FISH Protocols

FISH Overview

The techniques described are taken from other people/papers please do not quote this manual as a direct reference. The aim of this manual is to present the in situ hybridization methodology in a straightforward manner, describing "in-house secrets" that are usually omitted from published works. We hope this manual will be particularly helpful to investigators setting up in situ hybridization de novo. Following references are recommended for original information of the techniques.
Chromosome Preparation for In Situ Hybridization

Squash Method

1. Germinate seeds on moist filter paper in petri dishes at room temperature.
2. Root-tips at 1-2 cm long are collected and placed in vials with water. Place the vials in a box with crushed ice in water and store box in a refrigerator for 22-24 hrs.
3. Root tips fixed in a 3:1 mixture of 95% ethanol:glacial acetic acid for 1-7 days.

Notes: better in situ hybridization results will be obtained if the fixation is not too long. However, it is always relatively difficult to make preparation if the fixation is less than two days. Try your best.

4. Stain the root tips in 1% acetocarmine for 10 min - 1 hr. Do not stain the root tips too long.
5. Make the preparation the way you like. But never try to soften the root tips by warming, or even boiling them.
6. Store the slide in a -70°C freezer. The slides can be stored up to years without seriously affecting the N-banding and in situ hybridization results in our hands.

Notes: Many experienced cytogeneticists love to say that making preparations is the most important step for any cytogenetic or molecular cytogenetic techniques. This is true for in situ hybridization. We introduce you to a very simple way to check the quality of your preparations for in situ hybridization. If the morphology of the chromosomes in most of the cells does not change (under a phase contrast microscope) before and after the ethanol series treatment (for drying), it means you are doing a good job.
Biotin/digoxigenin-labelling of Plasmid or Genomic DNA

1. Prepare following reaction solution:

10X nick-translation buffer	5 µl
dNTP solution	2.5 µl
dUTP solution (biotin or digoxigenin)	2.5 µl
Plasmid or Genomic DNA	1 µg
DNA Polymerase I	12 units
DNase I	
H2O	
Reaction volume (total)	50 µl

2. Use H2O to adjust the total volume to 50 µl. Carefully mix after each step of adding solutions and spin down at the end.
3. Incubate reaction tube in water bath at 14-15˚C for 2 hrs.

4. Stop the reaction by adding 5 µl stop buffer (200 mM EDTA).

Notes:

a. 10X nick-translation buffer: 0.5 M Tris HCl pH 7.5, 50 mM MgCl2. dNTP solution: containing 0.5 mM dATP, 0.5 mM dGTP, 0.5 mM dCTP. dUTP solution: 0.166 mM dUTP (biotin or digoxigenin-congugated) and 0.333 mM dTTP .

b. The most critical part for the labelling (if the DNA is clean) is the size of the final nick translation product. The best in situ hybridization results will be obtained when the size of the biotin-labeled probe is around 200-600 bp.

c. For determining the size of the probe, denature 15 µl of the nick translation product in boiling water for 4 min and cool down on ice immediately. Run the sample on a mini-gel with a DNA size marker.

d. The size of the nick translation product can be adjusted by adding different amount of DNase I in the reaction solution.

e. Random priming technique can also be used for labelling. Since the size of probe is relatively easier to control by nick translation, we prefer to use the nick translation technique.

f. We suggest you to buy a nick-translation kit if you don't have enough experience. Several companies sell such kits which include the biotin-dUTP or digoxigenin-dUTP.

II. Probe Purification

1. Start 20 min before the end of the nick translation incubation. Plug the bottom of a 1 ml tuberculin syringe with siliconized glass wool.
2. Fill the syringe with Sephadex G-50 up to the top using a sterilized pasteur pipette.
3. Place the Sephadex-filled syringe into a 15 ml Corex tube.
4. Centrifuge at 1500 rpm for 4 min to pack down the sephadex.
5. Repeat step 2 to 4 so that the packed sephadex column has a total volume of about 0.9 ml.
6. Wash the column twice with 55 µl TE by spin at 1500 rpm for 4 min each.
7. Place a sterilized 1.5 ml tube on the bottom of the 15 ml Corex tube, insert the packed and washed syringe into the corex tube such that the tip of the syringe is fitted into the 1.5 ml tube. Load the nick translation product (55 µl, with stop buffer) and spin at 1500 rpm for 4 min.
8. The probe DNA will be collected in the 1.5 ml tube.

Notes:

a. If you did not make any mistakes, the final volume should be close to 55 µl. Usually 0.5 µl of such biotin-labelled probe (1 µg/55 µl = 18 ng/µl) is enough for each slide for in situ hybridization. Thus you can use the probe for at least 100 slides.

b. You can check the size of the probe (by running a mini-gel) either before or after the spin-column purification.

c. The probe can be stored in a freezer for several years. You may store it at 4°C if you use it regularly.

d. The spin-column method can be replaced by other techniques for probe purification. You can use an easier ethanol precipitation method.

Hybridization of the Probe to Chromosomes

1. Remove the cover slips of the slides (stored in a -80°C freezer) with a razor blade and dry slides in an ethanol series (70%, 95%, 100% ethanol 5 min each at room temperature). The slides should be dried several hrs before in situ hybridization.
2. Prepare the hybridization mixture as follows:

deionized formamide	10 µl
20X SSC	2 µl
Sheared salmon sperm DNA (10 mg/ml)	2 µl
Probe DNA	1-2 µl
H2O	? µl
50% dextran sulfate	4 µl
Total	20 µl

Notes: Use H2O to adjust the total volume to 20 µl. 10 µl will be applied to each slide with a 18 ´ 18 mm cover slip. Make sure the solution is well mixed because the 50% dextran sulfate is very sticky.

3. Denature this mixture by placing at a 80°C heating block for 5 minutes and immediately chill the mixture on ice. Spin down the solution.
4. Add 150 µl of 70% formamide in 2X SSC solution on the dried slides and cover the slides with 22 ´ 40 mm cover slips. Place slides on a plate (Metal or glass) in a 80°C hot oven for 1.5 min. (10 ml stock solution of 70% formamide in 2X SSC: 7 ml formamide, 1 ml 20X SSC, 2 ml H2O)
5. Remove the cover slips by swing the slides and dip the slides immediately into an cold ethanol series (70%, 95%, and 100%, 5 min each at -20°C) and air dry the slides.
6. Apply 10 µl hybridization mixture to each slide and cover with a 18 ´ 18 mm cover slip. Seal the cover slip with rubber cement and place the slides in a wet chamber.

Notes: You can use any kind of boxes with 2 layers of wet 3 mm Whatman paper on the bottom, use plastic bars or something else to hold the slides.

7. Incubate the wet chamber at 37°C for minimum 6 hrs or overnight.

Notes:

a. You may check the dried slides under a phase contrast microscope to make sure the metaphase cells are still there and the chromosomes are still in good shape (flat). This is very important. You will never get satisfactory results if the chromosomes become ugly after drying.

b. The quality of formamide is important. The formamide from CMS is very good in our hands.

Detection of ISH Signals with Fluorochromes

1. Peel the rubber cement using a forceps. Dip the slides in a staining jar containing 2X SSC. Gently shake the staining jar until the coverslips fall from the slides.

2. Wash the slides with the following steps:

2X SSC, room temperature	5 min
2X SSC, 42°C	10 min
2X SSC, room temperature	5 min
1X PBS, room temperature	5 min

Notes: You may increase the temperature and time of the 2X SSC wash if you want to reduce background. The above washing series works fine for most of the probes in our hands.

3. Drain (never dry) the slides on paper towels and add 100 µl of FITC (or rhodamine) conjugated anti-biotin (or anti-digoxigenin) antibody (1:50 to 1:200 dilution depending on the product from different companies) cover with 22 ´ 40 mm coverslips, incubate at 37°C for 30 min.

4. Remove the coverslips by tilting the slides or dipping the slides in a beaker with 1X PBS. Wash the slides three times in 1X PBS (5 min each) at room temperature. Notes: You can change the time and temperature for the washing to adjust the stringency.

5. Drain the slides on paper towel. Add a thin layer of antifade solution (from Vector) containing 1 µg/ml propidium iodide or DAPI (4',6-diamidino-2-phenylindole), cover with a coverslip.

6. Check your in situ hybridization results using a fluorescence microscope. The FITC-detected probes will be in a yellow-green color. The rhodamine-detected probes will be a red color.