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SPECIAL ISSUE: NEW/EMERGING TECHNIQUES IN BIOLOGICAL MICROSCOPY

Out with the old and in with the new: rapid specimen preparation procedures for electron microscopy of sectioned biological material

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Abstract This article presents the best current practices for preparation of biological samples for examination as thin sections in an electron microscope. The historical development of fixation, dehydration, and embedding procedures for biological materials are reviewed for both conventional and low temperature methods. Conventional procedures for processing cells and tissues are usually done over days and often produce distortions, extractions, and other artifacts that are not acceptable for today's structural biology standards. High-pressure freezing and freeze substitution can minimize some of these artifacts. New methods that reduce the times for freeze substitution and resin embedding to a few hours are discussed as well as a new rapid room temperature method for preparing cells for on-section immunolabeling without the use of aldehyde fixatives.

Keywords Biological specimen preparation · Electron microscopy · High-pressure freezing · FS · Resin infiltration · Resin polymerization · LR White · On-section immunolabeling

Abbreviations

CF	Conventional fixation
CLEM	Correlative light and electron microscopy
EM	Electron microscopy
FS	Freeze substitution
HPF	High-pressure freezing
LN_2	Liquid nitrogen
LRW	LR White
RF–FS	Rapid freezing–freeze substitution
RT	Room temperature
RTS	Rapid Transfer System
UA	Uranyl acetate

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Introduction

The purpose of this article is to review and present the best current practices for preparation of biological samples for examination as thin sections in an electron microscope. It will only cover methods aimed at producing samples embedded in resin. It will differ from previous articles or books on the same subject because it will focus on low-temperature fixation methods and emphasize how to achieve sectionable material by much quicker methods than those that are typically used.

The basic specimen preparation procedures for resin embedding of biological specimens were worked out by the mid-1960s and many laboratories today are still using these methods without significant change to produce their cell fine structure data. Other laboratories have chosen to use equipment and techniques to physically fix cells and tissues by cryoimmobilization, sometimes referred to as cryofixation. In particular, high-pressure freezing (HPF) is the technique that has allowed resin-based electron microscopy (EM) to take a leap forward in cell preservation that is comparable to the shift in quality that took place when glutaraldehyde was introduced as a primary fixative (Sabatini et al. 1963). While the application of HPF was initially for freeze-fracture work, the use of freeze substitution (FS) methods to transition into resins has evolved as the main way that HPF is used today.

Specimen preparation for obtaining sections of biological material is done in three basic steps: fixation, dehydration, and embedding in resin. Each of these three major processing operations will be considered in historical context and new procedures that are considerably faster than conventional

methods will be presented, along with examples from a diverse array of organisms. Some basic suggestions for improving HPF results are included as well as a new room temperature (RT) method for preparing cells for on-section immunolabeling without the use of aldehyde fixatives.

Fixation

Historical context

Conventional fixation methods The first images of biological material in electron microscopes were not fixed in the usual sense, but simply dried down onto a grid before viewing (Rasmussen 1997). Most of these images were not much better than good light microscopy but did reveal information about viruses and other cell components of small size. The best early EM images of a whole cell were the whole mounts of tissue culture cells by Keith Porter (Moberg 2012) that were fixed in osmium vapor before drying. It soon became clear that most cells, and certainly tissues, were too thick to be observed as whole mounts and that some type of sectioning would be required. However, it took a long time to put all the pieces in place so this could be done reliably. A good fixative was needed, as was an embedding medium that was hard enough for thin sections, and a microtome and knife combination that would produce those sections. The development of buffered osmium fixative known as "Palade's pickle" (Palade 1952) was one such landmark development for fixation and this method and variations were used up to the mid-1960s until they were replaced by double fixation with glutaraldehyde and osmium tetroxide (OsO_4) (Sabatini et al. 1963; Franzini-Armstrong and Porter 1964; Tormey 1964). This fixation strategy is still the method of choice for many laboratories today. Typically, cells are fixed for about an hour in a buffered glutaraldehyde solution, rinsed with buffer, then post-fixed with OsO_4 for another hour more or less, then rinsed with buffer and/or water prior to dehydration in an alcohol or acetone series. There are many variations on this theme with regard to the timing, concentrations, buffer composition, pH, osmolarity, and so forth (Hayat 2000), but this is the essence of the double fixation procedure commonly used by many laboratories today.

En bloc fixation/staining with uranyl acetate (UA) has also become common practice in conventional electron microscopy. UA was first used by Kellenberger et al. (1958) to stabilize viral and bacterial DNA and subsequently found to decrease protein and phospholipid extraction in a variety of tissue types (Farquhar and Palade 1965; Karnovsky 1967; Brightman and Reese 1969; Silva et al. 1968, 1971; Terzakis 1968). At least two studies (Valentino et al. 1985; Erickson et al. 1987) have used UA as a secondary fixative, replacing OsO_4, prior to on-section immunolabeling of material embedded in LR White (LRW) and/or Lowicryl K4M resins.

Cryofixation About the same time that early electron microscopists were drying down cell fractions and particulate samples, other researchers were exploring freeze drying and metal shadowing as an alternative method of specimen preparation (Müller 1942; Williams and Wyckoff 1944; Wyckoff 1946; reviewed in Muhlethaler 1973). But after sectioning techniques were worked out for resin material, the idea of working with sections of material that had been rapidly frozen was tested. At first, freeze-dried tissues that were infiltrated with wax was tried (Bretschneider and Elders 1952), but the most useful conclusion from these studies was that plunging tissue into cooled mixtures of propane and isopentane resulted in ice crystal damage. Fernandez-Moran (1952) cut frozen sections that were immediately freeze-dried or air-dried that were useful for histochemical studies but not for morphological work. Moor and Muhlethaler developed a cryomicrotome that worked in a vacuum (Moor et al. 1961) but they could not produce thin sections of frozen material. Instead, they found they could apply the replica technique of Steere (1957) to the fractured surfaces in a method that became what we know as freeze etching (Muhlethaler 1973). This made it feasible to look at the interior of cells, as with thin sectioning, but without the distortions introduced by fixation, dehydration, and resin embedment. But the reality was that unless large cells and tissues were infiltrated with a cryoprotectant such as glycerol, ice crystals would destroy the fine structure during freezing. What was needed was a way to freeze samples larger than a few micrometers without ice damage. This need was fulfilled with the development of HPF. Working in Hans Moor's laboratory at the Swiss Federal Institute of Technology (ETH-Z) in Zürich, Udo Riehle explored the possibilities of freezing biological samples under approximately 2,100 bar of pressure (Riehle 1968; Riehle and Hoechli 1973; Moor 1987). Moor and Hoechli (1970) developed a more reliable version of the apparatus and in the mid-1980s Balzers AG, Liechtenstein made the Balzers HPM 010 high-pressure freezer commercially available. Today, there are four models of high-pressure freezer that are commercially available, and their details will be given in a later section.

Why is cryofixation better than conventional fixation methods?

Perhaps first and foremost a fixative must act quickly, arresting movement of all molecules in their place as fast as possible. It has been estimated for plant cells immersed in glutaraldehyde that the fixatives may penetrate at the rate of 1–2 μm per second (Mersey and McCully 1978). But we also know from personal experience and from the literature (Shepherd and Clark 1976) that nematode worms can swim for hours in

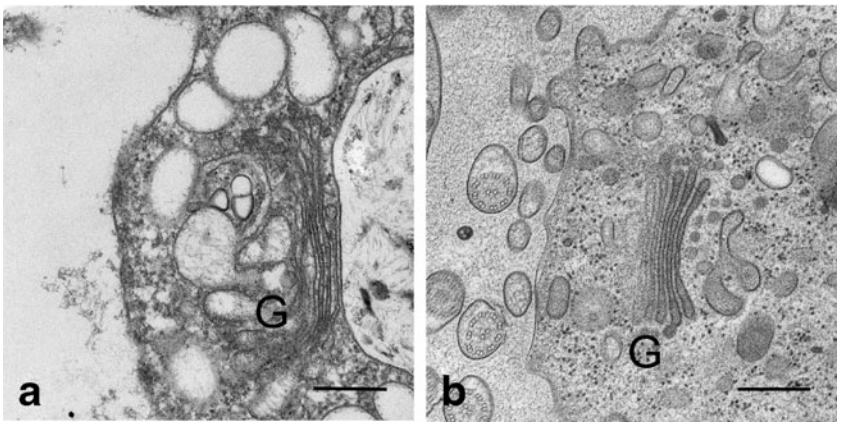

Fig. 1 Comparison of *Oscarella carmela* cells prepared by conventional methods (**a**) or HPF/FS (**b**). The Golgi apparatus (*G*) in particular shows distortions in (**a**) as compared to the regular stacking of cisternae seen in (**b**). Because there is significant extraction of cytoplasm by conventional methods, the cell in (**a**) shows higher contrast than that of (**b**) which has less extraction. Cells courtesy of Scott Nichols and Nicole King, University of California, Berkeley, and Colorado State University, Denver, respectively. *Bar* = 300 nm

glutaraldehyde fixative solutions. By contrast, nematode worms and all other samples can be physically arrested in place in about 30 ms by HPF (Mueller and Moor 1984). Because no chemical fixation of molecules in the usual sense takes place, some refer to this initial freezing step as cryo-immobilization instead of cryofixation. However, the latter term is widespread in the literature and the terms will be used interchangeably in this article.

A second role for fixation is to stabilize molecules against disruption by subsequent procedures such as dehydration and resin infiltration. Aldehyde and osmium fixatives are selective in their cross-linking and during dehydration membranes can become swollen and/or vesiculated, and proteins and ions in the cytoplasm can be extracted or displaced from their original positions at the time the fixatives were added. Bajer and Mole-Bajer (1972) noted that chromosomes in cell division moved as much as 0.5 μm after fixation began. In cryo-immobilized cells, dehydration and fixation with added chemicals such as OsO_4 or aldehydes take place at low temperatures (−25 to −90 °C) where larger molecules do not have enough thermal energy to move around but smaller molecules such as water can be exchanged for organic solvents. For an excellent discussion of the physics of specimen cryopreparation see the article by Kellenberger (1987). For a graphic representation of the differences between conventionally prepared and cryo-prepared samples, see Fig. 1 and illustrations in the following references (figure 3 in Steinbrecht et al. 1987; figure 1 in McDonald 1994; figure 1 in Mobius 2009). Membranous elements display the most obvious problems, but the distribution of the microtubules in the moth antenna (Steinbrecht et al. 1987) is also adversely affected.

Another line of evidence for why cryo-immobilization is the preferred method for retaining the best preservation of molecular detail comes from how today's structural biologists analyze molecules down to near-atomic levels of resolution by electron microscopy. Single particle averaging of molecules and macromolecular complexes is most effectively done by imaging them in vitreous ice in a cryo-electron microscope (Frank 2006; Boekema et al. 2009). For microscopy of subcellular detail within whole cells, the preferred method is imaging of vitreous sections by cryoEM (Al-Amoudi et al. 2004) or, if the whole cells are small enough, by cryo-electron tomography (Gan and Jensen 2012). The principal idea here is that the water molecules that determine the tertiary structure of proteins are retained even when the water is frozen. Other types of processing for EM that require replacing that water with an organic solvent and resin inevitably lead to some distortions at the highest levels of resolution. But if you compare the same structure prepared by conventional methods in resin, cryomethods in resin, and cryomethods in vitreous frozen water, you will see that cryomethods in resin sections are much closer to the vitreous state than the conventionally prepared sections (Al-Amoudi et al. 2004).

Do the arguments cited above mean that every sample for EM should be prepared by cryomethods? The answer is a clear no. In practice, there are times when conventional fixation (CF) may be fine for the task at hand and occasionally better. If some distortions and/or extractions of the cytoplasm do not interfere with the interpretation of the data, then regular methods will work. In other instances, rapid freezing followed by freeze substitution (RF–FS) can give images that are harder to interpret than those from conventionally prepared preparations. For example, counting synaptic vesicles in certain nerve cells is easier by CF than RF–FS because so much material is retained by RF–FS that the contrast between vesicles and background is much less than with CF, where extraction leads to even greater contrast (cf., figures 4 and 5 in McDonald 1994). In another example, RF–FS of some plants results in chloroplast thylakoid membranes that appear as white lines against a dark background and it is difficult to distinguish individual membranes, while with CF they look like membranes we are used to seeing in electron micrographs (Fig. 2).

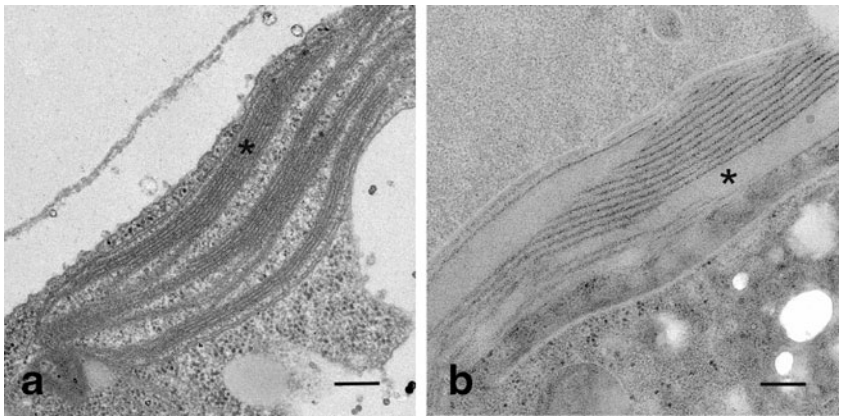

Fig. 2 Comparison of *Chlamydomonas reinhardtii* cells prepared by conventional methods (**a**) or HPF/FS (**b**). The thylakoid membranes (*asterisks*) show up as dark lines in Fig. 1a whereas they are light lines against a darker background in Fig. 1b. Cells courtesy of Jose Gines, University of California, Berkeley. *Bar* = 200 nm

But in general, if you are going to be doing high-resolution work such as electron tomography or making quantitative assessments of cytoplasmic elements, you should strongly consider using cryomethods for specimen preparation.

How does HPF work?

During HPF, cells or tissues are pressurized to about 2,050 bar and then cooled by liquid nitrogen within milliseconds. The goal is to extract the heat from a sample before cell water can rearrange into ice crystals. Under this level of pressure, the freezing point of water is lowered to about −20 °C, and the nucleation of ice crystals as well as their growth is slowed down. For more details about the theory behind this method, see the article by Riehle and Höchli (1973), Moor (1987), and articles by Shimoni and Mueller (1998), Studer et al. (2001), and Vanhecke and Studer (2009). As a word of caution, do not take these theoretical calculations too literally. For example, according to Moor (1987), HPF should theoretically provide ice-free (vitreous) frozen cells to a depth of 600 μm, but we now know that this condition is rarely satisfied even at depths of 200 μm in most tissues. What we have instead is microcrystalline ice a nanometer or so in size that does not seem to create significant distortions for cells embedded in resins. The main problem with all theoretical estimates for rates of freezing as a function of depth is that we do not accurately know the coefficient of heat transfer for cytoplasm, plus the fact that each cell type has a different cytoplasmic composition. Vanhecke and Studer (2009) show how rates of cooling inside the specimen cup diminish as a function of the distance from the metal specimen carrier increases. The key thing to remember about this idea is that the cells closest to the metal of the specimen carrier will cool the fastest. This has a practical application in that the thinner the sample the faster it will freeze. For an excellent discussion about the nature of cytoplasm and the response to cooling at a macromolecular level, see the article by Kellenberger (1987). For an informative discussion of the physics of rapid cooling and the implications for freezing cells, see the article by Dubochet (2007).

Another good source of information for thinking about what happens during ultrarapid freezing are tables that show some of the properties of metals, solvents, cryogens, and so on. There are two books that are particularly useful for finding this information. They are Echlin's (1992) "Low Temperature Microscopy and Analysis", Plenum Press, New York; and Robards and Sleytr's (1985)"Low Temperature Methods in Biological Electron Microscopy", 551 pages, Elsevier Press, Amsterdam. They are still available from some book sellers such as Amazon.com and others.

HPF machines

As mentioned above, the Balzers HPM 010 was the first HPF machine on the market and, until about 2000, the only one that was readily available. In 2000, Daniel Studer worked with Leica Microsystems, Vienna to produce the Leica EMPACT (Studer et al. 2001). Aside from the fundamental differences inside the machine concerning how the cooling and pressure was delivered to the sample, the EMPACT was much smaller than the Balzers machine, gave cooling and pressure curves for each freezing event, and was placed on a cart so that it could be easily wheeled around if necessary. In 2005, the EMPACT was modified to include a semi-automatic loading system and some upgrades were made to the internal freezing and pressure systems. The Rapid Transfer System (RTS) is an optional feature of the EMPACT 2, as it is known, and was designed in collaboration with Leica Microsystems by Paul Verkade (now at the University of Bristol in the UK) to enable a 4 to 5 s transfer from a light microscope to the HPF machine for correlative light and electron microscopy (CLEM) studies of dynamic cellular events (Verkade 2008). Leica microsystems also sells another HPF machine, the HPM 100, which is also on wheels, but much heavier than the EMPACT and of fundamentally different design from either the HPM 010 or EMPACT. One distinguishing feature are the specimen carriers which will be discussed next. The final model of HPF machine that is commercially available is the Wohlwend HPF Compact 02. It is somewhat similar to the

original Balzers HPM 010 internally but with improvements in nitrogen usage, synchronization of freezing and pressure systems, and the variety of specimen carrier types available.

Specimen loading is the key to freezing success

One's ability to produce well-frozen cells and tissues with HPF is directly proportional to the care taken in loading the samples. The HPF machines are "dumb" machines and will work the same, time after time after time. If one puts poorly prepared samples into the machine, one will get poor results out. Unless it is not working properly, one cannot therefore blame the HPF machine for delivering poor results. To get the most out of the HPF machine, give serious consideration to the following when loading the samples:

1. Work only with healthy cells

   Cells/tissues must be in an optimal physiological condition. Cells in culture should be in early to mid-log phase growth, if possible. They should be at the right temperature, in the most physiologically appropriate medium, etc. Cells should not be concentrated and allowed to sit on ice for long periods of time. Whole organisms should be well fed, at appropriate temperature and humidity, etc. Cells that are stressed will not generally freeze well (Hess 2007). Stress can include transfection, infection, gene silencing, high-speed centrifugation, age, and treatment with drugs as just a few examples.
2. If possible, use the shallowest depth specimen carrier available and have both sides of the sample directly in contact with the metal of the carrier

   This may be the single most important factor for achieving high yields of well-frozen cells. Because it is known that the rate of heat transfer is greatest where the sample touches the metal, the ideal carrier should be as shallow as possible while touching the tissue/cell on both sides. For example, *Drosophila* embryos tend to be about 175 μm wide by about 500 μm long. By using a 150-μm deep well, and slightly compressing the embryo, good thermal contact with the embryo will be attained and excellent freezing can be expected.

   The BAL-TEC HPM 010 (now the ABRA HPM 010), Wohlwend Compact HPF 01, and the Leica HPM 100 machines all take identical 3-mm-diameter specimen carriers. One can order specimen carriers from Leica, but the best variety and price for carriers is from Wohlwend Engineering. Wohlwend has carriers available in the following depths: 25, 50 100, 150, 200, 250, 275, and 300 μm. When used in combination, one can get additional depths of 75, 125, 175, 225, 325, 350, 375, 400, 425, 450, 475, 500, 525, 550, 575, and 600 μm. In practice, depths greater than 200 μm are less likely to give good freezing and should be avoided unless the tissues are heavily cryoprotected. All of these carriers have a well that is 2 mm in diameter. Additionally, one can use EM grids as variable depth spacers between two flat-sided carriers to add more variety (McDonald et al. 2007). One can also have special carriers made for particular needs (Craig et al. 1987; Sawaguchi et al. 2005).

   In contrast to the Wohlwend-style machines, the Leica EMPACT systems have a more limited variety of specimen carriers. There are carriers of 100 and 200 μm depths, a tube system with a 300-μm interior diameter, biopsy carriers that are 300 μm deep, and one cup-shaped carrier that is 400 μm deep. All the cup-shaped carriers are 1.4 mm in diameter, except for one that is 1.2 mm in diameter.
3. Work quickly but carefully, and do not let the samples dry out

   For all types of preparations, it is good to strive to go from living to frozen cells in less than a minute. This is not always possible with some preparations, but for others it is easy. The rapid loader for the EMPACT2 with RTS is a useful feature for increased speed of loading.

   The volume of the specimen carriers of nearly all shapes and sizes is less than a microliter. This volume will dry out very fast, especially if the humidity around the sample is low. Try working in a moist chamber, an agarose pad, or otherwise keeping your material moist over the time one is taking samples for freezing.
4. Avoid mechanical damage

   Sloppy cutting of tissues or overfilling specimen cups can damage your cells. Use biopsy needles where feasible; always use the sharpest scalpels and razor blades for bulk cutting. Disposable dermatology biopsy punches in 1.5- and 2.0-mm diameters are useful for leaves and other hard materials. It is better to have too little material and fill in the spaces than have sample sticking out the top of the carrier that will get crushed when the top piece is put on. Ideally, as stated in Rule 3, one should have both sides touching metal, but not all cells can take compression like fly embryos so it may be necessary to have a thin layer of filler on one side.
5. Do not use highly aqueous solutions as "fillers"

   When your sample does not completely fill the specimen cup cavity, you will have to add something to ensure that there is no air space in the cup during freezing. Air will insulate the sample from heat transfer and also collapse, possibly collapsing your material. Water has a very low thermal conductivity value and will prevent the rapid removal of heat from your sample. Frozen water is actually an insulator that will prevent the rapid extraction of heat from your cells.
6. Practice loading before you start freezing

   Because loading is so critically important to the success of HPF, one should practice getting samples into the

specimen cups before starting up the freezer. Once the freezer is running, a lot of liquid nitrogen is consumed and is just wasted if one spends time working out how to load. This is especially important if one goes to another laboratory to do the freezing. Practice at home until proficient before wasting time and money at another laboratory. If special tools or equipment for a particular sample is needed, be sure to have these at hand when ready to freeze.

7. Get help if needed

   This is especially important if one is doing a rapid time course experiment. However, at other times, such as with complicated dissections of organs, or whenever the biological material requires constant attention, having two or even three people involved in the freezing effort makes everything go much smoother and faster.

Other modes of rapid freezing

HPF is the preferred method for freezing cells larger than a few micrometers. However, for cells smaller than 3–5 μm, it is possible to use plunge freezing, spray freezing, propane jet freezing, or impact freezing on a cooled surface to immobilize cells prior to FS (Gilkey and Staehelin 1986; Echlin 1992). There is also a method called self-pressurized rapid freezing where larger volume cells or tissues can be frozen with simple equipment (Leunissen and Yi 2009). The limitation here is that the freezing vessel is a copper tube that has an inner diameter of 300 μm and samples have to be drawn up into the tube prior to sealing and freezing it. Then the frozen tube containing specimens has to be cut into smaller pieces in order for the FS media to exchange freely with the cells. Studies have shown that about half the cells inside are vitrified while the other half are badly ice damaged (Yakovlev and Downing 2011; Han et al. 2012). Nevertheless, for samples that can be fitted inside the copper tubes, having a 50 % yield of well-frozen cells is sufficient to collect good data.

Specific HPF methods for different cell types

Above some general rules were given to increase the likelihood of successful HPF. However, each type of cell or tissue may require its own set of conditions for optimal results. Space does not permit a discussion of all these subtleties, but there are numerous techniques articles in the literature that may be helpful in this regard (Studer et al. 1993, 2001; McDonald 1994, 1999, 2007, 2009; McDonald and Müller-Reichert 2002; Hess 2007; McDonald et al. 2007; Vanhecke and Studer 2009; Kaech 2009; Vanhecke et al. 2008). There are also hundreds of published papers using HPF on a wide variety of tissues, and these can be used to suggest particular procedures for freezing. It is also worth noting that freezing of fixed material is also useful for specimens that might be too delicate for fresh freezing such as nerve tissues (Sosinsky et al. 2008; Mobius 2009). Another strategy is to infiltrate fixed samples with 2.3 M sucrose or 30 % glycerol; freeze them by plunging into liquid nitrogen, cooled ethane, or propane; then FS and embed for either morphology or immunolabeling [van Genderen et al. 1991; van Lookeren et al. 1991; Voorhout et al. 1991; Oprins et al. 1994 (see Mobius 2009)]. Note that for samples infiltrated with sucrose that FS will have to take place in methanol because sucrose is not soluble in acetone (Mobius 2009).

Dehydration

Conventional methods

Historically, the dehydration methods used by the early electron microscopists drew on the precedents set by light microscope histology going back to the early nineteenth century. The organic solvents differed, but the principle was the same. Following double fixation with glutaraldehyde and OsO_4 at RT, specimens for EM are typically dehydrated through a series of increasing percentages of organic solvent, usually ethanol or acetone. The increments of solvent concentration can be small or large depending on the nature of the tissue. If the cells are in a monolayer, the time can be very brief. For example, tissue culture cells can be rinsed for 30 s in 80 % ethanol before going into a graded resin series (Robbins and Jentzsch 1967).

Freeze substitution dehydration

The process by which cryoimmobilized samples are dehydrated is called freeze substitution (FS). Rapidly frozen specimens are placed in organic solvents at low temperatures and then warmed over a period of hours to days to higher temperatures where they can be embedded in resins for sectioning. Traditional fixatives such as OsO_4 or glutaraldehyde can be added to the solvents so that chemical cross-linking or stabilization can take place. The type of organic solvent varies (Robards and Sleytr 1985; Echlin 1992), but in most cases acetone or methanol is used. Specimens warmed to RT are usually embedded in either epoxy resins or LRW. Lowicryl or LR Gold resins can be used to infiltrate and polymerize resins at lower temperatures, usually for the purpose of on-section immunolabeling, but in more recent times for electron tomography (O'Toole 2002) because the resins are more electron transparent.

The early history of freeze substitution

The first person to describe low temperature methods for improving preservation of cell structure was Altmann (1889) who used a freeze-drying process for light microscope studies. The term "freezing substitution" can be traced back to

Simpson (1941) who froze animal organ tissues by plunging into isopentane cooled by liquid nitrogen (LN_2) or into LN_2 alone, or onto a cooled metal block cooled in LN_2. Tissues were transferred to organic solvents at low (−40 to −78 °C) temperatures for periods from a few hours to several days before embedding in paraffin for light microscopy analysis. He recognized that ice crystals could cause distortions and that the extent of damage could be correlated with the rate of freezing and to the tissue water content.

It was Fernandez-Moran (1959, 1960) who first explored the potential of FS for EM. His articles do not have very specific details so what follows is Bullivant's (1960) variant of his method. Specimens were frozen in liquid helium, dehydrated at −75 °C in three changes of methanol over 3 weeks, infiltrated with methacrylate resins at −75 °C for 3 days, then polymerized by UV light at −5 °C for an unspecified amount of time. Sections were viewed either unstained or post-stained with either OsO_4, lead acetate, or the Gomori lead method for acid phosphatase (Bullivant 1960). Helium turned out to be less effective at freezing than originally thought and most subsequent freezing was done with LN_2 as the coolant. Throughout the 1960s, 1970s, and 1980s, there were hundreds of papers on all aspects of low-temperature methods for biological EM including different freezing methods, different FS times, FS temperatures, solvents, and additives that could be used during FS, and different embedding resins and immunolabeling strategies. Most of these papers are now mostly of historical interest, and the interested reader can find details and original citations in the following references: the review by Shiurba (2001) is particularly thorough, but see also the books of Robards and Sleytr (1985), Echlin (1992), and the section on FS in Pease (1973). Table 1 is a selection of important papers on HPF and FS from 1889 to 2001. It is not meant to be exhaustive and readers will no doubt have their own favorites.

Current freeze substitution practices

With the wider distribution of the high-pressure freezers in the biological EM community during the 1990s and beyond, FS became a more common method for dehydrating and

Table 1 Key references in the development of cryomethods for biological cytology

Year and citation	Development
1890 Altmann	Freeze-drying for improved light microscopy histology
1941 Simpson	Analysis of Altmann's method; first uses the term "freezing-substitution"
1958 Feder and Sidman	Review of methods and principles of FS for light microscopy; osmium–acetone fixative
1952 Fernandez-Moran 1959 1960	Low temperature preparation methods for electron microscopy including: freeze substitution for EM, progressive lowering of temperature dehydration, polymerization of methacrylate resins by ultraviolet light, and cryo-sectioning of frozen specimens
1960 Bullivant	Applied the freeze substitution technique of Fernandez-Moran to a variety of tissue types
1961 Rebhun	Plunge freeze invertebrate eggs in Freon, FS in osmium acetone
1966 Zalokar	Studied rates of substitution with dyes frozen in filter paper
1964 Van Harreveld and Crowell	Rapid freezing on a metal surface and substitution fixation
1967 MacKenzie	The "collapse phenomenon" in freeze substitution
1968 Moor and Riehle	Development of the high-pressure freezer
1972 Rebhun 1973 Pease	Reviews of freeze substitution
1980 Kellenberger et al. 1982 Armbruster et al.	Development of Lowicryl resins and the progressive lowering of temperature (PLT) dehydration method
1982 Newman et al.	Development of LR resins
1984–1985	Balzers AG releases the first commercial high pressure freezer, the HPM 010
1985 Robards and Sleytr	*Low Temperature Methods in Biological Electron Microscopy* book
1986 Gilkey and Staehelin	Summarize advances in ultrarapid freezing
1983;1986 Humbel and Müller	Experiments on freeze substitution and low temperature embedding
1987, 1991 Kellenberger	Potential of cryofixation and freeze substitution: theoretical considerations
1987 Steinbrecht and Müller	Freeze substitution and freeze drying
1987 Moor	Theory and practice of high-pressure freezing
1992 Echlin	*Low Temperature Microscopy and Microanalysis* book
1993 Studer et al.	High-pressure freezing comes of age
2001 Studer et al.	The development of the Leica EMPACT high-pressure freezer
2001 Shiurba	Freeze-substitution: origins and applications, a comprehensive history of low-temperature methods for light and electron microscopy to this point

chemically fixing cells prior to resin embedding. The commercial availability of semi-automated FS machines from Balzers, Leica, and others also helped to push FS applications forward. In 2001, Leica Microsystems produced the "Leica EM AFS Recipe Book" (a PDF is available at http://otolic.stanford.edu/documents/Recipe_book.pdf). The booklet is a compilation of many of the published procedures for FS up to that time. What is immediately obvious from a quick perusal of the contents is that there were no well-agreed-upon procedures for doing FS, and this is still true today. Like many methods that are poorly understood mechanistically, researchers tend to use what they were first taught and what they know will work. The most-used fixatives are OsO_4 (with or without UA) in acetone for morphological work and low (0.1–0.5 %) concentrations of glutaraldehyde in acetone for on-section immunolabeling of resin sections. UA can also be added in combination with these fixatives or by itself. Times and temperatures for FS are extremely variable. In general, most people start at −90 °C if they are using an AFS machine or −78 °C if they are using dry ice. Holding the cells at −90 or −78 °C for about 3 days is common though time at this temperature can be shorter or much longer (up to a week or more). Then the specimens are warmed slowly (about 5 °C/h more or less) to either to RT or some intermediate temperature where they are held for more time. A common practice is to start a FS run on Friday and have it warm to RT by Monday. In general, plant cell biologists tend to do longer FS runs than other biologists.

Freeze substitution in 3 h or less

The wide variety of FS methods in the literature suggests that it is a very forgiving procedure. In 2010, we began to test some of the basic assumptions about FS, especially the timing issues. The result was a paper that showed how FS could be carried out on diverse types of biological tissues in 2–3 h (McDonald and Webb 2011). This is the method presented here, including modifications of the original publication that make the procedure even simpler. Specifically, we no longer recommend using the dry ice method (called QFS in McDonald and Webb 2011).

In Table 2, the materials needed for 3-h FS, their costs in 2013, and some suggested suppliers are listed. Another alternative is to buy a kit from Electron Microscopy Sciences. Kits come with (EMS Cat. No. 34500; $1,595.00) or without (EMS Cat. No. 34500-S, $775.00) a shaker, or components can be purchased separately.

The freeze substitution procedure*

(modifications from the SQFS procedure in McDonald and Webb 2011 are indicated by **bold** type).*

Table 2 Materials needed and supplier information for quick FS processing

Material	Supplier	Cost (as of 2013)
1. Foam box or ice bucket with lid (min 8 cm deep)	Found in most laboratories	Little or no cost
2. Dry block heater module of anodized aluminum with 13-mm holes	Various biological supplies vendors, e.g. Cole-Parmer Cat. No. S-03132-00 VWR Cat. No. 13259-130	 121.00 USD 82.00 USD
3. Cryotubes with O-ring seal	Genesee Scientific. www.geneseesci.com	
	Tubes=cat. no. 21-265	26.80 USD/500
	Caps=cat. no. 21-266	49.00 USD/500
4. Datalogger type EL-USB-TC-LCD[a]	www.lascarelectronics.com microdaq.com	80.00 USD 70.00 USD
5. Type T thermocouples	Omega Engineering, Inc. www.omega.com/ Part #5TC-TT-T-30-36	46.00 USD/5
6. Freeze substitution solutions	Use your usual solutions	
7. Some kind of agitation device[b]	See footnote	N/A

[a] This device is highly recommended but not essential. However, some method of monitoring temperature is needed

[b] We prefer a rotary shaker with enough stability to take the weight of the box and contents. One could also use some kind of rocker if it were sturdy enough. These vary widely in lab availability and cost if you have to order something. We have no recommendation for ordering at this time. Our suggestion is to find something you can borrow to try the method, then buy if you have to

1. Have frozen samples in frozen FS medium on hand in LN_2.
2. Place the metal block in the foam box and fill with LN_2. Wait until the block reaches LN_2 temperature which is indicated when the LN_2 stops boiling. About 5 min, adding more LN_2 may speed up the process.
3. Place the samples into the holes of the block. **Any hole will do as they will all maintain the same temperature during FS** (Richard Webb, University of Queensland, Australia, unpublished results).
4. **Insert the tip of the thermocouple probe on the datalogger between the cryovial and the wall of the 13-mm hole. If preferred, the probe can be taped to the side of a cryovial containing acetone only and that placed into one of the holes.** In the published article, we suggested sealing the thermocouple tip into a dummy cryotube, but these inevitably leak.
5. Use a PC (Macs will not work) to start the temperature recording in the datalogger.
6. Pour off LN_2 back into a storage dewar.

7. Turn the block on its side so that the cryovial tops are up against one side of the foam box. Place some wadded up paper or aluminum foil behind the block so it will not move around when shaking.
8. Place the box and contents on a shaker and set to rotating at 100 rpm. Do not put the lid on the box unless you wish to extend the time of the FS run by about 50 %.
9. Monitor the temperature on the datalogger and remove the cryovials/block from the shaker when the temperature reaches 0 °C, or later if coming to RT is preferred. The samples are now freeze substituted and ready for rinsing and infiltration with resin. **Note:** the length of time for FS will vary considerably depending on the particular set-up and location used. The original SQFS method done by Rick Webb (McDonald and Webb 2011) took 90 min, but using a different combination of metal block and foam box it takes about 2.5 h in my laboratory. The main variable seems to be the velocity of airflow in the fume hood. If you always put the shaker in exactly the same spot in the fume hood and lower the hood door to the same place, you should get consistent warm-up times.
10. Stop the datalogger and store the file.

Safety notes

1. It is extremely important not to trap any LN_2 in the cryovials when loading them with the frozen specimens. If you use a pair of sturdy forceps or a hemostat, you can turn the vial and pour out any liquid if you do it in the vapor phase right above the LN_2.
2. When putting on the cap after loading specimens onto the frozen fixative, use a warm cap because the O-ring will be pliable and seal more securely. It is essential to use cryovials that are leak proof, e.g., with a hard rubber O-ring. If you choose to use another type, always check for leaks by agitation with pure acetone before using FS media with fixatives.
3. When using osmium fixatives in FS media, the shaker should be in a fume hood. If the vials are sealed correctly, this should not be necessary but leaks may happen and it is better to err on the side of caution.

Resin infiltration and polymerization

Conventional methods

History of resin development The development of resin embedding of biological specimens was driven by the realization in the 1940s that most cells were too thick to be viewed directly in the electron microscope and that some kind of ultrathin sections were needed. Some development of microtomes and thin sections took place in the early 1940s, but they were not generally successful and it was not until after the end of World War II that fruitful work was begun on microtomes, glass knives for cutting, and resins hard enough for cutting ultrathin sections (summarized in Pease and Porter 1981; Newman and Hobot 1999). The introduction of epoxy resins by Maaløe and Birch Andersen (1956) to replace the less stable methacrylate resins (Newman et al. 1949) was a major advance, and later formulations of Araldite and Epon resins by Glauert (1956) and Luft (1961) set the stage for the modern era of ultrathin sectioning of biological material. While many resin variants were developed in these times, the resins besides Epon and Epon–Araldite that seem to have survived in common use to the present include Spurr's (Spurr 1969), LR White (Newman et al. 1982, 1983), and the Lowicryls (Kellenberger and Garavito 1980; Armbruster et al. 1982). A history of resins for immunolabeling and/or CLEM can be found in Newman and Hobot (1999).

Infiltration Infiltration of resins usually takes place in incremental concentrations of resin/solvent mixtures at RT (Epoxy and LRW resins) or low temperatures (LR Gold, Lowicryls). Textbooks on biological EM (Hayat 2000; Bozzola and Russell 1999) recommend 25 % increments for an hour each with agitation, and an overnight incubation in pure resin is often suggested for the epoxy and LRW resins. Lowicryl guidelines included with the resin kits recommend 60 min each in resin/ethanol concentrations of 50 %, 67 %, and pure resin, plus overnight or 4–16 h in pure resin at the temperature appropriate for the particular Lowicryl. RT methods also suggest agitation with each step, but with low temperature methods in an AFS machine, this is not usually possible. However, the Leica AFS2 unit with FSP robot (Leica Microsystems, Vienna) allows some mixing.

Polymerization There are two basic modes of resin polymerization: heat and UV light. The former for epoxy and LRW resins, but it is also possible to polymerize Lowicryls with heat by changing the accelerator (Newman and Hobot 1993). The LR and Lowicryl resins can be polymerized at temperatures between −35 and 25 °C with chemical catalysts, but the Lowicryls are designed primarily to be hardened by UV light. Recommended times for Lowicryls vary with the type, but for the popular Lowicryl HM20 indirect UV for at least 24 h is suggested, with an additional 2–3 days of direct UV at RT. Both the LR resins and Lowicryls are generally polymerized in an oxygen-free environment.

Rapid methods

Early studies It has not always been the case that resin infiltration and polymerization times were measured in days. For

monolayers of tissue culture cells, Robbins and Jentzch (1967) dehydrated and infiltrated them with Epon resin in about 20 min. In the early 1970s, there were several papers published showing that resin infiltration and polymerization could be done on a variety of tissue types in different types of resins in 2–3 h. Details and references can be found in Hayat (2000) and Glauert 1975), but the method of Hayat and Giaquinta (1970) is representative. Small pieces of animal organ tissues (mouse liver, spleen and kidney, and embryonic chick heart) were infiltrated with a mixture of 50 % Epon/acetone for 15 min, changed twice in pure Epon for 10 min each, and polymerized at 100 °C for 1 h. Specimens were constantly agitated during the infiltration steps. Altman et al. (1984) introduced a 4-h procedure for Lowicryl K4M. Hayat (2000) also mentions without showing data that in his laboratory rapid methods have been used with success on tissue culture cells, algae, bacteria, and fungi. These all tend to be small volumes and Hayat and Giaquinta (1970) emphasize that small sample size is crucially important.

Microwave processing This technology goes back quite a few years (reviewed in Login and Dvorak 1994), but in my opinion the point where it became reproducibly useful was after the commercial development of a research microwave by Ted Pella, Inc. (Giberson et al. 1995; reviewed in Giberson and Demaree 2001). Resin infiltration times in the microwave are short (about 15 min each in 50 % resin/solvent and two changes of pure resin) as are polymerization times (about 1.5 h) for resins. But one limitation of rapid polymerization is that it has to be done underwater and this limits the types of resin molds one can use.

Ultrarapid infiltration with centrifugation The method we recommend for resin infiltration is similar to the methods developed in the 1970s but differs in that it uses moderate centrifugation to speed resin infiltration into cells and tissues.

Fig. 3 A mini-centrifuge of the type used for rapid resin infiltration. This type of centrifuge operates at a fixed speed of 6,000 rpm or 2,000×*g*

This work is published elsewhere (McDonald 2013), but can be easily summarized here. We had been routinely using microwave infiltration and polymerization in my laboratory following FS, but we wondered if the microwave was really necessary. Our methods were developed independently of the early work mentioned above, which we only discovered when writing up the experimental results.

Materials and equipment

The only additional equipment that you will need beyond what you normally use for infiltration is a mini-centrifuge (Fig. 3) or variable speed centrifuge that spins at 6,000 rpm. These mini-centrifuges are inexpensive and readily available (cf., http://www.southwestscience.com/).

Quick infiltration procedure

Although this procedure should work with any material ready for infiltration and polymerization, for purposes of continuity the assumption is that one has just removed the samples from the quick FS apparatus as described in the previous section.

1. Start by making up epoxy resin, and remove frozen resin from the freezer or LRW resin from the refrigerator. The Epon formulation we used is 23.5 g Eponate12 (Ted Pella, Inc., Redding, CA, USA), 12.5 g DDSA, 14 g NMA, and 0.75 ml BDMA. The LRW is hard grade. The Epon–Araldite formula is 6.6 g Eponate 12, 4.4 g Araldite 502, 12.2 g DDSA, and 0.8 ml BDMA.
2. Remove the samples from the rocker and rinse in pure acetone in the cryotubes. After three to four quick rinses over 5–10 min, remove the samples from the specimen carriers and cut or break into smaller pieces if possible.
3. Make up the appropriate volumes of resin in mixtures of 25, 50, and 75 % resin/acetone. One needs approximately 1.5 ml of each solution per cryotube.
4. Remove all but enough acetone to cover the samples, add 25 % resin/acetone mixture, and resuspend the samples. Resuspension is a critical step and material should be brought up into the column of the new resin mixture with a toothpick, needle, or some other implement. Put on a rocker for 3–5 min and then spin for 30 s at 6,000 rpm (2,000×*g*) in a mini-centrifuge (Southwest Scientific, Phoenix, AZ, USA) or variable speed centrifuge. This is all the time required for infiltration.
5. Repeat step 4 with 50, 75, 100, and 100 % resin. More viscous resin solutions with smaller sample pieces may require longer centrifugation times to reach the bottom of the tube. If processing a large number of tubes, place them on a rocker or rotator while changing resin in the remaining tubes.

6. For the final change in pure resin, pour out the resuspended sample from the second pure resin change into a weigh boat or other vessel, making sure to label which sample it is. Fill a new cryotube or Eppendorf tube with pure resin and, using a toothpick or needle, transfer the specimens from the weigh boat to the top of the new resin tube. Spin down once more in the mini-centrifuge.
7. Resuspend and pour out specimens into another weigh boat and transfer them to the polymerization containers of choice.

Quick polymerization procedure

1. Set up a polymerization oven to 100 °C.
2. Place the samples in the oven and remove after 2 h.

Types of polymerization containers that can be used include polypropylene capsules (e.g., Ted Pella, Inc., product no. 133-P), latex flat embedding molds, and thin-layer embedding between two PTFE-coated microscope slides (Reymond and Pickett-Heaps 1983; Mueller-Reichert et al. 2003). Standard BEEM® capsules will not work as they deform at 100 °C.

On-section immunolabeling

Conventional methods

Processing methods for on-section immunolabeling are thoroughly covered in a book by Newman and Hobot (1993) and in a history of resin development for immunochemistry by the same authors (Newman and Hobot 1999), so only a general overview will be included here. Chemical fixation for immuno-EM (iEM) is generally with low (0.1–0.5 %) concentrations of glutaraldehyde and no OsO_4 because it is known that for most antibodies successful labeling is inversely proportional to fixative concentration. The duration of fixation is also important and Hobot and Newman (1991) suggest that times longer than 15 min can be harmful to labeling density. Dehydration with ethanol is common though acetone can be used and partial dehydration is thought to lead to increased labeling (Newman and Hobot 1993). Cryosubstitution by the progressive lowering of temperature (PLT) method and embedding in Lowicryl resin by UV polymerization is considered by many to give the best morphology and labeling density.

Rapid FS in 0.2 % uranyl acetate/acetone and rapid embedding in LR White at 100 °C for 90 min

The rapid FS and embedding methods presented above can be adapted for EM on-section immunolabeling (McDonald 2013). The modifications include FS without the traditional glutaraldehyde or osmium fixatives but using only UA in acetone. During rapid FS, the specimens are warmed to RT and then embedded in LRW resin and polymerized for 90 min in a 100 °C oven.

FS with UA only and embedding in Lowicryl at low temperatures is a proven strategy for on-section immunolabeling (early work is reviewed in Hippe-Sanwald 1993 and Nicolas and Bassot 1993). More recent work (Hawes et al. 2007) suggests that a semi-rapid FS with UA alone in acetone produces excellent membrane morphology in Lowicryl HM20 though high concentrations (2 %) of UA reduce antibody labeling compared to lower (0.2 %) concentrations. Evidence from Nixon et al. (2009) and Kukulski et al. (2011, 2012) shows that FS in acetone–UA and Lowicryl HM20 embedding will preserve fluorescence of cloned probes in resin sections. It is even possible to FS in solvents alone to retain good morphology and antigenicity if the resin is polymerized at low temperature (early work summarized in Nicolas and Bassot 1993; Monaghan and Robertson 1990; Monaghan et al. 1998). Some investigators have freeze substituted specimens in solvent only and then embedded in LRW (Lancelle and Hepler 1989; McCurdy and Pratt 1986) or even Epon (Nicolas and Bassot 1993) for purposes of immunolabeling. There is at least one case where UA in acetone was used for FS to RT and embedding in epoxy resin (Porta and Lopez-Iglesias 1998), and they report that morphological preservation was good. We find that good immunolabeling and morphology can be achieved by FS in acetone–UA alone, warming to RT for quick infiltration with LRW followed by quick polymerization at 100 °C. The procedure has been published (McDonald 2013) but is summarized here.

Freeze substitution and LRW resin infiltration

1. Freeze substitute in 0.2 % UA in acetone over 2–3 h by the SQFS method (McDonald and Webb 2011; see also modifications in the previous section) and warm to RT. Specimens can be removed at 0 °C and warmed immediately to RT.
2. Rinse three to four times in pure acetone over about 10 min.
3. Resuspend samples in 25 % LRW (hard grade) resin–acetone mixture, agitate for 5 min, then spin for 30 s in a mini-centrifuge at 6,000 rpm.
4. Repeat step 3 with LRW–acetone mixtures of 50 and 75 %.
5. Make three changes in pure LRW, rocking or rotating for 5 min each and spin down at 6,000 rpm (2,000×*g*) in a centrifuge for 30–60 s in between changes.
6. Transfer to the polymerization container of choice. We use either flat-bottom polypropylene capsules (Ted Pella, Inc., prod. # 133-P) with a piece of Aclar (EMS, Hatfield,

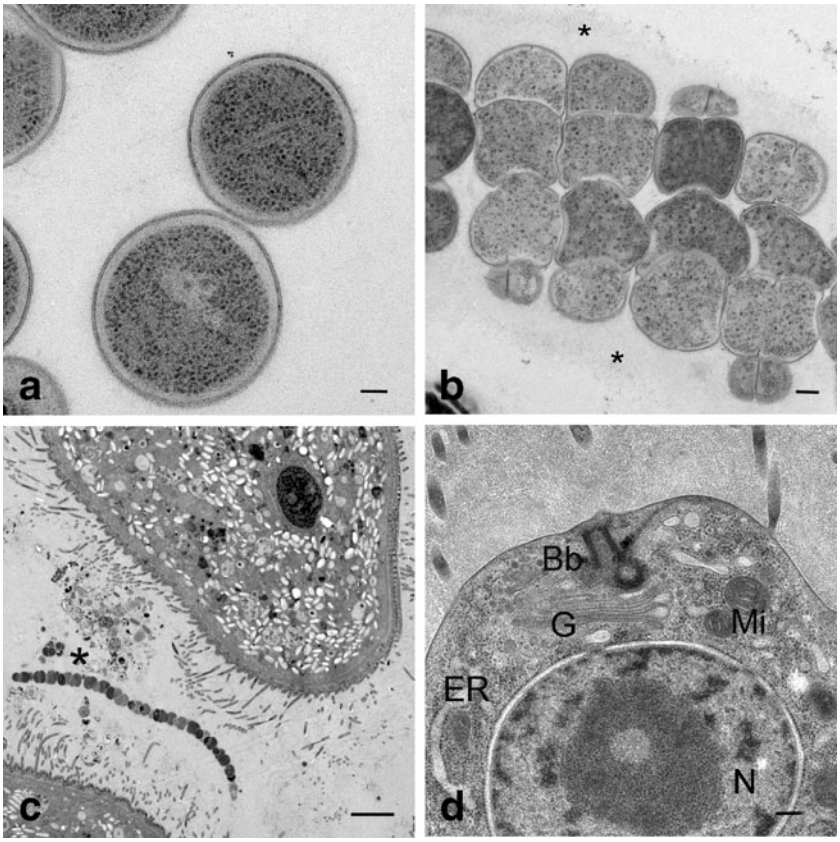

Fig. 4 Small cells prepared by a 2.5-h FS procedure and rapid resin embedding with Epon resin in 3 h. In (**a**), bacterial cells (*Agrobacterium tumefaciens*) show good preservation as indicated by smooth cell wall profiles and uniform density of cytoplasm. Cells courtesy of John Zupan and Patricia Zambryski, University of California, Berkeley. *Bar* = 100 nm. Panel (**b**) is of unidentified bacterial colony cells from the rumen of a cow. Note the preservation of a fine sheath (*asterisks*) around the cells. Cells courtesy of Marissa Hirst and Scott Dawson, University of California, Davis. *Bar* = 200 nm. Panel (**c**) shows portions of two unidentified ciliates from the cow rumen and a region containing numerous bacterial cells (*asterisk*). Cells courtesy of Marissa Hirst and Scott Dawson, University of California, Davis. *Bar* = 5 μm. Panel (**d**) is a choanoflagellate cell (*Salpingoeca rosetta*) containing typical eukaryotic organelles such as a nucleus (*N*), endoplasmic reticulum (*ER*), basal bodies (*Bb*), mitochondria (*Mi*), and a Golgi apparatus (*G*). Cells courtesy of Pawel Burkhardt and Nicole King, University of California, Berkeley. *Bar* = 200 nm

PA, USA, cat# 50425) in the bottom. The Aclar pieces can be cut out with an office paper punch. Note that containers such as BEEM capsules are not recommended because they deform during polymerization.

Polymerization

Samples are placed in an oven set to 100 °C and 50 ml liquid nitrogen is put in the oven so that the subsequent expansion of nitrogen gas will displace any oxygen through the thermometer hole in the top of the oven. Make sure your oven is ventilated before putting in liquid nitrogen. If it is not ventilated, then you can try putting in some dry ice to create less oxygen. If you are using embedding capsules that are well sealed then neither nitrogen nor dry ice may be necessary. The specimens are removed after 90 min.

Microtomy and microscopy

Polymerized blocks of cells and tissues were sectioned on a Reichert-Jung Ultracut E microtome at 70 nm thickness and post-stained with aqueous UA for 5 min and Reynold's (1963) lead citrate for 3 min. Sections were observed in a Tecnai Biotwin electron microscope (FEI Inc., Hillsboro, OR, USA) operating at 120 kV. Images were recorded on an Ultrascan 1000 CCD camera (Gatan Inc., Pleasanton, CA, USA).

Examples

The images included here are intended to illustrate several key points: (1) the excellent quality of morphological preservation in both epoxy and LRW resins, (2) the absence of resin infiltration problems, and (3) immunolabeling of the LRW sections.

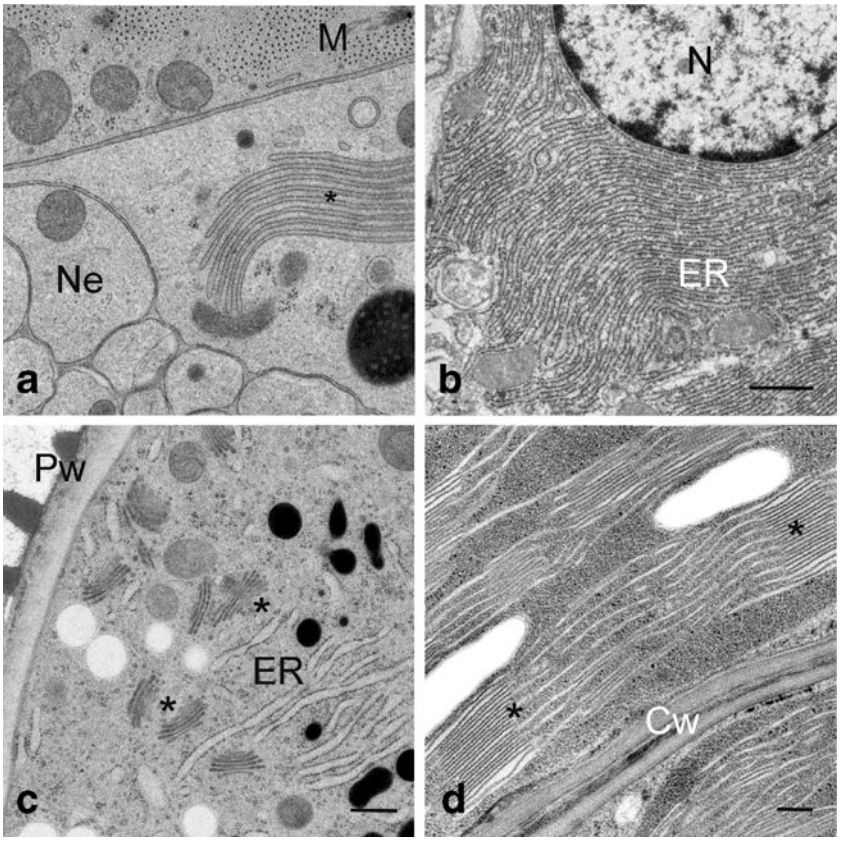

Fig. 5 Whole organisms and tissues processed as in Fig. 4 and embedded Epon–Araldite resin. Panel (**a**) is from the head region of the nematode *Caenorhabditis. elegans* and shows some typical animal tissues such as muscle (*M*), a bundle of nerve cells (*Ne*), and an array of smooth membranes (*asterisk*). Worms courtesy of Denise Lapidus and Barbara Meyer, University of California, Berkeley. *Bar* = 200 nm. Panel (**b**) shows a chief cell from the stomach of a mouse with a large array of endoplasmic reticulum (*ER*) and a portion of nucleus (*N*). The stomach tissue was fixed by immersion in 2 % glutaraldehyde prior to high-pressure freezing. Tissue courtesy of Susan Hagen, Beth Israel Deaconess Hospital, Boston. *Bar* = 1 μm. Panel (**c**) is a portion of a pollen grain inside a whole anther of *Arabidopsis thaliana* frozen by HPF. Despite the pollen wall (*Pw*), cells are well infiltrated and show numerous Golgi (*asterisks*) and endoplasmic reticulum (*ER*). Cells courtesy of Michael Melzer, Leibnitz Institute of Plant Genetics and Crop Plant Research, Gatersleben, Germany. *Bar* = 0.5 μm. Panel (**d**) is from leaf tissue of *Oxalis* sp. plants collected on the University of California, Berkeley campus. Stacks of thylakoid membranes (*asterisks*) are well preserved and there are no infiltration problems at the cell wall (*Cw*). *Bar* = 200 nm

From the dozens of different specimen types that we have prepared using the methods described above, we have selected those that span a wide range of size and complexity, from small single cells to whole organisms and large tissues. For more examples, see the articles on FS in 3 h or less (McDonald and Webb 2011) and rapid resin embedding methods for morphological analysis and on-section immunolabeling (McDonald 2013). We believe that these results compare favorably with published examples that take much more time to process. With the exception of Figs. 1b and 2b, all the examples presented in this article were high-pressure frozen in either a Bal-Tec HPM-010 (Bal-Tec AG, Liechtenstein) or Leica (Leica Microsystems, Vienna) EMPact2-RTS high-pressure freezer.

Three-hour embedding in Epon or Epon–Araldite

Small cells While bacteria and other small cells might be expected to infiltrate with resin easily and quickly, the cell wall can sometimes be a serious hindrance. Figure 4a shows a section through a pellet of well-infiltrated bacterial cells. Figure 4b is a colonial bacterium from the gut of a cow. Note the excellent preservation of the sheath material around the colony. In Fig. 4c, portions of two ciliates from the cow gut are shown plus an assortment of smaller bacteria in between the two. The higher magnification of a single choanoflagellate cell shows more details of the subcellular organelles.

Larger cells and tissues In Fig. 5, cells and tissues that are more challenging to infiltrate rapidly are shown. Whole *Caenorhabditis elegans* worms have diffusion barriers that are so effective that live cells can swim for hours in 2 % glutaraldehyde solutions (Shepherd and Clark 1976). But as shown in Fig. 5a, even a resin as viscous as Epon–Araldite can infiltrate the organism completely in about 30 min. Mouse organ tissues are best high-pressure frozen fresh, but in some cases fixed tissues can be used as in the stomach cells shown

in Fig. 5b. Pollen grains are another type of cell with significant diffusion barriers to fixatives and other solutions. However, after HPF and rapid FS of whole *Arabidopsis* anthers, the infiltration with Epon–Araldite resin was very effective (Fig. 5c). We believe this is particularly significant because it shows that low-viscosity resins such as Spurr's (Spurr 1969) are unnecessary. Finally, sections of whole leaves of *Oxalis* plants were prepared after 6 h of processing and the details of chloroplast structure are well preserved (Fig. 5d).

Two- to three-hour embedding in LR White following FS without traditional fixatives

High-pressure-frozen cells that are freeze substituted to RT with only UA show remarkably good preservation of morphology as well as antigenicity. Figure 6 shows examples of both. The choanoflagellate cell in Fig. 6a does not show membranes as well as the comparable osmicated and Epon-embedded cell in Fig. 4d, but the overall preservation is certainly suitable for most immunolabeling work. The whitish areas near the cell apex are most likely glycogen-like food storage granules and not extraction artifacts. See images in McDonald (2014) for more examples of this cell type including immunolabeling. Figure 6b is from the cytoplasm of a ciliate in the cow rumen. The cytoplasm is well preserved including bundles of microtubules characteristic of these organisms. Basal bodies and microtubule bundles in cross-section label well with anti-tubulin antibodies (Fig. 6c). On-section antibody labeling with anti-actin also works well as shown in Fig. 6d and McDonald (2013).

Discussion

High-pressure freezing

The value of HPF has been so thoroughly documented and reviewed over the past quarter century that no further

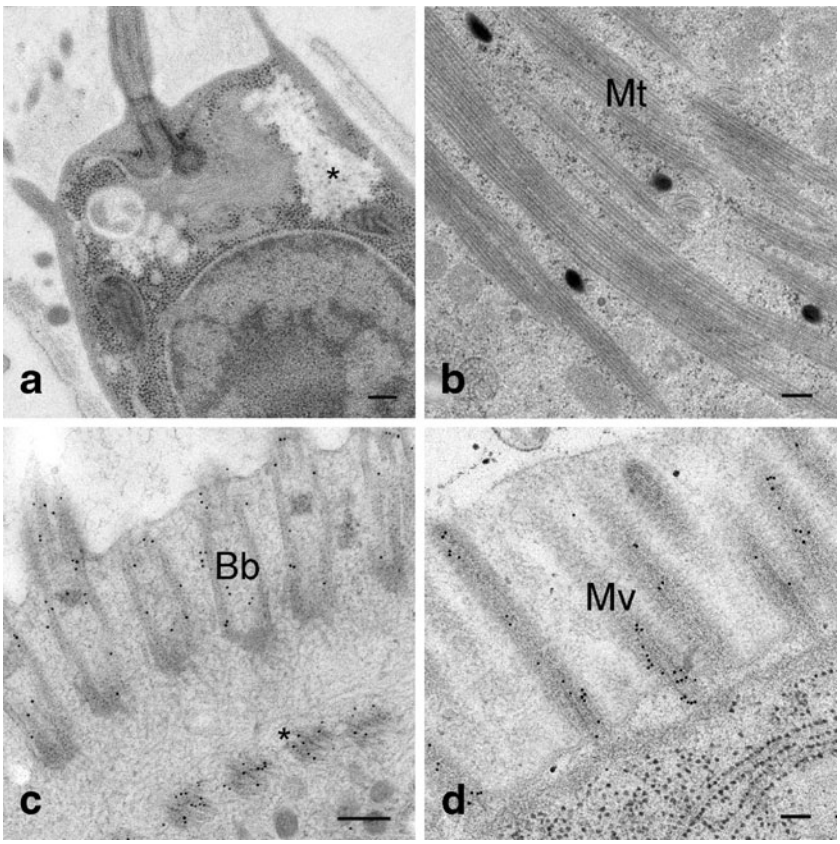

Fig. 6 Cells prepared by FS in 0.2 % uranyl acetate in acetone and embedded in LR White resin at RT. Panel (**a**) is a choanoflagellate cell (*Salpingoeca rosetta*) kindly provided courtesy of Pawel Burkhardt and Nicole King, University of California, Berkeley. Comparison with the Epon-embedded cell in Fig. 4d shows that the organelles are well preserved but lack membrane contrast because no osmium tetroxide was used during FS. *Bar* = 200 nm. Panel (**b**) shows bundles of microtubules (*Mt*) in the cytoplasm of an unidentified ciliate in the cow rumen. Cells courtesy of Marissa Hirst and Scott Dawson, University of California, Davis. *Bar* = 200 nm. Panel (**c**) is from another ciliate in the cow rumen with basal body (*Bb*) microtubules and rootlet microtubules (*asterisk*) labeled with 10 nm gold recognizing an anti-tubulin primary antibody. Cells courtesy of Marissa Hirst and Scott Dawson, University of California, Davis. *Bar* = 200 nm. Panel (**d**) shows labeling of actin inside microvilli (*Mv*) of the *C. elegans* gut with 10 nm gold. Worms courtesy of Denise Lapidus and Barbara Meyer, University of California, Berkeley. *Bar* = 100 nm

discussion about its general merits needs to be included here. However, the point needs to be made that for success with rapid FS and embedding, the specimens must be very well frozen. If problems with the final images are encountered after using these rapid methods, the most likely cause is poor specimen freezing. In well-frozen samples, the ice crystals that form are probably very small and do not interfere with morphology as viewed in resin sections. Likewise, if recrystallization (sometimes called devitrification) occurs during FS warming, it may do little harm if the crystals are small. The fact that we can warm specimens to RT only in the presence of acetone and UA but still get acceptable morphology validates this idea. These and other topics related to quick FS and embedding procedures are discussed in more detail below.

Freeze substitution

A prevailing myth about FS is that it needs to be done over long periods of time, usually 2 days or more. This is an interesting evolution because the early FS procedures practiced by Humbel and Müeller (Humbel et al. 1983; Humbel and Mueller 1986; Steinbrecht et al. 1987) were relatively short, lasting a little over 24 h. This was primarily because they used methanol as the FS solvent and their tests of dye extraction from paper showed that substitution was quick to replace water and that methanol could absorb considerably more water than acetone at −90 °C (Humbel et al. 1983; Humbel and Mueller 1986). However, when it was realized that methanol extracted more lipids and proteins than acetone (Weibull et al. 1984; Humbel and Schwarz 1989), there was a tendency to switch to acetone. Some studies (McDonald 1994; Monaghan et al. 1998) showed that compared to acetone methanol did a poor job of preserving ultrastructure. The fact that the model calculations (Humbel et al. 1983; Humbel and Mueller 1986) showed that even 1 % water in acetone delayed substitution by many hours was probably one reason why FS times were extended to days instead of hours, just to be safe. The irony is that we now know that FS will proceed quickly and safely with even 20 % water added to the solvent and that 5 % water in acetone can be used routinely to improve membrane contrast in some cell types (Walther and Ziegler 2002; Buser and Walther 2008). Another study that contributed to lengthening the times of FS was by Steinbrecht (1982; Steinbrecht et al. 1987) who found that 7 days was a useful time for FS of moth antennae.

The other rationale for extended FS times was the concern over recrystallization of cellular water during the warm-up phase. When Steinbrecht (1982) added 5 % water to acetone for FS, he concluded that it caused ice crystal growth during rewarming, a conclusion that is at odds with what we know about FS with water in acetone now (Buser and Walther 2008). Recrystallization in pure water begins as low as −135 °C but the critical temperature for most biological systems was not known but it was assumed that it was probably warmer than −78 °C because FS without noticeable ice damage was possible if the specimens were held at this temperature or lower (usually −90 °C) for periods thought long enough to guarantee water replacement. But recent comments by Dubochet (2007) suggest that even though hexagonal ice is probably formed during warming there is no significant movement of the molecules (segregation). In his words, "Then the result is that all the water of a biological specimen is crystallized into large hexagonal crystals **without any segregation** [my emphasis], and the structural preservation is excellent down to molecular dimensions". To understand this, you have to imagine a large, perhaps single crystal in the entire cell but the water molecules are not all contiguous but dispersed finely throughout the cell. Think of a finely branched bush or tree (Dubochet 2007). In other words, recrystallization takes place by fine ramifications through the cell, but it does not damage the cells for FS if they are well frozen in the first place. If this is so, then the speed of substitution can be much faster as our results confirm (McDonald and Webb 2011; McDonald 2014).

Another reason that FS can be made faster than previously believed is continuous agitation. Think of film development. As fresh developer encounters a silver halide crystal, it rapidly becomes exhausted at the surface. To speed the development, we agitate film so there is a continuous supply of developer available to the silver halide. In this way, film is developed in minutes. It is possible to develop film by allowing diffusion to drive the reaction, but it takes much longer. In photography, it is known as "stand development". By continuous agitation of the FS solution, we may be speeding up the exchange of solvent for water in much the same way.

Resin infiltration

One of the long-held beliefs about resin infiltration is that viscous resins take longer to infiltrate into cells. This idea seems like common sense and has long been stated as a truism in basic textbooks (Glauert 1975; Hayat 2000), but there seems to be little published evidence to back up this statement. Spurr's low-viscosity resin (Spurr 1969) was readily adopted by most plant biologists because it was supposed to penetrate botanical specimens more readily than other epoxies. Even if this were true, it does not mean that the more viscous resins which have better sectioning properties and beam stability could not be used as well. There is even some evidence that Spurr's resin does a much poorer job than more viscous epoxy resins in preserving cell structure of certain plant tissues following FS (cf., Figs. 1 and 2 with figure 8 in Hess 2007). Results in my laboratory (McDonald 2013, and those presented here) show that Epon or even Epon–Araldite resins can infiltrate many types of tissues in very short periods of

time, even those with diffusion barriers as substantial as pollen cell walls (cf., Fig. 5c in this article).

It may well be that centrifugation helps infiltration with more viscous resins, but early studies of rapid infiltration (Hayat and Giaquinta 1970; Bain and Goves 1971) do not seem to mention it. Nevertheless, most infiltration schemes use some kind of agitation and that may be sufficient. We use centrifugation because it is quick, and we know from mixing up 20 % BSA solutions (McDonald et al. 2010) that it certainly speeds up the solubilization of that reagent. Perhaps a more critical factor for achieving rapid infiltration times is the size of the sample.

Even if the speed of penetration of all resins turned out to be about the same, it would still take longer to infiltrate a large sample than a smaller one. The quick methods presented here have the advantage that all the specimens were high-pressure frozen so they are very thin (25 to 300 μm) in one dimension. In their paper on rapid embedding, Hayat and Gianquinta (1970) stress that using small pieces of tissue is important. One rule of thumb that we use is to cut the pieces into a size that will fit into the trimmed blockface for sectioning. In my laboratory, this is less than about 250 μm for most tissues. Embedding large pieces only to discard portions during trimming makes little sense and may unnecessarily lead to infiltration problems.

FS in 0.2 % uranyl acetate and embedding in LR White at 100 °C

A typical FS fixative for immunolabeling is a low (0.1–0.2 %) concentration of glutaraldehyde plus some UA in acetone. The problem with that strategy is that even small amounts of glutaraldehyde will block antibodies from binding to antigens. It is not true for all antibodies but the number that work for light microscopy and EM is probably on the order of 25 % or fewer. Embedding cells without aldehyde fixatives should, in principle, increase that number. We have tested relatively few antibodies on tissues prepared only with UA and LRW but plan to make this a priority in future experiments. We will also be testing to see if FS in UA and embedding in LRW will preserve fluorescence in the polymerized resin. Preliminary results have shown that fluorescence is preserved but more tests need to be done on a range of fluorescent probes and tagged proteins expressing at different levels.

Is uranyl acetate a fixative? The answer is yes or no depending on the type of procedure being used. For negative stain, it can be considered a primary fixative. For conventional EM processing at RT, it is frequently used as an en bloc tertiary fixative/stain following glutaraldehyde and osmium fixation (Glauert 1975). It has also been used as a secondary fixative substituting for osmium when immunolabeling in LRW is carried out (Erickson et al. 1987). Its action as a fixative in conventional processing has been discussed by Silva et al. (1968, 1971) and Terzakis (1968). However, in general, the EM community seems to think of UA as more of an en bloc fixative or post-stain rather than a primary fixative (Hayat 1981, 2000). The situation for low temperature methods is somewhat different. In freeze substitution, UA is known to stabilize lipids against extraction (Weibull et al. 1984) and is used in many FS solutions as an additive to either glutaraldehyde or osmium. It has frequently been used alone with acetone or methanol for low-temperature embedding (early references in Nicolas and Bassot 1993; Hawes et al. 2007; Nixon et al. 2009; Kukulski et al. 2011). However, for FS warming to RT, UA is not considered a fixative in the same sense that aldehydes and OsO_4 are. If it were then the literature of FS should be filled with examples of FS to RT with only UA and solvent. We could only find one such example (Porta and Lopez-Iglesias 1998) that was reported as preserving good ultrastructure, but no images were shown. This lack of FS procedures using UA as the sole fixative for embedding at room temperature leads us to conclude that the FS community does not consider UA as an EM fixative in the usual sense. The general belief seems to be that one needs an aldehyde or osmium fixative in the FS medium if you are going to embed at RT (Möbius 2009; Hurbain and Saches 2011).

Lowicryl resins are known to cause contact dermatitis and the vapors are very volatile. Embedding samples at low temperatures can be tricky, especially if one prefers to have oriented specimens in the polymerized resin. Furthermore, it is difficult to keep the resin and molds free of condensed water (frost) when embedding at low temperature. Embedding in LRW at RT is much easier and, if desired, partial hydration can be maintained with LRW because it will polymerize with up to 12 % water present (Newman and Hobot 1999). For these reasons, the methods presented here and in McDonald (2013) offer a safer and easier alternative for embedding without aldehyde fixatives. It may be that Lowicryl preserves the ultrastructure better, but this remains to be demonstrated.

Finally, there is a general belief that heating specimens to 100 °C will cause uneven polymerization (Glauert 1975) or interfere with antibody labeling if the resin is LRW. In my laboratory, we have found neither assumption to be true. In fact, LRW polymerized at 100 °C does not come out of the oven “sticky” at the top as it does in a 60 °C oven and it sections very well. Those who use research microwave ovens to polymerize resins also do so close to or at 100 °C because the resin is sealed in capsules in boiling water. One can also use microwaves for quick infiltration with resins but our results show that they are unnecessary.

Summary and conclusions

Obtaining results on sectioned material with EM has a reputation for taking a long time, whether it is by conventional

methods or HPF and FS. In this review, we show that it does not have to take so long, and that it is possible to achieve excellent results with procedures that can be completed in 1 day. But there are many advantages to the rapid processing methods presented here beyond just being able to see your results quickly: (1) If multiple users in a laboratory or core facility want to do different FS procedures they do not have to wait until the AFS machine is finished in several days to a week to start their own experiments; (2) for time-sensitive material, multiple experiments can be finished before the specimens are no longer available; (3) if adjustments need to be made to the ways specimens are frozen or freeze substituted, they can be done in a reasonable time frame; (4) the equipment for rapid FS and embedding is inexpensive compared to automated systems; (5) more researchers would use EM if they realized that experiments did not have to take such a long time; (6) these procedures will be useful in workshop settings because students can get results before the course ends; and (7) rapid methods may actually give better results than those where samples sit in solvents and resins for long periods of time because there is likely to be less extraction from the cells and tissues.

Some pathology laboratories already use fast turnaround methods using microwave processing, but the procedures we present here are simpler and just as fast without the equipment expense. Positive results have been achieved on a reasonable number of different cell types, but we consider most of the data so far to be proof of concepts. We expect that these rapid methods will also have a role to play in developing new and better procedures for correlative light and electron microscopy. We encourage everyone who does freeze substitution and embedding in resin to try these procedures then publish their results so we can begin to better understand the limitations as well as the positive applications.

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Conflict of interest The author declares that there is no conflict of interest.

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