A Manual of Applied Techniques for Biological Electron Microscopy

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Preface

There are a number of laboratory manuals and textbooks on biological electron microscopy available today. Some present a small number of techniques and offer limited examples of their application (such as where a buffer formulated with cacodylic acid would be appropriate and a buffer containing sodium phosphate would not). Hayat's books on techniques (1981, 1989), on the other hand, are extensive compendia of most of the techniques encountered in the literature and provide encyclopedic scope, but without specific recommendations as to which techniques are most successful. Many of the books available are cumbersome to use at the bench because they do not lie flat when opened.

What this manual does is present a series of techniques used routinely or semiroutinely in our service-oriented electron microscopy laboratory. The methods described have, in most cases, been used by us for years on a broad variety of tissue and cell samples and thus represent a laboratory-tested set of procedures. This book is designed to make it easy to locate a technique and to read it with the manual lying open on the laboratory bench.

This manual represents our standard operating procedures that have been adopted over the years after extensive testing of the multitude of techniques utilized in biological electron microscopy. Specific procedures have been included because the reagents used are typically readily available and comparatively easy to formulate, and the results achieved are reproducible for a variety of tissue and cell types. These methods are not presented as the only way to approach problems, but they are offered as procedures that work in most situations.

In our laboratory, investigators are always encouraged to consult up-to-date literature in their area of interest before deciding on a specific technical approach

to sample handling. If the literature presents a number of different approaches, we usually fall back on our standard operating procedures to begin the investigation.

It is hoped that the material in this manual will offer, at the least, a starting point for a variety of investigations and that the underlying rationale presented for each method will help investigators choose appropriate protocols for their problem-solving efforts.

Each major subject area begins with a general explanation of the problems being addressed and then presents the specific technical approaches employed in our laboratory. Each technique describes applications, objectives, formulations of stock solutions, specific procedural steps, results expected, and specific references where appropriate. For further in-depth explanations of sample preparation, microtomy, instrumentation, photography, cryotechniques, cytochemistry, and other topics, modern textbooks covering theoretical aspects of biological electron microscopy should be consulted (Dykstra, 1992; Bozzola and Russell, 1992).

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CHAPTER 1

Specimen Preparation

PRINCIPLES OF FIXATION

Fixation is an attempt to quickly arrest biological activity and to stabilize cellular components with minimal distortion of conformational and spatial relationships between cellular constituents. In addition, for immunocytochemistry and cytochemistry, it is often desirable to retain as much biochemical reactivity as possible.

One of the first concepts to consider before deciding on a fixation regimen is the difference between killing and fixing. Most of the time, the term *fixing* is used to include both processes, which may occur at about the same time, but they are not one and the same process. Cells and tissues immersed in typical aldehyde fixatives can exhibit physiological and physical changes before cellular death occurs. In some cases, it may be necessary to kill cells with toxic fumes (osmium tetroxide) before immersing them in aldehyde fixatives to prevent structural change. The rapidity of some cryofixation methods ensures that killing and fixing are essentially simultaneous events.

Biological electron microscopy utilizes chemical and/or physical fixation. While chemical fixation remains the most common method employed, physical fixation coupled with chemical fixation (in the form of cryosubstitution or microwave processing) is gaining increasing favor. A brief discussion covering the salient features of fixation follows.

Chemical Fixation

Chemical fixation for transmission electron microscopy helps to prepare cells for the traumas of subsequent washing with aqueous solvents, dehydration

with organic solvents such as ethanol or acetone, embedment in plastic resins, polymerization of the resins by heat, exothermic catalysts, or ultraviolet radiation, and subsequent imaging with high-energy electron beams in an electron microscope. Materials prepared for scanning electron microscopy are not infiltrated with resins but are subjected to high pressures while in solvents during critical point drying.

An ideal fixative would transform the viscous colloidal protoplasm of a cell into cross-linked and stabilized cellular components. The spatial relationship between all organelles and cellular structures would not be altered, the cellular components would not be solubilized, and the biological activity of complex proteins like antigens and enzymes would remain undiminished. The perfect fixative that can best maintain "a frozen moment in the eternal flux of life" (Bracker, 1967) in a cell has yet to be developed. There are a variety of fixatives and procedures that can achieve some of the desired effects. Part of the decision-making process for choosing processing protocols involves selecting which cellular characteristics are most important to the study being undertaken (structure or biological function). Some of the parameters that must be considered when preparing fixatives are discussed next.

1. Rate of Penetration

The speed of penetration, to a large extent, determines the success of a fixation procedure. Small fixative molecules (formaldehyde) penetrate more rapidly than larger ones (glutaraldehyde), but the larger molecules possess larger numbers of reactive sites and can thus cross-link and stabilize cellular components more thoroughly.

2. Buffers

The buffering system is used typically to maintain the pH of the tissue somewhere near physiological levels during fixation and to maintain near-isotonic conditions. With cytochemical procedures, on the other hand, the optimum pH for an enzymatic reaction sometimes dictates the type of buffer and the pH that must be maintained during processing. For structural studies, most cells fix well within a pH range from 7.0 to 7.4. Certain highly hydrated tissues fix better at a more alkaline pH (i.e., 8.0–8.4) whereas plant cells, nuclear material, and the fibrils of mitotic spindles fix better at a more acid pH (6.0–6.8) (Hayat, 1981). The tonicity of a solution can be adjusted by adding an electrolyte (e.g., sodium chloride) or a nonelectrolyte (e.g., sucrose). Electrolytes are favored over nonelectrolytes because the latter tend to decrease the rate of fixative penetration and increase extraction of cellular components (Hayat, 1981, 1989). Most commonly used fixative formulations are prepared as slightly hypertonic solutions.

3. Temperature

Fixation at 4°C is often recommended to prevent the "leaching" of cellular components. Reduced temperature decreases the mobility of cellular components and minimizes the activity of hydrolytic enzymes that begin leaking out of cellular compartments as membranes become permeabilized during aldehyde fixation. At the same time, low temperatures stimulate depolymerization of microtubules and may produce other cytoplasmic alterations. In practical terms, room temperature fixation for 1–2 hr works well for most tissues and cells.

4. Sample Size

Most fixatives do not penetrate much farther than 0.5 mm from the sample surface in 1 hr at room temperature. If cells are not fixed within about 1 hr, noticeable autolysis will begin to occur in most samples. Thus, a specimen should have at least one dimension that is less than 1 mm thick to achieve good fixation.

In general, proteins are fixed by aldehyde primary fixation, lipids by post-fixation with osmium tetroxide, and nucleic acids and carbohydrates are preserved most readily if they are associated with either proteins or lipids (see Hayat, 1981; Dykstra, 1992).

5. Types of Primary Fixatives

Primary fixation is typically undertaken with aldehydes such as acrolein, formaldehyde, or glutaraldehyde. The first two penetrate more rapidly than glutaraldehyde, but they all penetrate to the center of most 1-mm-thick tissue specimens within 1 hr. The choice of stocks is controversial, with some workers insisting that only the highest purity materials, usually stored in sealed ampules under inert gas (nitrogen), should be used. For structural investigations, empirical evidence suggests that this concern is overstated.

If concentrations of glutaraldehyde greater than 25% are needed for a given dilution series, it is imperative to purchase stocks sealed under inert gas to prevent the natural polymerization of concentrated glutaraldehyde when it is exposed to air. Otherwise, biological-grade glutaraldehyde works well as long as it has not become yellow or the pH has not dropped below about 3.5. If either of these changes occurs, discard the stock.

In the past, 37% formaldehyde stocks were considered inadequate for use in electron microscopy because they are formulated with small amounts of methanol to prevent polymerization. McDowell and Trump's (1976) fixative recipe, which calls for the use of Fisher 37% formaldehyde (F-79) stocks, should have

made it clear to electron microscopists that the small amount of stabilizer in commercial formaldehyde presents no apparent problem.

Another important consideration when choosing fixatives is that typical glutaraldehyde fixation of samples for electron microscopy (using 2% or stronger glutaraldehyde) produces materials that are difficult to section if processed for light microscopy. In addition, various common histological techniques such as periodic acid–Schiff (PAS) reagent staining for polysaccharides do not work well with materials so fixed (in fact, PAS staining can be used to monitor the depth of glutaraldehyde fixative penetration into a tissue sample). Because many cell biology studies and diagnostic samples in medicine require the evaluation of materials by light as well as electron microscopy, this aspect of fixative choice must be considered. It should also be remembered that glutaraldehyde binds to cellular components essentially irreversibly whereas formaldehyde bound to cellular components can be washed out in aqueous media.

The three most commonly used fixatives that are at least partially suitable for light and electron microscopy are:

	37% formaldehyde	25% glutaraldehyde	Na_2HPO_4	NaH_2PO_4	NaOH	H_2O
Carson's (Carson *et al.*, 1973)	20 ml	—	—	3.72 g	0.84 g	180 ml
Trump's 4F:1G (McDowell and Trump, 1976)	20 ml	8 ml	—	2.32 g	0.54 g	172 ml
10% buffered neutral formalin (Luna, 1968)	20 ml	—	1.3 g	0.80 g	—	180 ml

Karnovsky's fixative (1965) is called for in some procedures, particularly for perfusions. Unfortunately, there are a variety of "Karnovsky's" cited in the literature so that it is necessary to look up the particular formulation that was used by an author if that technique is to be duplicated. The original Karnovsky's recipe contains 5% glutaraldehyde and 4% formaldehyde, as opposed to the 1% and 3.7–4%, respectively, in Trump's 4F:1G. The high osmolality of Karnovsky's fixative (over 2000 mosmol) along with its lack of suitability for PAS staining because of the high concentration of PAS-reactive glutaraldehyde make it a poor choice as a general fixative. In addition, it is tedious to prepare and does not store for more than a week or so. Trump's 4F:1G is easy to prepare, has a lower tonicity (176 mosmol) than Karnovsky's, keeps for months at 4°C, and can be used for tissue storage at 4°C for several years (Dykstra, 1992). It functions as an excellent perfusate as well as immersion fixative and sections nearly as well as

formalin-fixed materials embedded in paraffin or glycol methacrylate (JB-4® resin).

Physical Fixation

Cryofixation utilizes extremely low (−190 to −196°C) temperatures applied to very small samples (less than 0.5 mm in most cases) to rapidly remove heat from tissues and cells. The combination of physical and chemical techniques for fixation (cryosubstitution) has been widely employed during the last 10 years. With this technique, materials are quick-frozen in a suitable cryogen in liquid nitrogen and then the cryogen is exchanged with a solvent such as acetone or methanol held at low temperature (typically −80°C) containing chemical fixatives that are not chemically active at such low temperatures. Thus, water in the sample is replaced by the solvent selected, which serves as a carrier for the fixative components. The fixative components diffuse throughout the specimen during the substitution process without chemically reacting with the sample because of the low temperature that is maintained. Subsequently, the solution of acetone and fixatives is slowly brought to room temperature, which results in each fixative component reacting with cellular materials at a specific point in the heating curve. The rapidity of cryofixation coupled with the stability offered by subsequent chemical fixation produces images significantly different from those produced using conventional chemical fixation.

Microwave fixation and staining has become popular in recent years for light and electron microscopy (Leong *et al.*, 1985; Leong and Gove, 1990). In some cases, cells or tissues are fixed directly with microwaves (after suspension in a suitable fluid such as phosphate-buffered saline) whereas in other cases, the samples are put into a chemical fixative (aldehydes or osmium tetroxide) that is then subjected to microwaves to reduce fixation time from hours to a matter of minutes or seconds. The latter procedure is reported not only to require much shorter fixation time, but also to provide better fixative penetration than conventional chilled or room-temperature chemical fixation for 1–2 hr.

ORGANIC DEHYDRATION

Dehydration is designed to remove all water from a sample and to replace it with a solvent that is miscible with the final embedding medium. Most embedding media are immiscible or only partially miscible with water. Ethanol and acetone are the two most common dehydration fluids, typically used in a graded series beginning with 25–50% solutions and culminating in 100% ethanol or