HISTOLOGY-RELATED PROTOCOLS

H1: Fixation Issues and Frozen Block Generation

Books are written on this subject, and opinions are many. One major issue that people agree on is that either poor fixation or overfixation can be absolutely detrimental to a histological technique. Various fixatives also differ a lot in how they fix the tissue, and whether they are irreversible or not. The major fixative used in histology labs are 10% neutral buffered formalin. This can be bought as is, and has a very long shelf life at room temperature. For most techniques this is adequate. We routinely use freshly prepared, or frozen stocks, of 4% paraformaldehyde – a very similar fixative. Fixation reactions with aldehydes are progressive, so the longer in fix, the stronger the fixation. Formaldehyde fixation is not irreversible. We believe the most important aspect of fixation is to keep things constant: for that reason we always fix overnight – no less, no more. At least with this decision, the tissue, even between experiments, should display some degree of consistency, which is the most critical aspect when comparing various tissues.

Safety issues: Remember to use gloves while working with formaldehyde – it is carcinogenic. Vapors are generated; so keep it to the widest extent possible in a hood. Dispose of formaldehyde using a dedicated waste container – never in the sink.

Embedding Tissue Into Frozen Blocks

1. Isolate the tissue in cold 1xPBS. *Keep cold, especially if you plan to do in situ hybridization of the sections later.*
2. Transfer the tissue to freshly thawed 4% PFA in PBS for 4 hours-overnight (not longer than overnight) at 4ºc (you can make 4% PFA, aliquot, and store at –20c)
3. Transfer tissue to 30% sucrose in PBS solution until the tissue sinks to the bottom of the tube (a couple of hours) at 4ºc. *Sucrose is needed to cryopreserve the tissue*.
4. Remove half the volume, add 30% OCT making a 1:1 30%sucrose/OCT solution for 1-2 hours at 4ºc. *This step facilitates OCT penetration.*
5. Transfer tissue to 100% OCT for 1 hour at 4ºc.

Embed tissue into blocks as follows:

1. Label plastic mold with what the tissue information. *Use a permanent ink pen. If you label later, the mold is cold, and you cannot write on it.*
2. Place the plastic mold onto a piece of dry ice and apply a layer of OCT into the mold. It will turn white as it freezes. While it freezes, place the tissue in the middle of the block and place a layer of OCT to cover tissue. Be careful not to make any bubbles in the OCT. This can cause minor problems with the cutting later. Orient the tissue, if needed, such as early embryos. *Remember, it is possible to embed multiple tissues in the same block, to save cutting time later.*

When all of the OCT is white place the tissue (still in the plastic mold!) in the –80ºc freezer immediately. Store in a plastic bag, sealed, or use a box with desiccant.

H2: Fixing Cells with Fresh Paraformaldehyde

Hazards: Carcinogenic at low doses. Fumes and contact are dangerous.

Protection: wear gloves when handling formaldehyde. Wear mask and goggles when preparing from powder. Mix in chemical waste hood.

Waste: Discard formaldehyde by emptying into hazardous waste container. Store in chemical waste hood until picked up by waste disposal.

Spill clean-up: refer to MSDS. Any spill over 25 µl is considered hazardous.

Storage: Store powder on chemical shelf. Store liquids in opaque containers at 4ºC. Use within five days. Label all containers as carcinogenic.

Procedure

For up to $5x10^6$ cells:

1. Paraformaldehyde preparation:

    A) From 16% sealed vials. Break vial, dilute to desired concentration using 10x PBS and ddH2O to a final concentration of 1xPBS. (To make 12 ml of a 1% solution, add 750 µl 16% PFA to 1.2 ml 10xPBS to 10.05 ml ddH2O.
    B) Make 4% solution from powder: Add 4 g of PFA to 96 ml PBS in 250 ml beaker. Place on hot plate set at approx 4.5 (don't bring temp above 80ºC) with stirrer at 2.5. Mix for approx 30 min. Cool
    C) Store in opaque container at 4ºC. Use within five days. Otherwise will decompose.
3. Start with a cell pellet
4. Resuspend pellet in 1 - 4 % PFA (either 200 µl in 96 well-plates, or 1 ml in eppendorf tubes).
5. Let sit 20 min at RT.
6. Pellet cells and wash with 1x PBS. These cells can be embedded in agar (see below) and prepared for staining.

H3: Preparation of Frozen Sections

After the embedding, and possible storage, you will need to cut sections. This is a skill that is mastered over time, and although it is not difficult, certain problems can arise. Frozen sections are prepared on a cryotome, which consists of a movable holder for the tissue, and a fixed knife – all cooled down to between –15°c-20°c. To mount the tissue block on the specimen holder, add a little OCT on top of the metal holder inside the cryotome, and as it starts to freeze, pop out the frozen block containing your tissue and position flatly on the OCT. This will glue your tissue onto the specimen holder. You can, although it is not necessary, trim the edges of the block with a scalpel. We normally do not do that, as it may give problems while sectioning. Then, mount the specimen in the machine, and start sectioning. Start out by 20 um sections, trimming until you see tissue. If the sections do not flatten or have cracks/splitting, adjust either the: 1. Angle of sectioning, or 2: speed of movement, or 3: position of the knife (you may have a crack in the knife edge), or 4: the position of the anti-roll plate. Maybe you have to try out all to get good results. When you start picking up sections, adjust the section thickness to 7-8 µm

(others make 3-4 μm sections with success), and position as many as you want on the slide (we normally take two), by moving the slide down onto the section lying on the knife. It will attach immediately to the slide. Use Superfrost-type slides, as if the slides have not been pretreated to bind tissue, you may loose it during the histological staining technique later. Between sections, clean the knife for OCT traces. You may be able to get sections without the use of the anti-roll plate. Label your slides as you prefer, but at least with a section number, and ID of the tissue block. Use a marker pen certified for histological use – any other might fail to be visible during the later steps while staining. Having taken the sufficient number of slides for your experiment (always take more than you think you will need – as it generally turns out that you will need more later…), then store the sections at -80°c. We use slide containers for 100 slides, which are nicely labeled, and the container is stored in the -80°c freezer inside a zip-lock bag containing dessicant. Remember that too much moisture/ice will not make your sections look nicer when you are going to stain them later.

H4: Immunohistochemistry

There is a multitude of ways of performing immunohistochemistry. We do not want to go into detail with this here, and there are many more than listed here. Generally, we split up IHC into three main types of stainings, which are:

1. Single staining, enzymatic. (Figure 1)
2. Double or triple, staining, fluorescent, non-amplified. (Figure 2)
3. Amplified staining with Tyramide conjugates. (Figure 1 and 3)

Each of the above serves individual purposes. The single staining is generally used to evaluate if an antibody works in immunohistochemistry. It is also routinely used if only a single antigen is to be detected, and with presentation quality of lesser importance. To do this, we take advantage of a very robust and inexpensive kit-solution offered by Zymed. This is called Histostain SP; SP is abbreviated for streptavidin-peroxidase. You will need a specific kit reacting to your primary antibody (either mouse or rabbit). The outline of this method is to detect your primary antibody with a biotin-conjugated secondary antibody, which is subsequently bound by streptavidin-peroxidase. A color reaction is then performed with the Peroxidase that is present at the sites where your antigen is located. A bright red precipitate is the result if you use the AEC substrate mix (which we prefer), or a dark brown-red precipitate if you use DAB (di-amino-benzidine, which we do not recommend, as this does not provide clarity next to a hematoxylin counterstain). The typical HRP application is as follows: (Figure 1)

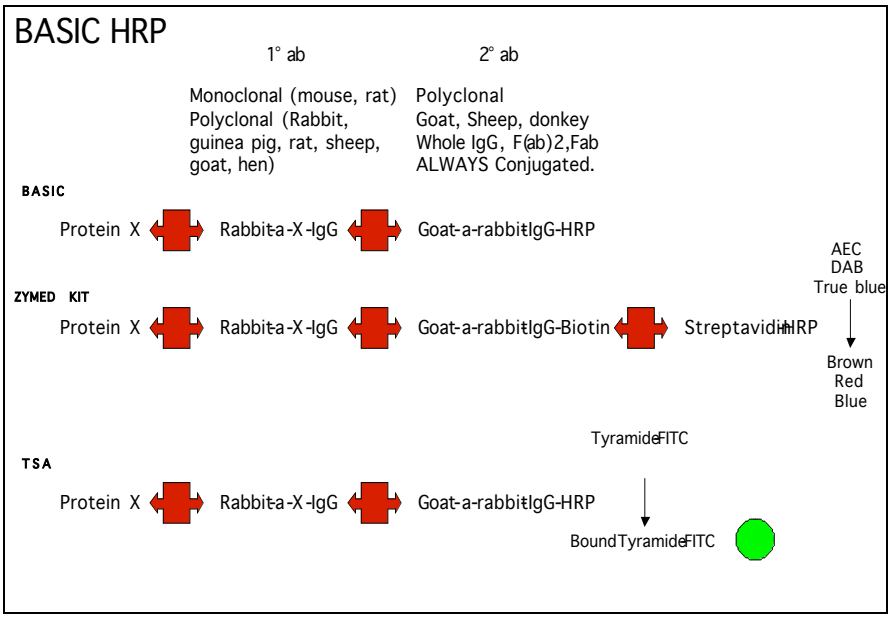


FIGURE 1. Overview of stainings involving Horseradish-peroxidase.

Alternatively, a very useful method is to perform triple immunofluorescence. Needless to say, information about co-localization is exactly what most people need to know, and for that reason, immunofluorescence is the only way to go. Below (Figure 2) is a schematic drawing of a setup of a quadruple immunofluorescence technique. Normally, one is limited by the number of different species primary antibodies are available from. Antibody concentrations are normally adjusted upward, so a rule of thumb is to use 5-10 fold more primary antibody.

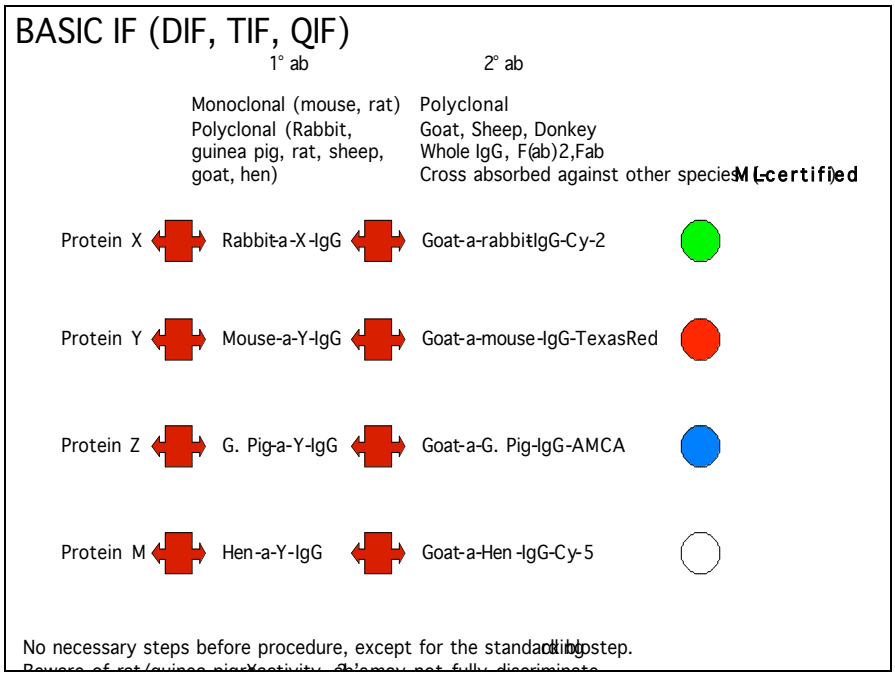


FIGURE 2. Overview of a quadruple immunofluorescence staining.

Lastly, it is possible to combine both HRP staining and immunofluorescence – and take advantage of amplifying one fluorescent signal. Below (figure 3) is an example of TSA amplification of a rabbit antibody, together visualized with red and blue immunofluorescence staining against two other primary antibodies from different species (Figure 3). Decrease the antibody concentration for the one that is being amplified, and maintain the 5-10 fold increase for the other two, as normally used in immunofluorescence (example 2)

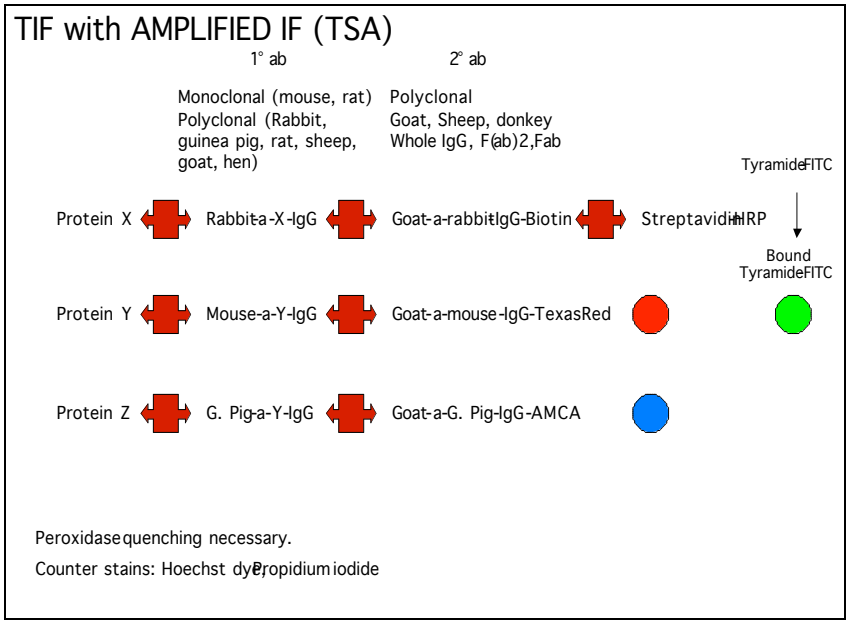


FIGURE 3. Overview of a staining built around one antibody visualized using TSA amplification, together with two others detected by normal immunofluorescence.PRE-STAINING MANIPULATIONS:

Irrespectively whether you are doing a HRP stain, immunofluorescence, or TSA-amplification, you will need to pre-treat your slides prior to adding antibodies. In general, the same steps are needed for all staining types (except for Peroxidase-blocking during non-amplified immunofluorescence, during which it is unnecessary).

Frozen sections

Frozen sections (8 µm thick), are taken out of freezer (- 80 °C). The sections can either be dried (30 minutes at 37°c), or post-fixed 15 min. in 1% Paraformaldehyde in 0.1M Phosphate buffer pH=7.4 before the staining, depending on the antigen to detect.

Paraffin sections

Paraffin sections (4µm thick) are de-waxed in following solutions left for 5 min. in each jar
3 X Histoclear.
3 X 99% Alcohol
1 X 96% -
1 X 70% -

Quenching of endogenous peroxidase in 1% H_2O_2 dist. water for 30 min., or in 3% H_2O_2 in dist. water for 5 min. Rinse in dist. water.

From here the sections could be stained directly (some do, but we never do) or they could be treated with enzymes or heated in a microwave oven (always performed).

Microwave treatment

In order to detect some antigens microwave-treatment of paraffin sections is required. It is important to standardize the conditions to be able to reproduce the results.

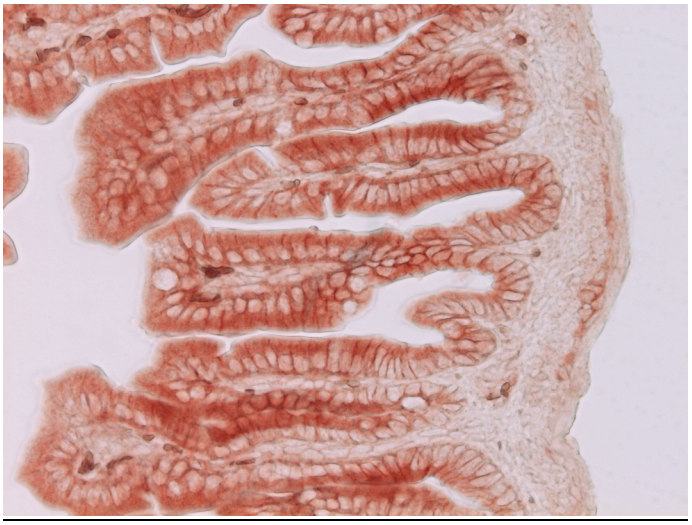
Rinse the sections in dist. water before soaking them in 200 ml of 0.01M Citrate buffer pH 6.0 in a glass or plastic jar. Microwave at 600 Watts for 2 x 5 min. The liquid will boil. Refill the jar with 40 ml. distilled water between the two treatments. After the second treatment sections are left for 20-30 min. in the buffer for cooling. And yes – it is possible to microwave frozen sections, if these have been dried for 30 minutes at 37°c.

Once the selected procedure has started the sections must never dry out. This could result in non-specific staining.

All incubation steps are carried out in a humidified chamber at room temperature.
Optimal concentrations of primary antibodies are dependent on sample preparation, antibody affinity, and amount of antigen and should be determined by the investigator.

H5: Zymed Staining Procedure

This procedure is chosen from the protocol Zymed provides with the Histostain-SP BULK^{TM} -kit.



After pre-treatment of the sections:

1. Rinse 3 X in PBS.
   If more sections on a single slide need to be stained with different antibodies, draw a hydrophobic circle around the sections with a PAP-pen (Zymed)

2. Apply SERUM BLOCKING SOLUTION (bottle 1A, this is a 10% non-immune goat serum). Incubate 10 min. Remove the solution by tapping the slide on a paper towel.

3. Apply 50-100µl primary antibody diluted in PBS supplemented with 0.25% BSA. Incubate for 60 min.

   To detect nuclear localized antigens primary antibody is diluted in PBS supplemented with 0.25% BSA and 0.3% Triton X-100. Incubate overnight.

4. Rinse with PBS 3 X 5 min.

5. Apply BIOTINYLATED SECOND ANTIBODY (bottle 1B). Incubate for 15 min.

6. Rinse in PBS 3X 5 min.

7. Apply STREPTAVIDIN- PEROXIDASE CONJUGATE (bottle 2). Incubate for 10 min.

8. Rinse in PBS 3X 5 min

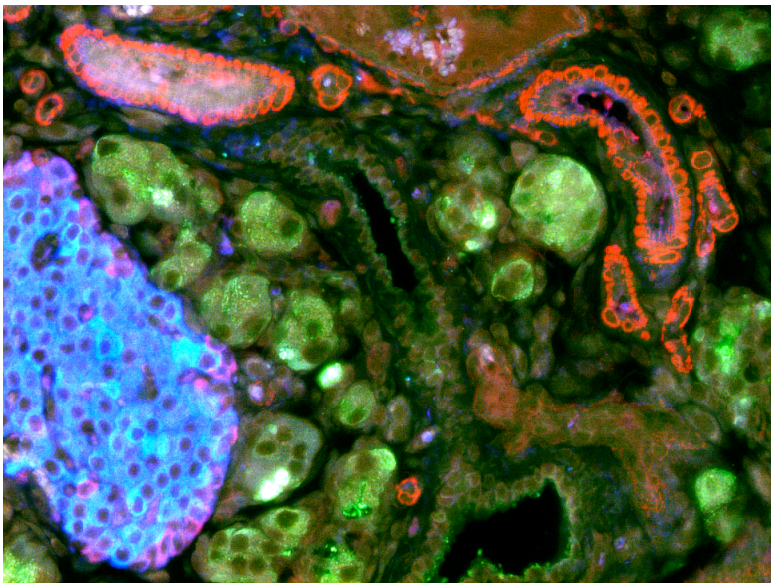
9. Apply chromogen solution: AEC SUBSTRATE - kit (Zymed no.00-2007). Prepare 1 drop Citrate buffer (A) + 1 drop Amino - Ethyl -Carbazole in DMF + 1 drop Hydrogen Peroxide in 1 ml. dist. Water. Incubate 10 min. protected from light.

10. Rinse well in dist. water.

11. Apply hematoxylin to counterstain the cells *(omit this step if the antigen is localized in the cell nucleus).* Incubate 3-5 min and Rinse well in tap water.

12. Mount a cover slip with Aquatex (Merck no. 8562), or 30% glycerol.

H6: Double and triple immunofluorescent staining on paraffin or frozen sections



The double-immunofluorescence technique is based on the principle that you use two primary antibodies from hosts of different species. For example mouse-anti-glucagon and guinea pig-anti-insulin. These are mixed before they are put on the sections. The detection of the primary antibodies requires highly specific secondary antibody to avoid cross-reaction. Those are coupled to different fluorophores (e.g. Cy2 and Texas Red). Jackson ImmunoResearch Laboratories provides whole series of affinity-purified secondary antibodies.

Optimal dilutions of primary and secondary antibodies are dependent on sample preparation, antibody affinity, and the amount of antigen, which should be determined by the user.

Depending on the type of tissue-sections slides should be pre-treated like this:

Follow the same steps as listed above for single antibody detection.

1. Dry sections or de-paraffinate

2. Microwave treat in Citrate buffer.

General comments. Once the procedure has started the sections must never dry out. Drying out results in non-specific staining.

All incubation steps are carried out in a humidity chamber at room temperature.

Use a PAP-pen® to draw a hydrophobic circle around the section to reduce the volume of antibody used.

Use 50-100µl blocking solution or antibody per section- or just sufficient to cover the tissue completely.

3. Rinse the sections 3 times in PBS.

4. Apply 10% non-immune donkey-serum (serum from the same host as the secondary antibody is raised in) in PBS to block unspecific staining. Incubate for approximately 10 min*. THIS STEP may be critical that you work a little with. 10% goat serum is not a highly efficient block. Instead, you may try other types of blocking and longer incubations. We have had particular success with the TNB-block supplied with the TSA kits from Perkin-Elmer. Alternatively, there are certain anti-mouse blocks, which are made to help stainings using monoclonal mouse ab's on mouse tissue (E.g. Zymed's BEAT-blocker)*

5. Drain off the serum blocking solution and apply the mixture of the two primary antibodies (f. ex. mouse-anti-glucagon + guinea pig-anti-insulin) diluted in PBS supplemented with 0.25% BSA and 0.3% Triton X-100.

6. Incubate overnight. *Really good ab's do not need to incubated o/n. But it never hurts.*

7. Rinse in PBS – 3 X 5 min.

8. Apply the mixture of the two secondary antibodies (e.g. Texas Red-donkey-anti-mouse + Cy2-donkey-anti-guinea pig) diluted in PBS supplemented with 0.25% BSA and 0.3% Triton X-100. Incubate 45 min. Protect from light.

We always use the secondaries at a concentration of 1:100. As listed by the vendor, Jackson, good storage is to aliquot the secondaries in a 50/50 glycerol solution. We do that, meaning that we in fact use the secondaries in 1:200. (E.g add 500 ul PBS to a new tube from the vendor, then add 500 ul Glycerol (87%), and aliquot in 100 ul sizes in the –20°c freezer.

9. Rinse in 3 changes of PBS - 5 min. each.

10. Apply 20-25 µl Tris-glycerol mounting medium pH = 8.4 to each section and mount the coverslip. (You can also use permount, but allow this to dry completely before taking to the microscope. Permount is like glue..)

11. Examine with a fluorescence microscope. Store at 4°c and and take pictures within the next month. Fluorescent signals fade over time. Best results are immediately after staining.

Controls

Always include sections of control tissue where the actual antigen is known to be present.

Test the primary antibodies one at a time and the dilution-buffer on the control block developed with the mixture of secondary antibodies to check for potential cross-reactivity. Specific secondary antibodies (e.g. Cy2-donkey-anti-guinea pig+ Texas Red donkey-anti-mouse) should look like this in the microscope using the relevant filter-combinations:

Primary antibody	Cy2-filter	Texas-Red filter
Guinea pig-α-insu	green	blank
Mouse-α-glucagon	blank	red
PBS/BSA	blank	blank
GP-α-insulin + M-α-glucagon	green	red

Notes on fluorophores and secondary antibodies:

We always use Cy2 instead of FITC, as Cy2 is stronger, does not fade, and has the same spectral characteristics as FITC. FITC is a thing of the past. For the RED channel, you can use TexasRed or Cy3, and in blue we prefer AMCA. These fluorophores are conjugated to IgG's bought from Jackson ImmunoResearch. It is possible to use secondary antibodies from other vendors such as Vector Labs, or Molecular probes. The Alexa-series of conjugates are good. Remember that if you want to use Cy-5, this fluoresces in the non-visible range, and you will need a specific cube, and camera to pick up the signal, and pseudocolor this for visualization. However, this way you can do a quadruple labeling, with E.g AMCA, Cy2, Cy3 and Cy5. All you need is 4 good primary antibodies from different species to distinguish these.

When you dig down in the number of secondary antibody types available form e.g Jackson immunoresearch, it may seem like a maze. Often, an antibody type, e.g an anti-rabbit secondary comes in 6 different types, and each is possibly conjugated to 10-12 various fluorophores (Cy2,3,5, texasRed, FITC, AMCA etc, or enzymes (HRP, AP, streptavidin etc.). Some of these may have been generated in different species too, such as either goat, or donkey. Do not despair – this is great! The main difference is that you will see that some secondaries differ in what species they have been pre-absorbed against. Some are labeled "**ML**", meaning that these are qualified for multilabelling techniques, and these have been preabsorbed against a whole range of species. Preabsorption serves to reduce crossbinding to IgG's from other species, and thus reduce cross talk between the colors in a triple staining setup. However, it also reduces total reactivity, and for that reason you may want a non-absorbed antibody if you only want to detect a faint antigen singly.

Also, you may find that you can get either full IgG's, F(ab)2, or Fab-fragments of a given secondary antibody.

Fab-fragments do not contain the Fc-region of IgG, and for that reason will not bind to Fc-receptors that may be present in a given tissue. Therefore, you may also find reduced background if you use those of the

Fab/F(ab)2-type.
We have not found that you need to use secondaries raised in the same species – it is perfectly fine to combine goat and donkey antibodies in the same setup. Also, you do not have to match up the blocking serum to the secondary – often a 10% goat non-immune serum works perfectly, even if all the secondaries are raised in donkeys. Just make sure that you never block with a serum from a species of the same type as one of you primary antibodies.

	RABBIT	MOUSE	GOAT	SHEEP	Guinea PIG
Cy2	111-225-152		705-225-147		706-155-148
Fab2-Cy2		715-226-150			706-226-148
Texas Red		715-075-150	705-075-147	713-075-147	706-075-148
Fab2-Texas Red	711-076-152				
AMCA					706-155-148
Fab2-AMCA	711-156-152	715-156-150		713-156-147	
Cy-5					
Fab2-Cy-5	711-176-152	715-176-150			
HRP	111-035-006	115-035-006			106-035-003
Fab2-HRP			705-036-147	713-036-147	
Fab only	111-007-003				
Fab only RRX	111-297-003				
Fab only Cy2	111-227-003	115-227-003			
No absorbtion, HRP	111-035-006				
No absorbtion, Cy2	111-225-144				

Table, above: *List of current secondary antibodies used in the Jensen Lab*.
Fab2's are used interchangeable with normal IgG's. Fab only ab's are used in double stains of e.g. Rabbit/Rabbit as these can mask completely the first primary ab. No absorbtion ab's are used only in single stain setups.
Numbers refer to Cat#'s from Jackson Immunoresearch

H7: TSA-AMPLIFICATION DURING DOUBLE- and TRIPLE-IMMUNOFLUORESCENCE STAINING

TSA (Tyramide-stimulated amplification) is a way to enhance a peroxidase based staining reaction. The second benefit is that it results in a peroxidase reaction product that may be fluorescent, rather than precipitating. This staining procedure is highly sensitive, so that it often results in very low consumption of a primary antibody – typically, antibody concentration has to be reduced 10 fold or more, to suit a TSA technique. We have obtained beautiful results with a □-cell nuclear reacting serum against nkx6.1 at a dilution of 1:20000. However, with the enhanced sensitivity of the technique care should always be taken to reduce background, so more attention should be given to blocking, endogenous peroxidase blocking, and endogenous Immunoglobulins. Too often, excessive background staining is observed, if conditions

are not optimized. However, even with this in mind, TSA-amplification is in certain cases the ONLY way that poorly reacting antibodies may be successfully applied in histology.

More information about the TSA kits, and cat#'s for the various types of TSA-conjugates can be found on the NEN/perkin elmer website.

We have found that a combination of kits (ZYMED's histostain, and NEN/Perkin Elmers TSA kits) is both easy to use, and very successful.

1. Pre-treat the sections (microwaving and peroxidase blocking)

2. Rinse 3 X in PBS.

3. Apply *TNB Blocking Buffer*. From the TSA kit. Incubate 30 min. Remove the solution

4. Apply 30-60µl primary antibody diluted in *TNB Blocking Buffer*. Incubate overnight. *At this step you can add two other primary antibodies, that are not amplified. That way, you can do a triple IF, where only one channel is amplified. TSA does NOT interfere with the presence of other primary ab's against other species (except for rat/guinea pig, where IgG's from these two species are too similar) We normally use FITC-TSA for rabbit sera, and visualize e.g insulin (guinea pig) and glucagon(monoclonal mouse) - no need to amplify these, with blue AMCA anti-GP, and Texas-Red-anti-mouse.*

5. Rinse with PBS 3 X 5 min.

6. Apply BIOTINYLATED SECOND ANTIBODY (1B). Incubate for 30 min.

    6a. If you added other primary ab's, then add the respective secondaries here (e.g AMCA anti-GP, and Texas-Red-anti-mouse. No need to wash between 6 and 6b.

7. Rinse in PBS 3X 5 min.

8. Apply STREPTAVIDIN- PEROXIDASE CONJUGATE (2). Incubate for 15 min.

9. Rinse in PBS 3X 5 min

10. Apply *Fluorophore Tyramide (Amplification Reagent)*. Incubate 10 min. protected from light.

11. Rinse in PBS 3X 5 min and mount a cover slip with Tris/Glycerol pH=8.4

Comments

We generally use the TSA-regular kits, as compared to the TSA-plus kits, as the plus kits are simply too sensitive, and background is a problem. We always run controls with the TSA kit reagents alone, to see how much background the kit generates in absence of primary antibodies. An alternative way of proceeding compared to the above kit-combination, is to purchase HRP-conjugated secondaries against the primary antibody to be amplified. This method bypasses problems with endogenous biotin, which with the Zymed kit can give

problems. In this case, step 6 and 6b are combined, and all secondary antibodies are applied at the same time.

H8: Counterstaining Procedures

Counterstaining your tissue is a sound method to visualize your tissue during a staining procedure. For enzymatic stainings using HRP, hematoxilin is a good counterstain, as it contrasts nicely the red AEC deposits. However, bear in mind that you do not want to do a hematoxilin stain if you are staining for a nuclear antigen, as the blue nuclear staining will mask you red immunostaining. Also, do not overdo the hematoxylin – normally, 1-2 minutes is enough. Just add commercially available hematoxylin to your section after staining, and wash for a few minutes with running tap water. Coverslip and look.

For immunofluorescence stainings, there is generally no need to counterstain. Almost all tissues have a certain level of autofluorescence, and you will normally have a certain amount of background, that a camera easily can pick up. However, a very helpful counterstain is to use either DAPI, or Hoechst 33258 (which is brighter than DAPI), to visualize all nuclei. This is a very strong staining method that is extremely specific for DNA. It complements nicely other fluorophores, and do not mask, as hematoxylin. Use it at 0.001 mg/ml for 5 minutes. Wash extensively, and coverslip.

H9: ß-Galactosidase and Alkaline Phosphatase Staining protocols

Solutions

0.1 M NaPO4 buffer: (for 500 ml)
11.5 ml 1 M monobasic NaPO4
38.5 ml 1 M dibasic NaPO4
450 ml water

lacZ fix: (for 50 ml)
0.4 ml 25% glutaraldehyde
1.0 ml 250 mM EGTA pH 7.3
5.0 ml 1 M MgCl2
43.5 ml 100 mM sodium phosphate, pH 7.3 or PBS
Note: if doing a wholemount hAP stain, a mixture of 0.2% glutaraldehyde in PBS + 0.02% NP-40 + 0.01% NaDC may improve penetration of stain

lacZ wash buffer: (for 500 ml)
1. ml 1 M MgCl2
5.0 ml 1% sodium deoxycholate (NaDC; make up in water; store in fridge)
5.0 ml 2% Nonidet-P40 (make up in water; store in fridge)
489 ml 100 mM sodium phosphate (pH 7.3)

lacZ stain: (for 75 ml)
72.0 ml wash buffer

3.0 ml 25 mg/ml X-gal (dissolved in DMSO)
0.159 g K-ferrOcyanide
0.123 g K-ferrIcyanide

AP wash buffer: (for 100 ml)
10 ml 1 M Tris-HCl, pH9.5
2 ml 5 M NaCl
1 ml 1 M MgCl2
87 ml water
NBT/BCIP stain: (for 20 ml)
2 ml 1 M Tris-HCl, pH9.5
0.4 ml 5 M NaCl
1 ml 1 M MgCl2
200 µl 1% sodium deoxycholate (final 0.01%)
200 µl 2% NP-40 (final 0.02%)
70 µl NBT (to get 337 µg/ml)
70 µl BCIP (175 µg/ml)
16 ml water

H9a: lacZ Wholemount Staining

1. Rinse embryos/tissues in 100 mM sodium phosphate (pH 7.3) or PBS

2. Fix:
   - small embryos (<E9.5) in lacZ fix for 15 to 30 minutes on ice, with gentle shaking,
   - large embryos in 2% paraformaldehyde and 0.2% glutaraldehyde in PBS for 30 minutes on ice with shaking, then bisect and fix for an additional 30 to 60 minutes on ice in lacZ 2b fix
   - large tissues in lacZ fix for 4 hours on ice with shaking, bisecting the tissue after the first hour to allow penetrance of the fix solution.

3. Wash 3 times for 15 to 30 minutes in lacZ wash buffer.

4. Stain in lacZ stain solution at 37oC or room temperature for 30 minutes to overnight, with shaking and protected from light.

5. Wash 3 times for 10 minutes in PBS and store in lacZ wash buffer at 4 C.

H9b: hPLAP Wholemount Staining

1. Pre-heat a waterbath to 70 C and place a bottle/tube with the required amount of PBS in it
2. Fix: For Alkaline Phosphatase staining, embryos and tissues are fixed as for the lacZ wholemount protocol above. For better penetration of the Alkaline Phosphatase substrates, 0.02% NP-40 and 0.01% sodium deoxycholate can be added to the fix solution.

3. Rinse in PBS
4. Heat inactivate endogenous alkaline phosphatases by incubation in PBS at 70 to 75oC for 30 minutes.
5. Rinse samples in PBS
6. Wash in AP buffer for 10 minutes
7. Stain in NBT/BCIP stain, protected from light, at room temperature for 10 to 20 minutes or at 4 C for 0.5 to 36 hours.
8. Wash extensively in PBS containing 0.1% Tween-20 and 2 mM MgCl2.

H10: LacZ and hPLAP Slide Staining

This method allows detection of the enzymatic activities of b-galactosidase and Alkaline phosphatase in individual cells in a tissue. This staining has been optimized for the lineage-reporter Z/AP mouse line from C. Lobe's Laboratory.

Slide preparation:

1. Dissect samples into cold PBS

2. Fix samples as for wholemounts. Petri dishes or 6-well plates work well for containers
1. Wash samples 3 x 20' in PBS

2. Cryoprotect in 15% sucrose/PBS for 1 hour at 4oC
3. 30% sucrose/PBS overnight at 4oC.

4. Place in Tissue-Tek OCT (Sakura) at 4oC for several hours (at least 1 hour)

5. Embed in OCT:
6. put OCT into a plastic mold (write a label on the mold first)
7. place the tissue/embryo into the mold and orient it
8. place the mold onto dry ice (best to have some dry ice in a plastic tray) and hold it level, being careful to maintain the orientation of the embryo
9. STORE: When all samples have been prepared, wrap them in saran wrap, place in a labeled box and store at -80
10. Cryosection blocks at 7 to 10 µm (give the blocks about 30 minutes in cryostat chamber to equilibrate to —20). Place the sections onto poly-lysine coated slides (Fisher Scientific)
11. Dry slides for 1 to 4 hours at room temperature
12. STORE: Store at -20oC in a slide box, with a little dessicant (Dry-rite, should be blue) and taped shut

To stain:

13. Take slides out of —20 and equilibrate to room temp before opening the box
14. If doing hAP stain, place a large staining jar with PBS in a waterbath and pre-heat to 70 C

15. Place slides to be stained into staining jars (hold 15 slides and take 75 ml). Leftover slides can be placed back into —20 with dessicant.

lacZ Stain

1. Fix slides in cold PBS containing 0.2% glutaraldehyde for 10 minutes
2. Wash 3 times for 5 minutes in lacZ wash buffer
3. Stain in lacZ stain solution for 4 to 6 hours at 37oC, protected from light (wrap foil around the staining jar and place in a TC incubator).
4. When the staining is complete, rinse slides 3 x 5' in PBS
5. If only doing lacZ stain, at this point dehydrate the slides and mount coverslips (see below)

hAP Stain

1. For hAP staining alone, start with fix, then 3 times 5' washes in PBS, then proceed as follows:

2. Inactivate endogenous alkaline phosphatase by incubating slides in PBS at 70 to 75oC for
3. 30 minutes, in the preheated staining jar.

4. When finished, let slides cool at rm temp. for 1 to 2 minutes.

5. Rinse with PBS

6. Wash in AP buffer for 10 minutes

7. Shake excess liquid from slides and place on the rack in a Tupperware "pizza box"

8. Overlay with NBT/BCIP stain (requires 0.5 to 1 ml per slide). Put the cover on the box and cover with foil to protect from light

9. Stain for 10 to 30 minutes at room temperature

10. Wash the slides 3 times for 10' in PBS/2mM MgCl2

11. Dehydrate through an ethanol series:
    5' PBS
    5' 70% EtOH/PBS
    5' 90% EtOH/PBS
    5' 100% EtOH
    5' 1:1 EtOH/xylene (use xylene in the fume hood)
    5' xylene
    5' xylene

12. Mount with coverslips

    Normally counterstain is not a good idea, but if desired insert the following two steps before the dehydration steps above:

5' PBS
30'' to 5' Nuclear Fast Red (5% AlSO4, 0.1% Fast Red)
Length of stain depends on freshness of NFR

H11: General Materials for Histology

10 X PBS (Phosphate Buffered Saline), You can make this as listed below. However, we prefer to buy 10X solutions (1 gallon at a time).

NaCl	(Merck no. 6404)	80 g
KCl	(Merck no. 4936)	2 g
Na_2HPO4, 2 H_2O	(Merck no. 6580)	14.2 g
KH_2PO4 (Merck no. 4873)		2 g
Dist. H_2O up to		1000 ml.

Dilute 10 times and check pH to be 7.2

0.01M Citrate Buffer, pH=6.0

Citric acid monohydrate ($C_6H_8O_7.H_2O$)(Merck no. 244)	2.1 g
Dist. H_2O	900 ml
Adjust pH to 6.0 with approximately 13 ml of 2 M NaOH	
Add dist. water to	1000 ml

TRIS - glycerol mounting medium (for fluorescent staining)

Trizma -base	(Sigma no. T 1503)	0.49 g

Dissolve in 70 ml H_2O. Adjust pH to 8.4 with conc. HCl and adjust volume to 80 ml. Add 20 ml. glycerol (Merck no. 4095), mix well and filter (Millex-HA 0.45µm)

Other Materials

Aquatex Mounting medium	Merck	cat#. 8562
Bovine Serum Albumin	SIGMA	cat#. A 4201
Hydrogen Peroxide - 30%	Merck	cat#. 7209
Triton X-100	SIGMA	cat#. T-6878
Zymed Histostain, Rabbit.	ZYMED	cat# 85-6143
Zymed Histostain, mouse	ZYMED	cat# 85-6543
PAP-Pen	ZYMED	cat# 00-8888
Normal donkey-serum	Jackson ImmunoResearch lab.	no. 017-000-121
FITC-donkey-anti-guinea pig IgG	Jackson ImmunoResearch lab	no. 706-095-148

Texas Red-donkey-anti-mouse IgG	Jackson ImmunoResearch lab	no. 715-075-151
TSA kit, FITC.	Perkin Elmer	Cat# NEL-701

Immunohistochemistry Links

Histology - The Web Laboratory: if you need Online Help with understanding what's on your sections, this is a good site.

Histotechniques. Well Described, with further links. Good explanation of various fixatives

Immunology Protocols:immunofluorescence: Protocols Online, These are the Immunofluorescence protocols
Histology Lab: PROTOCOL INDEX – Histotechniques and protocols covering any histological staining

Internet Atlas of Histology, College of Medicine, University of Illinois at Urbana-Champaign –Not a bad site with H/E stainings of almost any tissue.

Fluorescence & Confocal Sample Preparation Links Web page

H12: DIG-RNA In Situ Hybridization (frozen sections)

In situ hybridization refers to the visualization of gene expression *in situ.*

We have found that it is not necessary to use DEPC treated water.

Probe synthesis

Template DNA is linearized with appropriate restriction enzyme

Cut 25 μg in a total volume of 50 μl.

Add up 150 μl Water, and extract with 200 μl Phenol:Chloroform:isoamyl alcohol (25:24:1)

Extract aqueous phase with 200 μl Chloroform:isoamyl

Precipitate DNA, wash in 70% EtOH

Resuspend at ~1μg/μl in ddH20 (add 20 μl sterile ddH2O), assuming a slight loss of plasmid during extraction.

To make DIG-labeled probe

Sterile ddH20 (not DEPC treated!*)	11μl
5X txn buffer (from frozen aliquot of stock, warmed to 37°C **)	4μl
DIG or Fluorescein labeling mix (Roche)	2μl
Linearized template DNA	1μl

RNAsin (promega)	1µl
SP6, T7 or T3 RNA polymerase (Roche)	1µl

-Incubate at 37°C for 1 hour
-Add 1µl of RNA polymerase
-Continue incubation for an additional 1 hour
-Run 2µl on 1% agarose gel to check

-Precipitate probe:

Add 1µl glycogen (20µg/ml; Roche)
7µl 7.5M ammonium acetate
75µl cold EtOH

Incubate 30 minutes at -20°C

Spin 15 minutes, pour off supernatant

Resuspend in 100µl sterile ddH20

Reprecipitate as above with 33µl ammonium acetate
300µl cold EtOH

After spinning down the pellet, wash with 1 ml 70% EtOH

Air dry pellet

Resuspend in 100µl sterile ddH20 (approx. conc. 100ng/µl); store at -20°C until use

*DEPC can interfere with enzymatic activity and prevent efficient RNA synthesis. Avoid its use in the labelling reaction.

**We have found that repeated freeze/thawing of RNA polymerase buffer leads to decreased enzymatic activity; thus, we aliquot the stock. If thawed at RT, DTT will not be soluble in the buffer and will appear as a white precipitate. The buffer is incubated at 37°C until the ppt disappears.

DAY1

This protocol starts with sectioned material (7 micron cryosections). Ask for a separate protocol for details about fixing, embedding, cryosectioning, etc.

OUR GLASS CHAMBERS HOLD 200 ML

Pre-Hybridization of sections:

1. Fix sections 10' in 4% PFA/PBS at RT

***JAN HAS FOUND THAT WE CAN USE FROZEN PFA DIRECTLY ON SECTIONS INSTEAD OF IMMERSING THE SLIDES IN THE GLASS CHAMBERS, THE TIME IS

INCREASED TO 15 MIN TO COMPENSATE FOR THE POSSIBLE LOSS OF PFA ACTIVITY WHEN USING THE FROZEN STOCK***

2. Wash 3' in PBS 1
3. Wash 3' PBS 2
4. Wash 3' PBS 3
5. Digest in Proteinase K (1µg/ml in 50mM Tris pH 7.5, 5mM EDTA)

   Adjust incubation time to each stage: 2 min/E10.5
   4 min/E12.5
   6 min/E 14.5

I have found that 5-10 min for adult pancreas leads to best signal with least background.

WE DO NOT DO STEP 5 – 9 IN THE OPTIMIZED PROTOCOL

6. Refix 5' in 4% PFA/PBS at RT
7. Wash 3' in PBS 1
8. Wash 3' in PBS 2
9. Wash 3' in PBS 3
10. Acetylate for 10' RT
    To 300 ml ddH20, add 4.08ml triethanolamine, 0.534 ml HCL, 0.763 ml acetic anhydride. Prepare immediately prior to use.
11. Wash 5' PBS 4
12. Wash 5' PBS 5
13. Wash 5' PBS 6
14. Add ~500µl hybridization buffer to each slide and incubate in humidifed chamber at 55°C for 1-2 hours.

Hybridization buffer:

50% formamide
5X SSC pH 4.5 (use citric acid to pH)
50µg/ml yeast tRNA (Sigma)
1% SDS
50µg/ml heparin (Sigma)

Hyb buffer can be stored at -20°C. It precipitates at RT, so thaw at 55°C immediately prior to use.

Hybridization:

1. Prepare hybridization buffer containing probe at 1ng/µl (1µl of probe per 100µl of hyb- you may have to adjust probe concentration)
2. Heat probe/buffer at 80°C for 5'
3. Cool at RT for 5'
4. Add to sections after removing prehyb: I use 80µl for a 20x40mm area
5. Coverslip with parafilm or glass coverslip
6. Incubate overnight in humidifed chamber at 70°C (water soaked paper towels are good)
7. Place 5X SSC (pH 7) and 0.2X SSC in incubator for day 2

DAY 2

Washes + antibody:

1. Transfer slides to rack submerged in prewarmed (70°C) 5X SSC. Move slides around in the 5XSSC before loading into rack in order to remove coverlsips; they should fall off easily. Incubate on rocker for 30' at RT.
2. Transfer to prewarmed 0.2X SSC (pH7) and incubate at 70°C for 3 hours.
3. Transfer to 0.2X SSC (at RT) and incubate for 5' at RT.
4. Transfer to 1X MAB and incubate for 5' at RT.

   5X MAB stock (1000 ml):

Maleic Acid	58g
NaCl	43.5g
10 N NaOH	~80 ml →pH to 7.5

   Maleic acid will not go into solution unless the pH is adjusted with NaOH. Alternatively, maleic acid disodium salt goes into solution easily; use 80g per liter.

5. Incubate slides in blocking solution for 1 hour at RT (longer is fine). I usually place the slides in slide mailers for this step.

   Blocking solution:

   2% Blocking reagent (Roche)
   10% heat inactivated sheep serum (inactivate at 56°C for 30 mins)
   0.1% Tween-20
   all in 1X MAB

   10% Blocking reagent:

   50g of Blocking reagent (Roche) dissolved in 500ml 1X MAB. Heat to dissolve and autoclave. Store at 4°C.

Replace blocking solution with fresh blocking solution containing anti-DIG or anti-Fluorescein antibody (Fab fragments; Roche) at a concentration of 1:5000. Incubate O/N at 4°C.

Day 3

Washes + staining:

A. Wash slides in 1X MAB with 0.1% Tween-20 for 15' at RT
B. Wash slides in 1X MAB with 0.1% Tween-20 for 15' at RT
C. Wash slides in 1X MAB with 0.1% Tween-20 for 15' at RT
D. Wash slides in ddH20 with 0.1% Tween-20 for 20' at RT
E. Place slides in mailers and add NBT-BCIP (precipitating; Roche) with 0.1% Tween-20. Wrap in foil and incubate at RT until desired signal can be seen. This can take from 2 hours to >3 days.
F. Change staining solution when it changes from yellow to purple or after 2 days of staining (which ever comes first).
G. Stop the reaction by transferring slides to 1mM EDTA in PBS.

H. Mount with aqueous mounting media or counterstain and mount with appropriate mounting media.

0.1% Nuclear fast red/5% aluminum sulfate is a good counterstain for BM purple signal. See http://www.vectorlabs.com/Protocols/Counterstains/H3403.pdf for staining protocol details.

For two color in situs:

The red color reaction is much less sensitive than the purple reaction. It is also very easy to wash away the red color. Thus, weaker probes should always be stained with BM-Purple and should be stained first.

Day 1: probe hybridization
Mix both DIG- and Fluorescein labelled probes (each at ~1µl/100µl hyb) in same hyb solution

Day 2: stain weaker probe first, use either 1:5000 anti-DIG or anti-Fluorescein

Day 3: washes and staining in BM-Purple as normal

Once purple color has come up:

1. Wash away BM-Purple and fix slides in 4% PFA for 10 minutes.
2. Wash slides in PBS for 3 minutes. Do 3 washes total.
3. Wash slides in 1X MAB for 5 minutes.
4. Block slides (as before).
5. Incubate slides in antibody appropriate for detecting second probe. Because the red staining is so less sensitive, it is essential to use a much higher concentration of antibody. I have found that a 1:500-1:000 dilution of anti-DIG AP or a 1:1000 of anti-Fluorescein AP works well. Slides can be incubated at 4°C O/N or at room temperature for 4-5 hours.
6. Wash slides 3x in 1X MAB/0.1% Tween for 15 minutes.
7. Rinse and then wash slides for 10 minutes in 0.1M Tris pH 9.5, 0.05M $MgCl_2$, 0.1M NaCl, 0.1% Tween-20 (staining buffer)
8. Add staining solution: 75µl of NBT/BCIP in staining buffer. Wrap mailers in aluminum foil and incubate at RT until brownish-red color comes up. Change the staining solution every 24 hours; it will have a dark red appearance.
9. Wash away staining solution with water and coverslip slides.

H13: Lectins

Lectins are plant-derived proteins that bind with high affinity to certain carbohydrate modifications on proteins. Certain mammalian proteins are highly glycosylated, including intestinal mucins. Lectins may bind to these, and as such proteins may be differentially expressed, the individual lectin may serve as a inexpensive and quick marker for certain cells in the develoing and adult gut – as well as other tissues.

Presently available reagents:

Short name	real name	Conjugate	Stock conc.	Working conc.
DBA lectin	Dolichos	RRX	2 mg/ml	25 ug/ml
SNA Lectin		FITC	2 mg/ml	25 ug/ml
GSII Lectin		FITC	2 mg/ml	25 ug/ul
UEA Lectin		FITC	2 mg/ml	25 ug/ul

All are obtained from Vector Labs. Price ranges from 50-75$/vial.

Do not go higher in working concentration than stated. Most likely, the best pictures are with a more diluted working solution

Procedure

Get slides into PBS (deparaffinate, or take frozen sections, after 30' drying time at 37c)

We block with 10 non-immune Sheep serum, but this step can possibly be omitted.

Add the lectin in PBS, incubate for 30 minutes at 37c,

Drain off Lectins

If Hoecst dye is added, then do so now for 5'

Wash 3x with PBS.

Mount with glycerol mount.

On E14.5 gut, staining is VERY limited with these lectins.

DBA:

Although Gittes et al postulate that DBA Lectin stains the precursor cells of the pancreas, this is not true. In the pancreas, DBA later becomes restricted to mature duct cells. There is no staining with DBA in the E14.5 pancreas

In the intestine, some DBA staining is observed along the forming brushborder of the enterocyte. However, the villi structure of the gut is still not properly established, and proliferating cells are scattered.

In the stomach, no staining is observed.

Certain cells of the hematopoeitic system stain strongly at E14.5. These can be noted particularly in the liver, and spleen. This is not the erythrocytes.

GSII:

In the pancreas, weak staining is observed in the recently formed exocrine acini. No staining is observed in other cells.

In the intestine, uniform, weak staining is observed in the epithelium.

In the stomach, uniform, weak staining is observed in the epithelium.

GSII also labels the same hematopoietic cells as those seen wit the DBA lectin in the liver and spleen.

UEA I:

In the pancreas, no staining is observed

No staining in the intestine

Weak,apical staining in the stomach epithelium.

SNA lectin:

SNA is not very specific, most cells stain with SNA. However, staining is localized to cell surfaces, which makes SNA an excellent contrast generator, outlining tissue structures beautifully.

In the pancreas, the outline of acinar structures are easily discernible. In the intestine, epithelium and distant mesenchyme condensing to smooth muscle stains most strongly. In the absolute periphery, strong staining is observed, making the outer mesenchymal layer, different from the smooth muscle easily recognized. In the spleen, the outer “epithelial-type” layer is readily recognized. These cells formed the splenic ridge, and can still be seen.

Strong staining is observed in a large fraction of the hematopoietic cell system. These cells are scattered inbetween other cells in all organs.

At E17.5 and later, significant staining is observed for all in the more mature tissues. Define staining based on co-staining analysis.

H14: Whole-mount Immunostaining

Given good antibodies, this protocol allows for the visualization of a protein of interest inside an organ. Use 10ml polycarbonate tubes for all wash steps, and in 1ml in a 48 well plate for all antibody and substrate steps. Rocking of tubes should be performed at all times. All steps are carried out at room temperature unless otherwise stated.

Tissue harvest

1. Dissect embryos in ice-cold PBS. Remove as much extraembryonic membranes as possible. Older than e10, micro dissect the pancreas/stomach.
2. Fix in 4% paraformaldehyde (PFA) in PBS at 4oC overnight.
3. Store tissue in 100% MeOH at -20 for up to a few months

Day 1

Incubate embryos in freshly prepared MeOH:DMSO:H2O2 (4:1:1) at room temperature O/N.

At this point embryos can be taken into 100% MeOH and stored at -200C at this point.

Day 2

1. Rehydrate the embryos in a series of 75%, 50%, 25% MeOH in PBS (15 min each) -> freshly prepared PBT (1 x 15 min, 2x2hours). (To make more holes in tissue do H2O 5x1 min, acetone at -20 for 5 min, H2O 5x1 min)
2. Incubate with blocking solution PBT + 2% BSA + 10% serum (has to be the same species as the secondary antibody, heat inactivate at 56oC if AP is used to develop) 1-2 hour.
3. Incubate with primary antibody in PBT at 4oC overnight.

The antibody incubation can be extended for several overnights without compromising the experiment.

Day 3

1. Wash with PBT (2x 15 min, 5x 1 hour).
2. Incubate with secondary antibody (AP, HRP or FITC coupled for green, or Cy5, Cy3 coupled for red) in PBT at 4oC overnight.

The antibody incubation can be extended for several nights without compromising the experiment.

Day 4

1. Wash with freshly prepared PBT (2x 15 min, 5x 1 hour).

For HRP:

a. In the dark make a pre-substrate incubation with AEC (Zymed) (NO H2O2!!) for 30 min
b. In the dark add H2O2 wait about 10 min.

For AP:

a. Wash with freshly made NTMT (3x15 min)
b. In the dark incubate with NTMT + 4.5ul NBT + 3.5ul /ml

2. Wash and post fix in 4% PFA in PBS.

Solutions

PBT: PBS + 0.2% BSA, 0.5% Triton X-100. Make fresh, lasts about 2 weeks.
H2O2: generally supplied as a 30% solution, stored at —20oC, lasts 6 months.
AEC: AEC SUBSTRATE - kit (Zymed no.00-2007). Prepare 1 drop Citrate buffer (A) + 1 drop Amino - Ethyl -Carbazole in DMF+1 drop Hydrogen Peroxide in 1 ml. dist.

NTMT: 50ml: 5M NaCl 1ml, 1M Tris-HCL (pH=9.5) 5 ml, 1M $MgCl_2$ 2.5ml, 10% Tween-20 0.5ml, H_2O to 50ml

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H15: Immunohistological Morphometry ImagePro Plus

Taking pictures

Choose magnification and pixel value (specify later for counting). Place FOVs randomly throughout the tissue - avoid overlapping pictures. Save pictures under Swimming Drive. Include in file name – experiment #, slide #, antibody/ antibodies, magnification, picture # (field of vision/ FOV). **Example:** Exp79 slide4_1 amy10x #1.

1. Click on the **Count and Size** icon on the toolbar (it's red and black).
2. Choose **Automatic Bright Objects** or **Manual** option.
3. Check **Measure Objects** and **Apply Filter Ranges**.
4. Click on **Count**.
5. Click on **View** in the Count and Size menu.
6. Choose **Statistics**.
7. Record the **Sum** value.

Taking area measurements

1. Click on the **Manual Measurements** icon on the main toolbar (a ruler and scissors).
2. Click on the **Create Polygon** icon.
3. Draw on the picture while left clicking; right-click to complete.
4. View the **Measurements** tab.

Verify calibration before counting

1. Click on **Measure** in the main menu.
2. Choose **Calibration** then **Spatial** or **Select Spatial** to make corrections.

Increase light on the picture in order to see the unstained tissue:
By clicking on the **Contrast Enhancement** icon in the toolbar.

Equipment

1. Olympus BX51 Upright microscope, equipped with fluorescence.
2. Olympus SZX12 dissection stereomicroscope.
3. Pixera CL600 Camera, cooled, full-color CCD digital camera.
4. Dell Precision 330 Imaging workstation (P4, 1 Gb RAM, 80 Gb SCSI HD).
5. Image-Pro Plus, version 4.5.0.19 for Windows.
6. Twain Viewfinder 3.0.1 to take pictures.

H16: Immmunocytochemistry of Cell Monolayers (Chamber slides)

1. Seed cells into chamber slides (Nunc, 8 well, at least a day before). The cells will always show heterogeneity in seeding density between corners and center. This is a benefit as various densities are thus imaged.

2. Wash 3x in 1xPBS

3. Permeabilize the cells by adding Ethanol: 70%-96%-99%-99%-96%-70%-5 min per step

4. Rinse 3X with PBS

5. Slides are blocked with TSA blocking solution. Pre-made in freezer (60 min.)

6. Primary antibodies are applied without washing, and left O/N overnight at room temp

7. Next morning, drain off primary abs, wash (PBS 3x)

8. Apply mix second Antibodies Cy2 a-rab, Tex-Red a-M. Incubate for 1 hr.

9. Rinse 3X with PBS, 5 min each time.

10. Mount slides using glycerol mount.