Adherent Cell Fixation and Hybridization – Slide Protocol

Reagents:

10µg/mL colcemid in Hank's Balanced Salts Solution (HBSS)
0.075M KCl hypotonic solution
1X Phosphate Buffered Saline (PBS)
Trypsin-EDTA in Hank's Buffered Salt Solution
Methanol
Acetic acid
Rubber Cement
FISH Wash Buffer 1
FISH Wash Buffer 2
DAPI with Antifade mounting medium
Ethanol

Equipment:

Water bath or oven
Incubator
Microscope slides / specimen slides
Glass coverslips
15mL conical tubes
Pipettes
Thermobrite or Hybrite with hydration cards (or hot plate and humidity chamber)
Inverted brightfield microscope

Preparation:

- Warm 0.075M KCl (hypotonic solution) to 37°C using a water bath or oven.
- Prepare Carnoy's fixative fresh on the day of use by mixing 3 parts of methanol to 1 part acetic acid. Store at 4°C until ready to use to keep ice cold.
- Program Thermobrite or Hybrite to denature at 75°C for 3 minutes, and 37°C for 24 hours.
- Soak hydration cards in deionized water.
- Heat FISH Wash Buffer 1 in Coplin jar with a lid to 73°C at least 2 hours prior to use.

Protocol:

1. Grow cells to ~80% confluency. Replace growth media with fresh media on day of harvest/processing.
2. Add 100µL colcemid solution to media and incubate for 30 minutes.
3. Aspirate the media. Wash cells with 5mL of 1X PBS, then aspirate.
4. Add 2mL of trypsin solution to cells and gently swirl container to cover the cellular area and incubate for 3-5 minutes at 37°C in incubator.
5. Examine cells using an inverted brightfield microscope. Neutralize the trypsin with 5ml of fresh media when most cells have started to lift. Gently pipette media to flush adhered cells off container walls.

6. Transfer cells suspended in media to a 15mL conical tube. Flush the original container with 5mL of fresh media, rinsing the sides, and transfer that to the conical tube as well.
7. Centrifuge cells at 1500 rpm for 5 minutes and aspirate the supernatant.
8. Add 1mL of 1X PBS to the pellet and resuspend the cells by gently pipetting up and down to agitate the pellet.
9. Add 1mL of hypotonic solution into the cell solution. Gently invert tube to mix (do not vortex or pipette).
10. Incubate at 37°C for 15 minutes.
11. Add 500µL of Carnoy's fixative by dripping the pipette into the cell solution, then close the tube and invert gently to mix. Centrifuge at 1500 rpm for 5 minutes.
12. Aspirate the supernatant.
13. Resuspend pellet in 5mL of Carnoy's fixative and gently pipette cells up and down to agitate cell pellet and mix thoroughly.
14. Centrifuge at 1500 rpm for 5 minutes.
15. Repeat steps 12-14 once more.
16. Add Carnoy's fixative to the pellet and resuspend cells by pipetting. Add enough Carnoy's fixative so the solution is translucent, almost opaque. This is typically about 1-3mL of Carnoy's fixative, but it could be more or less, depending on the size of the cell pellet.
17. Clean microscope slide or specimen slide with Carnoy's fixative, then drop 1-2 drops cell pellet suspension onto the slide while slide is still wet. Allow slide to dry on a hot plate warmed to 43°C. Check cellular area under inverted brightfield microscope for optimal cellular density per field of view.
18. Dehydrate slides for 2 minutes each in: 70% EtOH, 85% EtOH, 100% EtOH and air dry.

Hybridization:

1. Warm FISH probe and hybridization buffer to room temperature. Mix well by vortexing 10-15 seconds and spin down in a micro-centrifuge.
2. If not prediluted, the recommended probe mixture is 2µL of probe and 8µL of hybridization buffer to total 10µL per cellular area (1:4 dilution).
3. Apply working solution of probe mixture to cellular area and cover entire area with a glass coverslip. Using tweezers or a pipette, gently tap the coverslip to push out any air bubbles.
4. Seal the edges of the coverslip with rubber cement.
5. Place slides in Thermobrite/Hybrite, add the pre-soaked hydration cards, and run program specified in the **Preparation** section. Slides can be removed any time after 16 hours of hybridization, up to 24 hours.
    a. Alternatively, denature on a hotplate at 75°C for 3 minutes (ensure slide is completely protected from light), then incubate in a dark humidified chamber at 37°C for at least 16 hours.
6. Remove slides from Thermobrite and remove rubber cement and coverslip from each slide.
7. Place slides in FISH Wash Buffer 1 warmed to 73°C, agitate slides for 10-15 seconds in solution, then leave in solution for 2 minutes.
8. Place slides in FISH Wash Buffer 2 at room temperature, agitate slides for 10-15 seconds in solution, then leave in solution for 2 minutes.
9. Dip slide quickly in deionized water.
10. Allow slide to fully dry (protected from light), approximately 5 minutes, and counterstain with 10µL of DAPI with Antifade.
11. Visualize right away or store slides at -20°C until ready to view.

Notes:

- Carnoy's fixative should be ice-cold throughout the protocol, but cell pellet and slides can be dropped at room temperature.
- Cell confluency can be as low as 60%, but 80% is ideal for cell pellet sizing.
- After the last step in the protocol (step 16), the pellet can be stored in -20°C indefinitely. When moving onto hybridization, centrifuge the tube to create a cell pellet and then follow step 16 with freshly made Carnoy's fixative to prepare a stored sample for FISH.
- For strongly adherent cell lines, 0.25%-2.5% trypsin-EDTA in HBSS is used; some experimenting may be required to get the proper trypsin solution strength and incubation time for dissociating the cells from the wall or floor of the container (plate, flask, etc.) used.
- Cells are brittle when swelling in the hypotonic solution; avoid vortexing or pipetting at this step.
- Rubber cement and coverslips can be removed with tweezers or by gently rolling with fingertips. For particularly stubborn coverslips, soak for 1 minute in 2X SSC prior to post-hybridization washing.