In situ hybridization protocols

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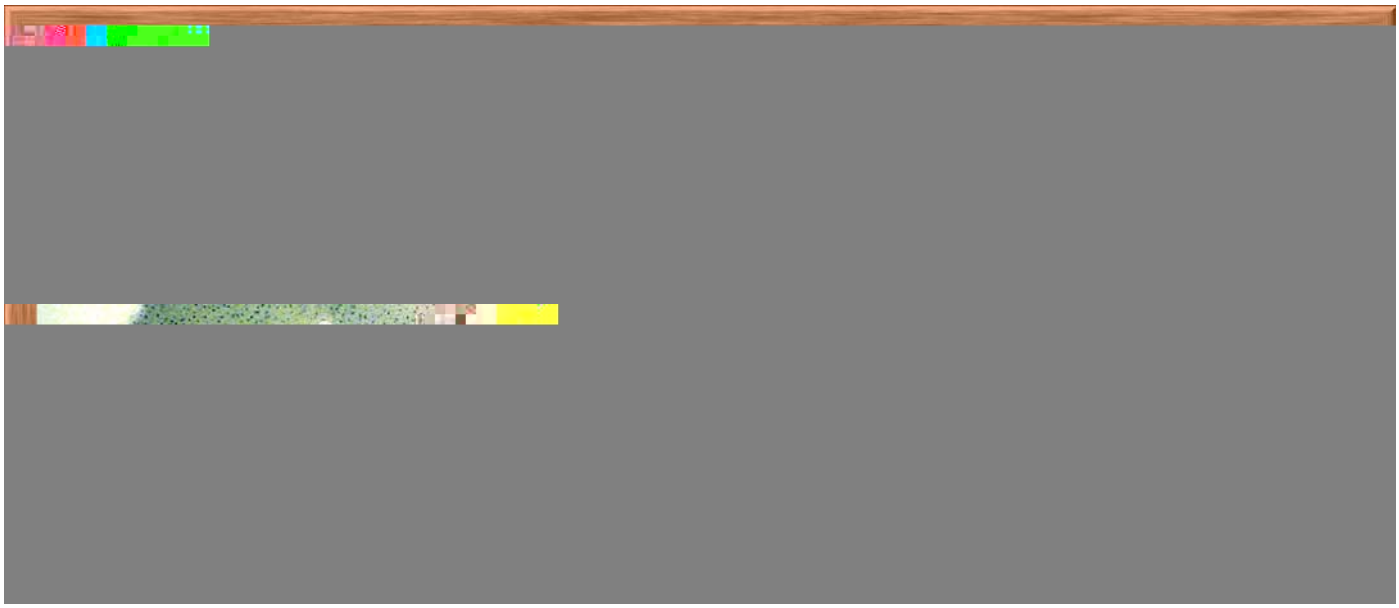

In situ hybridization showing labelling of individual cells expressing Fibroblast growth factor receptor 3 (Fgfr3) in an adult mouse brain coronal section developed with NBT/BCIP.

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,

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 µl per slide and lay a coverslip (oven baked) on top to spread the solution over the sections.
- Incubate at 65^{o}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^{o}C in 65

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

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^{o}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

Final concentrations	**Volumes of stock solutions**
3.5 mM DIG-11-UTP	25 µl DIG-11-UTP (or FITC-UTP)
10 mM ATP	7.1 µl ATP
10 mM CTP	7.1 µl CTP
10 mMGTP	7.1 µl GTP
6.5 mM UTP	4.6 µl UTP
	20.5 µl RNase free water
	Total volume 71.4 µl.

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^{o}C for most embryonic tissues. This might not be optimal for very small tissues. We dissolve paraformaldehyde in DEPC treated PBS at 65^{o}C, until the solution goes clear. There is no need to DEPC-treat the final solution as the paraformaldehyde will

Cryosectioning

Get a Sharp knife, collect sections on Superfros®Plus coated slides. A criticism of using fixed tissue versus unfixed is that fixed tiss

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^{o}C for inside chamber temperature and hold our specimens at about -16^{o}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

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^{o}

In situ detection methods, chromogenic and fluorescent

There is a choice of reagents for developing your in situs 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 situs or single in situs

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 noticeable 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 form 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.

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

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 (65oC) 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^{o}C or fresh unused coverslips) and hybridized overnight at 65^{o}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 hybridizatiByn0j-080(ed and ty-ed up h.0024 Tw 2u Tdaiili4c-0.0yn0

- O.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^{o}. 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 $NaH_2PO_4.2H_2O$
- 50mM Na_2HPO_4
- 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^{o}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^{o}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^{o}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 H_2O. 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^{o}C stringency washes. Some people omit these pre-65^{o}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^{o}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^{o}C wash buffer at 65^{o}C for 2 x 30 minutes (can be longer) Slides take approx 5-10 minutes to reach 65^{o}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 ETEMCTw 0fT50 12 56.7 1/3 Ting obbo 5 12 0 2 (

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 µl per slide and incubate overnight at 4^{o}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^{o}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 H_2O 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	**2 x Developing Buffer**
100mM Tris pH 9.8	200mM Tris pH 9.8
100mM NaCl	200mM NaCl
50mM $MgCl_2$	100mM $MgCl_2$

Developing solution
100mM Tris pH 9.8
100mM NaCl
50mM $MgCl_2$
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^{o}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 H_2O. Shake well before adding to slides and develop in the dark at 37^{o}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 "k0 1An be pe-id as fo]a024 Tw 19.89 0 Td(r, if you wish to ph1e8ifi 0 0 9eA,8lling 29.7

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^{o}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^{o}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 si

Fluorescent in s
(TSA).

Borate Buffer pH 7.4-9.2

FAST RED.

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

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" avadin which is supposed to give less background binding to lectins etc.