Adherent Cell Fixation and Hybridization – Wells/Plate Protocol

Reagents:

Fixation Buffer: 4% paraformaldehyde (PFA) in 1X Phosphate-Buffered Saline (PBS)
Permeabilization Buffer: 0.5% Triton-X 100/0.5% Saponin in PBS
Equilibration Buffer: 50% formamide in 2X SSC
Trypsin-EDTA in Hank's Balanced Salts Solution (HBSS)
0.1N hydrochloric acid (HCl)
1X Phosphate-Buffered Saline (PBS)
2X Saline-Sodium Citrate (SSC) buffer
1X SSC buffer
0.1X SSC buffer
Cell culture media
70% Ethanol
FISH Probe with Hybridization Buffer
DAPI

Equipment:

Multi-Well Cell Chamber or Multi-Well Plate
Hemacytometer or automated cell counter
Hot plate
Slide warmer
Humidified chamber
Incubator
Pipettes
Centrifuge
10mL conical tubes
Parafilm
Aluminum foil

Preparation:

- Prepare fixation buffer by heating 400mL of 1X PBS to 60°C, then adding 20g PFA powder. Drip 1N NaOH into solution until the powder dissolves and the solution is clear. Adjust final volume to 500mL and pH with HCl to 6.9. This solution can be frozen in aliquots indefinitely or stored at 4°C for up to 1 month.
- Prepare permeabilization buffer fresh on day of experiment by vortexing 500µL of Triton-X 100, 50µg of saponin, and 10mL of 1X PBS. Solution can be stored at 4°C for up to one week.
- Prepare equilibration buffer fresh on day of experiment by adding 5mL 50% formamide solution, 1mL 20X SSC, and 4mL deionized water together and mixing via inverting; do not vortex.
- Heat hot plate to 83°C prior to use.
- Warm 1X SSC and 0.1X SSC to 37°C prior to use.

Protocol:

1. Grow cells to 80% confluency in a multi-well chamber slide or a multi-well plate.
2. Dissociate cells with trypsin-EDTA in HBSS for 5 minutes.
3. Neutralize with cell culture media.
4. Agitate cells by gently pipetting in each cell a few times, then pipette cells into a 10mL conical tube.
5. Centrifuge at 250*g* for 5 minutes to pellet the cells. Count the cells.
6. Aspirate media and resuspend in fresh cell culture media to get ~140,000 cells/mL.
7. Add 50µL cell suspension to each well.
8. Spin plate down for 30 seconds to remove bubbles, then rest plate at room temperature for 30 minutes.
9. Grow cells for 24 hours at 37°C in an incubator.
10. Fix cells by adding 100µL of fixation buffer to each well and incubating for 15 minutes.
11. Rinse cells three times with 100µL per well of 1X PBS.
12. Permeabilize cells by incubating in permeabilization buffer (100µL/well) for 20 minutes.
13. Rinse cells two times with 100µL per well of 1X PBS.
14. Denature cells in 100µL/well of 0.1N HCl for 15 minutes.
15. Neutralize cell in 100µL/well of 2X SSC for 5 minutes.
16. Incubate cells in 100µL/well of equilibration buffer for 30 minutes.

Hybridization:

1. Warm probe and hybridization buffer to room temperature. Vortex well and spin down. If necessary, mix 1:4 dilution of probe concentrate to hybridization buffer to make a total volume that would allow application of 10µL per well. If predilute, then use 10µL of ready-to-use FISH probe per well.
2. Aspirate the equilibration buffer gently, taking care not to disturb the cells. Immediately add 10µL of probe mix to well. Do not let cells dry at any point; do only a few wells at a time.
3. Seal plate with aluminum foil to block light.
4. Tap plate firmly against benchtop to remove bubbles and spin down plate for 1 minute at 100*g*.
5. Place plate on 83°C-heated hot plate and denature at 83°C for 5 minutes. Ensure all wells are touching heated surface of the hot plate.
6. Immediately move plate to humidified chamber warmed to 37°C for 16-24 hours.
7. Remove plate from humidified chamber and rinse wells with 100µL/well of 2X SSC.
8. Rinse wells 3 times for 5 minutes each with 100µL/well of 1X SSC warmed to 37°C. Keep plate on 37°C-warmed slide warmer.
9. Rinse wells 3 times for 5 minutes each with 100µL/well of 0.1X SSC warmed to 37°C. Keep plate on 37°C-warmed slide warmer.
10. Stain with DAPI by diluting DAPI in 1X PBS, adding 100µL per well, and incubating for 10 minutes.
11. Rinse with 100µL/well of 1X PBS.
12. Plate with 100µL 1X PBS and seal plate with aluminum foil.
13. Image right away or store at 4°C.

Notes:

- When dissociating cells with trypsin-EDTA in HBSS, use an equal volume of the cell culture media for effective neutralization of the trypsin. The amount of trypsin used depends on its strength and the adherence strength of the cells being used.

- All incubations are done at 20-22°C unless otherwise specified.
- There are two pause points available in the **Protocol** section:
    - After step 13, cells can be stored in 1X PBS for up to 24 hours at 4°C or transferred into 70% ethanol and kept at -20°C for up to one week. In both cases, plates should be sealed to prevent evaporation or drying out in storage. Ensure cells are fully brought up to 20-22°C before continuing.
    - After step 18, cells can be stored in equilibration buffer at 4°C for up to one week. Plates should be sealed to prevent evaporation or drying out in storage.
- Do not fully empty pipette tip of probe mix, stopping at the first stop to avoid bubbles.
- Before imaging cells, ensure plate is warmed to room temperature and plate is clear of condensation. Wipe bottom of plate with 70% ethanol to clean before imaging.

References: A high-throughput DNA FISH protocol to visualize genome regions in human cells, Elizabeth H Finn, Tom Misteli