Sample preparation for DNA FISH

Considerations:

- Use #1.5 coverglasses.
- Depending on the cell type you may need to coat the cover glasses with poly-L-lysine or alike.
- Grow cells until they reach 80-90% confluency (they should not be reduced in size due to squeezing because of the neighbors).
- Make sure you optimize the seeding density for every cell line so that you end up with homogeneously spread cells all across every coverslip.
- If you use 6-well plates, always process one well at a time (when you exchange solutions).
- Use a suction pump for removing the liquids and plastic pasteur pipettes to add liquids.

Options:

1. Adherent cells for DNA FISH
    - 1.1 Purpose: preserve the 3D morphology of the nucleus
    - 1.2 Purpose: no need to preserve the 3D morphology of the nucleus
2. Suspension cells for DNA FISH
    - 2.1 Purpose: preserve the 3D morphology of the nucleus
    - 2.2 Purpose: no need to preserve the 3D morphology of the nucleus
3. Fresh frozen (FF) samples
    - 3.1 Purpose: preserve the 3D morphology of the nucleus
    - 3.2 Purpose: no need to preserve the 3D morphology of the nucleus

1. Adherent cells for DNA FISH

1.1 Purpose: preserve the 3D morphology of the nucleus

Fixation (using PFA):

1. Rinse quickly in 1 x PBS (+Ca, +Mg) (pre-warmed to 37°C) at RT, repeat once.
2. Fix in a generous amount of 4% PFA/1 x PBS (w/o Ca and Mg), 10 min at RT (prepared freshly right before use, 16%PFA from EMS or similar).
3. Wash 2 x 5 min with 125 mM Glycine/1 x PBS at RT.
4. Wash 1 x 5 min with 1 x PBS at RT.
5. If storage is needed, cells can be stored in 1 x PBS/0.05% NaN_3 at 4°C for up to a month. Caution: NaN_3 is toxic!

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

Fixation (using methanol/acetic acid):

1. Rinse quickly in 1 x PBS (+Ca, +Mg) (pre-warmed to 37°C) at RT, repeat once.
2. Fix with methanol/acetic acid (3:1 vol/vol) solution, 15 min at RT (exchange twice to fresh solution before incubation to make sure that what you fix with is not diluted with the PBS from the step before).
3. Rinse in 1 x PBS/0.05% Triton X-100 RT (exchange in a quick manner so that they don't dry).
4. Wash 3 x 10 min in 1 x PBS/0.05% Triton X-100 at RT.
5. Prepare a humidity chamber with a piece of a parafilm in it and pipette drops of 100-300 µl (150 µl for a 18 x 18 mm coverslip) of the RNase A solution (0.1 mg/ml, in 1 x PBS) onto the parafilm, then gently place the coverslip on top of the drop (very important: cell side downwards, facing the drop), incubate at 37°C for 60 min.
6. Take the coverslip to a 6-well plate (cells facing upwards) and wash in 1 x PBS, 2 x 5 min at RT.
7. Dehydrate the coverslip with the following EtOH series:
    A. 70% EtOH, 2 min at RT
    B. 85% EtOH, 2 min at RT
    C. 100% EtOH, 2 min at RT
    D. 100% EtOH, 2 min at RT
8. Let the coverslip air dry, for minimally 3h but preferably overnight at RT.
9. At this stage the coverslips can be stored in a dust-free environment for months or years at RT.

2. Suspension cells for DNA FISH

Considerations:

DNA (i)FISH for suspension cells is the same as for adherent cells and is performed on cells attached to the surface of a coverslip. Hence, one needs to identify a protocol for efficient attachment of cells to coverslips first. This can be done with live cells that are fixed once they start making contacts with coated coverslips, or else, fixation can be done in a tube using cells in suspension and fixed cells need then to be spotted onto coverslips and need to attach. This procedure needs to be optimized for each cell line.

2.1 Purpose: preserve the 3D morphology of the nucleus

Spotting of live cells before fixation:
Coat the coverslips with poly-L-lysine (PLL) or medium or alike (this needs to be optimized per cell line, though as a default one might start with the PLL).

PLL coating:

1. Immerse coverslips in 0.1% PLL (diluted in water) for 1 hour while shaking at 37°C.
2. Rinse 3 x with 1 x PBS.
3. Rinse 2 x with 70% EtOH.
4. Dry the coverslip in a hood.
5. Dispense cells (around 200-300µl) onto the coverslip that sits inside the 6-well plate. The concentration of the cells depends on the cell line and purposes. In general, we dispense 100,000 – 600,000.
6. Let the cells sit for 15-30 min at 37°C (the attachment depends on the cell line so monitor the attachment when you do it the first time to know which timing works best).
7. Gently add medium up to 1ml (pipette very gently, from the wall, try not to disturb the cells).
8. Spin at 500 g-800 g for 10 min.
9. Continue with fixing the cells (below).

Medium coating (for cells that attach easily):

1. Clean the coverslip with 70% EtOH followed by 100% EtOH, then dry it in the hood.
2. Place the coverslip into 6-well plates containing medium (medium used for the cell type you will be attaching).
3. Let the medium coat the coverslips for 1-2h at 37°C.
4. Add medium containing cells at a desired density (this needs to be optimized per cell line and per application) into each well.
5. Let the cells sit for 30-60 min at 37°C (the attachment depends on the cell line so monitor the attachment when you do it the first time to know which timing works best). If you would prefer the cells to spread and flatten, let them sit on coverslips overnight (recommended).

Fixation of attached cells (using PFA):

1. Once the live cells are attached to the coverslips, rinse quickly with 1 x PBS (+Ca, +Mg) (pre-warmed to 37°C) at RT, repeat once.
2. Fix in a generous amount of 4% PFA/1 x PBS (w/o Ca and Mg) for 10 min at RT (prepare freshly right before use).
3. Wash 2 x 5 min with 125 mM Glycine/1xPBS at RT.
4. Wash 1 x 5 min with 1 x PBS at RT.

5. If storage is needed, cells can be stored in 1 x PBS/0.05% NaN3 at 4 °C for up to a month. Caution: NaN_3 is toxic!

If one needs to attach fixed cells please follow this protocol:

Fixation of suspension cells before coverslip attachment (using PFA):

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, also monitor the number of cells that remain in the supernatant by pipetting a small droplet of the supernatant 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 (+Ca, +Mg).
3. Pipette 15 µl of cell suspension in a 1.5 ml eppendorf tube and add 15 µl of Trypan Blue, pipette very well.
4. Pipette 10 µl of the cell/Trypan Blue mix in each slot of an Invitrogen countess cell counting chamber.
5. Measure the cell death rate and cell concentration in each chamber and then average the values. Take note of the total amount of cells. Discard counting chamber and eppi tube.
6. Add 10 ml of 8% PFA/1 x PBS (w/o Ca and Mg) solution to the 10 ml of the cell suspension at RT (freshly prepared using the 16% stock from EMS Diasum or an equivalent).
7. Incubate at RT for 10 min while gently pipetting up and down or rotating.
8. Stop the PFA by adding 20 ml of 500 mM Glycine/1 x PBS solution to a final concentration of 250 mM Glycine, and immediately proceed to the next step.
9. Centrifuge for 5 min at 300–500 x g and exchange the PFA solution with 10 ml of 1 x PBS (w/o Ca and Mg), pipette up and down.
10. Repeat the same wash once.
11. If storage is needed, the cells can be stored in 1 x PBS/0.05% NaN3 at 4°C for up to a month. Otherwise proceed to spotting the cells onto coverslips. Caution: NaN_3 is toxic!

Spotting of fixed cells:

1. Coat the cover glasses with poly-L-lysine or alike (follow protocol above).
2. Spot your fixed cells at high concentration onto the coated coverslips and centrifuge.

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

Considerations:
Fixation with methanol/acetic acid can be performed after cells are spotted onto coverslips, or cells can be spotted onto coverslips after fixation with methanol/acetic acid in suspension (then spotting can be aided by cytospin). The order can be optimized for each cell line, but in general, we recommend fixation in solution and spotting the cells afterwards.

Fixation of suspension cells (using methanol/acetic acid):

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, also monitor the number of cells that remain in the supernatant by pipetting a small droplet of the supernatant 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 (+Ca, +Mg).
3. Pipette 15 µl of cell suspension in a 1.5 ml eppendorf tube and add 15 µl of Trypan Blue, pipette very well.
4. Pipette 10 µl of the cell/Trypan Blue mix in each slot of an Invitrogen countess cell counting chamber.
5. Measure the cell death rate and cell concentration in each chamber and then average the values. Take note of the total amount of cells. Discard counting chamber and eppi tube.
6. Centrifuge for 5 min at 100–300 x g at RT and resuspend in 1 ml of 1 x PBS (w/o Ca and Mg), pipette up and down thoroughly to achieve a single-cell suspension.
7. Add 20 ml of methanol/acetic acid (3:1 vol/vol) solution (RT) and pipette up and down multiple times (the volumes might need to be optimized).
8. Centrifuge for 5 min at 100–300 x g at RT and resuspend in 10 ml of fresh methanol/acetic acid (3:1 vol/vol) solution (RT).
9. Cells can be stored in the methanol/acetic acid at 4C for many months.

Note: Facility will do spotting when we do the hybridization

Spotting of live cells before fixation (using methanol/acetic acid):

Coat the coverslips with poly-L-lysine (PLL) or medium or alike (this needs to be optimized per cell line, though as a default one might start with the PLL), as described above.

1. Dispense cells (around 200-300 µl) onto the coated coverslip that sits inside a 6-well plate. The concentration of the cells depends on the cell line and purposes and needs to be optimized per cell line and application. In general, we dispense 100,000 – 600,000 per coverslip.
2. Let the cells sit for 15-30 min at 37°C (the attachment depends on the cell line so monitor the attachment when you do it the first time to know which timing works best). If you would prefer the cells to spread and flatten, let them sit on coverslips overnight (recommended).

3. Once the cells are attached, rinse them quickly with 1 x PBS (+Ca, +Mg) (pre-warmed to 37°C) at RT and proceed to fixation.

Fixation (with methanol/acetic acid):

1. Add methanol/acetic acid (3:1 vol/vol) solution (RT) onto the coverslips (make sure you add it quickly so the coverslips get covered by it immediately, instead of floating on top of it as may happen if you pour slowly).
2. Exchange to fresh methanol/acetic acid (3:1 vol/vol) solution (RT) and incubate at RT for 15 min
3. Rinse in 1 x PBS/0.05% Triton X-100 RT.
4. Wash 3 x 10 min in 1 x PBS/0.05% Triton X-100 at RT.
5. Prepare a humidity chamber with a piece of a parafilm in it and pipette drops of 100-300 µl (150 µl for a 18 x 18 mm coverslip) of the RNase A solution (0.1 mg/ml, in 1 x PBS) onto the parafilm, then gently place the coverslip on top of the drop (very important: cell side downwards, facing the drop), incubate at 37°C for 60 min.
6. Take the coverslip to a 6-well plate (cells facing upwards) and wash in 1 x PBS, 2 x 5 min at RT.
7. Dehydrate the coverslip with the following EtOH series:
    A. 70% EtOH, 2 min at RT
    B. 85% EtOH, 2 min at RT
    C. 100% EtOH, 2 min at RT
    D. 100% EtOH, 2 min at RT
1. Let the coverslip air dry, for minimally 3h but preferably overnight at RT.
2. At this stage the coverslips can be stored in a dust-free environment for months or years at RT.

3. Fresh frozen (FF) tissue samples

Considerations:

- 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.
- Depending on the source of the material, the tissue can be fixed before AND after freezing or ONLY AFTER freezing. It is critical to understand how the tissue was treated before the freezing to adapt the steps afterwards.
- If the tissue was fixed before freezing, 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:

- A block of a tissue is fixed in ice-cold 4% PFA for 1h prior to freezing – if this is the case proceed with methanol/acetic acid fixation right after drying and sectioning.

- The tissue is fixed in ice-cold 4% PFA O/N prior to freezing – antigen retrieval is needed after the sectioning.
- If the tissue was snap frozen prior to fixation (so no fixation before freezing), proceed to methanol/acetic acid fixation after sectioning and drying.

Sectioning:

- Depending on the application, one needs to choose the best thickness for the sectioning. The thicker the sections the more chances there are that the nuclei are intact so larger thickness is desirable. However, the thicker they are the trickier the hybridization becomes. The thickness should therefore be optimized.
- As a rule of thumb, start with a thickness of 10-12 µm and if you have difficulties in having the probe(s) to penetrate throughout, decrease to 6-8 µm, etc. Note: we have not experimented with the thickness extensively, perhaps a thickness of 20 µm could still work.
- We recommend coating coverslips with poly-L-lysine before placing the sections on.

1. Cut sections and mount them onto the PLL-coated coverslips.
2. Let the sections air-dry for 5-10 min.
3. Drying time should be sufficient to allow the water present in the freezing medium to evaporate as insufficient drying will cause section detachment.
4. Monitor the drying process to make sure the sections are well dried before proceeding to fixation.

3.1. Purpose: preserve the 3D morphology of the nucleus

Fixation (if no fixation was done prior to freezing):

1. Fix with a generous amount of 4% PFA/1 x PBS PBS (w/o Ca and Mg) for 10-15 at RT (prepared fresh right before use):
    - For thin sections (below or equal to 8 um): fix for 10 min.
    - For thick sections (above 8 um): fix for 15 min.
2. Wash 2 x 5 min with 125 mM Glycine/1 x PBS at RT.
3. Wash 1 x 5 min with 1 x PBS at RT.
4. If storage is needed, cells can be stored in 1 x PBS/0.05% NaN3 at 4°C for up to a month. Caution: NaN_3 is toxic!

Fixation (for cases where (mild) fixation was done prior to fixation):

1. Post-fix in a generous amount of 1% PFA/1 x PBS (w/o Ca and Mg) for 5 min at RT (prepared fresh right before use).
2. Wash 2 x 5 min with 125 mM Glycine/1 x PBS at RT.
3. Wash 1 x 5 min with 1 x PBS at RT.
4. If storage is needed, cells can be stored in 1 x PBS/0.05% NaN3 at 4°C for up to a month. Caution: NaN_3 is toxic!

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

Fixation

1. After drying, add methanol/acetic acid (3:1 vol/vol) solution and incubate for 15 min at RT.
2. Rinse in 1 x PBS/0.05% Triton X-100 RT (exchange to this PBS very quickly so that they don't dry)
3. Wash 3 x 10 min in 1 x PBS/0.05% Triton X-100 at RT.
4. Prepare a humidity chamber with a piece of a parafilm in it and pipette drops of 100-300 µl (150 µl for a 18 x 18 mm coverslip) of the RNase A solution (0.1 mg/ml, in 1 x PBS) onto the parafilm, then gently place the coverslip on top of the drop (very important: cell side downwards, facing the drop), incubate at 37°C for 60 min.
5. Take the coverslip to a 6-well plate (cells facing upwards) and wash in 1 x PBS, 2 x 5 min at RT.
6. Dehydrate the coverslip with the following EtOH series:
    E. 70% EtOH, 2 min at RT
    F. 85% EtOH, 2 min at RT
    G. 100% EtOH, 2 min at RT
    H. 100% EtOH, 2 min at RT
7. Let the coverslip air dry, for minimally 3h but preferably overnight at RT.
8. At this stage the coverslips can be stored in a dust-free environment for months or years at RT.