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

vi PREFACE

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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Contents

Chapter 1. Specimen Preparation	1
PRINCIPLES OF FIXATION	1
Chemical Fixation	1
Physical Fixation	5
ORGANIC DEHYDRATION	5
EMBEDDING MEDIA	6
MISCELLANEOUS TIPS FOR FORMULATING SOLUTIONS	
IN THE ELECTRON MICROSCOPY LABORATORY	9
A Routine Fixation and Embedding Schedule for Transmission	
Electron Microscopy Samples (Tissues or Cells)	12
A Routine Schedule for the Preparation of Cell and Tissue	_
Samples for Scanning Electron Microscopy	18
Preparation of Primary (Aldehyde) Fixatives	20
Preparation of Osmium Tetroxide	22
BUFFERING SYSTEMS	28
Cacodylate Buffers	29
Phosphate Buffers	30
Tris-HCl Buffer	32
Sodium Acetate Buffer	33
Dulbecco's Phosphate-Buffered Saline	34
RESIN FORMULATIONS	36
Spurr Resin	38
Poly/Bed 812 Resin	42
SPI-PON 812 Kit	44

x CONTENTS

	47
Epon 812	46
Araldite 6005 Resin	47
Mollenhauer's Epon/Araldite Resin	49
LR (London Resin Co.) White Resin	50
Lowicryl and LR Gold Resins	52
Polyethylene Glycol Method for TEM Sections	53
JB-4 (Glycol Methacrylate) Techniques for High-Resolution Light	
Microscopy	55
Agar Embedment of Cell Suspensions or Subcellular Particulates	
for TEM	57
Preparing Adherent Tissue Culture Monolayers in Situ	
for TEM	60
Flat Embedding of Cell Cultures Grown on Permanox® Tissue	
Culture Dishes for TEM	63
Preparation of Buffy Coats for TEM	64
Sperm Fixation	65
Central Nervous System Fixation (Brain, Spinal Cord)	67
Using Vacuum to Help Wet Fungal, Plant, or Insect Materials	
during Primary Fixation	69
Simultaneous Aldehyde/Osmium Fixation for Protozoan Samples	
or Samples with Large Amounts of Lipid	70
Killing Cells Prior to Chemical Fixation	72
Flat Embedding on Microscope Slides	73
Procedure for Deparaffining Tissues	75
Trocker to a particular to a p	
	79
Chapter 2. Sectioning Methods	
Making a Section Retrieval Loop	79
Making a Section Manipulation Tool	82
Making a Locking Ring for Forceps	8.
Making Glass Knives	8.
Making Glass Knife Boats	8.
Glass Knife Storage	8
Diamond Knives	8
Block Trimming	8
Sectioning Procedures	9
Semithin Sections	9
Grid Selection	9
Grid Cleaning	9
Ultrathin Sections	ç
Common Sectioning Problems	Ç

CONTENTS

Chapter 3. Specialized SEM Procedures						
Poly-L-lysine Technique for Attaching Particulates to Coverslips for SEM	10					
Organosilane Coverslip/Slide Treatment						
Drying Samples with Hexamethyldisilazane Drying SEM Samples with Peldri II™	10 11					
Sputter Coating Vascular Casting with Mercox CL-2B	11 11					
Chapter 4 Section Staining December 1						
Chapter 4. Section Staining Procedures	11					
Semithin Section Staining with Toluidine Blue O	12					
Polychrome Stain for Semithin Sections	12					
Staining Ultrathin Sections	12					
Chapter 5. Support Films	129					
Preparation of Formvar-Coated Grids						
Formvar-Coated Aluminum Bridges for Slot Grids	12					
Coating Grids with Butvar B-98	13 13					
Coating Grids with Collodion Films	14					
Making Carbon Support Films	14					
Chanter 6. Negative Staining						
Chapter 6. Negative Staining	147					
Negative Staining with Phosphotungstic Acid	14					
Negative Staining with Uranyl Acetate Negative Staining with Ammonium Molybdate	15					
Wetting Agents Used for Negative Staining	154					
stanning	15.					
Chapter 7. Preparation of Viral Samples for Transmission Electron Microscopy	159					
Ultracentrifugation Technique for Viral Sample Preparation	159					
Lyphogel Technique for Concentrating Virus	16					
Immune Electron Microscopy for Concentrating Virus	162					
Concentrating Virus with the Beckman Airfuge	16					

xii CONTENTS

Chapter 8. Vacuum Evaporation	167
Shadow Casting	170
DNA (Plasmid) Preparation for TEM	174
Chapter 9. Films and Photographic Methods	177
Kodak Electron Microscope (TEM) Film 4489	180
Films in 35-mm Format	181
Kodak Technical Pan 2415 for Photomicrography	183
Ilford Pan-F and FP-4	185
Kodak T-Max 100 Film	186
Kodalith Film	187
Kodak LPD4/Precision Line Film	188
Kodak Rapid Process Copy Film 2064	189
Kodak Recordak 2468	190
Polaroid Polagraph HC 35-mm Film	191
Polaroid Polaplan 35-mm Film	192
Tungsten-Balanced Kodak Ektachrome	192
Kodak Technical Pan 2415 for Making Copy Negatives	194
Polaroid Copy Negatives Using Type 55 P/N Film	195
Making Photographic Prints	196
Poster Preparation	200
	200
Chapter 10. Cytochemical Techniques	205
POLYSACCHARIDE STAINS	206
Ruthenium Red Staining	207
Silver Methenamine Staining for Polysaccharides	209
CALCIUM STAINING	213
Prefixation Calcium Staining in Muscle Tissue	213
Postfixation Calcium Staining with Pyroantimonate	216
COLLOIDAL GOLD TECHNIQUES	218
Preparation of 13-nm Colloidal Gold	220
Conjugation of Gold to Proteins	223
Indirect Immunolabeling Procedure for Sections of Materials	223
Embedded in LR White or Lowicryl K4M Resin	
(Acrylic Resins)	226
Procedure for Immunolabeling Intact Cells (Preembedment	220
Labeling of Cell Surfaces)	220

CONTENTS	xiii
Cryoultramicrotomy and Subsequent Immunolabeling (an Example for Muscle Immunolabeling)	229
Chapter 11. Autoradiography	235
of Cells or Tissues	236
Appendix A. Computing Micrometer Bar Sizes	243
Appendix B. Calibrating the TEM and the SEM	245
Appendix C. Materials and Methods Write-up Suggestions for Standard TEM and SEM Preparations	247
Appendix D. Safety	249
Appendix E. Electron Microscopy Supplies	251
Index	253

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 2 CHAPTER 1

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 nearisotonic 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

4 CHAPTER 1

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 ₂ HPO ₄	NaH ₂ PO 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 $(-190 \text{ to } -196^{\circ}\text{C})$ 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