CHAPTER 11

In Situ Hybridization

IN SITU HYBRIDIZATION NOTES

In situ hybridization (ISH) is a means of identifying where mRNAs are present in fixed tissue samples; as IHC identified proteins in fixed tissue, ISH identifies mRNA. ISH is performed by designing an antisense probe to your mRNA target, allowing your probe and mRNA to bind, and visualizing where your probe is in the tissue slice. *In situ* is Latin for "in location," and so you could consider ISH and IHC to be *in situ* techniques. ISH and IHC can be run together in the same tissue sample as an elegant control to show that your ISH probe and your IHC antibody are specific, but the two techniques have different requirements and so this control is difficult to pull off. Many online guides to ISH exist, but one good resource is from Dr. Wilcox,[28] and another is from Dr. Tom Houpt on his web site, the MagnetoWiki.

To get an ISH probe, you can design it much the same way as a PCR primer, except you're only interested in the antisense probe. For ISH, you can use cDNA probes, oligonucleotide probes, or RNA probes. RNA probes are reputed to be the best, followed by oligos, followed by cDNA. RNA probes are typically generated by introducing plasmids into bacterial cultures (like *Escherichia coli*) and purifying the probe. They are very sensitive (in every sense of the word) and require everything to be RNase free at every step before probe binding (after binding, the mRNA/probe is considered by RNases to be double stranded and therefore not a target). Oligonucleotide and cDNA probes are not as sensitive but don't come with the "everything must be RNase free and why won't you cater to my every whim" mentality that RNA probes require. Oligonucleotide probes are typically ordered from companies, unless you have a nucleic acid synthesis machine available. cDNA probes are generated from PCR; the PCR product is followed by asymmetric PCR (differing concentrations of forward and reverse primers) to generate a concentrated amount of probe.

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Types of ISH Probe Labeling

Once you have your probes, you must decide how to acquire a signal. Radioactive probes are the most sensitive, but nonradioactive methods have been making great strides in recent years. For radioactive probes, you have a couple of choices: ^{35}S, ^{125}I, ^{32}P, and some others. The iodine and phosphorus isotopes are "hotter" and have higher reactivity and shorter half-lives than the sulfur; sulfur seems to be the best to use unless you have a low abundance target, in which case the room is divided—some prefer to use hotter probes, others contend that sulfur is really just the best overall. Nonradioactive probes are currently split between biotinylated probes, digoxigenin (DIG) probes, and tyramide probes. Biotinylated probes are amplified using an ABC kit or streptavidin, similarly to IHC, though you can also use an anti-biotin antibody and a secondary antibody to amplify the signal. Tyramide probes are amplified using, unsurprisingly, a tyramide amplification kit. DIG probes are amplified using anti-DIG antibodies and other antibodies, if necessary. The draw of DIG probes is that DIG itself is found in plants, and so you should have zero background in your animal tissue from the anti-DIG antibodies. Biotin and tyramide are pursued because, while not as sensitive as radiation, you can still get quite a lot of amplification out of those probes. According to Dr. Wilcox, RNA probes are more sensitive than oligonucleotide probes, which are more sensitive than cDNA probes. Additionally, he claims that ^{35}S is more sensitive or equal to ^{33}P, which is more sensitive than ^{32}P, which is more sensitive than biotin and DIG. He also says that frozen tissue works better than paraffinized tissue. Your mileage may vary, but this is a good start.

You can order probes with these signal-generating molecules attached, or you can attach them yourself using different kits. Either way is costly. Be aware that some kits and some companies will attach molecules via a dideoxynucleotide base; this means you can attach only one molecule, and your signal/noise better be awesome for you to get anything for your time. It is better to have a regular nucleotide, so that you can add many signal molecules to your probe. If you use multiple types of signal molecule, you can perform multiplex ISH, using a combination of different probes (biotin, DIG, fluorescein) and antibodies (anti-biotin, anti-DIG, anti-fluorescein) to generate signals (or different fluorescent molecules on the probes, but this doesn't allow as much amplification or long probe storage). As with fluorescent

immunoblot or IHC, make sure that your fluorophores emit nonoverlapping wavelengths of light. For one protocol, see Ishii et al.[29]

Visualizing Your ISH

The process of visualizing your data depends on what kind of probe you use. For radioactive probes, you can submit your tissue to liquid emulsion followed by photography, or autoradiography film. Use of the film has the same issues here that it does for immunoblotting; you need a minimum amount of signal to get the silver grains to react, and there is a point where the film can't detect any further changes. For nonradioactive probes, you have many of the same options you have for IHC: fluorescence and colorimetric staining. Colorimetric stains require that you include an enzyme at some point in your experiment, whether conjugated to streptavidin or a secondary antibody (I've not heard of someone attaching HRP or AP directly to their probe, and you won't get much amplification that way). For fluorescence, you can attach a fluorophore to your probe, streptavidin, or secondary antibody. Afterward, you can visualize your colorimetric or fluorescent ISH reactions using the same methods of microscopy that you used for IHC.

ISH PROBE DESIGN FOR OLIGONUCLEOTIDE AND PCR PROBES

Before performing this step, check the literature and online databases to make sure that someone else hasn't developed a working probe already.

1. Using the BLAST algorithm from ensembl.org or NCBI, enter your sequence of interest.
2. Select "Blast against cDNAs" and "Allow some local mismatch." Look at the generated schematic to identify areas of the cDNA that do not also correspond to other parts of the genome.
3. Find a part of the diagram that has only a single bar, click on the Alignment tab, and make sure it's your gene of interest.
4. The number of bases for your probe depends on what kind of probe you want to design. Oligonucleotide probes can range from 18–50 bases, while PCR-generated probes are much larger (hundreds of bases).

5. Submit your chosen amplicon as a BLAST query to make sure it doesn't bind any other mRNAs. You can, at this step, determine if your probe will bind splice variants and the like. If mRNAs other than your target come up, start over at a different site on the cDNA.

ISH PROTOCOL (DEVELOPED FROM TWO SOURCES[30,31])

1. Cut brain sections fresh or remove them from cryoprotectant. Wash in ice-cold 2× saline-sodium citrate buffer (SSC). If from cryoprotectant, wash three times for 10 min each in 2× SSC or RNase-free PBS (see Chapter 10; RNase-free PBS is only necessary for RNA probes).

   I have used samples from both cryoprotectant and samples from fresh-cut sections. I prefer cryoprotectant, because I can use fewer slices than fresh-cut samples (or else I'm splitting between cryoprotectant and experimental slices and can't save them for later); and I know the tissues are safe in the freezer, while I'm less sure of samples sitting in sucrose solution. Typically, these washes are performed in scintillation vials, with an excess of solution (except prehybridization buffer) to properly wash tissue. The pipetting of solutions using a Pasteur pipette is what really makes this technique take a lot of time. Just make sure you wash in an excess of fluid. Additionally, some researchers use Proteinase K to "free" the mRNA for reaction. Some swear that it works; some swear that it's a waste of time. I avoid proteinases because I've not found them necessary and incubating too long can destroy your tissue!

2. If tissues were fixed with acrolein or glutaraldehyde, incubate tissue for 15 min in 1% sodium borohydride and wash in RNase-free PBS.

   Note that this is only important if you are using HRP to visualize the reaction.

3. Optional: to increase some probe signals, tissue can be incubated in 0.5% Triton X-100 solution at 4°C overnight.
4. Optional: if you are using RNA probes, rinse twice in 0.1 M triethanolamine (TEA; inhibits RNases).
5. Optional: if you are using RNA probes, incubate slices in 0.25% acetic anhydride in 0.1 M TEA for 10 min, followed by three 10 min rinses in 2× SSC.
6. Block in prehybridization buffer for 2 h at 37°C.

7. Add labeled probe to prehybridization buffer (no need to switch in fresh solution) and leave overnight at 37°C.

   Make sure to perform probe titrations as you do with antibodies. You should note that different protocols specify different heats for hybridization, with some as high at 70°C. This may not work well, as high heat can induce artifacts.[32] Determine which heat works well for your experiment. I prefer 37°C, as that is the body temperature of the animal and therefore should work just fine.

8. Rinse sequentially in 2× SSC, 1× SSC, and 0.5× SSC for 10 min each, 37°C.
9. Optional: if using RNA probes, incubate in RNase for 30 min at 37°C.

   This step eliminates any excess probe that did not bind a target, as your RNA probe is, without binding, single stranded and thus a target for RNase. Bound probe-target complexes are double stranded and not targets for RNase.

If using nonradioactive probes, begin staining methods (ABC kit for biotin, anti-DIG antibodies, for example). If using radioactivity, mount on slides, air dry, and expose to X-ray film or emulsion.

ISH CONTROLS[23,33]

Controls for ISH are a little less clear than other techniques. While positive and negative controls are still beneficial, there is a history of using the "sense" strand as a control. The appeal is that it sounds like a control, but it really isn't. The idea is that your sense strand shouldn't bind to anything and is therefore a signal of general, nonspecific binding. What you're really testing there, however, is whether your sense strand is antisense to anything else. As such, the sense control is really meaningless. What should replace it? A "hot/cold" control (named for the slang denoting radioactivity). The idea of a hot/cold control is that there are only so many mRNA strands your probes can bind to; anything extra is nonspecific binding. So you apply an excess of unlabeled ("cold") probe and your typical amount of labeled probe ("hot"). If you see nothing, the cold probe displaced the hot probe at binding sites, and there was nothing left to bind to. If you see

"staining," then your probe binds nonspecifically (perhaps specifically as well, but that's cold comfort to you; enjoy the pun).

1. *Positive controls*: as ever, including samples where your target should show up are helpful to determine if there are issues with your fixation or your tissues.
2. *Negative controls*: as ever, including samples where your target mRNA is absent can be helpful to determine specificity of the probe.
3. *Hot/cold probe competition control*: as explained above, tests specificity of the probe.
4. *Multiple probes*: similar to using multiple antibodies, multiple probes against different parts of the mRNA can be compared. If they show similar staining, that suggests that the probes are specific and targeting the same mRNA. Combine with positive and negative controls.
5. *Colocalization with protein*: combining IHC and ISH for the same target can make for a very convincing control. It can be difficult to perform, however, as the heat and chemicals required for hybridization can be incompatible with optimal conditions for IHC.
6. *Northern blots*: another sort of specificity test, though I have no experience with it.
7. *Poly$_{d(T)}$ control*: an offshoot of the normal positive control, poly$_{d(T)}$ binds mRNA tails (all of them, so expect a lot of background staining), and so tests whether you have introduced mRNA degradation to your tissue samples.
8. *Reference gene control*: an offshoot of the normal positive control, this directly tests whether you have introduced mRNA degradation to your tissue samples. This uses a new set of probes but can be very helpful to establish how damaged or undamaged your tissue is.
9. *RNase control*: suggests that your probes are only binding RNA, but this isn't necessarily a great control. RNAs, like proteins, are fixed in three dimensions by your fixative agent (paraformaldehyde, for instance, generates disulfide and other bonds in both proteins and mRNA), and so RNases are a danger to RNA probes but not necessarily to fixed RNA in the tissue.

SOLUTION RECIPES

20× SSC

NaCl	87.65 g
Citric acid	441 g
DEPC-H_2O	To 500 ml

pH to 7.0, autoclave, bring to 500 ml.

Nuclease-free H_2O

See RNA extraction section.

RNase-free PBS

Make 1× or 5× PBS as in the immunohistochemistry section, but add 1 ml DEPC and autoclave.

0.1M TEA

Triethanolamine (TEA)	1.49 g
DEPC-H_2O	To 100 ml

pH to 8.0.

0.5% Triton X-100

1 M Tris pH 8.0	5 ml
0.5 M EDTA pH 8.0	5 ml
Triton X-100	250 µl
DEPC-H_2O	To 50 ml

Prehybridization buffer

Formamide	60%
Tris pH 7.4	0.02 M
EDTA	1 mM
Dextran sulfate	10%
Ficoll	0.8%
PVP-40	0.8%
BSA	0.8%
2× SSC	To volume
Dithiothreitol	0.1 M
Herring sperm DNA	1.6 mg/ml

1 ml of prehybridization buffer per vial. Ficoll, PVP, and BSA are together in Denhardt's solution. Tris, EDTA, and dextran can be combined into a TED stock solution.

0.25% acetic anhydride

Acetic anhydride	10 µl
0.1M TEA	To 4 ml