PEP-NET

European Commission

Predictive Epigenetics: Fusing Theory and Experiment

Title of the protocol: Single molecule RNA-fluorescence in situ hybridization (smRNA-FISH) optimized for mouse embryonic stem cells (mESC)

Authors & Affiliations: Jana Tünnermann (Giorgetti lab, FMI, Basel) – ESR 5

Introduction:
Single molecule RNA-fluorescence in situ hybridization (smRNA-FISH) is a widely used tool for the detection of single RNA molecules in fixed samples. A variety of fixation and hybridization protocols exist, often optimized for specific cell types or application. This protocol focuses on the fixation of dissociated mESC and hybridization using Stellaris® RNA FISH probes.

Description of the protocol:

Fixation of dissociated cells

Reagents and consumables

- 22 mm x 22 mm coverslips
- 6-well tissue culture plate
- Nuclease-free water
- 0.01% Poly-lysine in water
- 1x PBS
- 3% Formaldehyde (PFA) solution in PBS
- 70% Ethanol

Protocol

Prepare coverslips

1. Place coverslip in a 6-well tissue culture plate
2. Put ~1 mL of 0.01% poly-lysine on the coverslip and leave it for at least 30 min at RT
3. Aspirate and let dry for at least 10 min before adding cells

Harvest cells (6-well format)

1. Aspirate medium
2. Wash with PBS
3. Add 400 µL accutase and incubate for 3 min at 37°C
4. Add 2 mL medium and mix
5. Centrifuge cells for 3 min at 950 rpm
6. Aspirate supernatant and resuspend in 1 mL medium
7. Count cells and make a dilution of $3*10^6$ cells/mL

Cell fixation

Be careful to never let the coverslips air-dry

1. Put 50 µL of cells in one drop at the center of the glass slide and 25 µL drops around
2. Let cells attach for 20 min
3. Wash 1x with PBS
4. Fix by adding 3% PFA for 10 min
5. Wash 1x with PBS
6. Wash 1x with 70% ethanol
7. For permeabilization, add 5 mL 70% ethanol, leave at RT for 1 h
8. Leave at -20°C over-night
9. Store at -20°C

smRNA-FISH

Reagents and consumables

- Stellaris® RNA FISH Probes of interest
- 6-well tissue culture plate
- Hybridization buffer: 1x SSC, 10% formaldehyde, 10% dextran sulfate
- Wash buffer: 1x SSC, 10% formaldehyde
- Humified chamber: 150 mm tissue culture plate with parafilm on the bottom and wet tissue at the corners
- 2x SSC buffer in water
- Glass slide
- Mounting medium (e.g., ProLong™ Gold Antifade Mountant)

Protocol

Prepare probe solution

1. Prepare 25 µL hybridization buffer per sample
2. Add 0.5 µL of 6.25 µM Stellaris® RNA FISH probe stock solution and vortex (working solution of 125 nM)

Hybridisation

1. Take coverslip out of freezer and place it in a fresh 6-well tissue culture plate
2. Add 1 mL wash buffer at RT and incubate for 5-10 min under constant agitation
3. Prepare probe in humifided chamber: drop 25 µL probe solution on parafilm
4. Transfer coverslip, cells down, on top of the probe solution in the humified chamber
5. Incubate in the dark at 37°C over night (ca 18 h)
6. Transfer coverslip to a fresh well containing to 37°C pre-warmed wash buffer
7. Incubate for 30 min at 37°C
8. Aspirate buffer and add 1 mL DAPI nuclear stain (500 mg/mL in PBS), leave 2 min
9. Aspirate DAPI, add 2 mL wash buffer
10. Incubate for 30 min at 37°C
11. Aspirate wash buffer, add 2 mL 2x SSC buffer for 2-5 min
12. On a glass slide, add 15 µL mounting medium and place the coverslip cell side down on the drop, if necessary cure
13. Seal the coverslip with nail polish

References:

Raj, A., et al. (2018). Imaging individual mRNA molecules using multiple singly labeled probes. *Nature Methods,* 5(10), 877-879. Pubmed

Femino, A. M., et al. (1998). Visualization of single RNA transcripts in situ. *Science,* 280(5363), 585-590. Pubmed

Horvathova, I., et al. (2017). The Dynamics of mRNA Turnover Revealed by Single-Molecule Imaging in Single Cells. *Molecular Cell,* 68(3), 615-625. Pubmed