Cryosectioning

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ABSTRACT

This unit describes sample preparation and sectioning methods for frozen tissue. Sections of this type are used in a variety of light microscopic procedures including in situ hybridization, immunohistochemistry, and enzyme histochemistry. *Curr. Protoc. Cytom.* 48:12.15.1-12.15.7. © 2009 by John Wiley & Sons, Inc.

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SPECIMEN PREPARATION AND SECTIONING

BASIC PROTOCOL

Samples are quick-frozen and then sectioned. This protocol produces sections that are particularly suitable for immunohistochemistry. See Commentary for critical parameters on specimen size before freezing tissue. However, if the tissues are not correctly frozen, artifacts will result. For the majority of studies, investigators generally use the paraformaldehyde/sucrose infusion method, suggested as a support protocol below. The major advantage of direct freezing is that it is extremely quick and the functional state of proteins is not compromised, such that sections may be cut and used for biochemical or molecular studies. This is not possible following fixation.

Materials

Liquid N_2
Isopentane
OCT compound (Tissue Tek II, Miles)

50-ml Pyrex beaker
Small Dewar flask or expanded polystyrene box
Filter paper cut into 1 × 7–cm strips (e.g., Whatman 50)
Forceps
Metal rod
Cryostat and microtome equipped with plastic roll plate (Fig. 12.15.1)
Cutting chuck (metal platform that supports specimen during sectioning)
Heat sink or CO_2 jet freezer
Fine brush and $^1/_4$-in. brush
Glass slides, preferably “Superfrost” (Fisher Scientific) or their equivalent

NOTE: All tools used in the cryosectioning, including the trimming razor blade, should be prechilled within the cryostat chamber.

NOTE: The slides should not be chilled.

Freeze tissue specimen and prepare for sectioning

1. Chill a 50-ml Pyrex beaker by immersing in a Dewar flask (or expanded polystyrene box) filled with liquid N_2.
2. Fill beaker with isopentane
3. Label one end of a filter paper strip with a pencil and place the specimen on the other end with forceps.

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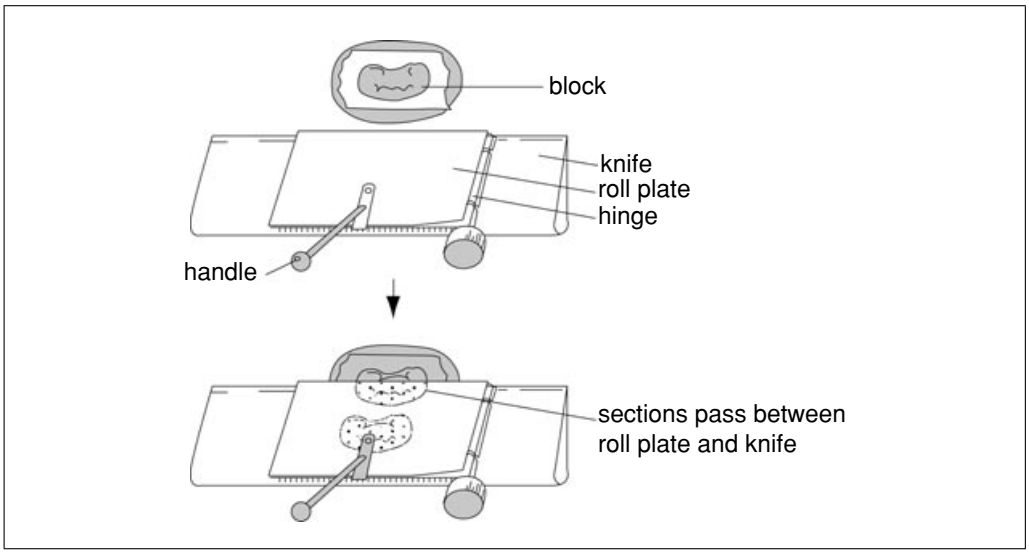

Figure 12.15.1 A plastic roll plate is hinged at the back of the microtome's knife holder and placed parallel to the plane of the knife edge. The plate touches the knife at the leading edge and is tilted up from the knife at the rear so that sections may pass between the knife and the plate.

4. Ensure the isopentane is liquid. If necessary, melt the isopentane by touching it with a metal rod.

5. Immerse the specimen in cold isopentane ~1 min. Some of the isopentane may solidify on the sample, but this will evaporate in the cryostat.

   At this stage, specimens may be stored in either liquid N_2 or in a −70°C freezer.

6. Place filter paper strips with tissue specimens in a cryostat chamber and mount on cutting chucks with a thin layer of OCT compound (Fig. 12.15.2).

   Do not use excessive OCT compound as this may lead to freezing artifacts; in particular, immersion of tissue in OCT should be avoided, unless small multiple specimens are being sectioned (see Commentary).

7. Cool the specimen/chuck mount within the cryostat with a precooled heat sink or a CO_2 jet freezer until the OCT compound solidifies.

   The specimen block must remain frozen throughout the mounting procedure to avoid formation of cryoartifacts (see Table 12.15.1).

8. Leave specimen within the cryostat for >10 min to reach the same temperature as the prechilled microtome.

9. Trim specimen block to a trapezoid shape—if it is convenient and does not compromise the morphology—using a prechilled razor blade. Tear off filter paper.

Section specimen block

10. Mount the chuck in the microtome with the parallel faces of the trapezoid in line with the knife and the wide edge toward the knife edge.

11. Retract the specimen block until it easily clears the knife edge.

12. Produce a smooth "face" on the block using the knife at rapid cutting speed (one section/sec).

    Choice of section thickness is important, as this will have considerable effect on the final resolution at the microscope. Generally, the thickest usable sections are ~10 μm and should not exceed 20 μm, since extremely thick sections will frequently shatter, rendering the specimen useless. It is best to reduce section thickness until sectioning is only just possible (~5 μm), as this will markedly improve quality.

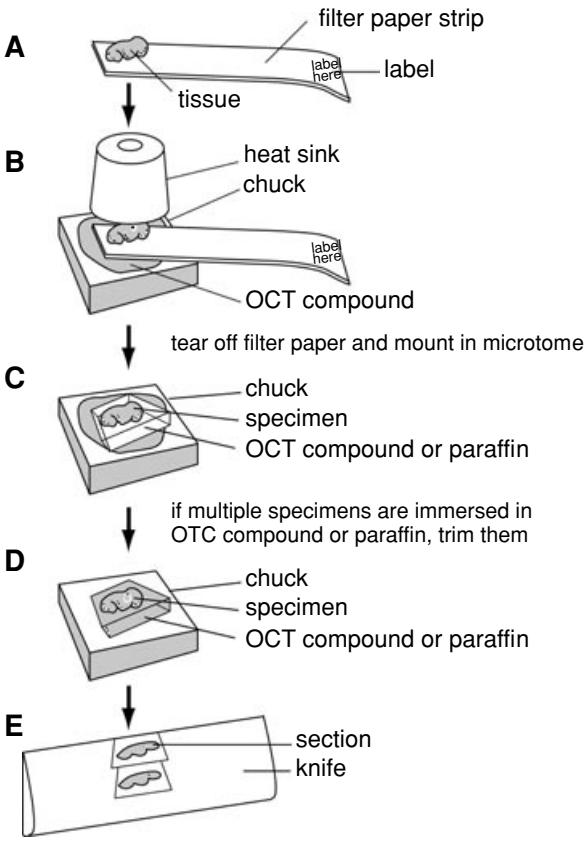

Figure 12.15.2 Sectioning procedure. **A** to **E** illustrate cryosectioning and correspond to steps 6 to 13 of the Basic Protocol; **C** to **E** also illustrate paraffin wax sectioning.

13. Once the specimen block has been cut so that either the structure of interest can be seen within the cut face or the block dimensions are optimal (between 0.5 and 1 cm on a side), sections may be collected.

Collect cryosections

14. The plastic roll-plate is used as it allows easy serial sectioning:

    a. Prior to sectioning, flip the roll plate down such that it touches the knife and lies parallel to the knife edge, though retracted slightly behind the cutting edge of the knife (Fig. 12.15.1).

    b. Turn the crank and cut sections, which under these conditions will roll up at the cutting edge of the knife.

    c. Lift the roll plate and, with a small brush, brush cut sections from the knife edge.

    d. Drop the roll plate and advance it slightly.

    e. Repeat steps 14a to 14d until sections pass smoothly between the roll plate and knife (generally when the roll plate leads the knife edge by ~0.5 mm), then proceed with step 15.

15. Lift the roll bar or plate and move the cut sections to the rear of the knife with a fine brush.

Table 12.15.1 Troubleshooting Guide for Morphological Problems Encountered During Cryosectioning

Problem	Possible cause	Solution
Poor morphological preservation throughout tissue	Inadequate care during dissection or excessive delay between dissection and freezing	Take great care during dissection not to damage tissue by stretching or excessive bending. Freeze tissue soon after dissection to prevent proteolysis by endogenous proteolytic enzymes. This is particularly important for delicate samples (e.g., small embryos or brain) or enzyme-rich tissues (e.g., liver). Fixation and infusion with sucrose may improve morphology considerably (see Support Protocol 1).
Holes in tissue	Ice crystal formation in tissue during freezing, or due to thawing of the block and subsequent refreezing and ice crystal formation in the cryostat	Ensure that blocks are frozen in isopentane rather than a gas such as nitrogen. The latter will boil on contact with the warm tissue giving a "shell freezing" effect, where the boiling gas insulates the outer layers of the tissue from the cold liquid nitrogen, thereby slowing freezing and allowing ice crystal formation. Ensure that when specimens are mounted in the microtome they are protected from warming by a large precooled heat sink. Ensure that you do not touch the specimens with your fingers or with warm tools.
Morphology appears fuzzy	"Pressure artifact" due to excessive pressure on the back of the slide while picking up the section	Use less pressure when picking up the section. A mixture of warmth and static charge will normally ensure that sections stick to the slide. If this is not the case, the slide is too cold and may be locally warmed by touching a finger to the back of the slide.
Morphology appears smeared	Slide was moved while lifting section	Hold slide against the rear of the knife and use this as a fulcrum while gently rocking the slide down to touch the section, thereby ensuring no lateral motion of the slide.
Sections do not stick to slide, they wash away during labeling	Treated slides were not used	Use coated slides
Sections blow away from knife edge during sectioning	A buildup of static electricity in the cryostat	There is no reliable cure. It is possible to buy polonium brushes, which may help. Antistatic guns (such as those designed for record players) have also been used successfully.
Sections have lines running up them and separate into ribbons on knife edge	Knife is dull	Replace knife, generally disposable knives are used routinely nowadays

16. Collect sections by gently touching to a warm Superfrost slide (see morphology troubleshooting, Table 12.15.1).

    Mounted sections may be left in the microtome until all sectioning is complete; the sections may be stored at –70°C for a limited time (overnight) in an airtight container (also see UNIT 12.16 on air dried sections for immunohistochemical study).

17. After lifting sections onto the slide, clear condensed ice from the knife using a thick ($^1/_4$-in.) brush.

    This brushing may need to be quite vigorous and, therefore, should always be toward the knife edge; otherwise the knife will be rapidly dulled and the brush ruined.

TISSUE FIXATION AND SUCROSE INFUSION

SUPPORT PROTOCOL 1

Frequently for cryosectioning, morphology is much improved if the tissue is fixed and infused with sucrose prior to sectioning.

Materials

Tissue samples *or* isolated embryos
2% (for immunohistochemistry) paraformaldehyde (PFA) fixative (3:1 dilution with PBS of 8% PFA fixative; see recipe)
Phosphate-buffered saline (PBS; *APPENDIX 2A*)
0.5 M sucrose in PBS

1. Fix small tissue samples or isolated embryos (e.g., day 7 or day 8 mouse embryos) in 2% (for immunohistochemistry) PFA fixative for 30 min at 4°C.

   Larger embryos, and some tissues, may require fixation for up to 4 hr. Larger organs should be fixed in situ within the animal by perfusion (see Support Protocol 2) prior to dissection.

2. Following fixation, wash tissue samples twice in PBS.

3. Infuse samples in 0.5 M sucrose in PBS until tissue sinks (~1 to 3 hr).

   This infusion may be extended to overnight, but no longer.

PERFUSION OF ADULT MICE

SUPPORT PROTOCOL 2

Perfusion of animals is essential for achieving good morphology and preservation of brains, kidneys, hearts, and many other organs. For the untrained person the first perfusion might not work—it is therefore advisable to learn this procedure using some control animals first, or to seek the advice of a trained animal pathologist or colleague familiar with this procedure.

The protocol described here for mice (applicable also to most other species) involves first exchanging the animal's blood with phosphate-buffered saline and subsequently exchanging the saline with freshly prepared ice-cold (4°C) 2% paraformaldehyde. This protocol gives reproducibly good results without the necessity of using a pressure-controlled perfusion setup. These more sophisticated setups (available in major Anatomy departments) need to be used if, for example, blood vessels or kidney glomeruli need to be fixed in situ without any possible collapse of capillaries.

Perfect perfusion requires working quickly and methodically. If large areas of grayish-white decolorization are not seen on the liver, spleen, and kidneys of a perfused animal, it is not worthwhile continuing with the procedure. The main causes of a failed perfusion are severing of major blood vessels during opening of the animal, improper insertion of syringe needle into the left ventricle (thereby penetrating into other heart chambers or going all the way through the heart), and slow perfusion, leading to blood clotting.

Materials

Phosphate-buffered saline (PBS; *APPENDIX 2A*)
2% paraformaldehyde (PFA) fixative (3:1 dilution with PBS of 8% PFA fixative; see recipe), freshly prepared at 4°C

Two 20- to 30-ml syringes equipped with 23-G needles
Container (bag) for mouse and CO_2 gas
Dissection instruments (scissors, forceps, etc.)
Labeled glass vials (filled with 2% PFA fixative)

1. Fill one syringe with 1× PBS, and another with 2% PFA fixative, 4°C. Set aside.

2. Kill mouse with CO_2 gas in a bag. Immediately after respiratory arrest, lay mouse on its back and open the thorax carefully to avoid excessive bleeding. Cut carefully through the rib cage and remove the diaphragm for access to the heart.

   It is important to work fast, but be careful to get a good perfusion. If the blood clots or the main blood vessels are harmed, perfusion will not work.

3. Carefully insert syringe filled with 1× PBS into the left ventricle. Cut open the right ventricle for drainage, allowing the 1× PBS to be slowly but constantly perfused into the heart.

   If the perfusion is working well, blood-rich organs such as liver, spleen, and kidneys will turn grayish-white.

4. After most of the blood has been flushed out, remove the syringe with 1× PBS and insert syringe filled with 2% PFA fixative (4°C) into the same puncture of the left ventricle. Slowly perfuse the mouse with ∼10 ml fixative.

   The start of the perfusion with 2% PFA is considered the starting point of the fixation process (t = 0).

   A sign of a good perfusion is a muscle tremor best seen on the limbs and tail. By the end of this procedure, the animal should be stiff. If perfusion is unsuccessful, it is not worthwhile continuing at this point (see Commentary).

5. Following perfusion, dissect out organs and tissues, transfer into well-labeled glass vials (filled with 2% PFA fixative), and store on ice (4°C). Further fixation via sucrose infusion is described in Support Protocol 1.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Paraformaldehyde (PFA) fixative, 2%

To make 2% paraformaldehyde, generally an 8% stock is made and diluted as needed. For 100 ml, measure 70 ml of water into a beaker and add 8 g of paraformaldehyde resin (Baker, Fisher, Sigma) while stirring with a stir bar. Cover, transfer the solution to a fume hood, and heat to 70°C. Add 1 drop of concentrated NaOH with a Pasteur pipet. The solution should clear immediately; if it does not, add a second drop of NaOH. There will be some fine particulates, which will not dissolve. Be careful not to overheat the solution. Remove from the heat, add 30 ml of 3× PBS, and cool to room temperature. Adjust pH to 7.2 with HCl, filter to remove particulates, and store at 4°C. This solution should last at least a month; in fact, if it remains clear (no sign of settled particulates in the bottom) you can continue to use it. Dilute to 2% with 1× PBS before use. Always check the pH before use, particularly if using to fix cells expressing fluorescent proteins, as the fluorescence will be quenched at low pH.

COMMENTARY

Background Information

Cryosections, which are rapidly and relatively easily prepared, provide a good system for visualizing cellular fine detail. There are currently three main sectioning methodologies available to study the morphology of cells and tissues at the light microscope level: cryosections, paraffin sections, and plastic (commonly methacrylate) sections. The latter two methodologies generally present a superior morphology than cryosections. However, the preparation procedures are more arduous, and neither method can be used reliably for immunohistochemistry where retention of native protein form and function may be necessary.

Critical Parameters

Fixation

As a general rule, for sections to be used in immunohistochemical studies, fixation should not proceed to the point at which antigenic activity is lost. This is rarely a problem if only formaldehyde is used as a fixative. For standard histological staining methods, where fixation is not important, the morphology may be optimized. If this type of methodology is the ultimate goal of the technique, paraffin or methacrylate sections may be the preferred methods of preparation, as the storage and handling of these types of specimens are both easy and cheap.

Size of specimens

The size of specimens to be sectioned may vary from groups of cells grown in suspension to whole animals—the upper size limit of the specimen is normally defined by the capacity of the standard laboratory cryostat, ~1.5 to 2 cm^3. The method of preparation prior to sectioning is important, as this will ultimately affect the final image quality.

Small, multiple specimens. This category includes samples ranging in size from cells (e.g., lymphocytes grown in suspension), to small animal samples (e.g., early mouse embryos or small nematodes). With specimens of this type, orientation is generally not possible. Instead, sufficient samples are included at random orientations in each specimen such that most or all orientations are present. Prior to freezing, specimens are mixed as a slurry in an inert supporting medium which is easy to section (e.g., OCT compound).

Large specimens. Larger specimens, such as organs or tissue samples, are generally sectioned individually. A factor that should be considered prior to freezing is whether the tissue sample has a homogeneous density, as this will make sectioning much easier. An example of extreme inhomogeneity is whole animal limbs, where skin, muscle, and bone may all be contained within the same sample, making sectioning very difficult; in this case, specific tissues should be dissected out prior to freezing. Small tissue samples, which are easily lost and are not available in large quantities, are best mounted in another tissue, such as in a "liver sandwich," which frequently serves as a negative control, as well as a tissue support.

Another feature, which should be considered prior to sectioning, is tissue orientation, as this may be indistinguishable in whole frozen tissue. This is important in tissues such as muscle or pieces of brain; here the type of information gathered from the labeled sections will depend entirely on orientation.

Sectioning

The cryostat must be thermally stable prior to sectioning. Fluctuations in temperature make sectioning very difficult as the specimen will either contract away from the knife or, worse still, expand into the knife, usually causing the sample to tear out from the chuck and be ruined. For this reason, it is necessary to allow the cryostat to stabilize for some time (30 min to 1 hr) after the chosen temperature is reached, or after altering the temperature, before proceeding with sectioning. Normal cutting temperatures are between −30° and –20°C. Usually, softer tissues require colder cutting temperatures. As a guide, softer samples (e.g., brain tissue) are cut at –30°C, while harder samples with a defined cytoskeleton (e.g., muscle) are cut at about –20°C. For very hard specimens it may be necessary to raise the temperature to –15°C, although temperatures any warmer than this may lead to excessively soft specimen blocks. It is quite easy to recognize temperature problems. Generally, if the sections appear brittle and crumble, the cryostat is too cold. If the sections wrinkle excessively, the cryostat is too warm.

Morphology

A variety of morphological problems may arise. Refer to Table 12.15.1 for a guide to troubleshooting some of the more common difficulties.

Anticipated Results

Cryosections should show good retention of morphology (Fig. 12.16.2), with no artifacts due to ice crystals and sectioning problems as described in Table 12.15.1. The sections provide an ideal substrate for immunohistochemistry (*UNIT 12.16*).

Time Considerations

Specimen preparation. If the specimen is to be fixed and infused with sucrose, this procedure may take several hours; otherwise, freezing the tissues takes only a few minutes.

Sectioning. With practice, this procedure becomes very rapid, taking <30 min for each specimen. However, it takes time to reach this degree of competence, so in the beginning, allot ≥1 hr for each specimen.