6 Microscopic Use of Fluorescent Microspheres

Histological studies of tissues and organs perfused with fluorescent microspheres require modification of most routine tissue processing methods in order to preserve fluorescent microspheres for microscopic visualization. For example, formalin fixation followed by routine embedding in paraffin is not feasible as the standard organic solvents used during tissue processing dissolve polystyrene microspheres. Several different ways of processing tissue were studied and the advantages and disadvantages of each are evaluated. These included: 1) formalin fixation followed by embedding in paraffin; 2) formalin fixation followed by embedding in glycol methacrylate; 3) air-dried lung followed by Vibratome sectioning; 4) formalin fixation followed by Vibratome sectioning; 5) rapid freezing followed by cryomicrotome sectioning. Methods for staining sections and the types of mounting media that can be used to apply coverglasses to slides are also evaluated.

Methods

Aldehyde fixation followed by embedment in paraffin or methacrylate produced sections of well-preserved tissue for microscopic examination. Paraffin blocks were sectioned at 7-10 μm with Sturkey disposable knives on a JB-4 microtome. Methacrylate blocks were sectioned with 12 mm wide triangular glass knives on a Sorvall (now Research & Manufacturing Co.) MT-2 microtome. The knives were made on a Sorvall Glass Knife Maker.

Unembedded tissue was sectioned with a Vibratome 1000 modified with a 1000 Ω rheostat in the cutting blade advance. This allowed a slow forward motion of the oscillating blade through the specimen. This rheostat modification was especially useful for specimens such as airways, which are tough and difficult to cut. A faster forward blade movement for these specimens resulted in deformation of the block and uneven section thickness. Without the rheostat modification, the forward motion of the razor blade carriage had to be stopped for short time periods with the speed control knob while the vibration of the blade continued. A blade clearance angle of 15-17° worked best for dry and ethanol-dehydrated specimens while 17-20° seemed better for gelatin-embedded specimens.

Sections were examined on a Leitz Diaplan compound microscope fitted with an incident mercury arc lamp. Three filter sets were used: 1) blue excitation light (450-490 nm) with a suppression filter for all wavelengths below 520 nm in the observation light path (commonly used for the fluorescein isothiocyanate (FITC) fluorophores); 2) green excitation light (530-560 nm) with a suppression filter for all wavelengths below 580 nm (commonly used for the rhodamine fluorophores); and 3) UV excitation (340-380 nm) with emission suppressed below 430 nm. The blue and UV excitation light caused microspheres of blue, green, yellow-green, and red emission colors to glow brightly (although most of our perfusion protocols used yellow-green and red microspheres only). The green excitation light was useful only for the red microspheres. With blue epi-illumination, microspheres photographed well against the green autofluorescence of tissue with Ektachrome 160T (tungsten) film. The UV illumination was used in combination with bright field transmitted illumination to provide better histological visualization.

A. Paraffin (Paraplast Plus) Embedding

Paraffin sections can be cut serially and 7-10 μm-thick sections provide good histological detail. An antimedium (or transition fluid)—a fluid miscible with both alcohol (used to dehydrate the tissue) and paraffin—has to be used to allow infiltration of the tissue with paraffin since alcohol is not miscible with paraffin. Hydrocarbons such as toluene and xylene have routinely been used for this purpose (Humason, 1979). Such hydrocarbons are now regarded as hazardous chemicals and as fire hazards due to their high vapor pressures (Lyon, et al., 1995). More pertinent to the present study, toluene and xylene also have the undesirable property of dissolving microspheres or removing the fluorophore from microspheres.

Several companies now sell proprietary mixtures as substitutes for hydrocarbons. Several of the proprietary mixtures were evaluated—Histoclear, Histoclear II, Americlear, and Hemo-De. Of these, only Histoclear II retained the fluorescent of the microspheres in paraffin sections. The reasons for the differences in the properties of these four substitutes are not known since they are proprietary mixtures. A schedule for processing with Histoclear II is given in Table 1. Based on the manufacturer instructions, infiltration times were increased approximately 50% compared to the usual processing times used with toluene.

Lyon, et al. (1995) recommended butyldecanoate (CH_3-$(CH_2)_3$-O-O=C-$(CH_2)_8$-CH_3, sold as "Estisol 220") as a substitute for toluene. They recommended clearing and infiltrating times about 4-6 times longer (up to 12 hours per change) than the typical toluene schedule. Paraffin is less soluble in butyldecanoate than in toluene; at 45° C, a saturated solution of Paraplast Plus in butyldecanoate is less than 40% paraffin. A schedule for processing with butyldecanoate is given in Table 2. The fluorescence of microspheres is not as intense as after processing in Histoclear II, particularly for the red

Table 1. Tissue Processing with Histoclear II Antimedium

Tissue blocks up to approximately 10 mm^3. Formaldehyde or glutaraldehyde fixation (not evaluated with other fixatives).

1. Wash in tap water, 3-4 changes of 30 minutes
2. To 35% ethanol – 2 changes of 15 minutes
3. To 70% ethanol – 2 changes of 15 minutes
4. To 70% ethanol – 30 minutes
5. To 95% ethanol – 2 changes of 15 minutes (or overnight)
6. To 100% ethanol – 6 changes of 15 minutes
7. To 100% ethanol + Histoclear II, 1:1 – 20 minutes
8. To Histoclear II – 2 changes of 30 minutes
9. To Histoclear II – 60 minutes (probably a minimum for this medium)
10. To 50% Paraplast Plus dissolved in Histoclear II at 45° C – 60 minutes
11. To pure Paraplast Plus at 60°C – 2 changes of 60 minutes
12. Transfer tissue to embedding mold under an infrared lamp

Table 2. Tissue Processing with Butyldecanoate Antimedium

Tissue blocks up to approximately 10 mm^3. Formaldehyde or glutaraldehyde fixation (not evaluated with other fixatives).

1.   Wash in tap water, 3-4 changes of 30 minutes
2.   To 35% ethanol – 2 changes of 15 minutes
3.   To 70% ethanol – 2 changes of 15 minutes
4.   To 70% ethanol – 30 minutes
5.   To 95% ethanol – 2 changes of 15 minutes (or overnight)
6.   To 100% ethanol – 6 changes of 15 minutes
7.   To 100% ethanol + butyldecanoate, 1:1 – 30 minutes
8.   To butyldecanoate – 2 changes of 60 minutes
9.   To butyldecanoate – overnight
       (alternative steps 8 & 9 – 2 changes of 12+ hours, agitating occasionally)
10.   To 35-40% Paraplast Plus dissolved in butyldecanoate at 45° C – 60 minutes
11.   To pure Paraplast Plus at 60° C – 2 changes of 60 minutes
12.   Transfer tissue to embedding molds under an infrared lamp

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microspheres. Yellow-green microspheres are still bright under blue excitation but red microspheres show fluorescence with green excitation only.

Biobond tissue adhesive was used to improve adhesion of paraffin sections to glass slides. Overnight drying after the sections are mounted on Biobond-coated slides is recommended for good adhesion. The Biobond coat is quite clear and does not stain as a gelatin-coated slide does.

B. 2-Hydroxyethylmethacrylate (Historesin) Embedding

This class of methacrylate resin is also known as glycol methacrylate. The embedding procedure we used was adapted from that developed by Chappard (1985) and it has proven very useful for uniform embedment of lung tissue that contains cartilage-reinforced airways and heavily muscled arteries. Chappard (1985) suggested infiltrating dense tissue with the fully catalyzed hydroxyethylmethacrylate (GMA) monomer at -20° C so that the catalyst can diffuse fully into tissue blocks. At room temperature, the fully catalyzed mixture will polymerize in minutes but polymerization is often incomplete in the center of dense tissue blocks larger than approximately 6-8 mm^3 as the catalyst cannot diffuse to the center of tissue blocks in such a short time. Tissue in catalyzed Historesin can be kept at -20° C for several weeks without polymerization if the monomer is changed every 24 to 48 hours. Even slightly higher temperatures (e.g., -16° C) allow polymerization in less than 24 hours. Blocks with cartilage or thick-walled arteries require 14 or more days in the catalyzed monomer. A schedule for processing with Historesin is given in Table 3.

Blocks, 4 x 6 mm in size, of fixed lung, heart muscle and kidney section well at 3-5 µm after 14 days infiltration at -20° C. Air dried lung may also be embedded in GMA with this procedure. Both yellow-green and red microspheres retain their fluorescence throughout the embedding process.

Table 3. Tissue Processing for Historesin Embedding

Tissue blocks 5 x 5 x 8 mm. Glutaraldehyde fixation (not evaluated with other fixatives).

1. Rinse in buffer used for the glutaraldehyde – 3 changes of 15 minutes
2. To 35% ethanol – 2 changes of 15 minutes
3. To 70% ethanol – 4 changes of 15 minutes
4. To 95% ethanol – 2 changes of 15 minutes
5. To 100% ethanol – 4 changes of 15 minutes
6. To 100% ethanol + Historesin Infiltrating Solution, 1:1 – 20 min (optional)
7. To Historesin Infiltrating Solution at 4° C – overnight (vacuum degas enough of the Infiltrating Solution for step 7 and to make up the required Embedding Solution as well; lung tissue may benefit by being kept under vacuum overnight instead of in a refrigerator)
8. To Historesin Embedding Solution at –20° C – 24 hours (always handle the Embedding Solution on ice and return to freezer as quickly as possible)
9. To Historesin Embedding Solution at –20° C – 6-13 days, changing to freshly made Solution every 24 to 48 hours (usually safe to leave tissue in the same solution over a week-end after the third day)
10. Transfer tissue to embedding molds on ice, fill the lower half of the mold with fresh Embedding Solution, and cover the tissue and solution with a piece of Parafilm; polymerize overnight in a refrigerator at 4° C or keep larger molds on a bed of crushed ice
11. Attach to specimen mounts with the last of the Embedding Solution

C. Vibratome Sectioning of Air-Dried Lung

Unfixed lungs can be dried over a period of several days by inflating with air at a known pressure. Dry lungs are sufficiently rigid to be sliced, and cubes of tissue, 10-15 mm on a side, can be cut out of the slices and fastened with a gel cyanoacrylate glue to 15x19 mm hardwood specimen mounts which fit in the chuck of the Vibratome. Of a number of glues tried, Quick Tite Super Glue Gel was the only really satisfactory product. "Non-gel" glues wick into much of the dry lung and render it too hard to section.

Sections were cut dry with the slowest advance of the cutting blade at a thickness of 100-150 μm and positioned without mounting medium under coverglasses on microscope slides. The coverglasses were then attached to the slides with narrow strips of Scotch Magic Tape.

D. Vibratome Sectioning of Unembedded Fixed Tissue

Tissue (lung, heart, kidney) was formalin fixed and then hardened in absolute ethanol for one week to two months. Cubes of unembedded tissue were glued to reusable, rectangular aluminum specimen mounts with Quick Tite Super Glue Gel. As absolute ethanol does not have the water to catalyze the cyanoacrylate glue, pieces were briefly wet in 95% ethanol before being glued to the aluminum specimen mounts. The glue deteriorates in absolute ethanol so that mounted blocks cannot be stored long term. Tissue blocks were kept wet during sectioning by periodic application of drops of ethanol with a Pasteur pipette.

A slow blade advance is required, especially through airways. Bronchi reinforced with cartilage and heavily muscled arteries will section down to 50-100 μm. For bronchi, it is necessary to section through the same cartilage ring that is glued to the aluminum specimen mount to keep the specimen from bending in front of the knife blade. The histology of fixed lung sections is better than that of unfixed, air dried tissue.

E. Vibratome Sectioning of Gelatin-Embedded (Bacto Gelatin) Fixed Tissue

Embedding irregularly shaped or subdivided specimens in a supporting medium allows the production of cohesive sections. Also, none of the specimen is rendered unsectionable by infiltrated hardened glue as happens with the unembedded material described above. Gelatin embedding does not degrade tissue histology or the fluorescence of the microspheres, but the processing time is lengthy. Gelatin-embedded specimens are usually frozen before sectioning, but Igarashi, et al. (1994) recently described a method for serially sectioning whole rat embryos using a rotating blade machine. We adapted their procedure for use with the oscillating-blade Vibratome.

Tissue pieces were fixed in buffered formalin. To prevent crosslinking (fixing) the gelatin in the infiltrating solutions, tissue was washed in water for at least one day with a minimum of 10-15 changes of water. Purified gelatin solutions of 15% and 30% (15 and 30 g in 100 ml distilled water) were prepared at 55° C. Thymol (0.1%) was added as a bacteriostatic agent. A processing schedule for gelatin embedding is given in Table 4.

Blocks of gelatin with tissue were cast in 22x40 mm Peel-A-Way disposable plastic embedding molds. Although disposable, they are durable enough to be used several times if desired. A 2-3 mm thick gelatin platform was cast in the bottom of the molds. This and the larger size of the mold allow for accurate orientation of the tissue on the specimen mount. The specimens and bottles of gelatin are handled under a red-coated infra-red lamp to prevent unwanted cooling while orienting the tissue in the molds. The warm tissue will sink into the platforms, however, if the latter are not thoroughly chilled beforehand in a refrigerator. Also, to prevent remelting the platform when the tissue is embedded, the molds are filled with the warm gelatin in two or more layers (allowing the first layer to gel at room temperature before adding the next layer).

Table 4. Tissue Processing for Gelatin Embedding

Tissue blocks up to approximately 10 mm^3. Formaldehyde or glutaraldehyde fixation (not evaluated with other fixatives).

1. Wash in tap water – 10-15 changes over 24 or more hours
2. To 15% gelatin (aqueous) at 37° C – 2 changes of 24 hours
3. To 30% gelatin (aqueous) at 37-40° C – 2 changes of 24 hours (more for dense tissue)
4. Transfer tissue to embedding molds (Peel-A-Way molds are particularly useful) under an infrared lamp
5. Chill gelatin and remove from molds
6. Fix tissue + gelatin block in 4% buffered formaldehyde – 24 hours
7. Warm gelatin block and wooden specimen mount; glue mount to block with molten 30% gelatin
8. Fix tissue + gelatin + mount in 4% formaldehyde – 24-48 hours

The filled molds are then chilled and the gelatin blocks removed from the molds. A thin spatula kept wet with water is inserted between the gelatin and mold wall freeing the sides of the block. Twisting the mold frees most of the bottom of the block and it can gently be pried out of the mold with the spatula. If the gelatin blocks are fixed in formaldehyde while still in the molds, they are somewhat prone to fracture during removal. In any case, the Peel-A-Way molds may simply be peeled apart freeing the gelatin block.

Tissue embedded in gelatin does not need much surrounding gelatin for sectioning. The travel of the specimen stage of the Vibratome limits the height of a piece of tissue to 10 mm. A block this tall needs a minimum length and width of about 10x10 mm for structural strength. After a gelatin block is released from its mold, its orientation to the cutting blade is determined and the larger excess dimensions cut away with a razor blade.

The gelatin blocks are then hardened in buffered 4% formaldehyde for 24 hours at room temperature (longer term storage is possible). Gelatin blocks and water-soaked, wooden mounts are then warmed in a 40° C oven and a bottle of 30% gelatin is warmed to about 55° C. The 30% gelatin is used as a glue to fasten gelatin blocks to the mounts. Mounted specimens are returned to formaldehyde for another 24 hours. Igarashi, et al. (1994) recommend 3 days or more.

The blocks, now fixed for a minimum of 48 hours, are given a final trimming with a razor blade. A rectangular cutting face works well, but the geometry of the face does not appear to be critical. The longer side of the block's cutting face should be parallel to the blade to better resist the lateral motion of the cutting stoke as the knife moves forward. The block and the razor blade holder are kept wet with distilled water.

Sections are cut using a slow blade advance. Cutting faces of 11x14 mm have been sectioned at 50 µm and smaller ones at 30 µm. With the larger faces, reliable serial sets have been cut at 100 µm, although it is difficult to avoid thick and thin zones in a small percentage of the sections.

F. Frozen Sections

Historically, this was the first technique we tried in our laboratory to preserve fluorescent microspheres in histological sections. In terms of tissue preparation, the technique is straightforward—fresh unfixed tissue is frozen and sectioned at 20-60 µm. It has the obvious advantage of avoiding organic reagents but the disadvantage that it requires the specialized technique of cutting frozen sections on a cryostat. We have largely abandoned this approach because of several additional problems which include poor histological detail due to freezing damage (which becomes greatly accentuated the larger the tissue size), the sections are unfixed and deteriorate rapidly, and the practical difficulty or impossibility of obtaining serial sections. A variation that we have not tried that would result in better tissue structure and more permanent sections is to chemically fix tissue before freezing.

G. Staining of Sections

Sections can be stained although the tissue autofluorescence with epi-fluorescence optics may be sufficient to allow sections to be viewed and analyzed without staining. This is particularly true for the thicker Vibratome sections and for sections of lung, which have more intense autofluorescent properties than sections of heart or kidney.

Paraffin sections. Sections can be stained with hematoxylin and eosin (Delafield's hematoxylin worked well) or with 0.5% aqueous methyl green (only lung tissue stained satisfactorily with methyl green). The methyl green staining procedure is followed with a rapid wash in absolute alcohol if immersion oil is used as a mounting medium. See the comments below about how mounting media cause fading of the stains.

Methacrylate sections. Tissue embedded in Historesin is not fluorescent so unstained sections are not easy to view with epifluorescence optics. But methacrylate sections can be stained routinely with Lee's stain and viewed with epifluorescence since basic fuchsin, a dye in Lee's stain, is fluorescent.

Vibratome sections of air-dried lung. Staining was not attempted.

Vibratome sections of unembedded fixed tissue. Staining was not attempted.

Vibratome sections of gelatin–embedded fixed tissue. Staining was not attempted.

H. Coverglass Mounting Media

A mounting medium is spread on sections mounted on glass slides before covering them with a coverglass (or cover slip). For routine paraffin and methacrylate sections, mounting media contain organic solvents, usually toluene. Such mounting media can be expected to degrade the fluoresence of microspheres as toluene-based antimedia do. We tested three organic solvent-based mounting media—Clarion, Cytoseal, and Histolyte 60—and ten water-based mounting media—Advantage, Aquamount, Aqua-Poly/Mount, Crystal/Mount, Fluoromount-G, Gel/Mount, H&E Mount, Hydromount, Omnimount, and Mount Quick. Since the most extensive testing of mounting media was done on Vibratome sections of unembedded fixed tissue, see that section below for the most detailed description of the mounting media.

Paraffin sections. Cytoseal preserved hematoxylin and eosin (H&E) staining but largely extinguished the fluorescence of red microspheres. Aquamount, Aqua-Poly/Mount, Gel/Mount, Hydromount, and Omnimount were used on unstained sections and sections stained with H&E and aqueous methyl green. They are satisfactory with unstained sections, but eosin and methyl green are leached from tissue within a few hours, eosin yielding a uniformly fluorescent area under the coverglass. H&E Mount preserved both hematoxylin and eosin stains but was strongly fluorescent under blue excitation. The standard H&E procedure can be modified by substituting Histoclear II for toluene to preserve the fluorescence of the microspheres in stained sections. High viscosity immersion oil can then be used as a mounting medium as it does not affect fluorescence of the microspheres.

Methacrylate sections. Aquamount and Mount Quick aqueous coverglass mountants rapidly decolorize Lee's stain. Yellow-green and red microspheres remain fluorescent, although the medium shrinks badly.

Yellow-green microspheres remain fluorescent in Cytoseal mountant, but the fluorescence of red microspheres in the blue exciting light (450-490 nm) is extinguished within half an hour. Immersion oil works well as a coverglass mountant since it does not affect the stain or the fluorescence of yellow-green and red microspheres.

Vibratome sections of air-dried lung. Mounting media distort dry sections and were not used.

Vibratome sections of unembedded fixed tissue. Sections can be mounted under a coverglass in absolute ethanol and the preparation allowed to dry. Although lung tissue, for example, shows some damage, the section quality is better than that from air dried (unfixed) lung. The tissue shows good autofluorescence under blue excitation and the microspheres show up well. With the coverglasses secured after drying with narrow strips of Scotch Magic Mending Tape, this method provides a durable preparation with a minimum of labor.

Two of the organic solvent-based mounting media—Cytoseal, Clarion—were tried. Both diminish the fluorescence of yellow-green microspheres to some degree, extinguish the fluorescence of red microspheres under blue excitation, and leave only a faint remnant of the red microsphere's fluorescence under green excitation.

The water-based mounting media produced the following results.

Aquamount: This mountant shrinks considerably as it dries, and 100 µm sections need several applications of Aquamount in order to produce a durable, Aquamount-filled space under the coverglass. Sections are cut in 100% ethanol and usually require partial rehydration to facilitate the spreading of an aqueous mountant over sections placed on slides. Rehydration can be done by placing sections in a 100 mm glass Petri dish filled with 70% ethanol. A slide is placed in the dish with the label end resting on the side of the dish. A section is maneuvered onto the largely submerged slide with a good quality sable hair artist's brush (which work better than a camel hair brush). The slide is removed from the Petri dish and drained. The section is immediately covered with several drops of mountant, and allowed to dry for several hours to overnight. Repeated cycles of adding mountant and drying can be done before a coverglass is applied.

Sometimes, sections curl in Aquamount and need to be covered with a coverglass with the first application of the mountant. Aquamount will often shrink completely away overnight from a 22 mm^2 coverglass, essentially freeing the coverglass from the mountant-infiltrated section below. The coverglass can then be lifted or 'popped' off and more mountant added to evenly refill the space under a new coverglass. The initial drying or shrinkage step takes longer, about 1-2 weeks, with sections mounted under 22x40 mm coverglasses. Small bubbles sometimes form within the layer of Aquamount which do not refill with a new application of mountant; in such cases, briefly soaking the dried layer in distilled water may help to refill bubbles when more Aquamount is added. In our hands, Aquamount was the only aqueous mountant that allowed clean removal of coverglasses. With toluene-base mountants, additional mountant can be added by 'wicking' it under the coverglass which may or may not allow complete refilling of spaces that form due to shrinkage of the mounting medium. This is not a feasible method for aqueous mountants because of the pattern of shrinkage, i.e., the mountants do not shrink back evenly from the edges of the coverglasses.

Ringing coverglasses with nail polish prevented shrinkage and air bubble formation for up to a few weeks. Vacuum degassing Aquamount before use also helped. But even with a substantial amount of initial work, Aquamount is not a permanent mounting medium. The same problems with shrinkage and bubble formation also occur with 7-10 μm paraffin sections, although the initial bubbles are small and the process takes longer.

Aqua-Poly/Mount, Gel/mount, Hydromount, and Omnimount: These mounting media behave much like Aquamount. They preserve the fluorescence of both yellow-green and red microspheres and of the tissue. As with Aquamount, thicker mounts last much longer if drops of medium are put on sections several times before applying the coverglass. These media become firm in about an hour and, except Hydromount, last somewhat longer than Aquamount before air bubbles form under the coverglass. But they do not allow coverglasses to be lifted off the sections when they dry out. Overall, these four media behave much alike.

Mount Quick: When sections were infiltrated without a coverglass, Mount Quick formed many cracks upon drying. It is also not satisfactory for fluorescence imaging as it contains many small fluorescent particles not readily visible under brightfield illumination.

Advantage: This mounting medium showed substantial autofluorescence.

Fluoromount-G: Of all the aqueous mounting media tried with 100 μm-thick sections (five-week trials), this medium shows the least shrinkage from the edges of the coverglasses. The fluorescence of tissue and yellow-green and red microspheres was well preserved.

Crystal/Mount: This mounting medium is supposed to be used directly on sections and dried without coverglasses. For lung tissue, even with a generous amount of Crystal/Mount, the dried medium can form air pockets in alveoli which become bubbles when a second application is made. The dried preparations preserve the fluorescence of yellow-green and red microspheres, and the tissue shows bright autofluorescence. Instructions supplied with Crystal/Mount state that toluene-based media can be used to mount a coverglass over a dried layer of Crystal/Mount without affecting the section. A trial of 45 days with Cytoseal (60% toluene) over a thick layer of Crystal/Mount confirms that both yellow-green and red microspheres retain their fluorescence. Fluorophores diffuse out of microspheres in half an hour to overnight when coverglasses were mounted with Cytoseal over sections covered with a dried layer of Aquamount or Aqua-Poly/Mount.

Tissue autofluorescence and fluorescence of yellow-green and red microspheres under blue excitation appear the same with all of the above aqueous mounting media except Advantage. Kidney sections have somewhat less autofluorescence than lung or heart. Tissue autofluorescence may vary with the fixation procedure.

Immersion Oil: Several drops can be applied to the middle of a section in absolute ethanol and a coverglass added to prevent rapid drying. Immersion oil is slowly miscible with absolute ethanol but doesn't mix well with 95% alcohol. The coverglass may be removed after some hours to allow the ethanol to evaporate. This is risky, however, since sections are not bonded to the slide and section shrinkage and wrinkling may occur. Under a coverglass the small droplets of ethanol in the oil disappear after several weeks, but the preparation may require months to clear completely. No significant shrinkage of oil volume or gas bubble formation were evident for up to eight months.

Immersion oil does not seem to diminish the fluorescence of either the yellow-green or red microspheres. Tissue autofluorescence is about equal to that using the aqueous mounting media.

Vibratome sections of gelatin–embedded fixed tissue. Gelatin sections require an aqueous mounting medium because they shrink and distort if dried under a coverglass without a mounting medium. This also happens if drops of an aqueous medium, including Crystal/Mount, are dried on the sections without a coverglass. Aquamount dries under a coverglass in a few days and retracts forming air spaces around and over the sections although the coverglasses are not removable for several more days. By this time the sections adhere to the slides (although they are probably not infiltrated with the mounting medium) and more Aquamount can be added after the coverglass is removed.

I. Visualization and photographic recording

Paraffin sections. Photography of 7-10 µm paraffin sections with simultaneous (single exposure) UV excitation and bright-field transmitted light is an effective photographic recording method if the intensity of the transmitted light is reduced. Dewaxed, coverglassed sections do not photograph well with simultaneous Nomarski interference contrast and UV. Since paraffin sections have considerable tissue autofluorescence under blue excitation, they can be photographed with blue epi-illumination alone (Fig. 1). With experimentation, an exposure index can usually be found so that both the tissue and fluorescent microspheres could be recorded in a single exposure on Ektachrome color film. Fluorescence is also present in sections that have not been dewaxed; with blue epi-illumination, un-dewaxed, uncovered sections look surprisingly good. Microsphere counts can easily be made on such sections.
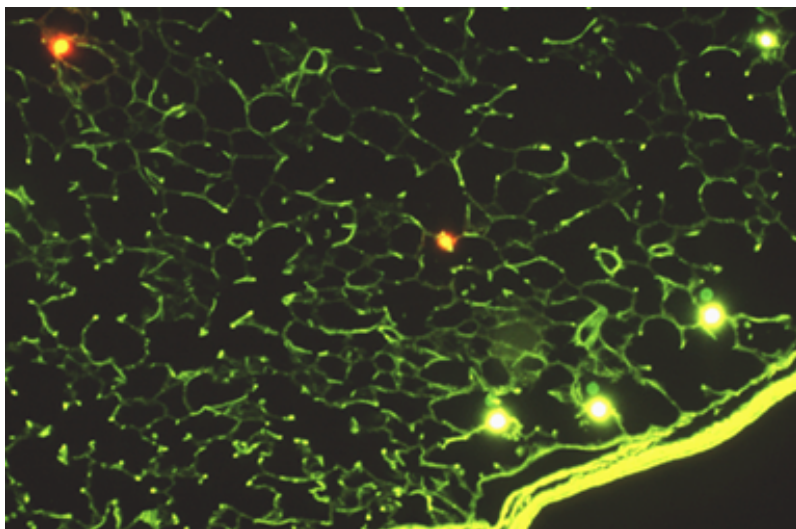

Figure 1. Paraffin section of lung showing several yellow-green and red microspheres in the parenchyma. This 7 µm-thick section was cut from a block after Histoclear II was used as the antimedium and the coverglass was mounted with Histoclear II as the coverglass mountant. This protocol preserves the fluorescence of the microspheres and the autofluorescence of the lung.

Methacrylate sections. Stained sections can be photographed with bright field illumination plus UV epi-illumination if the transmitted light is reduced. Unstained sections cannot be photographed using tissue autofluorescence since it is nonexistent. Unstained, uncovered sections can be photographed with simultaneous Nomarski interference contrast (using a sky blue background) and UV. Covering the sections markedly reduces the amount of interference contrast, depending on the refractive index of the mounting medium. Since we did not have a way of measuring the relative brightness of microspheres and tissue, bracketing exposures was necessary to obtain proper exposures.

Vibratome sections of air-dried lung. Sections under blue epi-illumination show considerable green autofluorescence. An alternative with sections up to 50 µm was a double exposure of blue epi-illumination only and bright field or Nomarski interference contrast transmitted light only. The exposure index used for the epi-illuminated exposure was about ten times that of the transmitted light exposure. This was the case even when a dark field correction was used with the epi-illuminated exposure. Experimentation was necessary to produce good photographs.

Vibratome sections of unembedded fixed tissue. The green autofluorescence of thick sections photographed much better than 10 µm paraffin sections although thicker sections show apparent loss of resolution. Simultaneous Nomarski transmitted illumination and UV epi-illumination or a double exposure of Nomarski illumination and blue epi-illumination produced colorful images with 35-50 µm sections (Fig. 2).
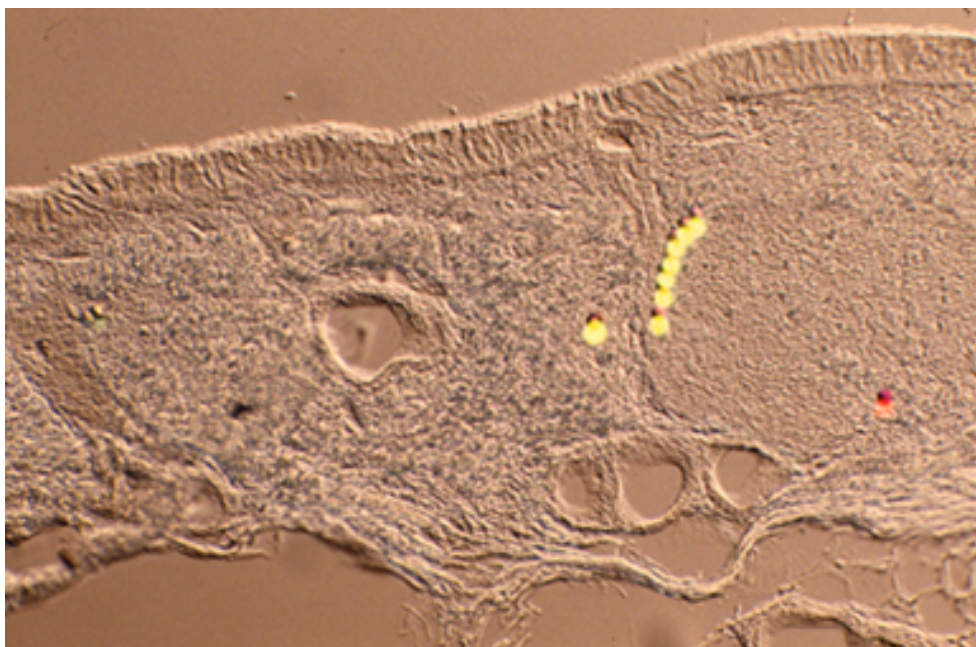

Figure 2. Section of a unembedded, formalin-fixed tracheal wall that was cut with a Vibratome. This 35 µm-thick section was photographed as a double exposure with epifluorescence and with Nomarski differential interference contrast optics. The tissue is visualized at moderate to good and the microspheres fluoresce brightly.

Vibratome sections of gelatin–embedded fixed tissue. Although gelatin has a faint fluorescence, sections show good autofluorescence under blue excitation, and yellow-green and red microspheres fluoresce brightly. The fluorescence is equal to that of unembedded, fixed tissue. Good color photographs may be made with blue epi-illumination although 100 µm sections are too thick to use a double exposure of Nomarski interference contrast and blue epi-illumination.

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