

Immunogold Labeling

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§
Infuse with (1.15) M sucrose & 10% PVP
§
Pretrim Block
§
Freeze
§
Ultrathin Cyrosection
§
Section: removal, thawing, and attachment
§
Blocking
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Gold Probe
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UA Stain
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Aldehyde Fix: Chemical fixation is essential for cryoultramicrotomy, and it is necessary for good ultrastructure, antigen immobilization, and prevention of protein extraction from thawed sections during the immunolabelling procedures. The di-aldehyde, glutaraldehyde, widely used together with paraformaldehyde (PFA), is a general-purpose fixative. Paraformaldehyde is freshly made by dissolving pfa powder in dH₂O and by heating the mixture to 65°C. Adding base to neutralize the solution causes the pfa to completely dissolve and clears the solution. The fumes of a hot pfa solution are also a good fixative and should not be inhaled, i.e. use a hood in preparing this solution and appropriate caution. Cover the pfa solution and allow cooling (RT) to occur, after which it should be filtered to remove any large particles. The cooled, filtered solution should be clear and have a neutral pH. A standard fix has: 3% PFA; 0.2-0.5% Glut (if any); and PBS pH 7.4 (8mM Na₂HPO₄, 1.5mM KH₂PO₄; 150mM NaCl, 3mM KCl). Combining the two fixatives in this way gives very good structure and acceptable immunolabeling. High concentrations of glutaraldehyde (used in routine EM, e.g. 3-4%)

will often obliterate the antigenicity of proteins. The limiting factor in chemical fixation is speed and this depends on the rate of fixation, and formaldehyde penetrates tissue (fixes) more rapidly.

PFA alone is a fine fixative for many purposes and rapidly enters cells. However, it does not crosslink proteins as well as glutaraldehyde and often gives poorer overall structure. But this lack of crosslinking can also preserve antigenicity among antibody/protein interactions. Therefore, certain tradeoffs have to be made between structure and the ability for the antibody to bind antigen. Obviously, it is meaningless if you cannot identify what you are looking at, but it is also meaningless if reagents do not work in a biologically meaningful way.

Formaldehyde is actually a gas and is purchased as a 37% (40%) solution. When it is diluted 10 fold it becomes a 4% formaldehyde solution or a 10% formalin solution. Formalin is a great fixative for gross structure preservation, but has little to do with most EM or any immunolabelling procedure. Furthermore, a 37% formaldehyde solution has a significant amount of alcohol (methanol), used as preservative, as well as other contaminants. These contaminants may interfere with fine structure preservation. However, freshly made formaldehyde, from PFA powder, greatly circumvents these problems. To simplify life, pfa can be made-up as a 16% sol., aliquoted, and stored at -20C for long time periods. Easier still is to buy ampoules of EM grade PFA solution. The mechanism of fixation that is used when fixing with formaldehyde is not clear. Some people believe that true solutions of formaldehyde cannot exist and all formaldehyde is rapidly converted into something like ethylene glycol which will simply precipitate protein from solution.

To fix tissue: For organs such as liver or brain, vascular perfusion is recommended. A 0.1% DMSO solution may improve the rate of fixative penetration. After fixation, the tissue is post-fixed for 1-24 hours in the same fixative, and then cut into 1-2mm³ pieces.

Suspension cells: A double concentrated fixative is mixed with equal volume of growing cells in culture (for E. coli $3-5 \times 10^9$ cells) for 30 - 60 min. These cells are next post-fixed for 30-60 min in standard fixative.

Monolayer of cultured cells: Tissue culture cells ($3-5 \times 10^7$ cells) are grown in flasks or dishes, the medium is removed and cells are fixed for 30 - 60 minutes using standard fixative.

For fixing NIH-3T3 cells:

- 1-tissue culture cells in flask or dish are grown to $3-5 \times 10^7$ cell.
- 2-wash cells 1X with PBS.
- 3-Fix with 8% PFA/250mM HEPES pH7.3 for 30min at RT.
- 4-Scrape cells into roughly 1ml of pfa
- 5-Spin cells at lowest possible speed, 1000rpm
- 6-Replace fix with fresh pfa, do not disturb pellet!
- 7-Leave 15min longer at RT
- 8-Spin at max eppendorf for 5min
- 9-leave at 4C, store

There are two basic problems with fixing cells: The first is the fact that fixatives can destroy the epitope of the antigen making it unrecognizable to the Ab molecule. Destruction of the epitope makes binding of the Ab to the antigen difficult. However, the two proteins might still be able to stick to each other in non-specific and non-meaningful ways. This loss of antigenicity is probably a result of steric hindrance or a conformational change in the protein molecule as the result of fixative binding to the epitope. This problem is most commonly associated with monoclonal antibodies having only one epitope recognition site. Polyclonal anti-bodies have several different antigen binding sites that have to be destroyed for complete reactivity to be abolished. Although, they often have dominant epitopes, which can make them, behave more like monoclonals from a functional standpoint.

The second problem involves that of a reduction in labeling efficiency due to poor penetration of the gold probes on the fixed and sectioned material. This problem has no solution. In essence, only the surface of cryo-sections can be labeled with gold probes. This is an important point, unlike light level immuno labeling a thicker section will not give more signal. A thin section will bind as much antibody as a thick section, but also has better spatial resolution. This also means that both surfaces of a section can be differently labeled under certain conditions, although this is not the way to approach routine double labeling as it can be extremely tedious requiring that primaries never touch alternate surfaces. This is hard to be sure of and is better to design an experiment where the problem is solved by the use of reagents.

Before proceeding to electron microscopy, it is advisable to verify the fixation

procedure by first looking at cell labeling with immunofluorescence. Alternatively, an ELISA dot blot assay can be used to allow the effects of several different conditions to be tested at one time.

If glutaraldehyde is destroying your epitope you can try to 1) omit the glut, 2) replace the glut with 0.1 % acrolein or some other fix, 3) compete the binding of glut to proteins with Ethylacetimidate (EAI) at 20 mM. The bad effects on antigenicity can be reduced if you pretreat your specimen with EAI. Pre-treatment with EAI and subsequent fix in 4% Glut retained the antigenicity of alpha-actinin in one study.

Indirect Intracellular Immunofluorescence opt. for Membrane Proteins

1) Fixative: 3% PFA in Ca/Mg free PBS, check pH with paper

2) Quenching with 50mM NH₄Cl in PBS

3) PBS/Sap/FSGel: 0.01% Sap, 0.25% gel, 0.02% NaN₃, in PBS. Store at 4C. FSG from Sigma comes as liquid, a 4-5x stock solution in 1X PBS can be made and diluted with PBS.

4) Mounting Medium: Mowiol (PVA), alt. use 10% glycerol in PBS. Add 2.5% DABCO to prevent bleaching of FITC. Phenylenediamine can be added to block red bleach, but this is usually not a big deal.

Procedure:

1-Wash cells with PBS briefly at RT

2-Fix ~20min to 1hr in PFA

3-Quench 3X in NH₄Cl, 3min ea.

4-Permeabilize cell with PBS/Sap/FSG for 20-30min at RT; this also blocks.

5-Meanwhile dilute Primary/secondary Ab in PBS/Sap/FSG. See notes. If dilution is not known bracket 10ug/ml, if possible. Spin diluted Ab, esp. secondary, in eppendorf for 5min, max. Do not disturb pellet!

6-Add Antibody to sample; coverslip, chamber slide, well, etc. For coverslips: invert coverslips on a 20ul drop of antibody solution applied to a layer of smoothed parafilm which is then kept in a closed, humidified chamber. Or, keep coverslips upright and incubate in a 24 well plate. However, this requires 200ul of solution to cover the coverslip properly. Incubate 30-60min at RT.

7-Wash 5X in PBS/Sap/FSG 3-5min ea. at RT. Transfer coverslips from drop to drop on parafilm surface with forceps. If in 24 well dish, the dish can be inverted and the coverslips will stay by surface tension probably.

8-Incubate for Secondary Ab as in step 6. However, you will have a different dilution from the primary. Wash as in step 7.

9-For a double label experiment, repeat steps 6-8. If primaries and secondary are

chosen carefully it is sometimes possible to mix the two primaries and two secondaries. This saves about 1 hour if it works, but wastes a day if it does not.

To a large extent it is worthwhile to determine how often you plan to double label and then it might be worth the test.

10-Mount the coverslips in Mowiol, dunk in PBS. but before mounting be sure to remove all salts by briefly dunking the coverslips in water first - this is very important or you will see phosphate crystals. Dry the back of the coverslip with a Kimwipe, not the front or you will wipe-off the cells and your experiment will be for naught. Place a drop of mounting medium on slide, usually 20ul, and place the coverslip gently down on the mounting medium. Blot away excess. If using glycerol, seal the coverslip with nail polish. If using Mowiol, let it dry for at least 20min and it will harden.

11-Store slides away from light, at 4C. Allow warming to RT before examining.

IF protocol from JKB 3/3/97

Gelatin Embedding:

The sample is often embedded in gelatin after fixation. Why? This improves both the sectioning and the adhesive properties. In addition, gelatin adds mechanical support that improves ultrastructure considerably, especially when the sample is full of many tiny holes. Lastly, it also makes it easier to handle individual cells. Other proteins can be used as well, but it is important to note that they should not be able to enter the cell. Proteins or other compounds entering the cell can also reduce the ability of gold to penetrate into the section. Good sectioning has been obtained with 5% acrylamide, but gold particle penetration was completely blocked.

Use 500ul of a 5-10% solution of gelatin (Merck; nr4070; Darmstadt, FRG) in PBS (pH 7.4) at 37C. The cells are spun at 14,000 x g for 1min and excess gelatin is removed. Note: embedding cells in gelatin **does** compromise surface labeling. When surface label is a goal, do not fix or post fix the gelatin. Instead, a 20min incubation on PBS at 37C, after sectioning of the gelatin embedded specimen will solubilize the protein and remove the gelatin coat.

Infusion With Sucrose:

Gelatin blocks are infused with 1M Sucrose for at least 1 hour at RT, the sucrose solution should include 0.5% Glut and 3% PFA. Before cryosectioning, specimens are incubated with 2.3 M sucrose in 0.1M phosphate buffer (pH 7.4) or 1.15M sucrose and

10% PVP in 0.1 M phosphate buffer (pH7.4) for at least 1 hour. PVP helps sectioning characteristics and facilitates section stretching after thawing. Note: some people (F. Wylie) suggest using a 1.8M sucrose with 10% PVP, and this information is from the original Tokyasu paper.

Cells that are not gelatin embedded are stored in 4C as a hard pellet.

To make samples:

- 1-Spin eppendorf tubes at max for 5min again, RT
- 2-Remove bottom of pellet carefully, into fix.
- 3-Slice into several 0.5-1mm pieces, place remaining cell pellet back into tube
- 4-Infiltrate pieces with sucrose/PBS (2.1M/1XPBS/GLY) for 3 X 5min or until samples fall to bottom of dish
- 5-Use transfer pipet to transfer pellet, do not allow sample to break-up
- 6-Place tissue on roughened, labeled copper stub
- 7-Use a paper point, or filter paper to remove excess sucrose.
- 8-Hold copper stub with hemostats and place sample upright into liquid nitrogen to freeze.
- 9-Wait for sample to stop bubbling, 5' and then place stubs sample side down into 0.5ml eppendorf tubes to protect samples.

Pretrim:

Specimens are mounted onto the specimen holder after roughening the surface with fine sandpaper to improve adhesion. The specimen is placed on the holder and excess sucrose is removed with a piece of wet filter paper. The block is pretrimmed with a razor blade until it is 1 cubic mm. A small block is easier to freeze and to cut. A large block, a large evil.

Freezing:

The specimen and the block are frozen by plunging them both into liquid nitrogen. A properly frozen block vitrifies and looks glassy. When frozen properly, samples can be stored for a long time interval.

Trimming:

To trim the specimen, allow the cryo-ultramicrotome temperature to stabilize for 30 minutes. The block face should be trimmed so that the top and bottom are parallel to each other. The actual block face should not exceed 0.2-0.5mm x 0.2-0.5mm, or about 1/5 to 1/10 the width of the glass knife. It is important that the block face and the knife-edge be as clean as possible. An eyelash can be used to remove waste material. However, using an eyelash can generate a lot of static electricity, which may need to be reduced using some sort of anti-static device. The problem of static electricity is more pronounced when you are using a diamond knife. **When using the CR-21 do not use a static gun, as this will damage the electronics!**

Ultrathin Cryosectioning:

Three prerequisites need to be fulfilled for successful sectioning.

1. The knife and specimen need to be as clean as possible
2. The sectioning surface should not exceed 0.5 x 0.5mm
3. Temperatures and sectioning speed need to be adjusted for the optimum setting.

The optimum temperature is between -80 and -100 C and the optimal cutting speed is between 2-3mm/sec (alternatively, some groups use a temperature of -120 C and cutting speeds slower than 1mm/sec). Increased speed facilitates sections, but compresses them as well.

There are two major problems associated with sectioning. One problem is wrinkling and the other is section fragmentation. Wrinkling is easily seen as a loss of interference colors under the stereo microscope. Ideal sections should show interference colors between gold and blue. When sections wrinkle, the temperature should be reduced or section thickness increased slightly. When the sections fragment, on the other hand, the temperature should be increased or section thickness should be decreased. If the problem is only slight, then you can solve the problem by adjusting the sectioning speed. Increasing sectioning speed reduces wrinkles, and

reducing sectioning speed helps prevent section fragmentation. The section thickness varies between 80-100nm.

Again, keep the knife and the specimen clean for best sectioning results!

Section Removal:

The sections are removed by using a 2mm diameter wire loop made from 0.2mm copper wire and dipped into 2.3M sucrose. Place the loop close to the section you wish to pick up, using the sucrose drop as a magnifier. The static electricity properties of the sections will cause them to jump onto the sucrose drop without actual touching. The drop should not completely freeze, allowing sections to stretch. Once the sections have adhered to the drop, they are removed from the cryochamber and allowed to warm up. In warming up, sections stretch somewhat and reduce some of their compression. When sections are picked up they should have a glassy appearance, if they do not look good at this gross level, they seldom look good under the EM. It is best to spend some time with the microtome and get the best looking sections possible. Generally, only three to five grids are needed for each sample. Obviously, the amount of sectioned material needed depends on your experiment. It usually takes less than one hour per sample to get the number of grids that you want. Long ribbons of sections tend to curl when you pick them up, so separate long ribbons into smaller ribbons of 5-7 sections each and pick up several side by side at once. By adding 10% PVP (10,000 MW) to the sucrose infusion solution, sections are supposed to stretch much better. Once good sectioning has begun, as judged by the light level, it is usually easy to pick up several grids rapidly. It is also a good idea to pick-up 2-3 more than you think need, if possible. Just calculate for yourself the amount of time it took to get the microtome set-up and sectioning the kind of sections that look good enough to collect. If you can set-up in 30min and have good section within 15min of sectioning then it is no big deal. On the other hand if it took an hour to set-up (a more likely time from scratch) and 30-40min to begin to get good looking sections then another 15min to get only 2 more grids could be time well spent.

I can't emphasize this enough, if you think that you do not have sections or cells on your grids, do not proceed with the labeling procedure. If you are not sure, it is a good idea to recheck some of your sections. Use sections that you will label and the same thickness. Stain them with UA and MC, see end of notes. And examine grids for

sections and presence of cells. This is also a good time to get a rough idea of stain parameters and overall ultrastructure. Most of the time this step is not necc, as cell pellets are whitish and most of the excess sucrose is removed prior to freezing. Once the fixation protocol is working, this is not a problem rather anti-body reactivity is the problem. However, structural preservation is a matter of degree sometimes and a question becomes how much structural detail can you give up to preserve antigenicity. There is not simple answer here and this is often where looking at sections, prior to labeling, can help. You may also want to play with different buffer conditions, which will extract more or less cytoplasm. Extraction of dense cytoplasm can sometimes help to see the labeling of membrane compartments more clearly; it can sometimes show components attached to the membrane more clearly.

After the sucrose drop has been warmed, the loop can be placed onto Formvar and carbon coated 100 mesh hexagonal grids. 100 mesh hex grids are used to give the most film support for the area that can be viewed. Copper grids are used because they are cheap and, unlike Nickel grids, are non-magnetic. Nickel grids can be also be used, but they cost a bit more and stick together more because of their magnetic attraction. Generally, this can make them harder to use. The Formvar film is loosened around the grid before section placement. Grids will adhere to the copper loop, and touching the surface of PBS at a 90 degree angle to the grid will release hold of the loop such that the sections remain hydrated by floating section side down on the PBS solution. Some people have suggested using 2% gelatin in PBS instead of the PBS alone. Sections can be stored overnight in PBS at 4C. Sections on copper grids are not stored more than a few days as the copper may oxidize turning green. Nickel and gold grids have the advantage of not oxidizing as rapidly and some investigators have stored sections cut on nickel grids as long as one week with no diminution in staining.

Quenching and Blocking:

Incubations are done on a sheet of Parafilm using droplets, 0.2-0.5ml in size. If sections have to be placed on gelatin first, the gelatin needs to be removed first by incubating the sections at 37C for 20minutes, and then diluting away the gelatin by adding PBS at 37C. There are two basic ways to quench and block sections:

- 1, incubate sections in 10% FCS with 0.15M Glycine / 1 X PBS for 10-20minutes at RT; This is the basic blocking solution we use, but there are a number of combinations of blocking compounds and not all antigens behave the same way.

- 2, incubate the grids in 50mM glycine drops for 2 x 5min to quench the fixative,

followed by PBS containing 0.5% BSA and 0.2 gelatin (incubated twice for 5min each on 200ul droplets).

Before using FCS, it should be heat inactivated to remove functional complement. In practical terms, we treat new FCS solutions in a 60 degree water bath for one hour, after which time they can be labeled as heat-inactivated FCS and then frozen in aliquots. Aggregates of protein should be removed by centrifugation or filtration prior to use.

Antibody Incubation:

Antibody incubation may be done at RT or at 4C for O/N. Optimal conditions need to be determined empirically for each antigen - antibody combination. Generally, well characterized anti-bodies (read here, affinity purified especially polyclonals) are used in the range of about 2 - 20 ug / ml. The size of the drop is about 10ul, but some people have used 5ul for precious reagents. Using ab's that have had other protein-A binding components removed will probably give you better signals.

Specificity, most anti-bodies will not label the nucleus and it should appear clean in your samples. Mitochondria are sticky organelles, perhaps due to their high protein content????, and may non-specifically bind some of your antibody. It is a good control. You will want to use the lowest dilution, or highest concentration, of antibody you can that does not give non-specific background label. When using protein-A, if your dilution is too low then you will see protein-A stuck all over your section and your solution, again, is to use a dilution that gives you a signal with no background staining.

Double Labeling-

There are two basic ways to double label your samples:

One, use antibodies from different sources and use the appropriate secondary reagent.

Two, use the standard labeling procedure with protein-A gold. Then saturate the remaining protein-A binding sites by incubating the section with 20ug/ml protein-A for 10minutes. Subsequently, the other antigen is labeled with a specific antibody / Protein-A gold of a different sized gold particle from the first incubation.

Only IgG reacts with protein-A for Immunocytochemistry and even then not equally well for all species. Rabbit IgG is probably the best example of binding for immuno-EM studies. Other examples include guinea pig, human, dog, some mouse

(IgG 2A and 2B) and some rat IgG (IgG 2C and IgG1). On the other hand, poor binding species include: goat, most rat (read most monoclonal ab's), most mouse, most sheep, donkey/horse, cow and protein-A has no binding to avian antibodies. There can be pros and cons to all of this.

Staining:

There are many different ways to achieve this. The most frequent staining procedure is that of adsorption staining. Here, the sections are rinsed on three droplets of dH₂O for five minutes each. This removes most of the ions from the section, next the sections are incubated in 0.2-0.5% UA in 2% Methyl Cellulose for 2-5 minutes at 4 C. Make a 2% methyl cellulose solution (25 centi poise), methyl cellulose takes a while to go into solution and is more soluble at lower temperatures, keep it on ice while you are using it. Spinning the MC for 1 hour at 100,000 x g at 4C removes any aggregates. However, some people prefer to filter this solution through a 0.22-0.4um syringe filter and some people prefer to do both. Take 900ul of 2% Methyl Cellulose, keeping it on ice, and add 100ul of 3% Uranyl acetate (aq.) solution, filtered to 0.22um, to give 1ml of a 1.8% Methyl cellulose solution containing 0.3% Uranyl acetate. Changing the concentration of the stain (from 2%-5% to yield a final UA concentration between 0.2-0.5%) and by adjusting staining time (between 2-10 minutes) will give the appropriate staining properties.

Again, for routine use, 900ul of a 2% MC solution is combined with 100ul of a 3%UA solution to give a final solution of 1.8% MC and 0.3%UA. Keep this solutions cold or it will harden.

Drying in Methyl Cellulose:

The sections are dried until the Methyl cellulose has a blue to gold color. This is done by picking up a grid using a 3.5mm loop and turning the grid perpendicular to wet filter paper and allowing the stain to diffuse into the paper creating a spot about 3-4mm in diameter. It is probably better to leave more methyl cellulose behind than over dry the sections. The drawback with too much methyl cellulose is poor contrast. On the other hand, if you remove too much methyl cellulose, drying artifacts are created

that greatly modify the ultrastructural presentation of the membranes. You can also remove the methyl cellulose by turning the looped grid perpendicular to a piece of dry filter paper and streak the MC for 2.5-3.5cm. Also, you can use the pointed ends of filter paper cut into triangles to withdraw the excess MC from between the grid and loop.

Use methocel MC25 (Fluka AG, Buchs, Schweiz) or methyl cellulose (Sigma Chemical Company, St. Louis, MO.).

Abbreviated Protocol

1. Fix cells with primary fixative, 1hr RT

Something like 4 - 8 % PFA (freshly made) buffered with 0.2 M HEPES, pH 7.4, or use 1X PBS, pH 7.4, some add small amounts of glutaraldehyde to fix. The duration of fixation and the amount of glutaraldehyde depends on the antigen and the quality of your final fix. Some antigens are extremely sensitive to glut. and some tissue is extremely difficult to fix. Very small amounts, 0.1-0.2%, can have remarkable effects on ultrastructure and still only cause minor problems to most antigens. The time of fixation varies, but 1hr is a good place to start. The actual number depends on the cell size, but with HELA or MDCK cells then $3-5 \times 10^7$ cells is fine. However, if much smaller cells are used, such as E coli, then 100 fold more cells are needed to create a good pellet.

2. The cells are scraped, spun at very low speeds (500rpm) to remove excess fixative and then fresh fixative is applied fresh.
3. Cells are spun hard for five min to make a tight pellet and then left O/N at 4C. For more sensitive material, cells are processed through the sucrose immediately.
4. The next day the pellet is removed and cut into 0.5-1.0mm blocks and then infiltrated with 2.1 - 2.3 M sucrose as a cryo-protectant for 15-20min.
5. After about 3 changes and when the blocks no longer float in the sucrose they are hand frozen in liquid nitrogen for storage.
6. The blocks are cut using a cryo - ultramicrotome, -100C, at a section thickness of about 90nm.
7. Using the static electricity on the drop of sucrose sections are picked up and then transferred to a Formvar/carbon coated grid.
8. These grids can be floated off on a dish of PBS, section side down. Grids can be stored for awhile this way before labeling, but they never can dry out.
9. Labeling procedure includes: PBS; 10% Fetal calf serum to block; Primary Antibody; wash in PBS; Secondary Ab (Protein A - Gold); wash in PBS; water to remove salts
10. Embed grid with sections in methyl cellulose - Uranyl acetate to contrast specimen and prevent drying out.

11. View under the microscope

If you use a Lowicryl (i.e. resin based) technique instead,

There is no need to infiltrate in sucrose, so

4. Wash cells in buffer medium used in fixative 3-5 times. Or until fix is gone

5. It may be desirable to cut them up into blocks

6. dehydrate through a series of ethanol solutions,
Begin at 30% for 15 min at 0-4C
50% for 5 min at 0-4 C

7. Then shift solution to -25C for 10min

8. 70% Ethanol 15 min, -25 C
95% Ethanol 15 min, -25 C

100% Ethanol 30 min, -25 C

100% Ethanol 30 min, -25C

9. Lowicryl infiltration

1 : 1 / EtOH : K4M 30 min -25C

1 : 2 / EtOH : K4M 60 min -25C

100% K4M resin 120 min -25 C

100% K4M resin O/N -25 C

100% K4M resin 60 min -25 C

UV light 5 days

Resin recipe

crosslinker A	2.7g	5.4g	10.8g	
crosslinker B	17.3g	34.6g	69.2g	
Initiator		0.1g	0.2g	0.3g

This will make between 5 - 10 mls of plastic per sample.

Solutions

Make Epon mixtures and store at -20C

Perspective on the amount of cytoplasmic extraction varies; some microscopists believe that HEPES is almost as bad as phosphate regarding extraction, and believe that the lowest extraction occurs with cacodylate buffer. Others think that cacodylate extracts more than HEPES. Cacodylate is used now for mostly historical reasons, and it can be argued that there are other buffers, which surpass it in all respects.

2.1 - 2.3 M sucrose solutions in 1X PBS

10% FCS in 1X PBS containing 0.02M Glycine (0.15%)

5% FCS in 1X PBS containing 0.02M Glycine

1X PBS + 0.02M Glycine / filter

Distilled water