Have you ever been awed by the histologist’s ability to create a complete set of serial sections through an embryo without losing a single section? This may seem an impossible feat until you learn the relatively easy methods of paraffin sectioning. In this chapter you will have the opportunity to make your own set of serial sections, and in the process, you will be learning a technique that has recently regained the spotlight in embryological studies. With the advent of immunohistochemistry and fluorescence microscopy, developmental biologists have a valuable set of tools, and they need to know the art of tissue preparation to apply them. Regardless of the staining technique used, the basic process is the same: Tissues are first fixed to avoid degradation, embedded in paraffin, cut into serial sections with a rotary microtome, and stained to contrast cellular elements. The paraffin technique for tissue preparation is the most commonly used for routine study of tissue. You will be using either chick embryos that you obtained from your previous laboratory exercise (Chapter 11) or other tissue provided by your instructor.

**Fixation**

Fixation is the first step in any procedure in which tissue is to be preserved for histological study. Fixatives kill. They kill the tissue, as well as any bacteria that are present that otherwise would cause the tissue to rot. They also coagulate or cross-link proteins, making them insoluble. All fixatives distort tissue to a certain extent, but in general, proteins and cellular structure are preserved. Normally you choose a fixative containing several ingredients that balance out each other’s ill-effects. For example, alcohol shrinks tissue and causes excessive hardening. You can counter these effects by adding an acid such as acetic acid, which swells tissue and prevents overhardening.

One of the safest fixatives to use, which will not leave any toxic residues behind, is Carnoy’s fixative, a mixture of alcohol and acetic acid. It is not an ideal fixative. The addition of formaldehyde, for example, would give better preservation of cytological detail. Formaldehyde, however, and most other fixing agents leave highly toxic residues that are virtually impossible to remove from instruments and glassware. If you were making whole mounts, where cytological detail is not critical, or you cannot risk contaminating your work area for future live material, Carnoy’s is ideal for use.

**Carnoy’s fixative**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glacial acetic acid</td>
<td>100 ml</td>
</tr>
<tr>
<td>100% ethanol</td>
<td>300 ml</td>
</tr>
</tbody>
</table>

Some Carnoy’s recipes also include chloroform along with glacial acetic acid and absolute ethanol in the proportions 3:1:6. Chloroform increases the speed of fixation, making it useful for fixing tissues that are difficult to penetrate. Chloroform, however, is extremely hazardous and must be used in a ventilated hood while wearing gloves.
Glacial means 100% acetic acid; vinegar is 5% acetic acid. Ethanol is the type of alcohol that people get arrested for driving under the influence of; hard liquor such as whiskey is about 40%–50% ethanol. Rubbing alcohol is 70% isopropyl alcohol—don’t drink it, it’s toxic. If you were out in the boondocks with no scientific supplies and found the perfect specimen you needed to preserve, what makeshift fixative would you devise?

A commonly used fixative that is better at preserving structure than Carnoy’s is FAA, made of formaldehyde, alcohol, and acetic acid. (Formaldehyde is a gas, which is sold in solution as formalin, which is about 40% formaldehyde in water.)

**Dietrich's FAA**

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>95% ethyl alcohol</td>
<td>30.0 ml</td>
</tr>
<tr>
<td>formalin</td>
<td>10.0 ml</td>
</tr>
<tr>
<td>glacial acetic acid</td>
<td>2.0 ml</td>
</tr>
<tr>
<td>distilled water</td>
<td>60.0 ml</td>
</tr>
</tbody>
</table>

A fixative that is widely used for embryological material, since it will not overharden yolky material, is **Bouin’s fluid**. It has the advantage that tissues may be stored indefinitely in it. Its major disadvantage is that the picric acid, though an excellent fixative for lipid-rich tissues that doesn’t harden the tissues, also stains the tissue yellow. The yellow color must be removed before other stains can be applied. Usually, this is accomplished after sectioning as the slides descend through the alcohol series. Just leave the slides in 70% alcohol for a longer period of time if the yellow color doesn’t disappear using the normal dehydration schedule.

**Bouin’s Fluid**

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>picric acid, saturated aqueous</td>
<td>75.0 ml</td>
</tr>
<tr>
<td>formalin</td>
<td>25.0 ml</td>
</tr>
<tr>
<td>glacial acetic acid</td>
<td>5.0 ml</td>
</tr>
</tbody>
</table>

To fix your embryo or sample of tissue, place the freshly dissected specimen (it should ideally be no more than 1–2 cm on a side) into a screw-cap vial containing your fixative of choice. For fixation and all steps that follow that require a fluid, the rule of thumb is that the amount of fluid used should be 10× the volume of the tissue. **Label** your vial with your name and what the specimen is, or use a piece of cardstock as your label and place it in the vial with your specimen. **Always use pencil for labeling**—it doesn’t come off in the reagents.

The amount of time your specimen is left in fixative should depend on the size of the specimen and the type of fixative. Most specimens should be fixed 6–8 hours, or overnight. If the specimen is smaller than 5 mm on a side, then several hours will suffice. If the fixative severely hardens and shrinks the

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* Note that under current OSHA guidelines, solutions containing concentrations of alcohol above 24% must be discarded as hazardous waste.

** Under current OSHA guidelines, used fixatives containing formalin must be discarded as hazardous waste, as must solutions containing concentrations of alcohol above 24%.

*** Picric acid is sold saturated in water and must be stored that way, since in dry form it is explosive. (The exceedingly destructive explosion in Halifax harbor during World War I was caused by sparks from a careless collision into a munitions ship loaded with picric acid.)
specimen, as does Carnoy’s, then the minimum fixation time should be used. Specimens should not be left in Carnoy’s fixative more than 2–3 days. If the fixative is like Bouin’s, which is not harsh on the tissue, the tissue may be stored in the fixative for extended periods (months or more) without severe alteration. Tissues should be fixed and stored in the refrigerator at 4ºC.

### Washing

Following fixation, your tissue sample must be washed. This, as well as many of the operations that follow, can be done in the vial. To make a transfer of solution, you can either pour off the old solution into a finger bowl, or you can pipette off the old solution. The new solution is added directly to the vial. This avoids unnecessary handling that can damage the tissue.

Washing is usually done in water. For most fixatives, washing specimens in distilled water for 6–8 hours, or overnight, following fixation is sufficient. If you have chosen Carnoy’s as your fixative, then washing in 70% alcohol instead of water is both adequate and faster. You also can use the 70% alcohol as a holding solution.

Following washing, the specimen should be transferred to 70% alcohol and stored at 4ºC until it can be processed further. It can remain here for a number of weeks.

### Dehydration and Clearing

To prepare the specimen for paraffin embedding, the specimen must be dehydrated through a series of alcohols up to absolute alcohol (ethanol is preferable to methanol, since it is less harsh on the tissues). This removes all the water, which is immiscible with paraffin. By going through a graded series of alcohols, the convection currents that are set up by each transfer are minimized, thereby minimizing harm to the specimen. After the water has been removed, a clearing agent, such as xylene or toluene, which is miscible with both 100% alcohol and paraffin, makes a bridge between the alcohol and paraffin. Toluene is less harsh on tissue than xylene, causing less shrinkage and hardening, and so should be used instead of xylene if possible.

In transferring the specimen through any alcohol solution, the old solution can be poured off (into a finger bowl) and the new solution added. Remember that under current OSHA guidelines, solutions containing concentrations of alcohol above 24% must be discarded as hazardous waste. Xylene and toluene are toxic. They should only be used in a ventilated hood (or outside, if necessary) and also must be put into special waste jars and disposed of according to OSHA regulations as a hazardous material.

Making a graded series of alcohol: *Always use 95% ethanol (ETOH) to dilute from,* rather than 100% ETOH. Absolute (or 100%) ethanol is exceedingly expensive compared to 95%. Here is an easy method for making any percentage of alcohol below 95% that you wish. To make a 70% solution of ETOH, for example, pour 95% ETOH into a graduated cylinder up to the 70 ml line, then add distilled water until the solution reaches the 95-ml line. This gives you 95 ml of 70% ETOH. If you are making a 50% solution, pour 95% ETOH up to the 50-ml line, and add distilled water up to the 95-ml line. To make larger amounts, just use quantities that are multiples of the numbers above.

The following schedule can be used. Times are sufficient for specimens that are no more than 1–2 cm/side. If the sample is larger, longer time periods must be used.
70% alcohol (if starting from water) 1–2 hours
95% alcohol 1–2 hours
100% alcohol (first time) 1/2–1 hour
100% alcohol (second time) 1/2–1 hour
Toluene or xylene (first time)* 1/2–1 hour
Toluene or xylene (second time) 1/2–1 hour

* Warning: If there is any water left in the specimen at this point, it will show as a milkiness around the specimen when it is placed in the clearing agent. The specimen must be brought back to 100% alcohol in this case to remove the remaining water.

Special microwaving techniques that save time
Microwaving the specimen during fixation and dehydration cuts down considerably on the time needed in each solution and improves infiltration of the solutions into the tissue. A number of precautions must be taken, however, to make sure that the specimen doesn’t overheat, that penetration is uniform, and that toxic exhaust doesn’t enter the room. A large beaker of water should be placed in the oven to absorb excess microwave energy. The specimen should not be placed in a “hot” spot of the oven. You can figure out where the hot spots are by placing wet paper towels in the oven and turning it on. Just as the towels begin to dry, turn off the oven. Wherever wet spots remain are good places for placing your specimen vial. Alternatively, the specimen vial can be placed on a rotating table in the oven. To avoid overheating, the oven can be put through cycles of being run on “high” for short periods (7–10 seconds) alternated with being off for 20 seconds. The specimen should not be heated over 70ºC—this causes overdenaturation of regions that are relatively impermeable and stain nonuniformly. Putting the vial on a petri dish of ice during processing can prevent overheating.

After 5 minutes of microwaving, a specimen 5 mm × 5 mm × 2 mm can go on to the next step in the fixation or dehydration series. Since microwave ovens vary, you will need to experiment to determine what times and settings work best for your particular oven. It is exceedingly important that the seals on the oven be checked to make sure that microwave radiation and toxic fumes are not leaking out into the room. It is safest not to use microwave processing on steps that involve higher alcohols, or clearing agents such as xylene or toluene.

Microwaving can also be used in place of chemical fixation in order to avoid the toxicity of chemical fixatives. Specimens are placed in a saline solution, such as chick Ringer’s solution, and microwaved for a period of time, maintaining a temperature of 68–70ºC. Small specimens can be microwaved for as little as 6 minutes. Again, you will need to experiment to determine the times and settings that work best for your particular oven.

Paraffin Infiltration
From the second change of toluene, the specimen is to be moved through several changes of melted paraffin. The paraffin you use will have a specified melting point. A melting point of 54ºC is typical and is suitable for sections that will be cut at 8 µm or thicker. Higher melting points indicate harder paraffin that can be cut into thinner sections.

Typically, the melted paraffin is kept in a paraffin oven or paraffin bath that will maintain the paraffin at a constant temperature. The specimen can be transferred to a cage specifically designed for infiltration. Be sure to include a label in the cage, along with the specimen, that distinguishes your sample from other samples in the oven. The cage can then be transferred from one bath of melted paraffin to another using forceps or a string tied to the cage. Alternatively, deep-welled spot dishes containing melted paraffin can be used in a paraffin oven and the specimen transferred from one well
to the next. In this method, use a narrow piece of cardstock that you hold with forceps to lift the specimen for each transfer—this avoids the harm to the specimen that would occur if a metal instrument such as forceps were used to pick up the specimen.

The paraffin should be kept above its melting point during this process, but not hotter than necessary, since heat severely shrinks and hardens tissue. Use two to three changes of paraffin, 1/2–1 hour each. Under ideal circumstances, you would be able to infiltrate in a vacuum oven—this gives good infiltration and shortens the time needed in hot paraffin.

**Paraffin Molds**

One of the most successful molds for embedding is a paper box made such that the seams are watertight. Such a box can be made to any size appropriate for your specimen. It also has the advantage, over molds with rigid sides, of being pulled inward along with the paraffin as the paraffin cools and shrinks. This prevents a deep indentation from forming in the center of the block. More expensive manufactured molds made of plastic or metal can also be used, and these can be bought from supply houses.

I much prefer paper boxes to any manufactured mold. A paper embedding box should be made of heavyweight paper. It is made most easily by folding the paper over a block of wood that has one face that matches the size that you need. For a specimen approximately 5 mm³, use a rectangular sheet approximately 6 cm × 10 cm. Place this on a block of wood with a face approximately 2 cm × 2 cm (Figure 12.1A). First fold the long sides of the paper down alongside the wood (Figure 12.1B). Holding these in place with one hand, use the other hand to fold one of the remaining sides down (Figure 12.1C). Crease the two corners at a 45° angle (Figure 12.1D). This creates flaps, which are then folded inward, and a tab that is folded down (Figure 12.1E). Do the same to the remaining side (Figure 12.1F).
**Paraffin Embedding**

Fill the embedding mold with melted paraffin and transfer the specimen from melted paraffin immediately to the mold. The specimen can be transferred using a small strip of cardstock as a spatula. This prevents damage to the specimen. Position the specimen in the mold using a hot needle, heated in the flame of an alcohol lamp. The orientation of the specimen should be appropriate for later sectioning of the block. If using a paper box, this orientation can be marked on the tab of the box, along with information on what the specimen is and any other information necessary to identify it later.

After a film of solidifying paraffin has formed over the surface of the paraffin, the mold should be transferred to a bowl of cool (but not ice-cold) water (water that is too cold can cause the paraffin to crack). The water causes the paraffin to cool more quickly, making for smaller crystal formation within the paraffin as it solidifies and hence less distortion of the specimen. Once the block is hard, remove it from the water and store in the refrigerator. You should not try mounting and cutting the block until it has cooled for 6–8 hours, or overnight. This improves the cutting process and is one of those magic tricks one learns but can’t explain.

**Mounting and Trimming Paraffin Blocks**

The paraffin block is to be mounted onto a piece of metal, synthetic fiber, or wood, all of which can be referred to as “chucks,” which fit into the chuck holder of the microtome. When mounting the paraffin block onto the chuck, the chuck must be held securely. A heavy piece of wood with holes cut into it or even a chunk of clay into which you press the chuck are suitable holding devices.

*Rough trimming*  Remove the paraffin block from its mold, keeping track of the orientation of the specimen within it. Trim away excess paraffin using a safety razor blade. This should be done on a protective piece of wood or glass. Do not trim too close to the specimen. Leave at least 5 millimeters of paraffin on all sides and more than this on the bottom of the block. Paraffin scraps from trimming can be remelted, filtered, and reused. Paraffin improves with reheating, since impurities are volatilized off with each heating.

*Mounting the block*  Using the flame from an alcohol lamp, melt some shavings of paraffin on the blade of a metal spatula and pour the melted paraffin onto the surface of the chuck. In this way, you are to build up a layer (at least 1 mm thick) of paraffin that is firmly attached to the chuck. Heat the spatula again and use it to melt the top of this layer of paraffin. Then heat both sides of the spatula blade, hold it between the bottom of the paraffin block and the paraffin on the chuck so that it melts both surfaces of paraffin, and slide it away as you press the paraffin block against the chuck. Heat the spatula blade again, and use it to melt some paraffin on the four vertical sides of the paraffin block to ensure that it is attached firmly to the chuck. Set aside until the paraffin is firm.

*Fine trimming*  You now need to trim the face of the block so that it is in the shape of a trapezoid, with the top and bottom sides being parallel. The slanted sides help the sections that are cut to stick together into a ribbon rather than sticking to the knife. If the top and bottom sides are parallel, then the ribbon that is cut will be straight. There should be about 2 millimeters of paraffin left around the specimen on all sides.
Sectioning

You must be familiar with the controls on the rotary microtome before you can proceed with sectioning (Figure 12.2). Be sure you have been checked out by an instructor and received permission to go on to the next step.

The wheel of the microtome should be in the locked position. Clamp the chuck containing your paraffin block into the chuck holder on the microtome. Using the control screws, align the chuck holder so that the face of the block is absolutely vertical. The block, when you look at it straight on, should look like the picture shown above, with the short side of the trapezoid uppermost.

Insert the microtome knife into the knife holder, and tighten the clamping screws. This should always be done with great care. These knives are exceedingly sharp and dangerous. If you should drop one, let it go—never try to catch it. (You could lose a finger by attempting such a catch.) These knives are also very expensive, so it is worth taking care of them.

Advance the knife toward the block. This is done by unlocking the wheel, lowering the paraffin block until it is on a level with the knife-edge, and then carefully moving the knife carriage forward until the knife is just in front of the paraffin block. Lock the knife carriage in place.

Before cutting sections, check the thickness setting. Paraffin sections are typically cut at between 4 and 12 µm in thickness. For routine sectioning, set the thickness to between 8 and 10 µm.

To section, turn the handle of the microtome, which advances the block toward the knife. On each rotation of the wheel, the block is moved forward the number of microns that have been set for sectioning thickness. Turn the wheel in a smooth slow motion to ensure getting the best sections. When sections begin to come off the block, the first several may be incomplete. These can be brushed away with a camel’s-hair paintbrush—no other instrument should ever be used, since these would damage the knife-edge. And never allow your fingers to get anywhere near the knife-edge.

Figure 12.2
A rotary microtome used for cutting paraffin sections.
Once a **ribbon** of sections begins to form, the end of the ribbon can be lifted with a moistened camel’s-hair paintbrush. Do not pull on the ribbon; simply support its end with the brush. When you have a suitable length of ribbon (no longer than the length of your ribbon box), take a second moistened camel’s-hair paintbrush, and use it to lift the attached end of the ribbon away from the knife-edge. Place the ribbon carefully into your **ribbon box** (a flat stationery box does fine). Cover the box so that air currents don’t disturb your ribbon. Keep sectioning until you have all the sections you need. If you are making a set of serial sections, keep each ribbon you cut in order, laying them down so that the last section of each ribbon is on the right, and each successive ribbon is below the previous ribbon.

**Retrimming the block**  As you section, you may need to retrim the paraffin block if the block face becomes too large or if the ribbon is not straight. Remember, the ribbon will not be straight unless the top and bottom sides of the block are precisely parallel to one another. When retrimming the block, the knife **must** first be removed from the knife carriage. Do this without changing the position of the carriage. Retrimming with a safety razor blade may now be done on the paraffin block in place without removing the chuck from the chuck holder.

After retrimming the block, replace the knife, and back the paraffin block off a slight distance (an eighth of a turn) from the knife using the handle for this—it is on the opposite side from the sectioning wheel. This will avoid taking too thick of a section on the first rotation of the wheel.

**Cleaning the knife**  When you have finished sectioning, the knife should be cleaned of any paraffin that is sticking to it. First attach the knife handle to the knife. This will allow you to hold the knife without danger. Place a few drops of xylene or toluene on the edge of the knife, and with a Kimwipe®, wipe very carefully, moving from the flat of the knife off the edge of the knife, as you would to brush crumbs off a table. Never wipe along the edge of the knife or into the edge. Remove the knife handle and place the knife in its holder (sharp edge down).

**Mounting Sections**

No matter how carefully you cut your sections, the cutting process compresses the sections, and part of the mounting procedure is to **expand** the sections before they adhere to the slide. This can be done by floating the sections on warm water (5–10°C below the melting point of the paraffin). Some histologists float the sections in a water bath and then pick the expanded sections up on a slide. I find it easier to put water on the slide itself, float the sections on the water, warm the slide on a warming tray, and then pour off the water after the sections are expanded. This second procedure is the one we will use.
Select the number of slides you think you will need. Even though these have already been factory-cleaned, they should be cleaned again by at least rubbing them with a dry Kimwipe®. Then, using a diamond marker, etch into one end of the slide the information that is necessary to identify it (usually, specimen or experiment number and the number of the slide in the series that you are making).

An adhesive must now be applied to the slide so that the specimen sections will adhere properly and won’t fall off during further processing. If you are not trying to identify specific proteins in your staining procedure, then the best adhesive to use is Mayer’s albumen. This can be made easily and stored indefinitely. Mayer’s albumen can even be left out at room temperature, but it is better to store it in the refrigerator.

**Mayer’s albumen adhesive:**

Equal parts:
- glycerin
- egg white (shaken first to break it up and make it slightly frothy)

Add a few crystals of thymol as a preservative. Store in a dropper bottle, in the refrigerator.

**For use** Add a drop of adhesive to each slide. Using a Kimwipe®, smear the drop across the area of the slide where you will be placing sections. You should see a shiny film of adhesive when you tilt the slide into the light. It is important not to apply too much adhesive, since it will stain during the staining procedure and will cause too much background staining if applied too thickly.

Now add a dropper full of boiled distilled water from a dropper bottle. This water should have been recently boiled and cooled. The boiling removes air from the water and helps to prevent air bubbles from forming underneath your sections.

You can now place lengths of paraffin sections onto the slide. Using a scalpel or safety razor blade, cut your paraffin ribbons into lengths. These should be no longer than half the length of the coverslip that you’ll be using.

**Mounting the ribbons** Using two camel’s-hair paintbrushes to hold the two ends of each piece of ribbon, transfer pieces of ribbon to the water on the slide. Ribbons of serial sections must be placed in order, one under the other, until the width of the slide is filled (Figure 12.3). It is best if the ribbons are touching one another along their length so that they are rafted together. This makes it easier to manipulate the ribbons later.

Place the slide on a **slide warming tray** that is set at a temperature that is 5–10°C below the melting point of the paraffin. Usually this is a temperature that feels very warm to the touch but not hot enough to burn you. As the water warms on the slide, the sections will expand, greatly increasing the length of the ribbons.

When the ribbons look completely expanded (this takes about 1 minute), remove the slide from the warming tray. Tip one corner of the slide against absorbent paper, such as a Kimwipe®, to wick off the water without disturbing the ribbons. You need to remove only as much water as will pour off. This leaves a film of water behind that allows the repositioning of any ribbons that have become displaced.

Using a dissecting needle, reposition the ribbons so that they are arranged as you wish on the slide. Place the slide back on the warming tray for about 10 seconds. This will help flatten any slight ripple you might have caused when repositioning the ribbons. It is extremely important that you do not melt the paraffin, since this will seriously distort your sections. Remove the slide from the tray and place in a slide holder.
**Drying slides**  The slides must now dry for at least 6–8 hours (or overnight) before staining. If any water remains prior to staining, the sections will not stick properly to the slide. It is best to dry the slides in a slide-drying oven (set on warm) or in a jar that contains desiccant.

**Staining**

During the processing of the slides, the general scheme is that first the paraffin is removed from the sections using xylene or toluene, since the paraffin prevents staining of the tissues. The slides are then processed down to water, since most staining solutions are water-based. This requires a graded series of alcohols to prevent severe convection currents that would damage the tissues. Following staining, the slides are dehydrated again through a graded series of alcohol, cleared in xylene or toluene, and coverslips are applied using a plastic mounting medium that is miscible with the clearing agent.

For clearing agents, it is better to use toluene than xylene since it causes less shrinkage. For alcohol, it is better to use ethanol than methanol since it is gentler on tissue and is nontoxic.

**Setting up the staining series**  There are a variety of types of staining dishes that can handle a few slides up to many slides at a time. For up to nine slides, Coplin jars (Figure 12.4) are used (place every other slide at an angle so that it is sharing a slot with the slide in front of it on one side and sharing a slot with the slide behind it on its other side). It is best, however, to process no more than five at a time; each slide is slid into a pair of slots and is separated from the next slide by a pair of glass ridges. This ensures that no slide touches another slide. A distinct advantage to Coplin jars is that most have screw tops, which will prevent evaporation of solutions between use.

The Coplin jars should be set up and labeled prior to the beginning of staining. For a typical hematoxylin, eosin, and alcian blue staining process, set up the following series of Coplin jars and label both the jars and their tops. Use label tape and always label in pencil since toluene (and xylene) as well as alcohol typically dissolve the ink of other markers. On a jar that is in the “down” series, a downward pointing arrow can be used to indicate this, and on a jar that is in the “up” series, an arrow pointing upward can be used. In the down series, there are two jars of toluene (or xylene) to ensure that all paraffin is removed. On the up series, there are two jars of 100% ETOH to ensure that all water is removed from the sections. These are the two most critical steps in the series for determining success.

![Figure 12.4](image)

A Coplin jar used in staining slides. Slides are placed in slots between glass ridges to keep them from touching one another.
Coplin jars for a hematoxylin, eosin, and alcian blue staining series

- Toluene (or xylene) I — ↓
- Toluene (or xylene) II — ↓
- 100% ETOH — ↓
- 95% ETOH — ↓
- 70% ETOH — ↓
- Distilled water — I
- Alcian blue
- Distilled water — II
- Hematoxylin
- Tap water
- 70% ETOH — ↑
- Alcoholic eosin
- 95% ETOH — ↑
- 100% ETOH I — ↑
- 100% ETOH II — ↑
- Toluene — ↑

Using the hematoxylin, eosin, and alcian blue staining series

Hematoxylin and eosin (H&E) are the most commonly used general nuclear and cytoplasmic stains. Hematoxylin (a basic dye) stains acidic components, primarily nucleic acids, a dark blue and is therefore used as a nuclear stain. Eosin (an acidic dye) has an affinity for cytoplasmic elements. Its yellowish-red color makes it an ideal counterstain to hematoxylin. Alcian blue stains glycosaminoglycans (e.g., mucus, chondroitin sulfate of cartilage) and is a simple addition to the H&E series that adds useful information.

Reasons for the order of things  Because alcian blue is used at a very acidic pH, it must be used prior to hematoxylin in the staining series to avoid removing any bound hematoxylin. A tap-water bath is placed after hematoxylin as a “bluing agent.” Tap water is slightly basic, and a basic solution is needed to change the color of the bound hematoxylin from a rusty brownish color to a dark, almost black, blue. Moving up from eosin should be done as quickly as possible to avoid losing too much eosin from the tissues. Processing can be speeded up by gently dipping the slides in and out of the solutions to increase the speed of diffusion. This must be done gently to avoid detaching the sections from the slide.

Filtering stains before use  A precipitate forms in most stains, requiring that they be filtered before use. Hematoxylin will need to be filtered if it has been sitting for more than a day. Alcian blue should also be filtered but doesn’t need filtering as frequently—once a week should be sufficient. Alcoholic eosin forms little precipitate. Simply check for a precipitate and filter if necessary. (Unbleached coffee filters make inexpensive filters that are sufficient for removing the precipitate from stains.)

Remember that toluene and xylene are toxic, and these should only be used where there is good ventilation. Use in a fume hood (or handle them outside!).

Staining schedule

- Toluene (or xylene) I — ↓ 20 min.
- Toluene (or xylene) II — ↓ 20 min.
- 100% ETOH — ↓ 5 min.
- 95% ETOH — ↓ 5 min.
- 70% ETOH — ↓ 5 min.
- Distilled water — I 5 min.
- Alcian blue (filter before use) 10 min.
- Distilled water — II 5 min.
- Hematoxylin (filter before use) 2 min.
- Tap water — several changes 1 min. each
- 70% ETOH — ↑ 5 min.
- Alcoholic eosin 5 min.
95% ETOH — ↑
100% ETOH I — ↑
100% ETOH II — ↑
Toluene (or xylene)* — ↑
1 min. dipping
1 min. dipping
1 min. dipping
2 min. and hold

*On transferring your slides to the final toluene (or xylene), if you notice any white clouding of the fluid, this means that there is still water on the slide and in the tissues. You must immediately go back to 100% ethanol. If you don’t, there will be water droplets in your preparation that will make your slide look like salad dressing.

### Formulary for staining solutions

**Alcoholic eosin**
- eosin Y, saturated solution in 95% ETOH (approx. 0.5 gm/100 ml)
- 95% ethanol

**Alcian blue**
- alcian blue
- distilled water
- glacial acetic acid
- thymol (to prevent mold)

Filter before use.

**Hematoxylin**

There is true art to making hematoxylin solutions. You can buy them already made, or you can make them yourself. For any hematoxylin solution, it is important to know the shelf life of the solution. Some will keep for years (Delafield’s and Erhlich’s), and some last only a month or two (Harris’s).

**Ehrlich’s hematoxylin**
- hematoxylin
- ammonium alum
- (NH₄)₂SO₄·12H₂O
- 100% ethanol
- glycerin
- distilled water

Add 10 ml glacial acetic acid. Keeps for years.

### Mounting Coverslips

You are now ready to put a coverslip on your slide using a mounting medium. Since you are going from toluene (or xylene) to this mounting medium, this step should be done in a fume hood.

The mounting medium you use should be a neutral mounting medium such as Histoclad. Cheaper mounting media, such as Permount, are acidic. They cause the stain to fade and oxidize over time, making a cloudy ring around the edge of the slide that gradually works its way to the middle. Any slide that needs to be permanent is worth the extra cost of a neutral mounting medium.
The hardest part of mounting is devising a method to lower the coverslip without introducing air bubbles into your preparation. Once you start the mounting procedure, work with speed. Do not let your sections dry out. They will do so very quickly. The amount of mounting medium you should put on the slide depends on the length of the coverslip you are using. Use an eyedropper to put a pencil’s width of medium down the length of the slide to match the length of the coverslip. Hold one end of the coverslip with forceps, and lower the other end onto the slide, holding it in place with a dissecting needle. Slowly lower the rest of the coverslip. If an air bubble is introduced, it will usually move to the edge as you lower the coverslip. Once the coverslip is in place, very slight pressure on the top of the coverslip using the end of a pencil or dowel (you can use the handle end of your dissecting needle) will push out any bubbles on the edges. This must be done gently to avoid damaging the sections. In most cases, bubbles should just be left where they are. A few bubbles are better than smashed sections.

If you have too much mounting medium so that it is oozing out from under the coverslip, don’t touch it. After it dries for a week, excess mountant can be removed with a razor blade (carefully).

Allow your slides to dry flat in a dust-free environment (such as a small cardboard box used for stationery or a flat cardboard slide holder). These should be left at room temperature for several days before observing them under the microscope. After a week, once they are thoroughly dry, they can be stored on their sides in regular slide boxes.

**Accompanying Materials**

See *Vade Mecum: “Histotechniques.”* This chapter of the CD illustrates, with movies and still pictures, how to do each of the procedures described here.

**Selected Bibliography**


Humason, G. L. 1979. *Animal Tissue Techniques,* 4th Ed. W.H. Freeman, San Francisco. This is a classic, and still very useful, handbook on histological techniques. It gives very complete instructions on each step in the processing of tissue, as well as recipes for fixatives and stains, and explains what each is specific for.

H. Schiebler (eds.), *Prog. Histochem. Cytochem.* 27: 1–127. This guides you through steps needed to adjust the microwave protocol for processing tissues to the particular microwave oven you are using.


**Suppliers**

A large biological supply house, such as:

**Fisher Scientific**
585 Alpha Dr.
Pittsburgh, PA 15238
1-800-766-7000

[www.fishersci.com](http://www.fishersci.com)

A full line of histological supplies, including reagents, stains, staining dishes, slides, coverslips, embedding media, mounting media, microtomes and microtome supplies, slide warmers, and slide boxes.

**Ward’s Natural Science Establishment, Inc.**
P.O. Box 92912
5100 West Henrietta Road
Rochester, NY 14692-9012
1-800-962-2660

[www.wardsci.com](http://www.wardsci.com)

Ward’s offers an economical line of student microtomes for cutting single sections at a time, not suitable for serial sectioning. They also sell a selection of histological supplies and neutral mounting media.

**Glossary**

**Alcian blue:** A dye that, when in an acidic solution, stains glycosaminoglycans (e.g., mucus and chondroitin sulfate of cartilage).

**Bouin’s fluid:** A type of fixative that contains picric acid, formalin, and glacial acetic acid. It is particularly good for fixing lipid-rich tissues, and is therefore often used to fix embryonic specimens.

**Carnoy’s fixative:** A fixative that contains glacial acetic acid and ethanol. Though a harsh fixative on tissues, it has the advantage of not leaving any toxic residues behind once it has evaporated.

**Chuck:** In histological procedures, a chuck is the piece of wood, synthetic fiber, or metal on which the paraffin block is mounted. The chuck fits into the chuck holder of the microtome.

**Clearing:** The process in a histological preparation in which tissues become clear and translucent. Common clearing agents include xylene and toluene, which are immiscible with water, and require that the tissues be dehydrated prior to clearing. Glycerin is a clearing agent that is miscible with water, and can be used without dehydrating the specimen.
Coplin jar: A type of glass jar that is used for staining histological sections mounted on slides. The jar has sets of parallel grooves that allow multiple slides to be stained without their touching one another.

Coverslip: A thin transparent cover that is placed over a specimen on a microscope slide. They are usually made of glass and are of the same refractive index as the mounting medium used for making histological slides.

Cytological: Cellular.

Dehydration: The removal of water. In histological procedures, a specimen is usually dehydrated through a graded series of alcohol prior to embedding or mounting.

Dietrich's FAA: A fixative that contains formalin, alcohol, and acetic acid. Because of the acetic acid, it tends not to overshrink or overharden tissues.

Embed: The process of infiltrating a specimen with a substance such as paraffin so that the specimen can be subsequently sectioned for histological examination.

Eosin: An acidic dye that has an affinity for cytoplasmic elements. Its yellowish-red color makes it an ideal counter stain to hematoxylin.

Fixation: The process of fixing a tissue. Fixation kills the tissue, as well as any bacteria present, and coagulates (cross-links) proteins.

Fixative: Anything that fixes a living organism so that it can't be degraded by bacteria or other microorganisms.

Fluorescence microscopy: A type of microscopy in which molecules in the specimen are made to fluoresce—that is, emit light by being activated with short-wavelength illumination such as the ultraviolet light from mercury arc lamps or lasers. The fluorescing molecules can be fluorescent dyes that stain components of cells; some biological molecules, such as chlorophyll, fluoresce themselves.

Formaldehyde: A pungent gas, HCHO used in solution as a fixative. It is sold as a solution (about 40% formaldehyde in water) called formalin. OSHA has determined that formaldehyde is carcinogenic, so care should be taken to avoid fumes and gloves should be worn to avoid contact with the skin.

Glycosaminoglycan (GAG): A long, unbranched polysaccharide (formerly called mucopolysaccharide) made up of repeating units of a disaccharide in which one of the sugars is an amino sugar. Complexes with protein to make proteoglycans. Out in the extracellular matrix, they cause extensive swelling of spaces by absorbing water. Examples of GAGs are hyaluronic acid (hyaluronate), chondroitin sulfate, and heparan sulfate.

Hematoxylin: A basic dye that is used as a histological stain. It stains acidic components, primarily nucleic acids, a dark blue and is therefore used as a nuclear stain. Cytoplasmic ribosomes are also darkly stained.

Histological study: The study of tissues.

Immunohistochemistry: Using antibodies, specific to target molecules, complexed to histological stains in order to locate even minute quantities of the target molecules within tissues and cells.

Mayer's albumen adhesive: An adhesive that is used to affix paraffin sections to a glass slide. It is made from egg whites and glycerin.

Microtome knife: Any knife that fits on a microtome and is used to cut histological sections of a specimen.

Microtome: A machine used for making exceedingly thin sections of a specimen. Different types of microtomes include the sledge microtome and rotary microtome.
Mounting medium: The medium in which a specimen is mounted for observation under the microscope. A mounting medium can be water soluble, such as glycerin jelly, or immiscible with water, such as a plastic polymer dissolved in a solvent. When a mounting medium dries, it is firm and transparent, and typically has the same refractive index as glass.

OSHA: Occupational Safety and Health Administration, a division of the U.S. Department of Labor.

Paraffin embedding: Infiltrating a histological specimen with paraffin. A specimen is first dehydrated and cleared, and then immersed in melted paraffin. Once infiltrated with paraffin, the specimen is placed in a mold containing melted paraffin; when the paraffin cools, the paraffin block can be sectioned on a rotary microtome.

Picric acid: A yellow crystalline substance that is an excellent fixative of lipid-rich tissues. It is also highly explosive in dry form and therefore must be stored with water covering the crystals. (The destruction of the Halifax harbor during World War I was caused by an explosion of picric acid aboard a munitions ship.)

Rotary microtome: A machine used for sectioning embedded specimens into very thin slices. It holds a sharp steel blade and has a wheel that is turned to advance the specimen towards the blade.

Serial sections: Histological sections of a specimen that are taken sequentially, and kept in order. Usually every section is mounted in correct order for microscopic observations.

Slide holder: A simple apparatus for holding slides. Usually it is made of metal or glass, with slots that allow multiple slides to be loaded into the holder.

Slide warming tray: An electrically heated tray, much like a food warming tray. The rheostat allows the tray to be adjusted to an appropriate heat, warm enough to flatten paraffin sections, but not so hot that it melts them.

Stained: In histological procedures, this means dyed with histological dyes to contrast various components of cells and tissues.

Staining series: In histological procedures, the series of steps that first prepare sections for staining, then stain the sections, then prepare the sections for final mounting of a coverslip over the sections.

Washing: In histological procedures, this is the step following fixation in which the fixative is washed out of the tissue.