Animal Tissue Techniques

FOURTH EDITION

Gretchen L. Humason

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OAK RIDGE ASSOCIATED UNIVERSITIES

W. H. FREEMAN AND COMPANY
San Francisco

Sponsoring Editor: Arthur C. Bartlett
Project Editor: Pearl C. Vapnek
Copyeditor: Susan Weisberg
Designer: Robert Ishi
Production Coordinator: Fran Mitchell
Compositor: Bi-Comp, Inc.
Printer and Binder: The Maple-Vail Book Manufacturing Group

Library of Congress Cataloging in Publication Data

Humason, Gretchen L
Animal tissue techniques.

Bibliography: p.
Includes index.
1. Histology—Technique. 2. Stains and staining (Microscopy) 3. Histochemistry—Technique. I. Title.
QM556.H85 1979 578'.9 78-17459
ISBN 0-7167-0299-1

Animal Tissue Techniques, Fourth Edition

Printed in the United States of America

9 8 7 6 5 4 3 2 1

Preface

This book of basic and standard histological procedures (and some specialized techniques) was designed to meet the diverse needs of premedical students, medical technicians, zoology majors, and research assistants. Most histological reactions follow a logical and specific sequence, and I have attempted to include simplified discussions of the basic methods that are applicable both to normal and to pathological conditions in zoology and medicine.

It is not intended that this text should be a complete reference book on histology; the experienced worker knows of numerous such tomes, as well as journals that specialize in histology and related topics. However, special methods of wide usage and exceptional merit are included, particularly those that are not overly complicated or unpredictable. It is hoped that technicians, once familiar with the material covered here, will watch the literature for modifications and improvements of standard techniques; in this way, with this book as a foundation, their work can be kept up to date and, perhaps, simplified.

Methods for fixation are fairly well established, with only occasional variations. The section on fixation presented herein is as modern as I can make it, and it includes a brief description of the chemicals employed. Old staining techniques continue to be perfected and new ones developed; I have tried to include the best of these and, for the sake of the student, to adapt them to the standard three-hour laboratory period and to the kinds of equipment most widely available. Some special methods that are more time-consuming have been included for special projects and research. They have been simplified wherever possible to serve as introductory techniques for the student who plans to proceed to more complicated techniques later.

Some instructors may not agree with the way I have organized the text, but to me it is logical. Thus fixation is treated first because it is usually the first process in tissue preparation; this is followed by embedding in some kind of medium, sectioning on a microtome, mounting sections on slides, and, finally, staining them with the help of a microscope. A logical arrangement of staining methods is hard to come by, so I have followed my own inclinations: Some sections are organized by related tissues, others by related methods. The latter was considered desirable for such processes as silver impregnation, metachromasia, and the use of Schiff reagent. The final chapters include such specialized techniques as histochemistry, chromosome preparation, autoradiography, and invertebrate mounts. Wherever possible, I have referred to my own experience with these methods to help students succeed with their first efforts, and I have included modifications that might appeal to other adventurous technicians.

This book is in four parts. Part I covers those basic procedures and general considerations with which every tissue technician should be familiar. Part II provides detailed information about specific staining methods for most tissues. An instructor might choose a few favorite methods from this section to round out a course, while the professional technician will find here most of the specific methods required on the job. Part III deals with special procedures, those that are special in the sense that they are not common in most laboratories, although they may be very important in some. Although the discussion of some of these procedures is brief, references have been cited extensively for the benefit of those who might wish to refer to more thorough discussions. Part IV is devoted largely to laboratory aids and the preparation of solutions—useful information in any laboratory. In this edition some methods are removed and new ones added, others are modified. The arrangement of chapters has been altered by moving freezing, nitrocellulose, and other techniques to the Special Procedures section. This leaves the first two parts devoted almost exclusively to the paraffin method and the use of the microscope to examine the finished slides.

In the fourth edition, *Animal Tissue Techniques* has been extensively revised and updated. Many of the changes have been to improve its usefulness for graduate and undergraduate teaching. The typography has been altered and the design improved with an eye to making the book more readable and, hence, more useful to students and technicians alike. The list of references has been carefully emended to cover recent important publications in the field. Information on all suppliers cited in the text is consolidated for easier reference in one list beginning on p. 574.

Many of us have not regarded with proper respect the potentially dangerous materials that are used in the laboratory. Accidents do happen,

so a section is included on pp. 572–574 concerning the hazards of laboratory materials. This should be called to the attention of all students and technicians.

To have included everything necessary to satisfy everyone and still to have kept the price of the book within the means of the average student would have been impossible. Some topics, necessarily, have been treated only in passing. The electron microscope, for example, is much too specialized for students in beginning technique classes, and an entire book could be devoted to instructing students in its operation alone. The topic of photomicrography is equally complex. Methods for preparing plastic whole mounts have not been included; excellent leaflets on the subject are published by the companies that supply the materials necessary for their preparation. Good color photographs are helpful, but they are also, unfortunately, expensive—even a few of them can add appreciably to the cost of a book. In my teaching, I have used a demonstration set of slides to help my students recognize proper staining. The set started with a few of my own slides, and it was gradually enlarged by additions from the students in my classes. The students were happy to contribute examples of their best work, and the collection eventually increased to several hundred excellent slides. Other instructors might consider building a study collection of slides in the same way.

I have derived invaluable personal satisfaction from my association with students. I am grateful to them for helping me to develop my tolerance and patience—two qualities that are essential in my profession. I am grateful to them, too, for what they have helped me to learn, for there is no surer way to master a subject than to teach it to others. One former student in particular should receive credit for her encouragement and for prodding me toward writing this book—Marlies Natzler of the University of California at Los Angeles.

Grateful acknowledgments are also due to Marvin Linke, Jeanne Simmons, and Leta Burleson, the three artists who contributed to the four editions of this book; to Julie Langham, for help with photography; to Nellie M. Bilstad, for valuable suggestions; to the Cytogenetics Division of Oak Ridge Associated Universities, for information about late developments in chromosome preparation; to the Zoology Department of the University of California at Los Angeles, for the lessons I learned there as a student, a departmental technician, and a lecturer; and to Dr. C. C. Lushbaugh, for his continued encouragement.

September 1978 *Gretchen L. Humason*

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Part I
BASIC PROCEDURES

Chapter 1
Fixation

As soon as a tissue ceases to be alive, its cells start to change. Multiplying bacteria begin to destroy them, and the process of autolysis (self-digestion) by contained enzymes begins to dissolve them. The activity of these enzymes is reversed from that in live cells; instead of synthesizing amino acids into proteins, they begin to split proteins into amino acids. These amino acids diffuse out of the cells; as a result cell proteins are no longer coagulable by chemical reagents. These cell changes are called postmortem conditions and must be prevented if tissue is to be examined in the laboratory.

The prevention of postmortem conditions is the primary objective of tissue preparation, but it is also necessary to treat tissue to differentiate the solid phase of the protoplasm from the aqueous phase, to change cell parts into materials that will remain insoluble during subsequent treatment, and to protect cells from distortion and shrinkage when subjected to such fluids as alcohol and hot paraffin. Other important objectives of tissue preparation are to improve the staining potential of tissue parts and to alter their refractive indices for better visibility.

The procedure used to meet these requirements is called fixation, and the fluids used are called fixatives or fixing solutions. Any fixative should:

1. Penetrate rapidly to prevent postmortem changes.
2. Coagulate cell contents into insoluble substances.
3. Protect tissue against shrinkage and distortion during dehydration, embedding, and sectioning.
4. Allow cell parts to be made selectively and clearly visible.

Some fixatives may have a mordanting effect on the tissue—that is, they combine insolubly with it—and enhance the attachment of dyes and proteins to each other.

Tissues should be placed in fixatives as soon as possible after death. If delay is unavoidable, they should be put in a refrigerator, thus reducing autolysis and putrefaction to a minimum until the fixative can be applied.

Because a single chemical seldom has all the qualities of a good fixative, a fixing solution is rarely composed of only one chemical—familiar exceptions are formalin and glutaraldehyde. Most reliable fixatives contain one or more coagulant chemicals and one or more noncoagulant chemicals. Coagulants change the spongework of proteins into meshes through which paraffin can easily pass, thus forming a tissue of the proper consistency for sectioning. They also strengthen the protein linkages against breaking down during later procedures. Used alone, however, coagulants may form too coarse a network for the best cytological detail or may induce the formation of artificial structures (artifacts). Noncoagulants produce fewer artifacts, but if used alone they give the tissue a poor consistency for embedding.

Thus the most efficient fixing fluids are combinations of protein coagulants and protein noncoagulants. It should be emphasized, however, that no fixing solution is ideal: all chemical agents cause some chemical change in the protein structure of the cells. The choice of fixative will depend on the type of investigation to be undertaken, and an effort should be made to discover what adverse effect the fixing agent may have on the cellular components under study.

Because they contain ingredients that act upon each other, many mixtures are most efficient when made up fresh. The individual ingredients can usually be made up into stock solutions which can then be mixed together immediately before use. Among the frequently used chemicals are formaldehyde, glutaraldehyde, ethyl alcohol, acetic acid, picric acid, potassium dichromate, mercuric chloride, chromic acid, and osmium tetroxide (osmic acid). Since every chemical has its own set of advantages and disadvantages, each component should, whenever possible, compensate for a defect in some other component. For example, in the widely used fixative, Bouin solution: (1) Formaldehyde fixes the cytoplasm, but in such a manner that it retards paraffin penetration. It fixes chromatin poorly and makes cytoplasm basophilic. (2) Picric acid coagulates cytoplasm so that it admits paraffin, leaves the tissue soft, fixes chromatin, and makes the cytoplasm acidophilic. Its disadvantages are that it shrinks the tissue and that it makes chromatin acidophilic. (3) Acetic acid compensates for the defects of both formaldehyde and picric acid.

Another example: Birge and Tibbitts (1961), by adding 0.7% sodium

chloride to two fixing solutions (formalin and Bouin), reduced the amount of shrinkage caused in nuclei and cytoplasm.

When the future use of a tissue is in doubt or if it is to be stored for an indefinite time, formalin is usually the conservative choice for a fixative; it permits secondary fixation (postfixation) and will not harden excessively. If the primary objective of tissue preparation is the compilation of a simple anatomical study of cell components, then routine fixatives can be used: formalin, Gomori, Susa, Zenker, Helly, or Bouin. Special fixatives for cell inclusions are Carnoy, Flemming, Champy, Helly, Schaudinn, Regaud, and others. For histochemistry the researcher is limited to aldehydes, acetone, or ethyl alcohol (p. 392).

Most fixing solutions are named after the person originating them, e.g., Zenker and Bouin. If the same person originated more than one combination of chemicals, additional means of designating them have been used: Flemming weak and strong solutions; Allen B3, B15 series; etc. A few fixatives have been named arbitrarily: The name Susa was coined by Heidenhain from the first two letters of the words *su*blimate and *sä*ure.

CHEMICALS COMMONLY USED IN FIXATIVES

Acetic Acid

Acetic acid (CH_3COOH) is one of the oldest fixatives on record: in the eighteenth century vinegar (4–10% acetic acid content) was used to preserve hydras. In modern techniques it is rarely used alone but is an important component of many fixing solutions because of its efficient fixing action on the nucleus and its rapid penetration. It fixes the nucleoproteins, but not the proteins of the cytoplasm. It does not harden the tissue; actually it prevents some of the hardening that, without it, might be induced by subsequent alcohol treatment. In some techniques, however, acetic acid must be avoided because it dissolves out certain cell inclusions, such as Golgi and mitochondria, and some metals, such as calcium. Many lipids are miscible with acetic acid or are soluble in it. It neither fixes nor destroys carbohydrates. Pure concentrated acetic acid is called glacial acetic acid because it is solid at temperatures below 17°C.

Acids in general cause swelling in tissues—in collagen in particular—by breaking down some of the cross linkages between protein molecules and by releasing lyophilic radicles that associate with water molecules. An acid's swelling action can be a desirable property because it counteracts some of the shrinkage caused by most fixing chemicals. To curtail swelling after fixation with acetic or trichloracetic acid solutions, tissues should be transferred to an alcoholic washing solution rather than to water.

Acetone

Acetone (CH_3COCH_3) is used only for tissue enzymes, such as phosphatases and lipases. It is used cold and penetrates slowly. Only small pieces of tissue are fixed in this chemical.

Chromium Trioxide (Chromic Acid)

Crystalline chromium trioxide (CrO_3) is called chromic acid when it is added to water, usually in a 0.5% amount. Chromic acid is a valuable fixative but is rarely used alone. It penetrates slowly, hardens moderately, causes some shrinkage, forms vacuoles in the cytoplasm, and often leaves the nuclei in abnormal shapes. It is a fine coagulant of nucleoproteins and increases the stainability of the nuclei. It oxidizes polysaccharides and converts them into aldehydes—an action forming the basis of the Bauer histochemical test for glycogen and other polysaccharides. To fix water-soluble polysaccharides, it is better to use acetic acid and then posttreat them with chromic acid.

Chromic acid can also be used to partially oxidize fats to make them insoluble in lipid solvents. The oxidizing action may go too far, however, and potassium dichromate, which acts in a similar fashion, is safer for this purpose and is therefore more commonly used.

Excess chromic acid must be washed out, because it may later be reduced (undesirably, for our purposes) to green chromic oxide (Cr_2O_2). Because formalin and alcohol are reducing agents, they must not be mixed with chromic acid until immediately before use.

Alcohols

Alcohol cannot be used as a fixative for lipids because it makes them soluble. It does not fix carbohydrates, but neither does it extract mucins, glycogen, iron, and calcium. Alcohol is seldom used alone, although it is occasionally used for fixing enzymes.

Ethyl alcohol (ethanol, C_2H_5OH) hardens tissue but causes serious shrinkage. It is a strong cytoplasmic coagulant but does not fix chromatin. When alcohol is used, nucleic acid is transformed into a soluble precipitate and is lost in subsequent solutions and during staining.

Methyl alcohol (methanol, CH_3OH) is also used as a fixative, principally for hematologic tissues.