*B Sullivan September 2006*

**Fluorescence In Situ Hybridization (FISH) following Indirect Immunofluorescence**

1. After immunofluorescence (primary and secondary antibodies), crosslink antibodies to chromosomal proteins using 6-10% formaldehyde [formalin; Sigma F1635, diluted in KCM or PBS-Tween (0.05%)] at RT for 10 minutes. Fixing solution should be made fresh.

2. Transfer slides to 2X SSCT (2X SSC, 0.1% Tween-20) for 5 minutes at RT. Slides can be stored in fridge in this solution for 1 week. Let slides in 2X SSCT warm up to room temperature before proceeding with FISH (step 3).

3. Denature slides in 70% formamide/2XSSC, pH7 for 4-8 minutes at 75-78°C. Also denature probe at 75°C during this time. (4 minutes is long enough to denature chromatin fibers.)

4. Immediately wipe off excess formamide around area of interest (i.e. circular area from cytospinning) without disturbing cells. Quickly place slide on a slide warm and place denatured probe onto sample area. Cover with 18x18mm$^2$ coverglass or a 12x12 mm round coverglass if your target area is the circle from cytospinning.
*note: It is best to do only one slide at a time in the denaturing solution, as PFA/formaldehyde fixed protein-DNA complexes may not denature well or may re-anneal quickly. You must move quickly at this step.*

5. Transfer slides to a humidified chamber and incubate 24-48 hours at 37°C.

6. After hybridization, wash slides as follows (do not agitate slides during washes b/c cells may detach):

> 4X: 50% formamide/2X SSC, pH7, 15 minutes, RT, 37°C or 42°C.
> [For repetitive probes (such as centromere probes), use a higher stringency formamide wash of 60-65% formamide/2X SSC pH 7; you can also do washes at 37°C or 42°C to increase stringency]
> 4X: 2XSSCT, 5 minutes, RT, 37°C or 42°C
> 1X: 4XSSCT, 5 minutes, RT

7. Directly-labeled probes (i.e. Alexa Fluor 488, Alexa Fluor 594 or Texas Red) are now ready to be counterstained and viewed under the microscope. Counterstain with Vectashield (Vector Labs) or SloFade (Molecular Probes) supplemented with DAPI. For indirectly labeled probes (i.e. with biotin or dig), proceed to step 8.

8. If detecting biotin or dig labeled probes, block slides for 10 minutes at RT with blocking solution (4X SSC/5% nonfat milk - make fresh).

9. Detect biotin or dig with appropriate secondary antibodies diluted in blocking solution. Incubate overnight at 4°C or for 2 hours at RT.

10. Wash 3 x 2 minutes after each incubation in 4XSSC/0.1% Tween-20/0.02% Na Azide.
**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. I usually detect dig-labeled probes with FITC anti-dig/FITC anti-sheep and biotin-labeled probes with TR avidin, Cy3 avidin, Cy5 avidin or AMCA avidin.

11. Counterstain as in step 7.

**Notes and Solutions:**

The formamide for making the denaturing solution and the post-hybridization washes is just the regular stuff available from Fisher, Sigma, or American Bioanalytical, i.e. it is not ultrapure.

**Hybridization mixture for dissolving DNA probes (use blunt tips for pipetting as dextran sulfate is viscous):**

**50% formamide (ultrapure)**
**2X SSC**
**10% Dextran sulfate (from a 50% stock)**
**1% Tween –20**

**KCM**
120 mM KCl
20mM NaCl
10mM Tris-HCl, pH7.7
0.1% Triton X-100

**PBST (PBS + 0.05% Tween-20)**
136mM NaCl
2mM KCl
10.6mM $Na_2HPO_4$
1.5mM $KH_2PO_4$
pH to 7.4 then add Tween-20
add 0.02% Na Azide for long-term storage at RT