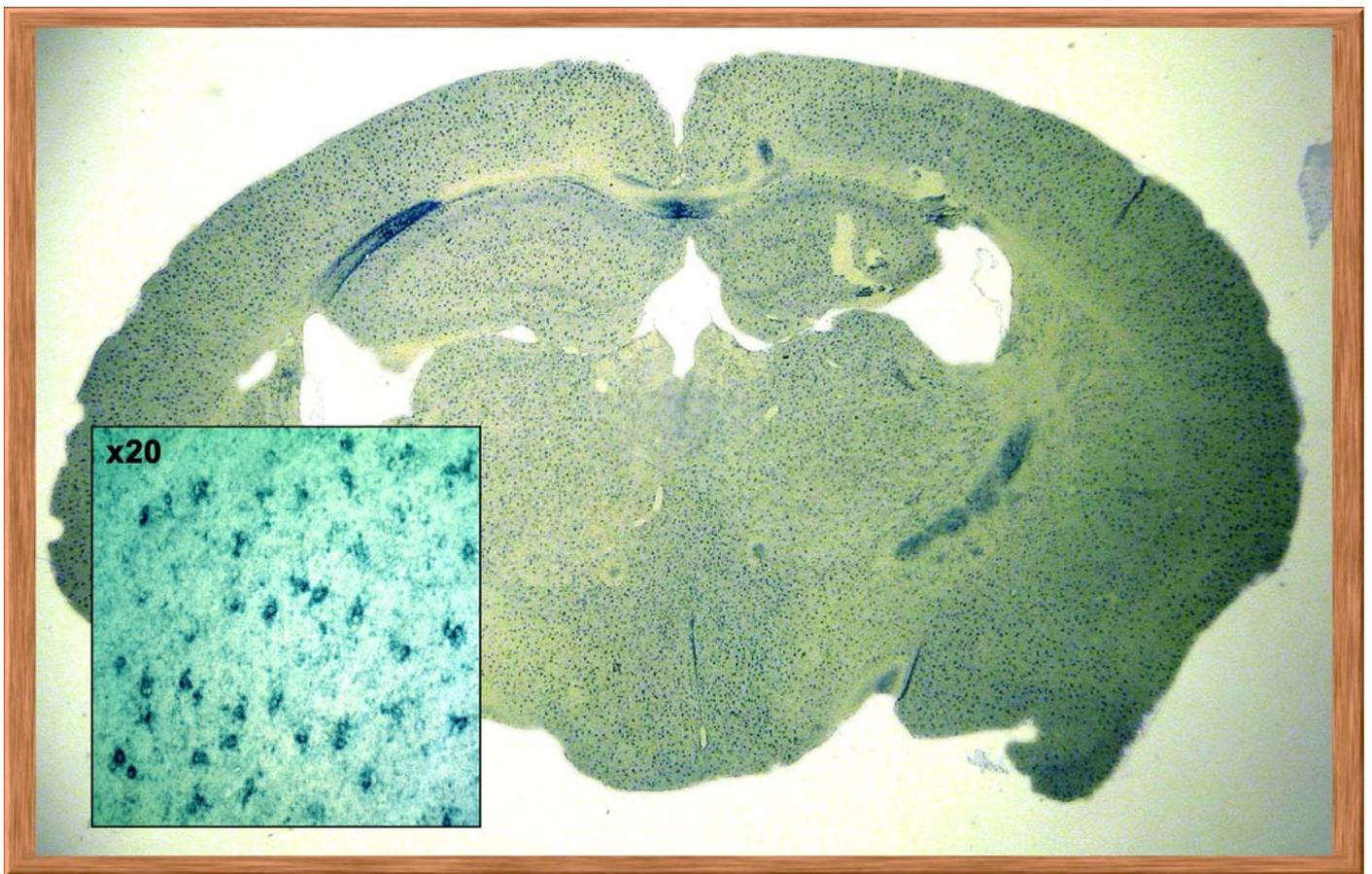


In situ hybridization protocols

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In situ hybridization showing labelling of individual cells expressing Fibroblast growth factor receptor 3 (Fgfr3) in an adult mouse brain coronal section developed with NBT/BCIP.

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We have used in situ hybridization in our laboratory since the early days of S³⁵ labelled probes (now superseded) and have extensive experience of the various methodologies used – and the many potential problems! This document contains detailed protocols for both single and double in situ hybridizations and lists various methods of visualizing your signal, both chromogenic and fluorescent.

In situ hybridization is a multi-step process with many potential pitfalls, which can have a cumulative effect on the sensitivity of detection. Over the last ten years or so we have encountered many of them - usually by trying to take shortcuts! The lesson is: don't take shortcuts, no matter how trivial they might seem. For example, you can sometimes substitute formamide "straight from the bottle" for de-ionized formamide in the hybridization mixture, without much effect on sensitivity. Problems start when you purchase a bad batch of formamide (formamide degrades to formic acid), your in situs stop working and it takes weeks of trouble-shooting to get back on track. Better to have stuck to de-ionized formamide in the first place.

It is rarely a single problem that causes poor in situs, usually several things combine to frustrate you. This makes it all the more important not to change things unnecessarily. With a complicated protocol if it ain't broke, don't fix it.

Overview of potential problems

There are at least four major areas to pay really careful attention to:

1. Probe

Choice of cDNA probe can be very important - size can and does matter. We prefer larger (approx 1 kb probes) although we do use smaller ones and larger ones. Small probes usually give less signal-to-noise. The level of expression of your target mRNA can become the limiting factor here.

We have found that cleaning our cDNA templates with phenol/chloroform and re-precipitating before in vitro transcription routinely generates good probes. Problems in this area have been traced to "dirty" templates. You can sometime get away with not cleaning your linearized cDNA, but this is not recommended.

Transcribing the RNA probe correctly is paramount. If your probe doesn't look like the examples shown in this protocol, start again. You should have a nice tight band, not a smear on the gel.

After transcribing our RNA probe we do not remove the cDNA template nor the free nucleotides. Experience has taught us that there is no advantage in doing these extra steps and in fact we have found they can decrease the sensitivity, perhaps because of loss of total probe.

If you use the quantities outlined in the transcription protocols you will find it unnecessary to quantify the amount of probe generated. Invariably they work at a 1/1000 dilution. Sometimes we lower the concentration. Higher concentrations (e.g. 1/500) tend to give a high background.

2. Tissue Preparation

We routinely fix in 4% paraformaldehyde and cryoprotect in 20% sucrose solutions which have been Diethyl Pyrocarbonate (DEPC)-treated, to destroy any residual RNase. Fixation times are important; over- or under-fixation can reduce sensitivity, perhaps related to reduced probe penetration into the tissue (over-fixed) and degradation or loss of mRNA (under-fixation).

Preparation of the sucrose solution is paramount. We always DEPC treat the solution and then autoclave. Dissolving sucrose in RNase free water might work some of the time, but leaves you vulnerable to an RNase contaminated sucrose solution, which is not a good idea. Problems have arisen in the past with non-DEPC treated sucrose solutions which have contained RNase and destroyed the mRNA in the tissue whilst cryoprotecting. Note that you cannot destroy RNase merely by autoclaving!

3. Hybridization buffer

You must use de-ionized formamide routinely. Sorry, but that ultra pure Analar grade is not de-ionized formamide. (Note this doesn't matter for the high temperature washes after you have hybridized your probe, just in the hybridization buffer). You must also take care to avoid any RNase contamination of the hybridization buffer. For example, you must ensure that the tRNA you use is RNase free (see protocol below). Buying "molecular biology grade" tRNA is no guarantee that it is RNase free. If it doesn't say it is RNase free on the label then it isn't.

All other solutions should be bought in as RNase-free and solutions should routinely be DEPC.

4. Correct pH

Use a Tris pH electrode. If your pH electrode is not specifically designed for Tris buffers your final pH will be incorrect (usually ~1pH unit out). Tris electrodes are made of glass, not the robust plastic ones seen in general use in most laboratories. Those are fine for most reagents and buffers, but not for Tris buffers. If you haven't got one use pH paper (do not dip the paper in the buffer but take a drop of buffer to the paper using a sterile pipet!). The pH optimum for calf alkaline phosphatase (AP, the enzyme that ultimately develops your colour reaction) is pH9.8 so it will help if your developing solution is at pH9.8. Of course alkaline phosphatase will work at anything above pH8, so solutions that are not at the correct pH will ultimately yield a colour reaction, but not as efficiently and sensitively as one at pH9.8.

Minimal protocol

- Make DIG or FITC labelled antisense probes.
- Dissect tissue, fix in 4% paraformaldehyde and cryoprotect in 20% sucrose solution before rapid freezing.
- Cut frozen sections and collect on SuperFrost plus coated slides. Allow to dry.
- Dilute labelled probe 1/1000 in hybridization buffer. Add at least 300 μ l per slide and lay a coverslip (oven baked) on top to spread the solution over the sections.
- Incubate at 65°C overnight in a humidified chamber.
- Remove coverslips and wash 2 x 5 minutes in MABT.
- Follow with 2 x 30 minute washes at 65°C in 65°C Wash Buffer.
- Wash briefly in MABT, 2 x 5 minutes.
- Block with blocking solution for 1 hour.
- Dilute AP-Anti-Dig antibody 1/1500 in blocking solution (note 1/500 for peroxidase conjugated antibodies). Add 500 μ l per slide and incubate at 4°C overnight.
- Wash in MABT, 3 x 10 minutes is suggested.
- Wash briefly in pre-development buffer pH 9.8 (2 minutes is fine).
- Incubate at 37°C in development buffer pH 9.8, 5% PVA containing NBT and BCIP.*** Note you can use other means of developing your signal here if you wish.
- Allow blue colour to develop to required intensity (2 hours to overnight) and stop reaction by washing in tap water.
- Dehydrate and mount in a xylene based mountant.
- Store in the dark.

*** Due to the viscosity of the PVA and its tendency to precipitate if made up in developing solution we make up two solutions, a 2x Development buffer pH 9.8 and a 2x PVA solution (10% in H₂O). In use we take 25 ml of 2x development buffer and add 50 μ ls of NBT and 50 μ ls of BCIP stock solutions (both made up at 1000X) and mix thoroughly. Then we add 25ml of 10% PVA solution and mix again before pouring over slides and allowing the colour reaction to develop at 37°C, in the dark. These concentrations of NBT/BCIP are lower than the usually stated ones, but we find it does not affect the development time in any way.

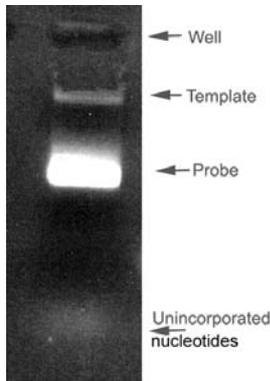
In vitro transcription of labeled probes

Perhaps the most important consideration in choosing a probe is the size of the cDNA you are using. Generally small probes (200-400kb) do not work as well as larger probes (1-2 kb). That is not to say they don't work, they sometimes just don't work as well. Probes larger than 2 kb can be used and we have found no problems (biggest we use is 4.7 kb). Not all probes work equally well. Occasionally we have found a 1 or 2 kb cDNA that just didn't work very well (possibly secondary structure?), these have been very rare. But on the couple of occasions we have come across them we have re-cloned either a different region of the cDNA or gone for some 3' UTR of the gene and this approach has (so far) worked fine.

If you wish to do double in situ hybridizations you need two differentially labelled probes; usually one labelled with DIG and the other FITC (or biotin; beware adult tissue is full of endogenous biotin). The FITC labelled probe is considered to be the least detectable and should be used for the probe where the target mRNA is expected to be highest in the tissue. This is usually developed first and the DIG labelled probe is usually developed second. However, in practise it doesn't seem to matter which way round this done. The important factor seems to be the levels of target mRNA expression in your tissue.

- Note it goes without saying that all solutions used for the transcription reaction and storage buffers should be RNase free.
- Make sure you are making the anti-sense probe, i.e complementary sequence to your target mRNA.
- Template DNA is prepared by linearizing your cDNA with the appropriate restriction enzymes. Usually we digest 10-20 μ g of cDNA in 100 μ l. After 1-2 hours at 37°C a small aliquot is taken and run on agarose gel to check that the plasmid is fully linearised.
- When you have a linear template you can now proceed to clean up the DNA.
- We have found the quality (and quantity) of our RNA probes is better if we clean our cDNA template before transcribing our DIG/FITC labelled RNA probes. Add 1/10 volume of 3M Na Acetate to the template DNA and extract once with equal volumes of phenol and chloroform/isoamyl alcohol (IAA) (24:1) and twice with equal volumes of chloroform/IAA. Precipitate by adding 2 volumes of ethanol and leave at -20°C for approx 1 hour. Spin down the pellet and wash with 70% ethanol and re-suspended at approx 1 μ g cDNA per 2.5 μ l in TE (10mM Tris-HCl, 1 mM EDTA pH7.5).
- In vitro transcription reactions are set up at room temperature in the following order (remember to keep all reagents RNase free, use RNase free tips etc etc!!):
 - 2.5 μ l linear cDNA (approx 1 μ g)
 - 4.0 μ l 5 x transcription buffer (supplied with polymerase)
 - 6.0 μ l of 100mM DTT
 - 2.0 μ l of 10 x DIG RNA labelling mix (10mM each of ATP,CTP,GTP, 6.5mM UTP, 3.5mM DIG-11-UTP or 3.5mM DIG-12-FITC (Roche).
 - 1 μ l RNasin (Promega)
 - 20-40 units of the appropriate RNA polymerase
 - H₂O to a final volume of 20 μ l.
- The mixture is incubated at 37°C for 2 hours. An aliquot (0.5 μ l) is removed and run on a DNA gel to check probe has been generated. Should see faint linear template and large incorporated probe beneath this. NOTE:- use RNase free reagents

wherever possible for making gel and buffer with. These do not have to be perfect so don't go to extremes such as treating the gel tanks etc. It is generally not a good idea to use a track on someone else's left over gel. A fresh gel should be used each time.



Occasionally the template is not visible and sometimes you get double bands of probe. Possibly caused by incomplete digest of cDNA, but the probes still seem to work fine. The example on the shown is of a DIG labeled probe. If you have labeled with FITC then the unincorporated nucleotides are highly visible (as they fluoresce on the UV light box). If you see a long smear rather than a tight band this would suggest a poor probe and perhaps it's a good idea to start again.

- Make the final volume of the labeled probe up to 100µl with 10mM DTT and store at -70°C in small aliquots (20µl). Sometimes people remove the DNA template with DNase, we have found this unnecessary. Many people will add a precipitation step after the reaction, again we have found this unnecessary and do not have problems with any background caused by unincorporated nucleotides. We also find that we often lose a considerable amount of our probe if we precipitate at this stage. So be warned if you do precipitate after incorporation you may need to do a series of dilutions to determine your optimum probe concentration. In our hands (without precipitation) the optimal dilution is routinely 1/1000. We don't actually know what this equates to in concentration, but this dilution factor has been remarkably consistent for over a 100 different probes used.
- We also make up our own DIG labeling mixture as this works out at about half the cost of buying in the already made up solutions from Roche.

DIG labeling mixture for in situs

Use chemicals from Roche- all are lithium salts

Stock solutions from Roche:	Roche catalogue number
100 mM ATP	1 140 965
100 mM CTP	1 140 922
100 mM GTP	1 140 957
100 mM UTP	1 140 949
10 mM DIG-11-UTP	1 209 256
10 mM FITC-12-UTP	1 427 857

The DIG-11-UTP and FITC-12-UTP are at 10mM and come in a volume of 25 µl (250 nM stock solution), therefore need final volume of 71.4 µl for 3.5 mM.

Final concentrations

3.5 mM DIG-11-UTP
10 mM ATP
10 mM CTP
10 mM GTP
6.5 mM UTP

Volumes of stock solutions

25 μ l DIG-11-UTP (or FITC-UTP)
7.1 μ l ATP
7.1 μ l CTP
7.1 μ l GTP
4.6 μ l UTP
20.5 μ l RNase free water

Total volume 71.4 μ l.

Tissue preparation

We have found that fixation time with paraformaldehyde is important. You can both under-fix and over-fix.

We prefer to fix tissue in 4% paraformaldehyde in PBS and routinely we usually fix overnight at 4°C for most embryonic tissues. This might not be optimal for very small tissues. We dissolve paraformaldehyde in DEPC treated PBS at 65°C, until the solution goes clear. There is no need to DEPC-treat the final solution as the paraformaldehyde will “fix” any contaminating RNases.

We have tried using fresh frozen unfixed tissue and find it is very difficult to detect our target mRNA in comparison to fixed. In tests I found that I could develop good signal in approx 3 hours on fixed sections, whereas it took approx 24 hours to develop a faint signal in unfixed tissue. So be warned unfixed works, but only just. Also DO NOT post-fix frozen sections with 4% paraformaldehyde, this decreases the sensitivity hugely.

We generally work with CNS tissue, so this is where we have the experience. Adult and postnatal brain is more difficult to fix than embryonic brain as the fixative has difficulties in penetrating the tissue. A protocol for adult brain or postnatal brain would be by perfusion of the animal with 5-10 ml PBS (DEPC treated) followed by 20-40ml of 4% paraformaldehyde. This is followed immediately by dissection of the brain and cutting it into 2-3 mm slices with a razor blade. Agar Scientific sell Perspex mouse brain moulds which allow slices to be made down to 1 mm in either coronal (L5050M) or sagittal sections. An added advantage of perfusion is that it also removes the background auto-fluorescent cells in the blood vessels making it easier to interpret fluorescence in situ.

After perfusion, fix the slices for 2-3 hours at room temperature (approx 20°C) in 4% paraformaldehyde. 1 hour is too short and 4 hours too long. These longer and shorter fixation times will still give a reasonable signal but they are not quite as sensitive as the 2-3 hour fix. This was tested with fluorescent tyramide probes on a low abundance mRNA. Note this was tested on 2mm brain slices, not whole brain.

At the other extreme, for very small pieces of tissue such as 2-3 day chicken embryos, 5-10 minutes at room temp can be adequate fixation and 1 hour at room temperature can render the target mRNA undetectable.

I suggest titrating the fixation time for your particular tissue. However, if in doubt, stick to 4°C overnight, except for very small pieces of tissues.

In every case this will be followed by cryoprotection in 20% (w/v) sucrose in PBS, usually overnight at 4°C. N.B It is very important that this solution is RNase free, we add Diethylpyrocarbonate (DEPC) (1ml per litre), shake vigorously until all the DEPC globules have disappeared then autoclave to degrade the DEPC (breaks down into CO₂ and Ethanol) and to sterilise the sucrose solution (turns yellowish on autoclaving). Do not assume that using RNase free water and reagents will give you RNase free solution every time. You can sometimes get away with not adding DEPC, but not all of the time.

Tissue is then removed from sucrose and placed on a paper towel to remove all the excess sucrose solution before surrounding by Tissue-Tec, usually in small foil boats

made by wrapping aluminium foil around either spectrophotometer cuvettes, 7ml bijoux or 30 ml universal's or whatever is at hand. These are then filled with tissue-Tec and the tissue placed and orientated inside the "boat". Make a note of orientation of specimen before freezing, either by cutting a small "v" in the boat, or marking with a felt tip pen. This is important because tissue-Tec turns white when frozen and you can no longer visualise your tissue inside it. Rapid-freeze your tissue by placing foil "boat" directly on dry ice or on a flat metal surface pre-cooled on dry ice (there are other ways of rapid freezing, but this is much less hassle and works sufficiently well that we use it routinely). N.B If using a silicon mould for very small specimens (available for EM specimens) place some aluminium foil directly over the tissue-Tec and place dry ice directly on top of this such that the dry ice is in direct contact with the foil above the tissue-Tec. Silicon is a poor conductor of heat so if placed directly onto dry ice it takes ages to freeze the tissue-Tec plus specimen i.e. too long!

A recent finding of plastic cryomoulds has saved us a considerable amount of aluminium foil origami, but because of poor thermal conducting properties they demand a different method of freezing. These cryomoulds are made by Tissue-Tec (available from Bayer <http://www.bayer.co.uk/>) Sizes 10 x 10 x 5mm (cat no 4565) or 15 x 15 x 5mm (cat no. 4566). We freeze directly into liquid Iso-pentane cooled to -70°C on dry Ice. Take an Ice bucket and find a largish beaker that you can drop the boats into. Place beaker in ice bucket and surround with dry ice, fill beaker with iso-pentane. You can tell when the iso-pentane is at the right temperature when a piece of dry ice placed in the iso-pentane no longer "boils".

Put tissue into dry cryomoulds, surround with tissue-Tec and drop into Iso-pentane. I try to "float" them on the surface and this results in them quickly slipping beneath the surface and freezing solid within a few seconds. Remove cryomoulds plus frozen tissue with long handled forceps and store at -80.

Cryosectioning

Get a Sharp knife, collect sections on Superfros®Plus coated slides. A criticism of using fixed tissue versus unfixed is that fixed tissue creases and folds when you collect it onto a slide. This is true, but the difference in the quality and sensitivity of the in situs will more than make up for this drawback. Also see end of this section for a method of generating high quality wrinkle-free frozen sections. It's tricky but it does work. However if you can't be bothered to do this you will still have lots of good areas on your ordinary section to photograph afterwards even on sagittal sections of adult brain. Most spinal cords should routinely cut without distortion or creases.

One could write a book about cutting good frozen sections. Some of the modern disposable blades and holders are quite good, but experience has shown that your own stainless steel knife will take some beating, particularly for those large brain sections. You will need to keep it sharp. Always check the edge of your knife under a dissecting microscope for damage before use and make sure you cut on "good bit" etc. If it's straight and smooth and doesn't look like a relief map of the Andes it is fine. If there are bumps, lumps, nicks etc you will struggle to cut decent sections.

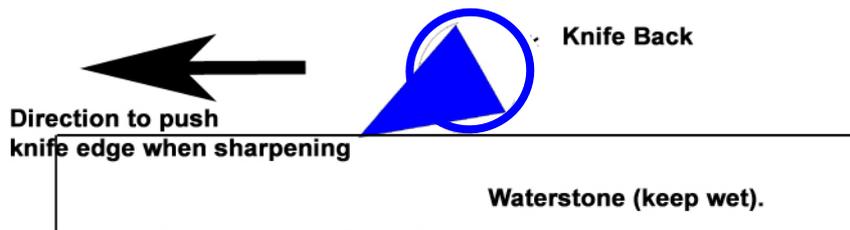
Knife Sharpening

Knife sharpening is not difficult if the right tools are bought. Cutting frozen sections damages knives; they wear and require re-sharpening at frequent intervals. We sharpen all our own knives; it's a relatively trivial process once you have the right tools. A knife back (available from Bright for a variety of knives) and a couple of water stones is all you need. We make the knife backs ourselves from stainless steel tubing with a slot cut out for the knife to sit in (see diagram below). Each knife should have its own back, as constant sharpening marries the knife back and knife edge (bevel) together. This means that each back and knife will wear together and should not be interchanged with other knives. Also make sure the knife and back are fitted together in exactly the same way, with side A of knife matched to side A of back, mark this with a diamond scribe to make it easy to match them.

To sharpen we use Japanese water stones of differing grades from 1000 grit (coarse) for grinding out nicks etc, 6000 grit (fine) for getting a good edge and 8000 grit (super fine) for polishing it even finer. These water stones are sold at most good carpentry supply shops or available on e-bay. They need soaking in water for 10 minutes before use and keeping wet whilst honing your knife edge. It's important to sharpen by pushing with the edge of the knife at the front, it seems counter-intuitive but it is the correct way to sharpen these knives. Keep as much pressure on the knives as you can whilst sharpening. Use a sweeping motion of left to right making sure the entire edge is sharpened in one motion (the water stones are not wide enough to cover an entire knife edge). Then flip over the knife and pull back towards you in the opposite direction. Start with the 1000 grit until all nicks have been removed (check on dissecting microscope), you should see dirty particles appearing as the knife frees abrasive particles of grit from the water stone. Examine under dissection scope to confirm nicks all gone. Wash knife and back to remove the coarse particles freed on the 1000 stone before moving on to the 6000 grit and ultimately the 8000 grit stones. Examine knife edge under dissecting scope and when a nice straight level edge is obtained...use for cutting.

1000/6000 combination stone can be bought from Classic Hand tools
http://www.classichandtools.com/acatalog/Japanese_Waterstones.html
(tel 01473424304) number TKK207 for the combination 1000/6000 grit (£32)

The 8000 grit waterstone can be bought from Dieter Schmid Fine tools <http://www.fine-tools.com/japwas.htm> order number 312093 for the 8000 grit Cerax sharpening stone (£50)



One tip is to sharpen one side of the knife more than the other so as to prevent an excessive bevel building up on both sides of the knife at the cutting edge. If you have ever sent a knife to be reground you'll know what I'm talking about.

Cutting Frozen Sections

Most problems with cutting frozen sections result from three major areas: blunt knife, anti-roll bar wrongly adjusted or wrong cutting temperature.

Everything needs to be set up in the cryostat as optimal as possible for the tissue you are attempting to cut. Temperature is very important and the temperature you choose will depend on many things, in particular the tissue you are cutting. We routinely use -18°C for inside chamber temperature and hold our specimens at about -16°C when cutting. In general if you are getting fracture lines along your sections whilst cutting you are cutting too cold. If the front of your block looks damp and wet you are too warm. Play around until you find the optimum conditions for your tissue.

Anti-roll bar needs to be clean and scratch free. Also note that very small adjustment to the roll bars height in relation to the top of the knife edge can have a significant effect on the quality of the sections so play around with this before altering temperatures etc. Check roll bar for scratches and nicks on its inside edge, as these can affect the quality of the section. When badly scratched replace. Also keep clean, washing with 70% ethanol or acetone can make a big difference as any dirt or scratches can "hold back" your section from sliding easily between the roll bar and the knife. Also when you change temperature gives the specimen about 15 minutes to equilibrate to the new temperature before going back to cutting sections.

Knife angle in the cryostat can make a difference but varies from cryostat to cryostat (we use Bright cryostats and keep the knife angle to $10-12.5^{\circ}$)

Sections can be cut from $5\mu\text{m}$ upwards, but for in situ I usually cut $15\mu\text{m}$ sections because this is a reasonably optimal thickness that provides good signal and detail at the single cell level. Thinner and the in situ signal decreases, thicker and more diffuse. Block

faces can make a big difference to section cutting and there are many thoughts and designs of block faces. I prefer this shape \ / where the top and bottom of block are parallel to the knife-edge and slightly v-shaped. Other shapes used are circles, squares and diamond shapes. Use whatever gets you find gives you the section quality you require. If you have uneven edges on your block you will often struggle to cut decent sections. Usually the major problem people encounter difficulties with when sectioning is that knife is not sharp enough....you did check it before use.....didn't you??

Collect sections on Superfrost®Plus coated slides. These are available from VWR International, catalogue number 631-0108. We make no additional treatments to these slides and presume that the surfaces of the slides in unopened boxes are RNase free. We try to keep them that way. Many people wear disposable rubber gloves when cutting sections to avoid contaminating the slides with RNase on your hands. If you ONLY handle by the white frosted bit that will not see any hybridization mixture you will be fine. Also a good idea to label your slides with pencil which seems to survive the whole process better than felt tip pen.

If there are any bubbles underneath the section (routinely occurs on large sections) they can be "burst" with a fresh non RNase contaminated hypodermic needle, but do this immediately after cutting before they dry out.

Obviously you are placing one side of your slide on an RNase contaminated surface, just don't put them section side down. It is not a good idea to remove a bad section from a slide by rubbing it off, either with gloves or particularly with your fingers. Leave it there. Some people recommend drying sections on a hot plate. I tested this but could not see any difference in air dried vs Hot plate (55°C) but note this might make a difference to your particular tissue.

We do not perform any further treatments to our sections. The next step is to apply the probe in your carefully made RNase free hybridization buffer. We have found that other treatments such as Proteinases etc do not increase the sensitivity on our sections. It is worth noting that if fresh frozen or pre-fixed frozen sections are then re-fixed with 4% paraformaldehyde after cutting then some form of proteinase treatment is almost essential to see any signal. Where we have found Proteinase treatment essential is for cell lines (covered at the end of this protocol) and whole mounts (not covered).

Wrinkle-free frozen sections

This is an adaptation of free floating sections and works well, although it is a little tricky to get it to work correctly. You usually need to cut 20-30µM sections, lift the roll bar quite quickly and allow sections to curl up. You now need an oven baked beaker filled with DEPC treated H₂O plus a fine artists brush. Wet the brush and touch gently onto the Tissue Tec surrounding your section. The section + Tissue Tec should stick to the brush and allow you to gently lift them out of the cryostat and place it in the water, where the section should float on the surface and the Tissue Tec dissolves away. Take a superfrost plus coated slide and place in beaker and gently manoeuvre the section (using the brush) onto the slide. You may have to hold the section onto the slide with the brush, and then remove slide with loosely attached section. You can now use the damp brush to iron out any underlying bubbles, or folds on your section under a dissection microscope. The sections are remarkably pliant at this stage. Allow to dry and proceed as per protocol. An example of a free floating section is shown on the title page.

In situ detection methods, chromogenic and fluorescent

There is a choice of reagents for developing your in situ after hybridization. I have outlined the most common ones with comments as to how we have found them. Which one(s) you use are dependant on what you need to see, whether you are doing double in situ or single in situ

1. NBT/BCIP. Nitroblue tetrazolium salt + 1. 5-bromo-4-chloro-3-indolyl-phosphate

If you are performing single in situ's then perhaps the most sensitive method for detection is to use Alkaline Phosphatase (AP) labelled detection with NBT/BCIP chromogenic detection which yields a blue colour. This is a very robust developing system which covers up a multitude of sins i.e. it will allow for the one or two shortcuts that you have probably introduced that have already affected the sensitivity of your detection.

You can also dehydrate and run this colour reaction through xylene and mount in xylene based mountant for permanently mounted sections. Despite contrary opinions this colour reaction does not noticeably dissolve in Xylene. However, the colour is light sensitive and will eventually fade if left in daylight over a period of several months, but seems stable (years) if stored in the dark.

2. INT/BCIP p-Iodonitrotetrazolium violet + Nitroblue tetrazolium salt

There is another colour (chromogenic) reagent that works well with alkaline phosphatase (AP) which provides a magenta colour (INT/BCIP). The magenta colour is alcohol soluble and can be removed from your sections at any stage of the process if required (important if performing double in situ hybridizations with chromogenic colours and showing co-localisation). I would say this is almost as sensitive as NBT/BCIP, but the label sometimes seems to be comprised of little crystals which do not photograph well at high power.

3. Fast Red

Fast Red is often used as a chromogenic reaction but is actually a better fluorescent colour. Be warned not all batches of Fast Red are the same. We routinely buy from Roche as Fast red bought from other companies has given us varying results (usually by not working) order number from Roche is 11 496 549 001. Needs alkaline phosphatase (AP) for development and gives really nice red fluorescent in situ's with rhodamine excitation. If your target mRNA is really abundant it will give you a nice red chromogenic colour.

4. Fluorescent Tyramide

There are at least four fluorescent colours (Fluorescein, Rhodamine, Cyanine 3 and Cyanine 5) than can be used, all of which utilise tyramide reagents and development with Hydrogen Peroxide (HRP). The HRP causes a deposition of fluorescent molecules from the tyramide reagents around itself. This developing system is not quite as sensitive as the chromogenic reactions. This is because the Alkaline Phosphatase enzyme will continue working for over 24 hours, whereas your hydrogen peroxide becomes swamped by its reaction products and will cease to work after 10 minutes or so.

The probes you are comparing will determine the choice of combinations of colours or fluorescence that you use. Below I've described the double in situ protocols as either

using both chromogenic colours together or both fluorescent colours together, but combinations of chromogenic and fluorescent colours work fine although it would be usual to develop the fluorescence second as the chromogenic reaction product will obscure the fluorescent deposit if both probes are co-localise.

Please note I have now included what has been termed as an enhanced sensitivity detection system that will yield a colour reaction within 20 minutes, but I'm not convinced it is any better than our normal methods. To date it has given us excellent signal, but has increased the background. I've included it as it may just be the answer you are looking for in your particular system.

Immunohistochemistry after in situ hybridization

Many target antigens actually survive the in situ process. Usually abundant proteins, such as Glial Fibrillary Acidic Protein (GFAP), and Nuclear proteins (such as NeuN) can all be detected by antibody staining. It's a case of try it and see if they work. Not all do though.

Single and double in situ hybridization with Dig and/or FITC-labelled probes

For summary of the various steps please see Minimal Protocol earlier in this document. This section is intended to be a more in-depth protocol with details of all solutions used and how to make them.

For double in situ hybridizations you need two probes, one DIG labelled the other FITC labelled. Mix together in pre-warmed (65°C) hybridization buffer at 1/1000 dilution for each probe. If performing single in situs you will only use a single probe diluted 1/1000.

Place approximately 300-400µl of hybridization mixture onto each slide before placing a coverslip over the top to spread the probe mixture over the sections. The slides are cover slipped (either with oven baked at 200°C or fresh unused coverslips) and hybridized overnight at 65°C. A VERY important consideration is to not allow the hybridization mixture to dehydrate or concentrate during the hybridization period, hence the large volume of solution used and the use of a humidified chamber for the incubation. You ignore this at your peril. If your coverslips don't slip off without any help in the morning then things have dried out and this drastically decreases the final signal. We presume because the concentration will have changed in the hybridization mixture affecting the ability of the probe to bind to its target mRNA. If you find you need to soak your coverslips off, or require force after these overnight hybridizations then you have screwed up and things are now sub-optimal. If you find a batch of in situs where some have worked well, some not so well.....the most likely explanation is that some have dehydrated.

We place our slides inside a plastic box awash with what we call 65°C wash buffer which contains 50% formamide. It is very important to have formamide present as the hybridization buffer contains 50% formamide so you need formamide vapour around otherwise there is a tendency for your formamide to evaporate into the humid chamber and this will slow the binding kinetics of your RNA/RNA hybridization. I now make plastic chambers with pairs of pipettes fixed with Araldite in to rest the slides on and have the liquid sloshing around underneath them. A lid is placed on top of this and the whole lot sealed with tape before placing at 65°C overnight. It's worth making sure everything is level both on the slide supports and also in the oven. Otherwise hybridization mixture will accumulate at one end of the slide and give a gradient of signal (when developed).

We have tried doing in situs at 55°C, but got no signal at all. We have tried a range of temperatures from 65°C up to 85°C and they have all worked well. I would suggest a minimum of 65°C but it might be worth experimenting if you have a difficult to detect target.



Hybridization buffer

- 1x "salts" (DEPC-treated)
- 50% formamide (deionized)

- 0.1mg/ml yeast tRNA (RNase free, phenol/chloroform extracted)
- 10% (w/v) dextran sulphate (Molec Biol grade, RNase free)
- 1x Denhardt's (Rnase/Dnase free).

Make up a large volume (100 ml will do around 200 slides) with highest quality reagents and water (DEPC-treated) and store in aliquots at -20°. Usually add and dissolve Dextran into buffer last to give a 10% solution. It also takes a little time to go into solution.

RNase free 50 x Denhardt's is from In Vitrogen (Cat no. 750018)

Dextran Sulphate is from Sigma (Cat no. D8906), tested for suitability in nucleic acid hybridizations. Does not state RNase free

10x "salts"

- 2M NaCl
- 50mM EDTA
- 100mM Tris-HCl pH 7.5
- 50mM NaH₂PO₄·2H₂O
- 50mM Na₂HPO₄
- N.B. This must be DEPC-treated and then autoclaved to remove any traces of RNase.

Note some protocols use dilutions of 20X SSC instead of the salts. If you do use this, check it gives the same concentration of NaCl as above and make sure it is DEPC treated and hence RNase free.

How to deionize formamide

To ensure that no RNase is present we use an oven baked beaker and stirrer (at least 180°C for a minimum of 24 hours). For each 100 ml of Formamide add 10gms of Amberlite mixed bed resin (Sigma A5710) and stir slowly and gently for 1 hour (do not stir for longer than 1 hour otherwise the resin breaks up and is difficult to remove). Filter off the resin beads through a sterile Nalgene tissue culture medium filter system. Aliquot into 50ml amounts and store at -20°C. Do not thaw and re-freeze, use once and discard. The ions present in ordinary formamide can interfere with the RNA-RNA binding on your tissue sections, so it is important that this reagent is deionized. You can sometimes get away with using non-deionized formamide but batches vary widely and it is recommended that you deionize routinely. Ignore this step at your peril.

Making RNase-free tRNA by phenol/chloroform extraction

RNase-free tRNA is incredibly expensive to buy and it's relatively trivial to make it yourself. Most commercially available tRNA is NOT RNase free and it is important to remove any RNase present using phenol and chloroform extractions.

Dissolve tRNA (brewer's or baker's) into RNase free water at approx 10mg/ml. Add 1/10 volume of 3M NaAcetate pH 4.5 and extract with an equal volume of phenol and equal volume chloroform/isoamyl alcohol (49:1 v/v) mixture. Vortex and then spin at 10,000 x G for 10 minutes. Remove aqueous phase and extract once more as above and finally once with the chloroform/isoamyl alcohol mixture only. Precipitate the tRNA by adding 95%

EtOH (Made with RNase free water) at the ratio of 1:2.5 (tRNA:Alcohol v/v) and leave at -20°C for 30 minutes. Centrifuge, wash tRNA pellet in 70% EtOH, centrifuge again, remove EtOH, and allow the pellet to dry before dissolving tRNA in RNase free H₂O. Take OD reading from spectrometer at 260nm and calculate the final tRNA concentration according to the formula below. I usually dilute 1/500 for the OD reading. Dilute to 10mgs/ml and store at -20.

Working out final tRNA concentrations

For RNA, a 1µg/µl (1000µg/ml) solution has an OD of 25 at 260nm

For RNA you take the OD and multiply by 25 and then multiply by the dilution factor, This will give you the concentration of your tRNA in µg/µl.

Post-hybridization washes

- Note the careful use of RNase free solutions is now over, your RNA/RNA hybrids should have now formed and are safe from the ravages of RNase. After the overnight hybridization your coverslips should just slide off the slides and the slides can now be washed. A sign of dehydration is when you need to soak them off or they need rubbing to remove them. If this has happened your sensitivity will be compromised. I usually start my washing with three washes (approx five minutes each) in MABT at room temperature. This will remove most of the hybridization mixture before the important 65°C stringency washes. Some people omit these pre-65°C washes without any noticeable affect on the final result. Please note if you are using probes across species then the stringency (NaCl concentration) and time of these 65°C washes will need to be determined. This method is for mouse on mouse probes, chick on chick; although they do work for most rat to mouse and quail to chick probes that we have used.
- Also note that your formamide for this wash buffer does NOT need to be de-ionized.
- Wash in what we call 65°C wash buffer at 65°C for 2 x 30 minutes (can be longer) Slides take approx 5-10 minutes to reach 65°C and need to be held at this temperature for at least 2 x 10 minutes, longer is fine, but this temperature and salt concentration is very important in removing non specific binding of your probe.
- The slides are then incubated for a couple of washes for a few minutes each in MABT to remove any traces of formamide (this last step may not be strictly necessary).

MABT

100mM maleic acid
150mM NaCl
0.1% (v/v) Tween-20
pH to 7.5

65oC wash buffer

1x SSC
50% Formamide
0.1% (v/v) Tween-20

Blocking of sections

The slides are transferred to a humidified chamber (can use the same chamber used for the hybridizations but fill the bottom with water this time) and incubated in blocking solution (MABT containing and) for 1 hour at room temperature without a coverslip.

Blocking Solution

MABT

2% blocking reagent (Roche, catalogue number 1 096 176)

10% Heat Inactivated Sheep Serum.

Follow manufacturer's instructions for dissolving the blocking reagent.

Antibody binding

Choice of antibodies at this stage really depends on how you wish to develop the in situ signal, either by chromogenic methods or by fluorescent.

Either add AP conjugated anti-DIG or FITC conjugated anti-DIG Fab fragments diluted 1/1500 in blocking buffer or Horseradish peroxidase (POD) conjugated anti-Dig or Fitc conjugated anti-Dig Fab fragments diluted 1/500. Usually 500 μ l per slide and incubate overnight at 4°C. Can use shorter times at room temp, but the overnight seems to have a slight edge.

AP conjugated anti-DIG Fab fragments	Roche, catalogue number 1 093 274
AP conjugated anti-FITC Fab fragments	Roche catalogue number 1 426 338
POD conjugated anti-DIG Fab fragments	Roche, catalogue number 1 093 274
POD conjugated anti-FITC Fab fragments	Roche, catalogue number 1 207 741

First Colour Reaction

In the example below we are going to develop the DIG labelled probe first and develop the blue colour first followed by a magenta coloration for our FITC labelled probe. Remember to include appropriate controls such as omitting labelled probe from some slides, or omitting to add the AP antibody to others. This controls for specificity and is required to show that killing the activity of the AP enzyme before adding second antibodies was successful (see below).

Note, if developing the magenta colouration first be sure to remove this staining product in alcohol before doing the blue colouration as the magenta reaction product always gathers a blue colouration in the NBT/BCIP reaction. Golden rule: if co-localisation is expected use 1. magenta, 2. photograph, 3. remove magenta, 4. develop blue, 5. re-photograph. If products are expected to be discrete use 1. blue, 2. magenta, 3. photograph.

Post-antibody washes and colour reaction

A really good tip here is to make sure that whatever containers you use for developing your colour reaction in are really clean. I routinely soak glass Coplin jars or whatever in 50% bleach overnight before washing thoroughly. Once you have some clean pots, keep them that way and only use them for the colour reaction development and keep them clean. Whilst your colour reaction is developing the solution should retain a yellowish tinge to it even if left over night. If you can see the actual solution turning blue then you have dirty developing pots and this will result in high background on your sections.

- The slides are transferred to coplin jars and washed 3 x 10 minutes in MABT; then 2 x 2 minutes in pre-developing buffer pH 9.8, see details below.
- The pre-developing buffer is replaced with developing buffer pH 9.8 containing BCIP and NBT and 5% Polyvinyl alcohol (PVA). NOTE various protocols suggest

varying pH's at this point, usually above pH 9. They all work, but the pH optimum for Calf Intestinal Alkaline Phosphatase is pH 9.8. Incubate in the dark at 37°C until the signal reaches a satisfactory intensity (usually a few hours to overnight, although exceptionally they can be left over the weekend). We make up a 2x developing buffer and to 25 ml of this add 50µl of stock BCIP and 50µl of stock NBT solution, then add 25ml of 10% PVA to bring final volume to 50 ml. This is sufficient for one coplin jar. Note the amounts for BCIP and NBT are approx 3 fold less than the concentrations recommended by Roche. This has been tested and we could detect no difference in intensity or time of appearance of the colour reaction. So use less and spend less at Roche's supermarket. Also note the reason for 10% PVA solution in H₂O is due to a precipitate forming with time if pre-mixed with the salt solution (Due, we think, to the magnesium ions).

Pre-Developing Buffer

100mM Tris pH 9.8
100mM NaCl
50mM MgCl₂

2 x Developing Buffer

200mM Tris pH 9.8
200mM NaCl
100mM MgCl₂

Developing solution

100mM Tris pH 9.8
100mM NaCl
50mM MgCl₂
5% Polyvinyl Alcohol
0.11mM BCIP (approx 0.1 mg/ml)
0.12mM NBT (approx 0.1mg/ml)

N.B. Use a special Tris-pH electrode or pH paper to ensure correct pH of Tris. Your normal pH meter will pH Tris wrongly, i.e. pH 9.8 will actually be something else, usually about a pH unit out. This is an important consideration as Calf intestinal Alkaline Phosphatase's pH optimum is 9.8, although it will still work at pH's above 8, but sub-optimally.

NBT/BCIP Stocks

1000 x NBT (nitroblue tetrazolium salt) is 100mg/ml in 70% dimethylformamide- dilute 50µl's per 50 ml final volume. Roche, order no. 1 585 002.

1000 x BCIP (5-bromo-4-chloro-3-indolyl-phosphate) is 50 mg/ml in 100% dimethylformamide- dilute as above. Roche, order no. 1 383 213.

These can be bought from Roche ready dissolved; 3 ml of NBT catalogue number 1 383 221 and BCIP catalogue number 1 383 213. But this is a more expensive than making them yourself.

INT/BCIP Stocks.

INT (p- Iodonitrotetrazolium violet) from Sigma (catalogue number I8377)

BCIP p-toluidine salt from sigma (catalogue number B8503 or from Roche).

For a 133 x stock solution dissolve both of them at 33mgs/ml in Dimethyl sulphoxide.

(Dilute 75 µl per 10ml of Developing buffer

Roche sell 3mls of a ready dissolved mixture (catalogue number 1-681-4600).

10% (w/v) polyvinyl alcohol stock solution (PVA). (av. Mw ~100k) from BHD or FLUKA. We have had problems with some batches from Sigma.

The most important step (apart from having clean developing containers) is the addition of PVA, this prevents the reaction product from diffusing from the reaction site and pushes sensitivity up by approximately 10 fold. We would not consider developing the colour reaction without PVA present; it really makes a big difference. However it is a real bugger to dissolve. To make a 10% solution in water (don't make up in developing buffer as it will precipitate after a few weeks) place in a water bath or oven at 80°C, keep shaking and inverting the bottle etc for approx 3 days until you have a very viscous clear solution. The final mixing can be with a magnetic stirring bar, but only after you have "melted" the PVA in the water. It takes time, so be patient.

N.B We usually make up a solution of 2x staining buffer, add just before use add BCIP and NBT and then add an equal volume of 10% PVA in H₂O. Shake well before adding to slides and develop in the dark at 37°C for as long as required. Depending on probes this can be between 2 to 8 hours. Occasionally slides need to be left overnight.

When the blue colouration has developed to your satisfaction wash slides in water.

Examine the degree of colour reaction at regular intervals using a dissecting microscope.

NOTE: - Folds and bubbles in the sections will give an intense blue colouration. This is not a specific signal, unless these distortions coincide with areas of labelling

Note: - this blue reaction product is stable in alcohol and xylene despite several claims they will dissolve it. However, the final product is light sensitive, so if you store your developed slides in daylight the colour reaction will fade over a period of several months. If stored in the dark, the colour remains indefinitely.

If you are only performing single in situ's then NBT/BCIP is the best colour reagent to use, as it is the most sensitive. For single in situ's you can now dehydrate through an ascending alcohol series (30,50,70,90,100% EtOH) for 30 seconds each followed by 2 x 2 minutes in xylene. Slides can then be permanently mounted in a xylene based mountant such as Xam (from BDH) or Depex. However, if you wish to perform double in situ hybridizations do not dehydrate just take your slides and proceed as follows...

Killing the first AP enzyme

You need to "kill" the AP enzyme before proceeding to detect your second labeled probe. There are two methods of killing AP, heat at 65°C or acid treatment. I use a combination of both to be really sure that the first AP is killed.

1. Heat at 65°C in MABT for 30 minutes (or longer)
2. Wash 2 x in room temp MABT
3. Incubate in 0.1 M glycine-HCl pH 2.2 for 30 minutes

4. Wash in MABT

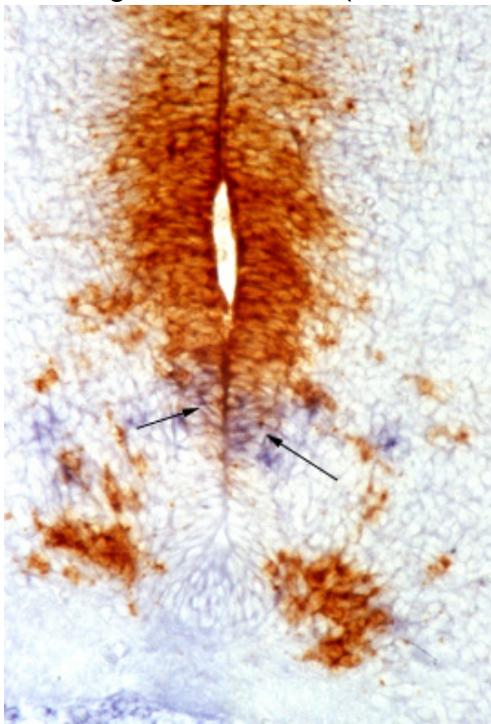
Slides are now ready for the next AP conjugated antibody.

Second Colour Reaction

Magenta:

1. Incubate sections for 1 hour in blocking solution.
2. Incubate overnight at 4°C this time in AP conjugated anti-FITC Fab fragments diluted in 1/1000 in blocking solution (or anti-dig if you used anti-FITC previously).
3. Wash extensively in MABT.
4. Wash in pre-developing buffer.
5. The pre-developing buffer is replaced with developing buffer + INT/BCIP diluted 75 µl per 10 ml. I make my own buying in INT (p-Iodonitrotetrazolium violet) from sigma (catalogue number I8377) and BCIP p-toluidine salt from sigma (catalogue number B8503) and dissolve both of them at 33mgs/ml in dimethyl sulphoxide (i.e one solution containing both reagents).
6. Place at 37°C and allow the sections to develop until a dark magenta colour forms. This usually takes from a few hours up to 24 hours depending on the signal strength of the probe used. If leaving the reaction product to develop overnight the stain often turns into a mass of crystals, which can be annoying for taking photographs at high magnification. Unfortunately I've not found a way round this problem, but changing the staining solution before leaving overnight seems to help in some, but not all, cases.
7. Wash slides before mounting with any non-alcohol/xylene mountant e.g 50% glycerol and sealing the coverslip with nail varnish.

NOTE: This magenta colour reaction is alcohol soluble so be careful. This is an advantage for doing double in situ's where the signals might overlap and you might wish to photograph the first layer before removing it and developing the next (If this is what you want then do the INT/BCIP colour reaction first, photograph remove stain by placing in ascending alcohol series (50,70,90,100% EtOH). Develop second blue colour and re-



photograph same section. This is quite tricky to do so if you can it is better to use fluorescent tyramide in situ's for co-localization. However, some mRNA's are expressed at such low levels that they are barely detectable by fluorescent in situ's' In this case you have no choice in the matter but to use the more sensitive AP chromogenic developing methods.

Example of double chromogenic In Situ

Platelet derived growth factor receptor A (PDGFRA) (Blue and arrows) amongst Pax 6 (magenta) labelled cells in a mouse E12.5 spinal cord.

Fluorescent in situ hybridization using Tyramide signal amplification (TSA).

The TSA plus fluorescent systems use Horseradish Peroxidase (HRP) to catalyze the deposition of a fluorophore labeled tyramine amplification reagent onto tissue sections or cells. The reaction is quick (less than ten minutes.... unbelievably fast!) its disadvantage is that it is not quite as sensitive as NBT/BCIP.

The kits are available from PerkinElmer Life Sciences and comprise of amplification buffer plus small tubes containing the fluorophore of choice that has to be dissolved in Dimethylsulphoxide (DMSO) before being diluting 1/100 in the amplification buffer just before use (They recommend 1/50 dilution, but 1/100 or 1/150 show no loss of intensity).

Protocol

1. The protocol is almost identical to that used for the chromogenic reactions described above. The probes used are the same, either FITC or DIG labeled.
2. The major difference is that instead of using Alkaline Phosphatase conjugated anti DIG/FITC you use HRP conjugated antibodies. Either Anti-DIG-POD (Roche, catalogue number 1-207-733) or Anti-FITC-POD (1-426-346). The technical data accompanying these reagents suggest they should be diluted 1/100-1/200. I use 1/500 routinely for both of them with no discernable loss of signal strength and it also lowers the background (although you may need to play around with these concentrations for your own particular probes).
3. Dilute the HRP conjugated anti-DIG/FITC 1/500 in blocking buffer and incubate overnight at 4°C.
4. Wash 3 x 10 minutes in PBS containing 0.1% Triton x100 (or some similar non-ionic detergent, tween 20 etc).
5. Immediately before staining mix TSA kit components together, this is recommended by the manufacturers to be used at 1/50 dilution of the fluorescent tyramide into the TSA amplification buffer provided. (dissolve small tyramide pellet in 150 µl of DMSO and store frozen....it lasts longer at -20 than at 4°C)
 - a. NOTE I use dilutions of between 1/100 and 1/150 with no discernable loss of signal. These tyramide reagents are very expensive!!
 - b. Add 200µls per slide and place a coverslip over each slide.
6. Incubate for 10 minutes at room temperature. I have found that longer incubation times (3 hours and overnight do not develop any higher signal than is achieved over the first 10 minutes (although this may be worth re-checking if you have a particularly weak probe).
7. Wash and examine under a fluorescent microscope.

TSA Amplification Buffer

0.1M Borate Buffer pH 8
+ 0.004% H₂O₂

Borate Buffer pH 7.4-9.2

Borax

(sodium tetraborate) $\text{Na}_2\text{B}_4\text{O}_7 \cdot 12\text{H}_2\text{O}$ MW = 381.37

Boric acid H_3BO_3 MW = 61.83

Make 0.1 M solutions of boric acid and borax and mix until desired pH is reached.

Add H_2O_2 and store at 4°C.

Killing HRP

Incubate slides in 3% H_2O_2 in PBS for 30 minutes at room temperature.

Wash and block with blocking solution for one hour before adding your second HRP conjugated antibody. Don't forget to include some controls where either the second probe or second antibody is omitted so as to control for specificity of the second layer colour reaction. i.e. did you really kill off the entire activity of the first enzyme! And proceed as above.

Four different TSA Plus kits are currently available from PerkinElmer Life Sciences INC currently (June 2005) they cost £993 for the 250-750 slides.

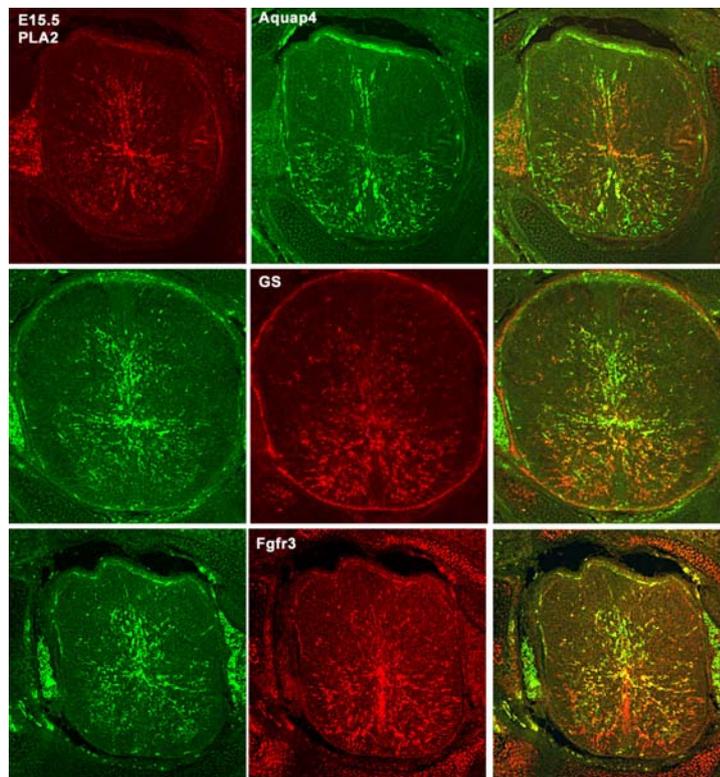
Fluorescein catalogue number NEL741 (50-150 slides) NEL741B (250-750 slides).

Rhodamine catalogue number NEL742 (50-150 slides) NEL742B (250-750 slides).

Cyanine 3 catalogue number NEL744 (50-150 slides) NEL744B (250-750 slides).

Cyanine 5 catalogue number NEL745 (50-150 slides) NEL745B (250-750 slides).

The example shown below is of E15 spinal cord combinations of PLA2 with Aquaporin 4, Glutamine Synthetase and Fgfr3 double in situ.

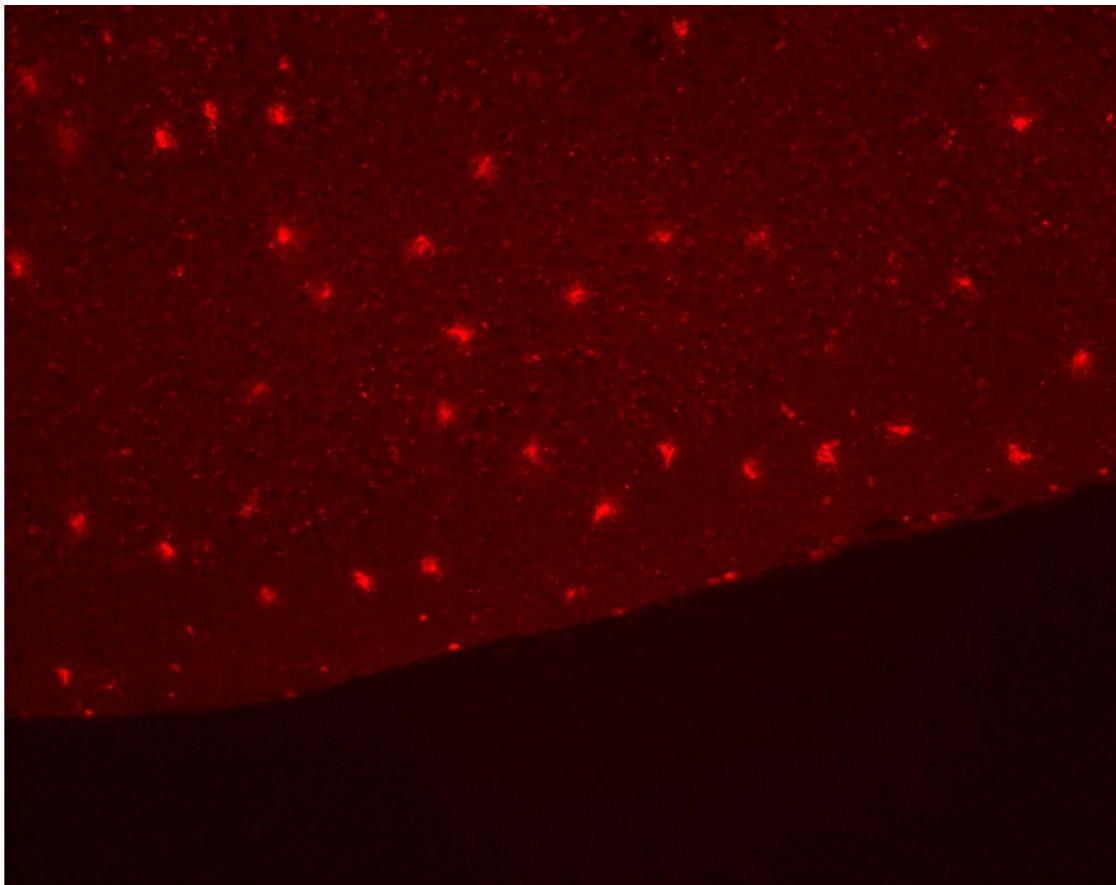


FAST RED.

Bought from Roche, catalogue number 11496549001 (20 tablets). Each tablet contains 0.5mg naphthol substrate, 2mg of Fast Red and 0.4mg of Levamisole . This blocks endogenous AP but doesn't affect the Calf Intestinal AP conjugated antibody.

In use, dissolve one tablet in 2 ml of 0.1M Tris-HCl, pH 8.2. Make fresh before use. Often find some particles remaining in solution, so filter through a small 0.22 μ M Nalgene filter or equivalent. Add to sections and allow to develop for a maximum of 30 minutes. Wash and examine under rhodamine fluorescent excitation. Or if your target mRNA is particularly abundant you will see a red coloration on the sections.

Note. We have found serious differences in the sensitivity of this protocol using different Fast Red sources. They quite often do not work, that is why we buy from Roche, as this source always works reliably.



Example of in situ hybridization developed with Fast Red. Probe is Platelet Derived Growth Factor receptor type A (PDGFRA) on adult mouse cortex. Individual cells expressing PDGFRA can easily be seen.

Improved sensitivity method.

This is based on methodology used by the Allen Institute for Brain Research, Mouse Brain Atlas, which has in situ gene expression patterns of 20,000+ mouse genes in adult mouse brains. Well worth a perusal to see if your gene of interest has already been “done”

<http://www.brain-map.org/welcome.do>

The basis of this technique is to use a peroxidase conjugated anti-Dig antibody, followed by a Biotinylated Tyramide, which deposits biotin molecules at the site of the peroxidase activity. Amplification is achieved by binding Neutravidin conjugated Alkaline Phosphatase to the biotin and results in development times of 30-40 minutes with INT/BCIP. This is supposed to give about a 100 fold amplification. In practice the developing times are improved, but in my hands results in a higher background and does not significantly improve sensitivity from our normal methods, which do of course, take longer for the colour reaction to develop.

- Bind 1/500 diluted POD conjugated anti-DIG (or FITC) overnight (or 2 hours at room temp) as per normal.
- Wash thoroughly in PBS + 0.1% Triton X-100.
- Dilute TSA-Biotin reagent 1/50 in dilution buffer supplied.
- Layer approx 200µl onto each slide and allow reaction to proceed for approx 15 minutes. Make sure the sections are covered.
- Wash extensively with PBS + 0.1% Triton X-100.
- Dilute Neutravidin 1/125 in MABT and incubate on sections for 30 minutes.
- Wash extensively.
- Develop with INT/BCIP as detailed above. Monitor colour reaction carefully, usually developed sufficiently within 30 minutes.
- Note A FITC conjugated Neutravidin is also available and could be used for fluorescent in situs.

Neutravidin-AP (£210) 2mg, catalogue number 31002 from Perbio Science UK
Neutravidin-FITC (£130) 5mg catalogue number 3106 from Perbio Science UK
TSA_Biotin System (£150) for the NEL700A (50-100 slides) from PerkinElmer (also NEL700 for 200-600 slides)

Note Neutravidin is an “improved” avidin which is supposed to give less background binding to lectins etc.

In situ hybridization on cell lines

This is our current protocol, we are trying to optimize the conditions and improvements will be added at future dates. Two important points, cells require a proteinase digestion (usually with Pepsin) and lower hybridization temperature. There is considerable scope for improving this protocol, It works, but could be further optimized.

NOTE. Until the probe has hybridized with your cells, make sure ALL solutions used to treat cells are RNase free. DEPC all water used for alcohols etc. Oven bake glassware etc.

Also include controls where no probe is added as some cells seem to be prone to giving false background positives.

1. Grow cells on glass slides/coverslips. Coverslips are more difficult to use because of the handling difficulties. The multiwell tissue culture chamber slides are ideal and allow several different treatments or experiments to be performed on one slide.
2. Wash cells in PBS then fix at room temp for 10-15 minutes in a solution of 4% paraformaldehyde in PBS (note the fixation times may need to be titrated).
3. Wash in DEPC treated PBS.
4. Dehydrate cells by incubating them sequentially in 70%, 90% and 100% Ethanol's, 2 minutes each. (Cells can be stored in 70% EtOH if required).
5. To remove residual lipids, wash cells in 100% Xylene, 2 x 5minutes each. Re-hydrate through ethanol's, 100%, 90%, 70% followed by PBS. 2 Minutes each. [NOTE, not sure how vital this dehydration and xylene steps are, some protocols omit it]
6. This is the vital step and we find that we get little or no signal if this protein digestion is omitted. You may need to vary the time of digestion to work out what is optimal for your probe to access your cells RNA. Essentially as below, but good idea to try a few different times of digestion. 10 minutes in 0.1% (w/v) pepsin in 0.1M HCL at 37°C for 10 minutes.
7. Alternatively one could consider using Proteinase K at this step, but you would need to titrate out digestion times.
8. Wash briefly in PBS (30 seconds).
9. Wash cells 4 x 2 minutes in PBS. Many protocols include a 4% paraformaldehyde fixation step at this point. We have found it decreases the signal and have omitted it.
10. Dilute DIG labeled probe 1/1000 in hybridization buffer, add 300/400 µl per slide and place coverslip over this.
11. Hybridize at 37°C overnight. (Strangely it doesn't seem to work at 65°C).
12. Wash 4x 10 minutes in 4x SSC followed by 4 x 10 minutes in MABT all at 37°C. If high background, increase temperature of washing in SSC or lower SSC concentration. We don't use this protocol routinely enough to be sure what the best washing procedure is.
13. Incubate in humid chamber in blocking solution (MABT containing 2% blocking reagent [Boehringer] and 10% heat-inactivated sheep serum) for 1 hour at room temperature without a coverslip.
14. Replace with anti-digoxigenin AP-conjugated antibody (Fab fragments; Boehringer) diluted 1:1500 in blocking solution, incubate overnight at 4°C (or with whichever antibody you wish to use to develop the signal with).

15. Transfer to coplin jars and washed 3 x 5 minutes in MABT; then 2 x 10 minutes in prestaining buffer (100mM Tris-HCl pH9, 100mM NaCl, 50mM MgCl₂).
16. The prestaining buffer is replaced with staining buffer (100mM Tris-HCl pH9, 100mM NaCl, 50mM MgCl₂, 5% (w/v) polyvinyl alcohol (av. Mw 70-100k, Sigma cat P-1763), 0.2mM 5-bromo-4-chloro-3-indolyl-phosphate (BCIP, Boehringer), 0.2mM nitroblue tetrazolium salt (NBT, Boehringer) and incubated in the dark at 37°C until the signal reaches a satisfactory intensity (usually a few hours to overnight). NOTE make up two solutions one with 100 mM MgCl₂, 200 mM Tris-HCl pH9, 200 mM NaCl and the other with 10% PVA in H₂O and mix 50:50 just before use. (MgCl₂ causes a precipitate to form with PVA over time).
17. Allow colour reaction to develop in the dark at 37°C until required level is reached. The time will vary depending on the abundance of the mRNA. Staining should normally be cytoplasmic. This is where the controls are important, as they should be clear. If they have "staining" as well may need to alter the conditions of the washes.

Note. Any real signal should be seen in the cytoplasm of the cells. Sometimes we get very blue nuclei, which are "false" positives.