# 22

# Conventional Specimen Preparation Techniques for Scanning Electron Microscopy of Biological Specimens

**John J. Bozzola**

**Summary**

This chapter covers conventional methods for preparing biological specimens for examination in the scanning electron microscope (SEM). Techniques for handling cells grown in liquid culture, as well as on substrates such as culture dishes, slide culture chambers or agar, are discussed. These methods may be used to process most cultured organisms as well as whole botanical and zoological specimens.

**Key Words:** Scanning electron microscopy; biological specimen preparation; conventional SEM preparatory techniques.

## 1. Introduction

Specimens for scanning electron microscopy (SEM) examination may vary from individual cells grown in culture to solid tissues to entire organisms measuring several centimeters in size. As was the case with specimen preparation for transmission electron microscopy (TEM), the basic steps for SEM preparation are very similar: fixing it in buffered aldehyde, postfixing it in osmium tetroxide, dehydrating it in ethanol, drying it, mounting it on a specimen stub, coating it with a heavy metal, and examining it in the SEM. Detailed considerations for the choice of buffer and type of fixatives are discussed in Chapters 1 and 2. In the present chapter, only the most commonly used reagents and procedures used to prepare specimens for SEM examination are discussed.

The major advantage of the SEM is that it permits one to study the morphology and surface detail of solid material. It literally permits an "in-depth" study of specimens with great relief because of the tremendous depth of field available to the operator. SEM studies typically investigate the external features of a specimen, in contrast to TEM studies, where intracellular exploration is the

From: *Methods in Molecular Biology, vol. 369*
*Electron Microscopy: Methods and Protocols, Second Edition*
Edited by: J. Kuo © Humana Press Inc., Totowa, NJ

major focus. However, the SEM can be used to probe internal cellular detail; if one removes the overlying material, perhaps by fracturing, cutting, or tearing into the specimen. An understanding of the operational principle of the conventional SEM is useful because the quality of images obtained will be affected by how specimens are prepared for microscopic examination.

The SEM is unlike most conventional imaging systems, including light microscopes and the TEM, because it does not contain image-forming lenses. The lenses in the SEM do not form images but act as a set of three condenser lenses, in a demagnification series, to focus an extremely small spot, or probe, of electrons on a solid specimen. When the high-energy, accelerated electrons strike the specimen surface, they generate different forms of energy, or signals. These signals include secondary electrons, backscattered electrons, X-rays, light, and heat. With the appropriate detector, these signals can be captured, yielding different sorts of information. For example, secondary electrons give information related to topography (the three-dimensional image), backscattered electrons give information primarily about differences in atomic number, X-ray signals reveal the types of elements present, whereas light and heat may reveal information about the compositional nature of the specimen area being probed.

The SEM probe is not static but is scanned rapidly over the specimen much like the scanning of the electron beam that takes place in a television monitor or cathode ray tube (CRT). When the SEM probe strikes the specimen, each point that is impacted will yield a certain number of secondary electrons, depending primarily on specimen topography. This information is displayed on a viewing monitor, or CRT, so the number of secondary electrons from the specimen is displayed in terms of brightness and contrast. Areas of the specimen that yield many secondary electrons will appear bright, whereas areas that yield fewer secondary electrons will appear proportionally darker on the CRT.

The quality of the image displayed on the CRT depends on the quality of the signal, or the overall yield of secondary electrons from the specimen. Stronger signals are the result of generating and collecting higher numbers of secondary electrons. The elements that make up the surface of the specimen are the source of the secondary electrons. Elements with a high atomic number yield a high number of secondary electrons and, ultimately, a higher quality image. Unfortunately, biological systems are composed of lighter elements (e.g., carbon, hydrogen, oxygen, nitrogen) and yield a poor signal in the conventional SEM. To overcome this obstacle, biological specimens are coated with a thin layer of a high atomic numbered element such as gold, palladium, platinum, or osmium. After fixation, drying, and coating with a heavy metal, specimens are ready for study in the SEM. For greater coverage of specimen preparation and SEM operation, several reference books are recommended ***(1–3)***.

## 2. Materials

1. Critical point dryer.
2. Sputter coater or vacuum evaporator.
3. Specimen collection supplies (tubes, pipettes, dishes, containers).
4. Buffers. A wide variety of buffer systems are available; however, the most commonly used buffer for SEM preparation is the phosphate buffer of Sorenson. Additional buffers are described in Chapters 1 and 2 on specimen preparation for the TEM. Sorenson's phosphate buffer consists of two parts: 0.2 *M* monobasic sodium phosphate, $NaH_2PO_4$, and 0.2 *M* dibasic sodium phosphate, $Na_2HPO_4$. The pH is adjusted by mixing the two components as shown in **Table 2**, in Chapter 1.
5. Fixatives (*see* Chapters 1 and 2 on specimen preparation for TEM). The usual fixative regime for SEM specimen preparation consists of 2% to 2.5% glutaraldehyde in phosphate buffer followed by postfixation in 1% osmium tetroxide, with or without phosphate buffer. It is especially important that one be aware of the dangers associated with using these chemicals.
6. Holders or chambers to hold small specimens during processing. The reusable, microporous chambers, sized at 12 × 12 mm, available through various microscopy suppliers (Electron Microscopy Sciences, cat. no. 70188; Structure Probe Inc., cat. no. 13215), are particularly useful to safely store very small specimens. Some are available in pore sizes ranging from 30- to 200 μm.
7. Poly-L-lysine-coated slides (commercial or self-prepared). Clean several conventional slides with soap and water, rinse in distilled water, and dry the slides in a dust-free environment. Prepare a solution containing 1 mg of poly-L-lysine (70,000–150,000 MW) per milliliter of distilled water. Flood or dip the slides in the poly-L-lysine solution for 10 min at room temperature. Rinse in distilled water, allow to air dry, and store until needed. Slides coated with poly-L-lysine are available from vendors of electron microscopy supplies.
8. Microfiltration apparatus consisting of micropore filter, holder, 10-mL syringe.
9. 1% Aqueous carbohydrazide (w/v).
10. Hexamethyldisilazane.
11. Mounting supplies (sticky tabs, carbon tape, conductive cement, china cement).
12. SEM specimen stubs.
13. Desiccated or dry compartment to store SEM specimen stubs.
14. Ethanol dehydration series (*see* Chapters 1 and 2 on specimen preparation for TEM).

## 3. Methods

### 3.1. Preparation of Cell Suspensions (see Note 1)

Cells that are grown as suspensions in culture liquids must be deposited onto a substrate of some sort. This step is accomplished either by allowing the cells to settle and adhere onto the substrate (*see* **Fig. 1**) or by trapping them on a microporous filter substrate.

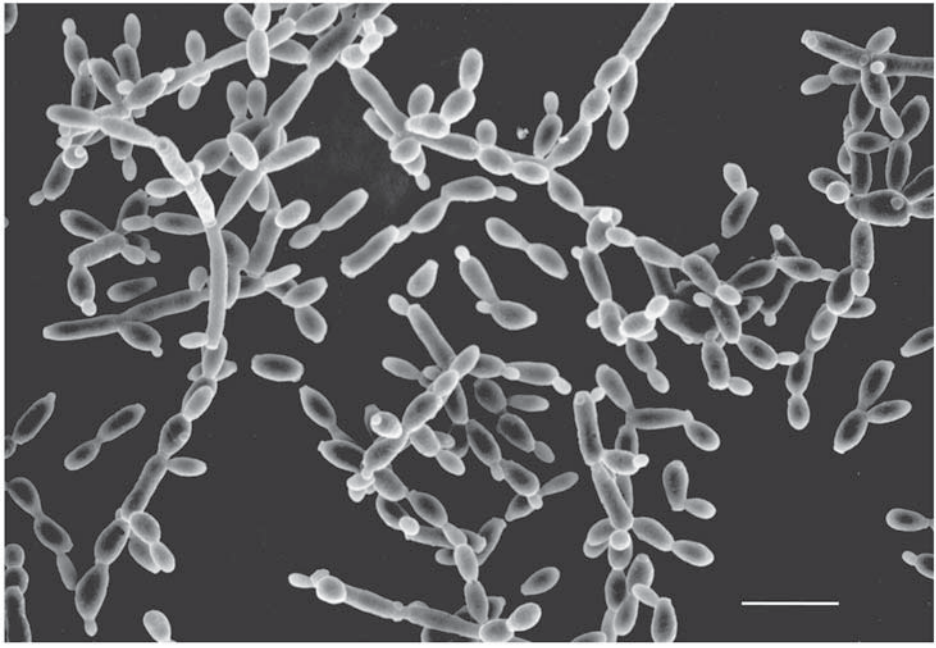


**Fig. 1.** *Candida albicans* yeast cells attached to the surface of a poly-L-lysine coated microscope slide. Specimen was prepared as described in **Subheading 3.1.1.** Bar = 10 μm.

### 3.1.1. Adherence of Cells to Poly-L-Lysine-Coated Slides

1. To adhere cells to a substrate, transfer several milliliters of culture to a microscope slide coated with poly-L-lysine and allow the cells to settle at room temperature for 1 hr. If cells do not adhere adequately using the poly-L-lysine procedure, then the organosilane ***(4)*** procedure should be used. Protect the slide from evaporation (*see* **Note 2**).
2. Tip the slide to drain the culture liquid, leaving some cells adhering to the surface of the slide.
3. Use a pipet to very gently flow 2.5% glutaraldehyde in phosphate buffer over the slide. Take care not to dislodge the attached cells. Allow the slide to stand undisturbed for 1 h at room temperature.
4. Drain the glutaraldehyde from the slide and rinse three times in phosphate buffer, each for 5 min.
5. Replace the distilled water with 1% osmium tetroxide in either phosphate buffer or distilled water. Allow slide to stand for 1 h at room temperature.
6. Rinse in distilled water and dehydrate the specimen using a graded ethanol series consisting of 25%, 50%, 75%, and two x 100% each for 10 min (*see* **Note 3**).
7. Transfer the slide, still submerged in absolute ethanol, to the critical point drying apparatus (*see* **Subheading 3.5.2.**) to complete the drying of the specimen.

8. Affix the slide to a specimen stub (*see* **Subheading 3.6.**) and apply a heavy metal coating using either a sputter coater or vacuum evaporator (*see* **Subheading 3.7.1–3.7.2.**).

#### 3.1.2. Cells Deposited on a Microporous Filter

1. Transfer several mL of culture into the barrel of a 10-mL syringe that contains an attached micropore filtration apparatus (*see* **Note 4**).
2. Place the plunger into the syringe barrel and apply pressure to the syringe to start the flow of liquid. If the flow stops immediately, too many cells were loaded into the syringe and a higher dilution is needed.
3. Remove the syringe barrel and associated plunger from the filter apparatus (*see* **Note 5**) and dispose of any unneeded cell culture.
4. Take up several ml of glutaraldehyde fixative into the syringe, place syringe onto the filter apparatus and press plunger gently to flow fixative onto the cells. Allow to stand at room temperature for 15 min.
5. Remove syringe from filter, discard unused fixative, and draw buffer into the syringe.
6. Rinse cells with buffer by attaching syringe to filter and pressing plunger.
7. Apply 1% osmium fixative using the syringe delivery method described in **steps 5** and **6**.
8. Discard unused osmium solution and rinse in distilled water using the syringe.
9. Dehydrate using a graded ethanol series applied onto the filter through the syringe.
10. After the cells have been rinsed in absolute ethanol, remove the filter holder and transfer the entire holder, including filter, into the critical point dryer.
11. Critical point dry the membrane, remove from filter apparatus, mount onto a specimen stub using double-stick tab and coat with heavy metal.

### 3.2. Preparation of Cells Grown on Substrates

#### 3.2.1. Cultured Cells on the Surface of Petri Dishes

Many cultured cells will grow onto the surfaces of Petri dishes (*see* **Fig. 2 A–C**), glass microscope slides or cover glasses, plastic flasks, or Permanox slide chambers. Attached cells are processed as follows ***(5)***:

1. Decant (or gently aspirate) the culture medium and replace with 2.5% glutaraldehyde in 0.1 *M* phosphate buffer (warmed to the same temperature as the culture). Fix at room temperature for 30 min.
2. Pour off fixative solution and rinse three times with phosphate buffer, each for 5 min.
3. Postfix for 1 h at room temperature in 1% osmium tetroxide in distilled water.
4. Rinse three times in distilled water, 5 min each.
5. Incubate in freshly prepared 1% carbohydrazide in distilled water for 15 to 30 min.
6. Rinse five times with distilled water over the course of a 15-min period.
7. Incubate in 1% osmium tetroxide in distilled water for 30 min.

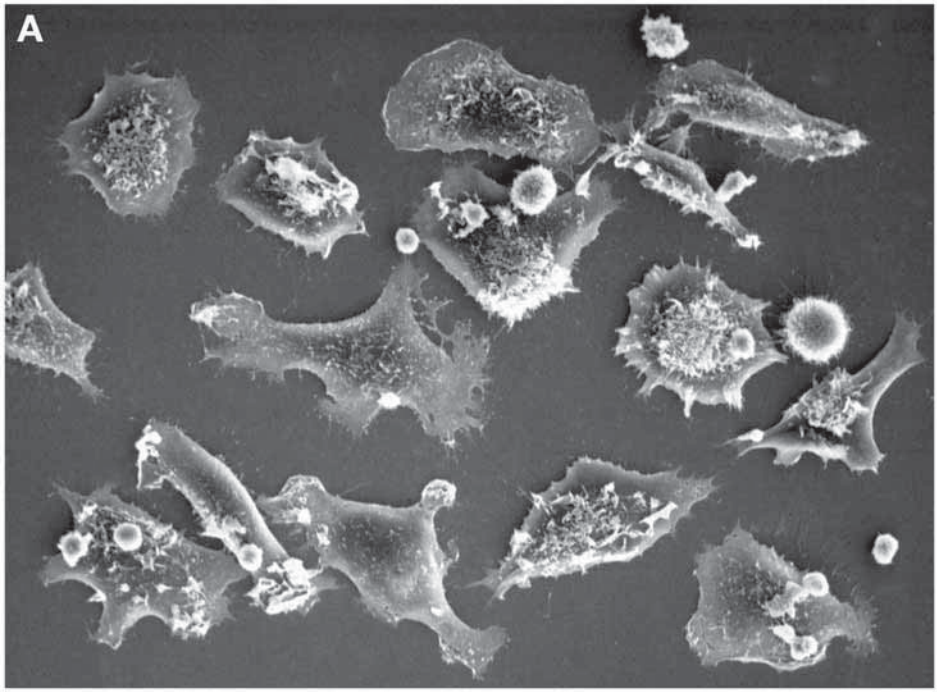


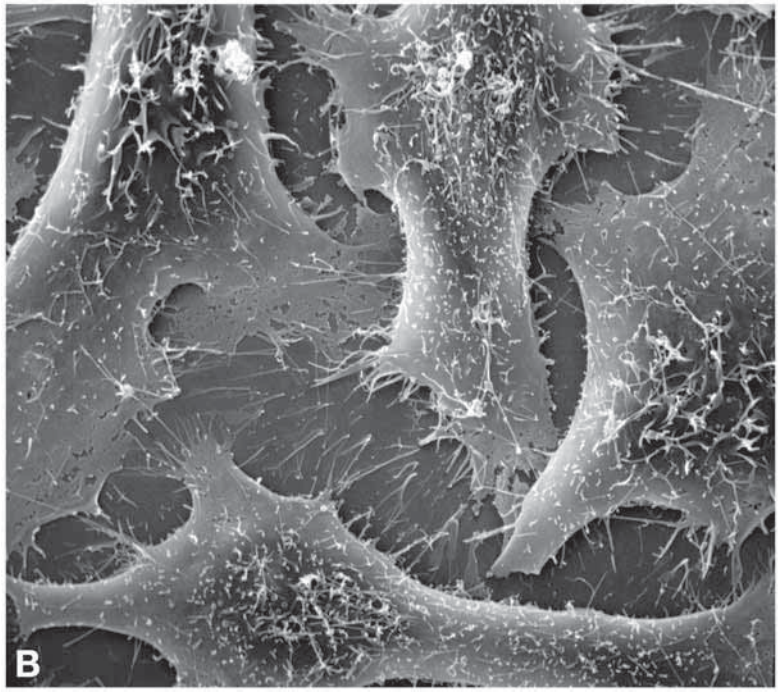

**Fig. 2. (A)** Monolayer of mammalian cells cultured on surface of Petri dish. Cells were prepared as described in **Subheading 3.3.1.** Bar = 25 μm. (Courtesy of William Kournikakis).

8. Rinse five times with distilled water over the course of a 15-min period.
9. Taking care not to let the specimen dry out, use wire cutters or clippers to cut the plastic dishes or flasks into pieces that will fit in the critical point dryer (*see* **Note 6**).
10. Place trimmed pieces, specimen side up, in another Petri dish.
11. Follow **steps 6–8** in **Subheading 3.1.1.**

### *3.2.2. Cultured Cells on an Agar Surface*

If the cells are growing on an agar surface (*see* **Figs. 3** and **4**), excise 1 × 1 1-cm pieces of agar containing the cells, removing as much underlying agar as possible.

1. Place excised agar pieces in a Petri dish or other vessel containing 2.5% glutaraldehyde in phosphate buffer to submerge the cells (*see* **Note 7**). Keep the cell layer uppermost.
2. After 30 min fixation at room temperature, rinse the specimen in three changes of phosphate buffer for 5 min each.
3. Post-fix the cells 1 h at room temperature in 1% osmium tetroxide, phosphate buffered or in distilled water.



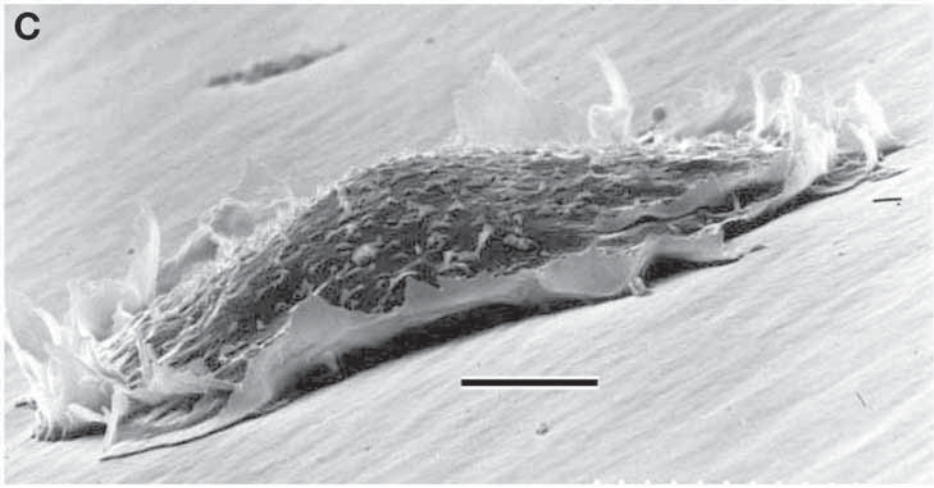




**Fig. 2. (B)** Overhead view of mammalian cells cultured on plastic surface. Cells were prepared as in (A). Bar = 10 μm. (Courtesy of William Kournikakis). **(C)** Low angle view of mammalian cell cultured on surface of Petri dish. Cell was prepared as described in **Subheading 3.2.1.** Bar = 5 μm. (Courtesy of Carol Heckman).

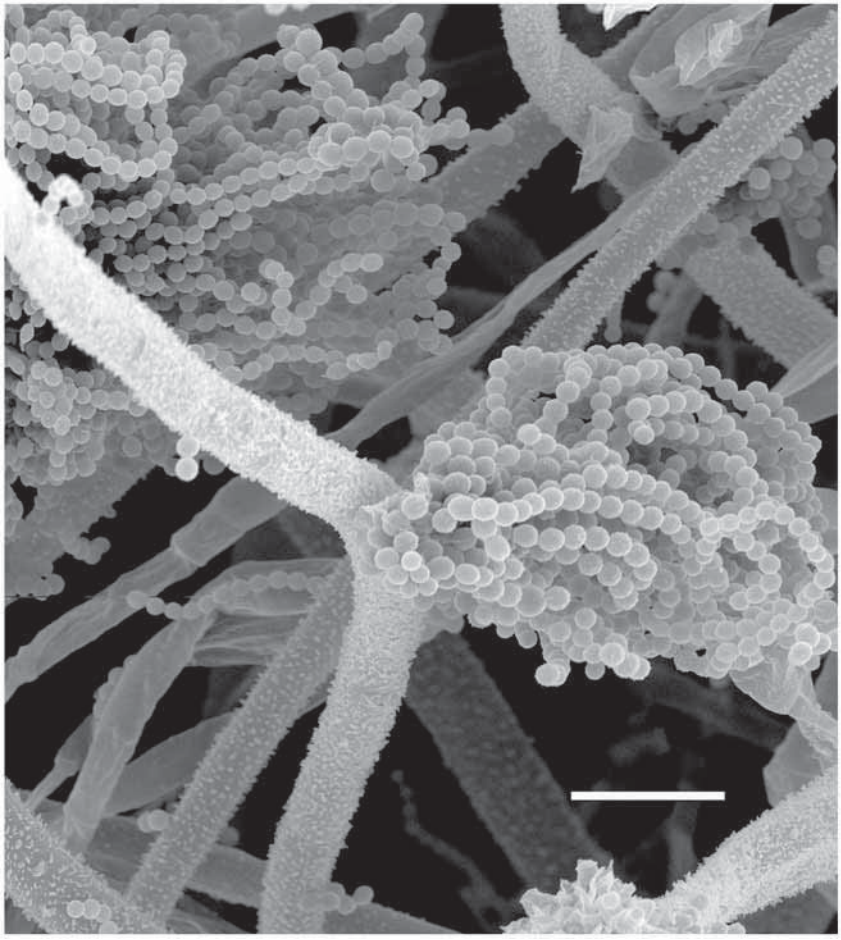


**Fig. 3.** *Aspergillus* fungi grown on agar medium. Specimen was prepared as described in **Subheading 3.6.2.** Bars = 20 μm. (Courtesy of Saara Mansouri).

4. Rinse three times in distilled water for 5 min each. After the last rinse, transfer the fragile specimens into holders, such as the microporous holders described in **Section 2**, for subsequent steps.
5. Follow **steps 6–8** in **Subheading 3.1.1.**

## *3.3. Preparation of Tissues and Large Pieces of Biological Material*

### *3.3.1. Normal Specimens*

Some organisms (insects, small plants) may be examined in their entirety using the following procedure (*see* **Note 8**):

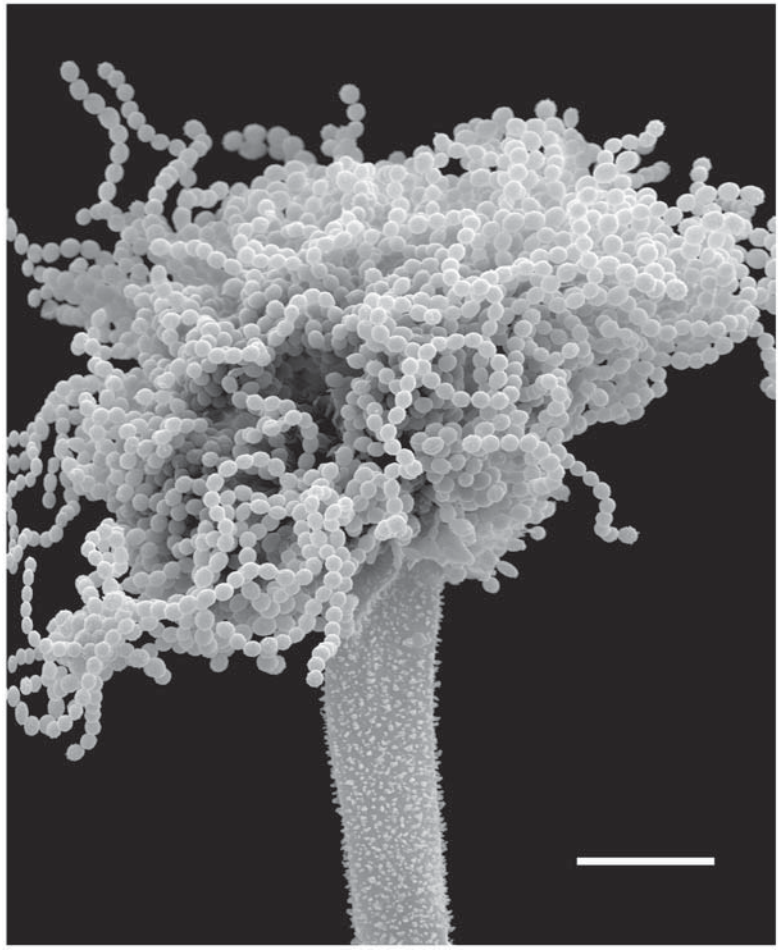


**Fig. 4.** *Aspergillus* fungi grown on agar medium. Specimen was prepared as described in **Subheading 3.6.2.** Bars = 20 μm. (Courtesy of Saara Mansouri).

1. Clean the surface of the organism using either mechanical means or chemicals (*see* **Note 9**) because surface debris may obscure surface features.
2. Transfer the specimen into a container of 2.5% glutaraldehyde in phosphate buffer so it is submerged. Fixation is normally conducted for 1 to 3 h at room temperature; however, specimens may be stored in this fixative for several weeks (*see* **Note 10**).
3. Rinse specimen in three changes of phosphate buffer, 5 min each.
4. Post-fix for 1 to 3 h at room temperature in 1% osmium tetroxide.
5. Rinse three times in distilled water, 5 min each.
6. Follow **steps 6–8** in **Subheading 3.1.1.**

### 3.3.2. Specimens Larger Than Several Centimeters

With larger specimens, it is necessary to excise pieces, which are then processed in the following way:

1. Clean the surface of the organism to remove any debris (*see* **Note 9**).
2. With a scalpel, or fresh razor blade, excise a piece of tissue from the organism (*see* **Note 11**) and transfer it to 2.5% glutaraldehyde in phosphate buffer.
3. Rinse specimen in three changes of phosphate buffer, 5 min each.
4. Post-fix for 1 to 3 h at room temperature in 1% osmium tetroxide.
5. Rinse three times in distilled water, 5 min each.
6. Follow **steps 6–8** in **Subheading 3.1.1.**

## 3.4. Storage of Specimens Before the Completion of Drying

One would normally process the specimen to complete dryness and store the specimen in a desiccator. However, it is possible to store the specimen for long periods of time at several points in the process. After fixation with glutaraldehyde, specimens may be stored in buffer for several weeks or months, if refrigerated. Storage in 75% ethanol (of the dehydration series) is possible for several days to weeks.

## 3.5. Drying the Specimen

Before specimens can be viewed in the conventional SEM, they must be completely dried because the high vacuum conditions in the SEM chamber will cause hydrated specimens to boil, thereby destroying the integrity of the specimen surface. Specimens may be dried in a variety of ways, depending on the nature of the specimen (e.g., intact whole organisms, cell suspensions, excised portions). If there is any doubt as to the proper procedure to follow, especially with an important specimen, critical point drying is the safest method to use.

### 3.5.1. Air Drying

Air-drying may be used with some unfixed, hardy specimens such as insects or botanical specimens such as seeds or pollen (*see* **Fig. 5**). Some bacteria, such as the thick-walled Gram-positive organisms, may also be air-dried following chemical fixation and dehydration in ethanol. Air-drying can be achieved several ways:

- Put unfixed specimens in a drying oven at 30 to 40°C for several days to weeks. This works well with insects with exoskeletons, some botanical specimens, and possibly some bacteria and fungi.
- Air-dry certain chemically fixed and ethanol-dehydrated specimens using a solvent with high vapor pressure such as hexamethyldisilazane.

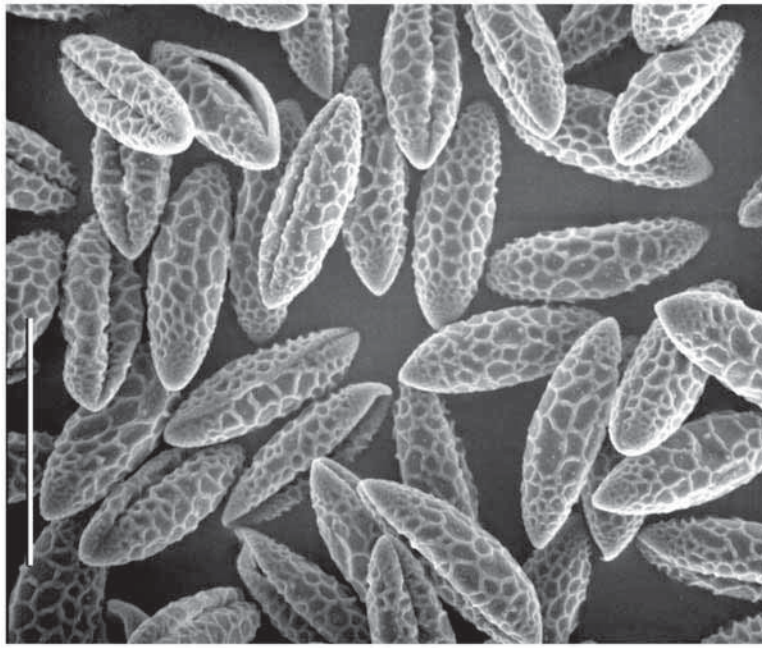


**Fig. 5.** Pollen from day lily. Pollen was shaken onto cover glass, air-dried and sputter coated as described in **Subheading 3.5.1.** Bar = 120 µm. (Courtesy of Steve Schmitt).

#### 3.5.1.1. Basic Steps for SEM Preparation of Air-Dried Specimens

1. Clean specimen surfaces and fix in glutaraldehyde/osmium fixatives following the standard schedule presented earlier in this chapter (*see* **Subheading 3.3.1.**).
2. Dehydrate in ethanol series up to absolute ethanol.
3. Transfer specimen into hexamethyldisilazane for 5 to 10 min.
4. Air dry specimen in dust free environment at room temperature or in a drying oven.
5. Mount specimen on SEM stubs, coat with heavy metal, and examine in SEM.
6. Store specimens in dry, dust free environment.

### *3.5.2. Critical Point Drying (CPD)*

CPD is the method most commonly used to complete the drying of chemically fixed and dehydrated specimens (*see* **Note 12**).

1. Transfer small specimens into holding devices (*see* **Fig. 6**) to prevent loss during CPD process. This step may be accomplished at any point after fixation in osmium tetroxide.
2. After the final change of absolute ethanol, place 10 to 15 mL of absolute ethanol in the prechilled chamber of the critical point dryer and quickly transfer specimens into the chamber.

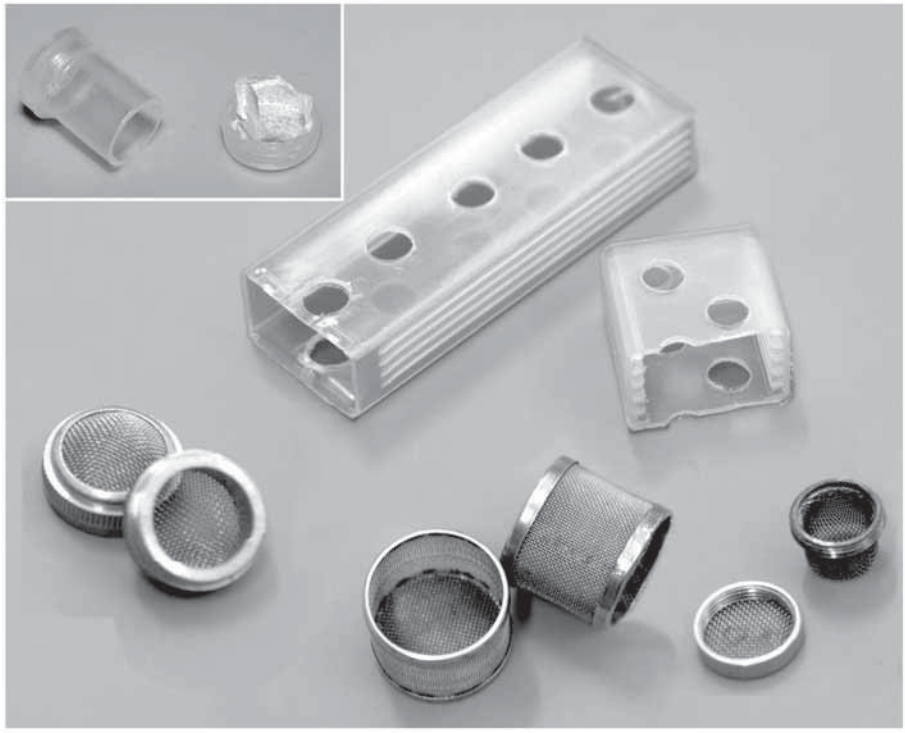


**Fig. 6.** Small holders used to protect small specimens during the critical point drying procedure. Inset, top left corner, shows holder fashioned from polypropylene embedding mold used in transmission electron microscopy. Large, rectangular container is a microscope slide mailer that has been modified to hold slides and cover slips. The three sets of stainless-steel mesh holders are commercially available.

3. Seal the chamber and fill with liquid $CO_2$, and maintain the recommended cold temperature, usually 0°C.
4. After several changes of liquid $CO_2$, to completely displace ethanol, raise the temperature of the CPD device to the critical point. For liquid $CO_2$, the critical point is 31.1°C at 1073 PSI.
5. After the liquid $CO_2$ is converted to gas, release the pressure while maintaining the elevated temperature to prevent re-condensation of the liquid $CO_2$.
6. Remove the fragile specimens, mount on specimen stubs, coat with heavy metal and view in the SEM.
7. After viewing, store specimens in dry, dust free environment.

### *3.5.3. Freeze-Drying*

Freeze-drying is occasionally used on specimens that would be damaged by the CPD procedure. Although chemically fixed specimens are often used, this

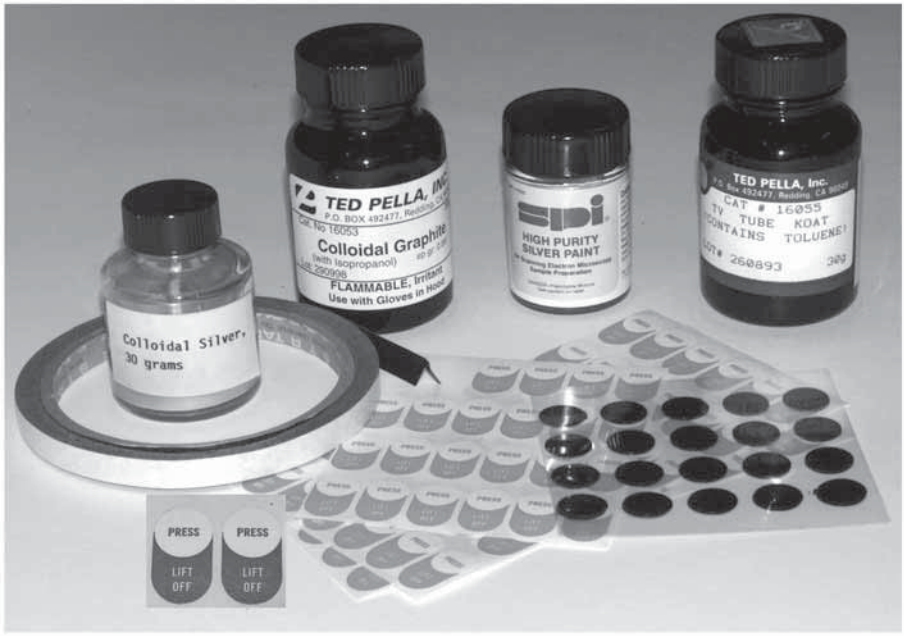



**Fig. 7.** Adhesives used to hold specimens on scanning electron microscopy stubs. These include conductive paints containing carbon or silver, conductive tapes containing carbon, sticky tabs (shown in lower left) that transfer a dab of adhesive material onto the stub, and carbon, double-stick disks.

is normally not needed since rapid freezing preserves the ultrastructure to a depth of 5 to 10 μm below the surface.

1. Rapidly freeze the specimen by plunging into isopentane or absolute ethanol that is chilled in liquid nitrogen to approx −85°C.
2. Transfer the specimen into liquid nitrogen (for storage) and then onto the −75°C cold stage of the freeze drying apparatus and activate the vacuum.
3. After several hours or days, depending on the mass of the specimen, warm the specimen stage to room temperature.
4. Remove specimens, mount on stubs, coat with heavy metal and view in the SEM. Store specimens in a dry, dust free environment.

### *3.6. Mounting Dried Specimen on SEM Stub*

After specimens have been fixed and dried, they are mounted on an aluminum planchet, or specimen stub (*see* **Note 13**). Several adhesives or glues are available (*see* **Fig. 7**).

- Liquid adhesives may be used to hold solid specimens such as glass or plastic substrates containing cells, large insects, or botanical specimens such as seeds or hard stems or leaves. Examples of these adhesives include cyanoacrylate, china cement,

silver- or carbon-doped conductive adhesives available through electron microscopy supply houses, quick-setting epoxy cement, and even hot glue from a glue gun.
- Tacky tapes, such as double-sided sticky tapes or special transfer tabs (from electron microscopy suppliers) are useful with specimens that might wick up liquid adhesive. Some double-sided tapes are electrically conductive since they contain carbon. The tapes and transfer tabs are used with small or porous specimens such as pollen grains, insects, and pieces of excised tissue.

### *3.7. Coating Specimens With Heavy Metal*

Mounted specimens are normally coated with a thin layer of heavy metal which serves as a source of secondary electrons and electrically grounds the specimen to prevent the build up of a high voltage static charge from the electron beam. The deposition of heavy metal may be accomplished in several ways.

#### *3.7.1. Sputter Coating*

Sputter coating is the most commonly used method because it is fast, reliable, and the apparatus is relatively affordable.

1. Transfer several stubs of mounted specimens into the chamber of the sputter coater. Most sputter coater chambers will accommodate 5 to 10 standard-sized specimen stubs. Do not crowd the chamber, because it will interfere with proper coating.
2. Close the chamber and activate the rotary pump to remove atmospheric gases. This may take 5 to 10 min if the coater has not been used in several days.
3. After the system has reached the recommended vacuum level, open the valve to flow dry argon gas into the chamber.
4. Adjust the argon level based on the reading from the vacuum gauge, activate the high voltage to the recommended setting and coat the specimen with the proper thickness of heavy metal such as palladium/gold or gold. Some coaters have thickness monitors whereas others estimate the thickness based on argon levels, high voltage, and time (*see* **Note 14**).
5. Store the coated specimen in a dry, dust-free environment.

#### *3.7.2. Vacuum Evaporation Coating*

Vacuum evaporation is sometimes used when a sputter coater is not available or if higher resolution coatings are needed.

1. Transfer several specimen stubs into the chamber of the vacuum evaporator. The chamber should be equipped with an electrode capable of carrying a current of 30 to 50 ampere (*see* **Note 15**).
2. Evacuate the vacuum evaporator to the recommended vacuum level, typically $10^{-4}$ Pa for most heavy metals. This step may take 15 to 30 min, depending on the efficiency and design of the system.
3. Slowly apply current to the electrode to melt the metal (platinum, palladium/gold, palladium) on the electrode. After the metal has formed a molten ball, raise the current rapidly to completely evaporate the metal.

4. Allow the electrode to cool several min and then admit air to the specimen chamber.
5. Store specimen stubs in a dry, dust-free environment.

### 3.8. Nonmechanical Coating of Specimens

Nonmechanical coating techniques do not require equipment such as sputter coaters or vacuum evaporators because osmium is chemically reduced on the specimen surface ***(6)***. Although more time consuming, the quality of coating is comparable with those obtained using mechanical devices.

1. Follow any of the protocols presented in this chapter to fix the specimens in glutaraldehyde and osmium tetroxide.
2. Rinse the specimen three times in distilled water, 10 min each.
3. Transfer specimen into freshly prepared (buffered or aqueous) solution of 8% glutaraldehyde and 2% tannic acid for 12 h. Change the solution three times during the 12-h period.
4. Rinse 3 times in distilled water, 10 min each.
5. Immerse in 2% aqueous solution of osmium tetroxide for 2 h.
6. Rinse three times in distilled water, 10 min each.
7. Repeat **steps 3** and **6**.
8. Continue normal processing of specimen: dehydrate, CPD, and mount on stub.
9. Store specimen under dry, dust-free conditions.

### 3.9. Storing SEM Specimen Stubs

Dried and coated or uncoated specimens must be stored in a clean, dry environment. Special plastic containers that firmly hold specimen stubs are available from microscopy supply houses (*see* **Fig. 8**). It is also possible to modify small cardboard or plastic boxes to safely hold stubs. These containers should hold stubs securely so that they do not spill out if the container is dropped or tipped. Stubs should be labeled on the bottom side using a permanent marker. Specimens must be stored desiccated, either inside of a sealed chamber with desiccant or in a drying oven set to 40 to 45°C. A sealed chamber can be readily fashioned from a large mayonnaise or other glass jar with a tight fitting lid. Desiccants, such as silica gel or calcium sulfate, should be placed inside the glass jar and changed when exhausted. If a large glass jar is not available, then specimens can be stored temporarily in a zip-lock plastic bag containing desiccant. Avoid contacting the specimen with the desiccant since this will transfer particulate dust onto the specimen surface.

## 4. Notes

1. Bacterial and fungal cells are processed in a similar manner as mammalian cultures with the exception that longer fixation times are used (several hours to overnight in each fixative).

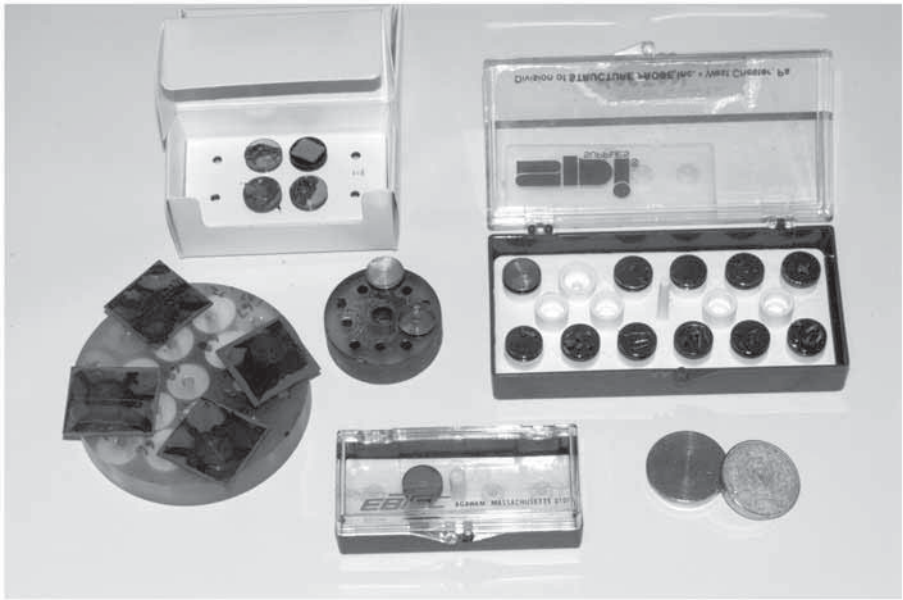


**Fig. 8.** Specimen stubs are best stored in rigid holders, shown. After securing the lids, the entire holders are placed in a dry, dust-free environment for storage.

2. To protect the cells from drying out during this procedure construct a humid chamber consisting of a Petri dish, or Tupperware® container, lined with paper towels soaked with water. Place several lengths of glass rod or applicator sticks on top of the paper to support the microscope slide. Cover the container during the incubation process.
3. The majority of cells are lost during the first dehydration step since the mixing of the ethanol with water generates vigorous swirling and heat. This can be lessened by using a very gradual dehydration, starting with 10% ethanol and slowly flowing the next alcohol (20%, etc.) in the series across the slide.
4. We have not been as successful with this procedure versus the method that uses a poly-L-lysine coated slide. Several causes of failure: clogging of filter because of an excessive number of cells, poor preservation of ultrastructure due to poor exchange of processing chemicals (especially in the CPD), curling or disintegration of the micropore filter. We have been most successful with the sturdier 13-mm silver micropore membrane, with 0.45-μm pores, available from Structure Probe, Inc.
5. The micropore membrane is fragile unless handled properly. Fluids must flow only toward the membrane. Never pull back on the syringe plunger with the filter attached since the back flow will rip the unsupported filter. The syringe must be loaded with liquid and then attached to the filter by means of a screw on lock rather than a friction fitting since the friction fittings do not hold the filter securely and it will fly off under pressure.

6. First, snip away the sides, then cut the bottom to size taking small cuts to avoid cracking the dish. A Dremel® high-speed rotary tool with a cutter wheel accomplishes this task quickly and neatly.
7. Be sure to maintain the orientation of the agar with the cells uppermost. Remove as much underlying agar as possible and handle the specimen very gently because the dried agar crumbles like a cigarette ash.
8. Hard organisms may not need to be fixed but simply air-dried. This includes specimens such as insects that have a sturdy exoskeleton, botanical specimens such as seeds or pollen, scales or hair from certain animals.
9. Because the surface is what one normally examines in the SEM, it is critical that all debris be removed. With some specimens, this step can be accomplished using light puffs of air, perhaps accompanied by sweeping with a soft brush. With wet specimens, buffer solution can be gently flowed across the surface. Sturdier specimens can sometimes be cleaned using a dental irrigation device set to dispense a low-pressure stream of buffer. In some cases, it may be necessary to use mild detergents, surfactants or enzymes to dislodge stubborn debris.
10. If the specimen is large, several cuts should be made in the specimen to allow fixative to diffuse into the interior or it should be cut into smaller, manageable pieces. The application of a low vacuum (from a faucet aspirator) may be applied to enhance penetration of fixative, especially in botanical specimens. With some botanical specimens, the inclusion of several drops of a detergent such as Tween-20® or PhotoFlo® will help break the surface tension and hydrophobic nature of the cell surface.
11. When specimens are so large that fixative cannot penetrate quickly, smaller pieces must be excised. The pieces should be no larger than 2 to 3 mm on one side. Place the specimen in the buffer solution and cut the desired pieces from the submerged specimen. Transfer specimens into fixative solution. To facilitate penetration, detergents may be added to the fixative and vacuum may be applied (*see* **Note 10**).
12. Most soft, biological specimens are damaged when they are allowed to dry by evaporation of water. The damage is caused by the passage of the air/water meniscus or interface through the specimen. This interface imposes surface tension forces of 2000 PSI and collapses most biological structure. The CPD substitutes ethanol, then liquid $CO_2$, for water. At the critical point, liquid $CO_2$ is converted to a gas that is then slowly released, thereby eliminating the air/water interface.
13. Because the specimens are quite fragile, special devices are used to transfer them onto the stub. Such devices include camel's hair brush, wooden applicator sticks, dissecting needles, jeweler's forceps, micropipettes, and eyelash probes consisting of a single eyelash glued to a wooden applicator. Electron microscope suppliers market a device called a vacuum needle that consists of a hollow, needle probe connected to a vacuum source generated by an aquarium pump. The operator controls the application of pressure by means of a hole in the handle. The specimen is picked up by touching the probe to the specimen, moving it onto the stub, and removing the finger pressure to release the specimen.

14. The thickness of the coating is very important: not enough and the specimen gives a poor signal and builds up an electrostatic charge; too much and fine details are buried under the metal coat. Some sputter coaters are equipped with a thickness monitor, a vibrating quartz crystal that accurately displays the thickness deposited on the specimen. Most manufacturers of sputter coaters provide a scale that correlates voltage and time to thickness of coating. It is also useful to construct a series of colormetric guides based on the deposition of metal on white paper. The colors change as the thickness increases, so that once one has a satisfactory coating (based on observation in the SEM), it is possible to approximate this coating for subsequent coating sessions.
15. In the thermal evaporation process, heavy metals are heated to their melting point in a high vacuum. Under these conditions, the metal is vaporized to a monoatomic state and travels in straight lines to condense onto the specimen stubs. The source of the evaporated metal is usually a fine gauge wire of pure metal such as palladium/gold, platinum, or gold. Typically, 1 inch of pure metal wire is wound around an electrode composed of a heavy gauge tungsten wire. The electrode is slowly heated under high vacuum, vaporizing the pure metal wire and depositing it onto the specimen. If the heat is applied too quickly, the metal will not melt properly and metal chunks will be ejected from the electrode, spoiling the specimen. Gold metal is easiest to evaporate, followed by palladium/gold alloy and platinum. The finest coatings are obtained with platinum, the coarsest with gold.

## References

1. Bozzola, J. J. and Russell, L. D. (1999) *Electron Microscopy Principles and Techniques for Biologists*. Jones and Bartlett Publishers, Sudbury, MA.
2. Dykstra, M. J. (1992) *Biological Electron Microscopy Theory, Techniques, and Troubleshooting*. Plenum Press, New York and London.
3. Goldstein, J. I., Newbury, D. E., Echlin, P., Joy, D. C., Fiori, C., and Lifshin, E. (1992) *Scanning Electron Microscopy and X-ray Microanalysis*. Plenum Press, New York.
4. Clayton, D. F. and Alvarez-Buylla, A. (1989) In situ hydridization using PEG-embedded tissue and riboprobes: increased cellular detail coupled with high sensitivity. *J. Histochem. Cytochem*. **37,** 389–393.
5. Heckman, C. A., Oravecz, K. I., Schwab, D., and Pontén, J. (1993) Ruffling and locomotion: role in cell resistance to growth factor-induced proliferation. *J. Cell Phys*. **154,** 554–565.
6. Takahashi, G. (1979) Conductive staining method. *Cell* **11,** 114–123.