RT.
8. Centrifuge for 5 min. at RT, resuspend the pellet in 1 ml of 1 x PBS and transfer the cells to a 1.5 ml protein LoBind tube (Eppendorf).
9. Repeat the wash with 1 ml 1 x PBS (w/o Ca and Mg).
10. Resuspend in 1 ml of 1 x PBS/RVC and store at 4°C. Note: use RVC only if you want to store the sample, if you proceed with hybridization the same day do not add the RVC. Storage is very risky as most likely you will keep on losing RNA gradually upon storage.

Note: we will do the spotting cells on coverslip once we receive your samples

2.2 Purpose: no need to preserve the 3D morphology of the genome

2.2.1 Need for methanol/acetic acid treatment

Fixing suspension cells with methanol/acetic acid (1 000 000 cells / 1,5 ml protein LoBind tube):

1. Take an aliquot of suspension cells (10^6-10^7) and centrifuge (100-300 x g for 5 min. at RT depending on the cell type (100 x g for relatively large cells like fibroblasts, 300 x g for small suspension cells like KBM7).
    - Always monitor the appearance of the pellet after centrifugation as well as how transparent the medium becomes in order to judge the centrifugation: to optimize this step monitor also the amount of cells remaining in the supernatant by pipetting a small droplet onto a microscope slide and observing it under a microscope.
    - If clumping occurs one may wash the cells in 1 x PBS containing 0.5 mM EDTA.
    - Depending on the cell concentration/amount needed do this centrifugation in a 50 ml, 15 ml or a protein low-bind 1.5 ml tube.
2. For a 50 ml falcon tube format, resuspend the cell pellet in 10 ml of 1xPBS.
3. Add 10 ml of methanol/acetic acid (3:1 vol/vol) solution to the 10 ml of the cell suspension.
4. Centrifuge for 5 min. at 300 – 500 x g.
5. Remove the supernatant leaving ~500 ul or so (depending on the pellet size) of the PBS solution to resuspend the cells to bring them to a single-cell suspension.
6. Exchange to a fresh portion of the methanol/acetic acid solution and leave the cells at RT for 15 min. while rotating.
7. Store at 4°C until needed (wrap the dishes with parafilm). Alternatively store in 70% ETOH at 4°C for up to a year (we have some evidence that storing in 70% Ethanol instead of methanol/acetic acid might yield better results).

2.2.2 No need for methanol/acetic acid treatment

PFA fixation followed by EtOH permeabilization for cells in suspension; 1 000 000 cells / 1,5 ml protein LoBind tube:

1. Take an aliquot of suspension cells (10^6-10^7) and centrifuge (100-300 x g for 5 min. at RT depending on the cell type (100 x g for relatively large cells like fibroblasts, 300 x g for small suspension cells like KBM7).
    - Always monitor the appearance of the pellet after centrifugation as well as how transparent the medium becomes in order to judge the centrifugation: to optimize this step monitor also the amount of cells remaining in the supernatant by pipetting a small droplet onto a microscope slide and observing it under a microscope.
    - If clumping occurs one may wash the cells in 1 x PBS containing 0.5 mM EDTA.
    - Depending on the cell concentration/amount needed do this centrifugation in a 50 ml, 15 ml or a protein low-bind 1.5 ml tube.
2. For a 50 ml falcon tube format, resuspend the cell pellet in 10 ml of 1 x PBS (+Ca, +Mg).
3. Add 10 ml of 8% PFA/1 x PBS (w/o Ca and Mg) to the 10 ml of the cell suspension at RT (freshly prepared using the 16% stock from EMS Diasum or an equivalent).
4. Incubate at RT for 10 min. while rotating
5. Stop the PFA by adding 20 ml of 500 mM Glycine/1 x PBS solution (RNAse-free, w/o Ca and Mg) to a final concentration of 250 mM Glycine, and immediately proceed to the next step.
6. Centrifuge for 5 min. at 300 – 1000 x g and exchange the PFA solution to 10 ml of RNAse-free 1 x PBS (w/o Ca and Mg), pipette up and down.
7. Centrifuge for 5 min. at 300 – 500 x g and remove the supernatant leaving ~500 ul or so (depending on the pellet size) of the PBS solution in order to resuspend the cells to bring them to a single-cell suspension before adding 70% Ethanol in the next step.

8. Add 10 ml of ice-cold 70% Ethanol.
9. Centrifuge for 5 min. at 300 – 500 x g to exchange the 70% Ethanol to fresh and ice-cold 70% Ethanol and store the cells in it at 4°C (can be stored for up to a year)

3. Fresh Frozen (FF) samples for RNA FISH

3.1 Purpose: preserve the 3D morphology of the genome

Considerations:

- Depending on the source of the material, the tissue can be fixed before AND after freezing or ONLY AFTER the freezing. It is critical to understand how the tissue was treated before the freezing to adapt the steps afterwards (to minimize the fixation time).
- If the tissue was fixed before being frozen, depending on how long that took and which % of PFA was used one might need to perform antigen retrieval after sectioning.

Here we provide different possible scenarios:

1. The tissue is fixed in ice-cold 4% PFA/1 x PBS for 1h prior to freezing – If this is the case minimize fixation after the sectioning to 5 minutes or even less (enough to have the tissue stably attached to the glass); one might even consider 1% PFA post-fix instead of 4%. After this post-fix proceed to do washes.
2. The tissue is fixed in ice-cold 4% PFA O/N prior to freezing – antigen retrieval might be needed after the sectioning.
3. If the tissue was snap frozen without prior fixation the steps after sectioning should be the same as for cell culture, as below:

Fixation on sections that have been snap frozen without any fixation prior to cryo-sectioning:

1. Remove the coverslips/slides with sections from the dry ice.
2. Place the coverslips/slides in a 6-well plate or a 10 cm petri dish.
3. Fix the sections by adding 4% PFA/1 X PBS (w/o Ca+ and Mg+) solution, RT (freshly prepared using the 16% PFA stock from EMS Diasum or equivalent). Note: Add the 4% PFA/1XPBS dropwise on the side of the dish, not directly onto each section, so as not to dislodge them from the glass.
4. Incubate at RT for 15 min.

Washes:

1. Rinse the sections twice with room-temperature 1 x PBS (make sure the PBS is RNAse-free).
2. OPTIONAL STEP if high background fluorescence is seen, one can quench the crosslinking reaction using glycine:
    - Wash the PFA away with 125 mM Glycine/1 x PBS (w/o Ca and Mg).
    - Wash 2 x 5 min. at RT while shaking (make sure PBS and Glycine are RNAse-free).
    - Wash 2 x with 1 x PBS (RNAse-free, w/o Ca and Mg).
3. Wash with 1 x PBS 2 x 5 min. at RT.
4. If you do not use the samples immediately for hybridization, store in 1xPBS/RVC at 4°C. Note: use RVC only if you want to store the sample, if you proceed with hybridization the same day do not

add the RVC. Storage is very risky as most likely you will keep on losing RNA gradually upon storage.

3.2 Purpose: no need to preserve the 3D morphology of the genome

Cryosectioning, fixation and permeabilization of the sections:

1. Remove the plates with sections on coverslips from the dry ice.
2. Fix the sections by adding 2 ml of RT 4% (wt/vol) PFA/1 X PBS (w/o Ca+ and Mg+) into each well. At this stage, add 4% PFA dropwise, not directly onto each section, so as not to dislodge it from the glass. Incubate the sections for 15–20 min at RT.
3. Rinse the sections twice with RT PBS, add 3 ml of 70% (vol/vol) ethanol to each well and incubate the mixture at 4°C for a minimum of 1 h (seal the plates with Parafilm to prevent ethanol evaporation).
4. PAUSE point Sections can be stored in 70% (vol/vol) ethanol at 4 °C for several months.