Non-Radioactive In Situ Hybridization to Sections (Frozen or Wax) Followed by 3c2 Immunohistochemistry
12/98 by Craig E. Nelson

Day 1

PBS	5'	
PFA	15'	4% (50ml of 20% PFA in 250ml PBS)
PBS	5'	
PK	5'	1 µg/ml (25l of 10mg/ml PK in 250ml PBS)
Glycine	5'	~2 mg/ml (~0.5g in 250ml PBS)
PBS	5'	
PFA	15'	4% in PBS
Acetic Anhydride	10'	0.25% (625l in 250ml of 0.1M TEA)
PBS	5'	
Hyb Buffer	15' @ 65°C	HybBff
Probe	ON @ 70°C	1 Cleaned Tx Rxn in 15ml of HybBff

Day 2 Color

Prewash	dip @ 65°C	Wash 1, I^{pre}
Wash I^1	15' @ 65°C	Wash 1, I^1
Wash I^2	15' @ 65°C	Wash 1, I^2
Wash I^3	15' @ 65°C	Wash 1, I^3
Wash III^1	15' @ 65°C	Wash 2, III^1
Wash III^2	15' @ 65°C	Wash 2, III^2
Wash III^3	15' @ 65°C	Wash 2, III^3
TBST Wash	3 x 10'	TBS, 1% TWEEN 20
Block	30'	TBST 1%, 5% Mixed Serum
aDig Ab	2hr @ RT or ON @ 4°C	1:2500 in TBST 1%, 1% Mixed Serum
TBST Wash	4 x 15'	TBST 1%
NTMT Wash	3 x 5'	Fresh NTM, 1%TWEEN
NBT/Xphos	From 1 to 4 days	250µg/ml NBT, 125µg/ml Xphos in NTMT

Day 3 Color

TBST Wash	3 x 15'	TBS, 0.1% TWEEN 20
Block	30'	TBST 0.1%, 5% Mixed Serum
3c2	1 hr	1:5 in TBST 0.1%, 1% Serum
TBST Wash	3 x 10'	TBST 0.1%, 1% Serum
Biotinylated aMus	30'	1:400 in TBST 0.1%, 1% Serum (Jackson Labs)
TBST Wash	3 x 10'	TBST 0.1%
Vectastain A/B	30'	10 ml TBST 0.1% + 2 drops A + 2 drops B, let stand 30'
TBST Wash	3 x 10'	TBST 0.1%
DAB Stain	1' to 10'	5ml H_2O + 2 drops Bff + 4 drops DAB + 2 drops H_2O_2
TBST Wash	3 x 10'	TBST 0.1%
H_2O	dip	
Mount		Gelvatol or Gelmount

Fluorescent Protocol

Day 1

PBS	5'	
PFA	15'	4% (50ml of 20% PFA in 250ml PBS)
PBS	5'	
PK	5'	1 µg/ml (25l of 10mg/ml PK in 250ml PBS)
Glycine	5'	~2 mg/ml (~0.5g in 250ml PBS)
PBS	5'	
PFA	15'	4% in PBS
Acetic Anhydride	10'	0.25% (625l in 250ml of 0.1M TEA)
PBS	5'	
Hyb Buffer	15' @ 65°C	HybBff
Probe	ON @ 70°C	1 Cleaned Tx Rxn in 15ml of HybBff

Day 2 Fluorescent

Prewash	dip @ 65°C	Wash 1, I^{pre}
Wash I^1	15’ @ 65°C	Wash 1, I^1
Wash I^2	15’ @ 65°C	Wash 1, I^2
Wash I^3	15’ @ 65°C	Wash 1, I^3
Wash III^1	15’ @ 65°C	Wash 2, III^1
Wash III^2	15’ @ 65°C	Wash 2, III^2
Wash III^3	15’ @ 65°C	Wash 2, III^3
TBST Wash	3 x 10’	TBS, 1% TWEEN 20
Block	30’	TBST 1%, 5% Mixed Serum
aDig Ab	2hr @ RT or ON @ 4°C	1:2500 in TBST 1%, 1% Mixed Serum
TBST Wash	4 x 15’	TBST 1%
NTMT Wash	3 x 5’	Fresh NTM, 1%TWEEN
Sigma FastRed	From 1 to 24 hours	1 tab buffer & 1 tab FR per ml H_20

Day 3 Fluorescent

TBST Wash	3 x 15’	TBST 0.1%
Block	30’	TBST 0.1%, 5% Mixed Serum
3c2	1 hr	1:5 in TBST 0.1%, 1% Serum
TBST Wash	3 x 5’	TBST 0.1%, 1% Serum
Flourescein aMus	30’	Use at dilution specified by manufacturer in TBST 0.1%, 1% Serum
TBST Wash	3 x 5’	TBST 0.1%
Alexa488 aFlour (Rabbit)	30’	Use at dilution specified by manufacturer in TBST 0.1%, 1% Serum
TBST Wash	3 x 5’	TBST 0.1%
Alexa488 aRabbit (Goat)	30’	Use at dilution specified by manufacturer in TBST 0.1%, 1% Serum
TBST Wash	3 x 5’	TBST 0.1%
H_2O	dip	
Mount		Gelvatol or Gelmount

Notes and Solutions:

All incubations that are not volume limited by reagents (expensive Alk Phos substrates and antibodies) are done in plastic staining buckets from TissueTek (Miles). These buckets use 250ml of solution and will accommodate 24 slides. I have a set of dedicated buckets for the pre-hybridization steps (5 buckets) and a set for the post-hyb washes (7 buckets).

For volume limited steps the slides are transferred to slide mailers from Ted Pella. The ones we use require 15ml of solution for each 5 slides. For even more volume limited steps incubations are done on the surface of the slide in a humid chamber. 150µl's is sufficient to cover the slide but =250µl is preferable.

All buckets and containers are rinsed thoroughly between uses.

We do not DEPC treat any of these solutions. If you are having trouble with your probe transcriptions you may want to DEPC treat the reagents you use for that reaction, but the rest of the solutions in this protocol can be made with lab quality DI water and would probably work fine with most tap water.

Probe Synthesis:

We use the Boerhinger RNA transcription reagents to run off digoxigenin labeled RNA probes. A typical rxn is done in 20l as follows:

H2O	14		
10x Bff	2		
10x Nuc Mix	1		
DNA (1g/l)	1		
RNAsin	0.5		
T3 or T7	1.5	>>>>>>>>	37oC for 2hr

I then remove the Tx rxn, add 80l of DEPC PBS and run it over a G-50 spin column. I run the flow through over another G-50 and then add the second flow through to 15 ml of HybBff. Pre heating the probe to 80 or 90°C does not seem to be necessary. I never store the probe in anything other than HybBff as I find that it is not stable in other solutions.

Day 1:

Wax Sections: The protocol as written is for cryo sections, to use it for wax sections simply dewax the slides and rehydrate then start at the beginning. Dewaxing and rehydration can be accomplished by the following steps: Xylene 2 x 10', 100% EtOH, 70%EtOH, 30%EtOH, PBS.

PBS: Stored in large quantities as a 10x stock. Make 2 liters of 1x PBS at the beginning of Day 1 and use it to prep the following solutions. I re-use the PBS rinses except for the last one which is fresh.

PFA: We use this as 4% paraformaldehyde in PBS. I typically dilute a 20% stock stored in 50ml aliquots at -20°C.

PK: We store 10mg/ml (1,000x) aliquots of Proteinase K at -20°C. If I were making stocks just for slice in situ's I would make 250 ml aliquots of 100x PK.

Glycine: I use =2 mg/ml. Typically I dump ~1g into 250 ml of PBS.

Acetic Anhydride: Many protocols call for constant stirring for this step. I find that mixing the acetic anhydride with the TEA buffer immediately prior to the incubation is sufficient and much easier. I do this by adding 625µl of acetic anhydride to 250 ml of 0.1M TEA in a 500 ml bottle, cap and shake vigorously, pour into bucket and add slides.

TEA: Triethanolamine buffer is made with powdered triethanolamine into a 1M (10x) stock.

Hyb Buffer: This recipe is from our whole mount protocol and has not been modified for the section in situs. We make this up in quantity and store it at -20°C.

50% Formamide (high quality, deionized)
5xSSC, pH 4.5 (pH with citric acid)
50 µg/ml yeast RNA
1% SDS
50 µg/ml Heparin

Day 2

The washes can be stored in amber bottles at room temperature and re-used indefinitely. High quality, deionized formamide need not be used to make up these washes, cheaper bulk formamide is adequate.

All of the washes are poured out at once into dedicated staining dishes. The first two are microwaved for 2 minutes (1min per 250ml in our microwave brings the solution to ~65°C) while the remainder are put into a 65°C water bath to come up to temp.

Wash 1:
50% Formamide
5x SSC, pH 4.5
1% SDS

Wash 2:
50% Formamide

2x SSC, pH 4.5

TBS: Dilute 10x to 1x and add TWEEN-20 to 1%. Make 2 liters.

10x TBS:

	for 1l
1.4 M NaCl	80g
27 mM KCl	2g
250 mM Tris, pH 7.5	250 ml of 1M Tris 7.5

Mixed serum: To block I use mixed serum from as many of the animals involved in the experiment as I can. This generally includes, chick , goat, rabbit, donkey, sheep and calf serums mixed in nonspecific quantities. Blocking solution and Ab incubation solutions are mixed to the appropriate concentrations of serum (5% to block and 1% to stain) and then filtered prior to use. If serum is precious a 5% solution can be stored at -20oC, thawed filtered and re-used as block and as a stock for the 1% sol'n. You will need 250 ml of 5% block.

aDig Ab: I use the BoeringerMannheim Ab at 2500x. I do these incubations in slide mailers that require 15ml of sol'n for every 5 slides. Typically I make up 60 ml of 1x Ab using 24µl of Ab (enough for 20 slides). The slides can stay in these slide mailers through the subsequent washes and, if your washes are good, can also be used for the color reaction.

NTMT: This buffer will acidify and precipitate over time so it is made fresh prior to each use. Make 1 liter to wash in TissueTek buckets and 250ml to wash in slide mailers.

	1 liter	250ml
100 mM NaCl	20ml of 5M NaCl	5ml
100 mM Tris, pH 9.5	100ml of 1M Tris 9.5	20ml
50mM $MgCl_2$	50ml of 1M $MgCl_2$	10ml
1% TWEEN-20	50ml of 20% TWEEN	10ml

NBT/XPhos: If more than 1 day of development is desired it is advisable to provide fresh 1x substrate each day to avoid the formation of large, ugly, insoluble crystals.

200x NBT: 50 mg/ml in dimethylformamide. Store aliquots at -20°C.

200x XPhos: 25 mg/ml in H_20 for Na salt or DMF for toluidine salt. Store as above.

1x reaction mix: dilute stocks to 1x in NTMT

Sigma Fast Red: To get a bright red fluorescent precipitate I use SigmaFast FastRed TR/Napthol AS-MX Tablet sets, product # F4648. A good signal will give a good brightfield red color and a very sensitive fluorescent red signal. I use this stuff at 200l/slide. Most of the reaction is typically complete after ~1hr but I

typically coverslip the slides and leave them overnight. This gives me better sensitivity without significant background but some minor loss of signal resolution at high mags.

Day 3

Vectastain A/B and DAB: We use Vectastain kits for these steps: Vectastain ABC *Elite* (#PK-6100) and Peroxidase Substrate Kit DAB (#SK-4100).

Flourescein aMus: This is a Jackson ImmunoResearch Antibody, code#: 715-095-150

Alexa488 aFlour (Rabbit) & Alexa488 aRabbit (Goat): These are from Molecular Probes Alexa488 Signal-Amplification Kit for Flourescein Conjugated Probes (A110-53). The Alexa488 moiety has very similar spectra to FITC but is supposed to be more intense and can be used with FITC filters. Wash the slides well between these steps as background will accumulate.

When Things Don't Work:

The first thing to replace when things don't work is the probe. If stored in HybBff at -20°C probes seem to be stable indefinitely. If your probe does not work the first time, redo your Tx rxn with clean solutions (DEPC) and check the product on a gel. If a probe stops working either just replace it with newly transcribed probe or try heating it to 80°C for 30' to relieve secondary structure that may have formed during storage. I have reused probes over ten times with no apparent dimunition in signal or increase in background. I simply store them at -20°C when not in use and take them out to 70°C for the hybridization.

The second thing to replace is the aDig Ab. This can go off leaving you with only the strongest signals or no signals at all. My feeling is that the antibody is quite stable but that public tubes are occasionally mistreated and lose their activity. Similarly, all of the antibodies used in the protocol are subject to going off so it pays to take good care of them and to use tested and trusted stocks.

If your reactions still don't work after replacing the probe and the aDig Ab, get some control probes and sections from a friend, replace all of your solutions and start again.