Beth Sullivan Lab June 2003 (updated June 2015)

Fluorescence In Situ Hybridization (FISH) on Methanol- Acetic Acid Fixed Preparations

Probe Precipitation:

25ng- 500ng labeled probe per 22x22cm^2 area
25-50 ng for repetitive sequences; 150-300ng for human, mouse or fly BACs, PACs, and fosmids or chromosome paints; 50-300ng for plasmids (depending on insert size)

10ug human or mouse Cot-1 DNA (available from Roche or Invitrogen; required for chromosome paints, euchromatic BACs and PACs, plasmids – it blocks repetitive sequences in BACs, PACs, fosmids, plasmids)

0.5uL salmon sperm DNA (stock concentration: 10mg/mL)

1/10 volume 3M NaOAc

2.5 volumes EtOH

Precipitate at –80°C for at least 15 minutes, or overnight at -20°C.
spin 14K in cold room or 4°C centrifuge, 15-20 minutes; remove supernatant without dislodging pellet
wash with 70% EtOH; remove supernatant without dislodging pellet
air-dry on bench
resuspend in probe hybridization mix (concentration of formamide depends on type of probe; see below)

Probe hybridization mix
per probe:
50% formamide (deionized) (note: use 50% for unique-sequence/locus-specific; 60-68% for repetitive probes, although 65% is standard "high stringency" for repetitive probes)
10% dextran sulfate (use blunt pipette tips – this is very viscous)
2X SSC
1% Tween-20
Bring volume to 100ul with dH_2O
Vortex and spin for a few seconds at RT

Note: We have 500uL stocks of each % formamide hybe mix (aliquoted from 10mL master stocks) that we store at -20°C but if you want, you can make the hybe solution fresh each time.

If you are going to make your probe hybe mix fresh each time, make up 100ul of mix, but bring probe up in only 10-12 ul of this mix per 22x22mm hybridization area, depending on the size of target area – i.e. for half of a slide or just the center of a slide, use 10-12ul; for an entire slide, use 20-25ul.

Target DNA: affix your target DNA (neuroblasts, salivary glands, disaggregated cells from tissue, TC cells, etc) onto clean glass slides.

Slide Denaturation:

1. Incubate slides in 100ug/ml RNase/2X SSC at 37°C for 60 minutes. This pre-treats the cells and removes RNA that may interfere with hybridization.
Note: This step is optional, however, we usually do it for at least 30 minutes.

2. After RNase treatment, dehydrate slides in an ethanol series: cold (on ice) 70%, 95%, 100%, 2 min each.

3. Air dry slides or dry slides on hot plate/slide warmer.

4. Denature slides in 70%formamide/2X SSC pH7 at 72°C for **50 seconds to 2 minutes**. 70% formamide needs to be pre-heated in a 75°C bath (check that temperature inside the jar is ~72°C).
If chromosome preps/squashes are freshly made (i.e. the same day that hybridization is done), denaturing time by 5-10 seconds or temperature can be dropped by 1-2 degrees. DO NOT exceed 2 minutes b/c chromosomes and nuclei will be over-denatured and look "hollow" and refractile!
Freshly made slides can be aged overnight at RT, for 30 minutes under vacuum at 80°C or in a oven for 1 hour at 60°C. This will reduce chance of over-denaturating target DNA. After I drop chromosomes on slides or make neuroblast/disc squashes, I use the slides for FISH within 2 weeks. If there is a lot of cytoplasm surrounding the metaphases, digest with pepsin before doing FISH.

5. Immediately dehydrate slides as above; you can use same ethanol series as you used after the pre-treatment step in 2X SSC, as long as coplin jars/tanks were kept covered. Air dry on hot plate. Slides that are denatured remain denatured for up to one week, however I recommend use within 1-2 days.

While slides are denaturing, you can also denature your probe or you can wait until your slides are denatured, dehydrated and keeping warm on the hot plate. Denatured DNA on slides will stay denatured for about a week (seriously).

Denature probes at 70-75°C for 10 minutes, spin quickly, and then apply to denatured DNA or to prewarmed coverslips (see below, Step 6).

Note: For mammalian BACs/PACs/plasmids, after denaturing, you must allow time to pre-anneal probe and unlabeled Cot-1 DNA to suppress repetitive sequences. We do this for 30 minutes at 37°C before applying to slides.

6. Add probe to dried slide on slide warmer, coverslip, seal with rubber cement and incubate 16-24 hours at 37°C in a humidified chamber (foiled covered plastic tray containing wet filter paper; use plastic pipettes to set your slides on, don't put them directly on the wet towels or filter paper).

7. After hybridization, wash slides in 50% formamide/2X SSC, pH7 at 42-45°C, 4 washes for 5 minutes each. Preheat all wash buffers in baths before doing washes. Depending on the probe, wash temperatures can range from RT to 42°C. The goal is to only see the fluorescent probe on the target sequence. If there is a lot of general fluorescence background, then increase washing temp from RT to higher temps (37 or 42).

For **repetitive probes except the pan centromere (all centromere probe)**, use higher stringency washes of 65% formamide/2X SSC pH 7 (and you may need to use temperature washes rather than RT to wash away any cross-hybe).

To put slides in coplin jars with wash buffer, I usually peel off the rubber cement around the coverslip and let it remain on (if it doesn't peel off with the cement). Don't worry, the coverslip will fall off during the first wash in formamide/2X SSC. This prevents inadvertently scratching and/or removing chromosomes/nuclei!

FROM THIS POINT ON, IT IS IMPORTANT NOT TO LET SLIDES DRY OUT.

8. Wash slides at 37°C in 2X SSC for 4 x 2 minutes.

9. OPTIONAL. Wash 1-3 times for 1 min in 0.1 X SSC at 60°C. This will remove background and is an important step to do at least **once** for repetitive probes.

10. Place slides in wash buffer (4X SSC/0.1% Tween-20) for 5 minutes at RT.***

11. For *in situ* with **directly labeled probes**, you can now counterstain with DAPI-antifade/SloFade or Vectashield-DAPI (V-DAPI) and visualize under the microscope. Take the slides directly from wash buffer, drain briefly and mount. For indirectly labeled probes (i.e. those that have been labeled with biotin or digoxygenin, proceed to step 12).

12. Block slides for 10-20 minutes in the dark (i.e. a foiled covered chamber) at RT with freshly made blocking solution (4X SSC/5% nonfat milk*). Use 100ul of blocking buffer per slide. Cover with a parafilm coverslip (parafilm cut to be about the size of the slide surface).

13. Detect with appropriate antibodies diluted in blocking solution. Use 100ul of antibody diluted in blocking solution per slide and cover with a new parafilm coverslip. All antibodies should be diluted in blocking solution and centrifuged at 14,000 rpm for 5 minutes at 4°C to remove unconjugated fluorochromes. Incubate 30 minutes at 37°C for each antibody, 1-2 hours at RT or overnight at 4°C.
The biotin-avidin complex can be amplified using layers of antibodies, i.e. FITC-avidin, followed by biotinylated anti-avidin, followed by FITC-avidin. I usually do the first antibody (i.e. FITC-avidin, Cy3-avidin or FITC-anti-dig at 4°C and the subsequent amplifications at RT). For double-labeled slides (i.e. TWO DNA PROBES), I recommend detecting dig-labeled probes with FITC anti-dig/FITC anti-sheep and biotin-labeled probes with TR avidin, Cy3 avidin, Cy5 avidin or AMCA avidin.

14. Wash with wash buffer 3 x 5 minutes with shaking/agitation.

15. After washes in wash buffer****, mount slides with SloFade-DAPI or V-DAPI. Coverslip with a glass coverslip. Slides can now be stored at 4 degrees C until visualization under the microscope.

**** steps where slides can be stored at 4°C before proceeding.