

**Manual of Histopathological
Staining Methods**

Manual of Histopathological Staining Methods

Frederick A. Putt
Department of Pathology
Yale University School of Medicine

A WILEY-INTERSCIENCE PUBLICATION

JOHN WILEY & SONS, New York • London • Sydney • Toronto

Copyright © 1972, by John Wiley & Sons, Inc.

All rights reserved. Published simultaneously in Canada.

No part of this book may be reproduced by any means, nor transmitted, nor translated into a machine language without the written permission of the publisher.

Library of Congress Cataloging in Publication Data

Putt, Frederick A

Manual of histopathological staining methods.

Bibliography: p.

1. Histology, Pathological--Technique. 2. Stains and staining (Microscopy) 1. Title. [DNLM: 1. Histological Techniques--Laboratory manuals. 2. Stains and Staining--Laboratory manuals. QS 525.P993m 1972]

RB27.P87

611.018

72-5671

ISBN 0-471-70246-3

Printed in the United States of America

10 9 8 7 6 5 4 3 2 1

2. 2. 2. 2.

FOREWORD

2. 2. 2. 2.

The production of a histological section requires teamwork. Usually the person who interprets the final product also selects the tissue sample, does the initial trimming, and starts the fixation. These are, of course, critical steps and their importance to the usefulness of the final product cannot be minimized. Between sending the fixed sample to the histology laboratory and receiving the finished stained slide, however, the average pathologist or researcher may give little thought to what is happening to his sample. The histology technician is entrusted to handle "routinely" a thousand details of utmost importance. Valid interpretation of the final product depends not only on the skill of the interpreter but also on the quality of the slide. The quality must be very good. The fairly thin, fairly well mounted, fairly well stained, and fairly well cleared slide is a murky forest laced with quicksand traps and treacherous pools. The excellent preparation may not provide all the answers, but at least it puts the investigator on firm ground. This alone justifies the efforts of a hard-working, skilled technician, but there is also a bonus. A fine section is a joy to see. What esthetic pleasure, what pure fun can come from looking through a microscope to discover crisply sharp images in bright clear colours!

For several years I have had the good fortune to have slides prepared under Frederick Putt's watchful eye. Many technicians have prepared these sections, as there have been many students trained by this master. That the extremely high quality has been sustained by all is testimony to Mr. Putt's skill as a teacher. The intricacies of preparation of any given stain are many. The chemical structure of the dye (when that is known) and the exact way it functions (when that is understood) are quite interesting and important. They are explained, discussed, and debated in many large volumes. But the histology technician's problem can sometimes be more immediate—he needs to know how to make a stain "work" right now. This manual concerns itself with how to make a stain work. The

small details and little tricks that make the difference are all included. Reading from this text is the next best thing to having a master at one's elbow.

I know from experience how useful this manual can be. My copy of the first draft proved to be invaluable when I was faced with setting up a laboratory in a new medical school four years ago. Since that first draft was finished each page has been read and reread by beginners as well as experienced technicians. This volume has thus been subjected to extensive bench trials. I am confident that student technicians who are just beginning to learn their trade and experienced technicians who want to refresh their memories will find this to be a manual of unequalled value in the laboratory. I predict that very few copies will rest long in office or library bookcases, but I do hope that its publication will enable me to keep a copy near my desk. It is most aggravating to discover that my copy has once again migrated back to the lab every time I wish to consult it.

Charles B. Carrington, M.D.
Associate Professor of
Pathology and Director of
Autopsy Services
Yale University School of
Medicine

PREFACE

The histopathological staining methods presented in this manual are those with which we have had personal experience. Most have been evaluated for consistency and reliability by members of the technical staff and have proved practical in routine application.

They include routine, special, and histochemical procedures as carried out in the histological laboratory in this department. Some have been modified to suit our own particular needs; others have originated from this laboratory. Classical methods which are still requested have been retained. A discussion of the all-important accessory procedures to staining, fixation, decalcification, dehydration, embedding, and sectioning is also included.

In order that a routine histological laboratory function efficiently, certain basic procedures common to various staining techniques, usually carried out in sequence, must remain fairly constant. In keeping with the needs of such a laboratory, the majority of staining methods outlined in this book can be completed during the course of a routine laboratory day. In general, the less complicated and more reproducible staining method has greater possibility of being accepted as a routine procedure. Methods that are unpredictable and that have been successful for only one or two individuals are soon rejected.

Many staining procedures share solutions in common and these could be listed together in a table. We have found it more practical, however, at the expense of repetition, to precede each method by the stated fixative of choice and the solutions necessary to complete the procedure. The reader who actually uses this manual in the laboratory will soon learn to appreciate this arrangement.

Routine, special, and histochemical methods to demonstrate related structures or tissue components are grouped together, but not in any given sequence. In some cases, more than one selection is presented in order that a choice can be made and results compared. In this manner, experience can be gained in a practical way. When checking a new or unfamiliar staining procedure, relevant control material is advisable. Because of their special applica-

tion, specific metallic and selected neuropathological staining methods are presented separately in Chapter 12. Many require special fixatives and are among the more difficult techniques to master for consistent results. A modified method to mount whole organs on paper as outlined by Gough and Wentworth is given in Chapter 14.

Very little automation has crept into the histological laboratory thus far. Preparing tissue for microscopic examination is still a practical and exacting art. Many staining methods can still be considered empirical, since the chemical basis on which many staining reactions takes place is not fully understood. Histochemical technique is based on specific reactions between chemicals or dyes and tissue components. Variation of procedure or staining method becomes necessary at times to bring about the desired results. This will depend more on experience and individual skill than on didactic description in a text.

Choice of tissue and proper fixative depends mostly on the pathologist; producing acceptable histological preparations depends on the technician. Cooperation is essential. Shoddy preparations, inadequate in detail for diagnostic purposes, are a constant source of frustration to the pathologist, all the more so if lectures, student teaching, or illustrations for publication are involved.

It would be quite impossible for any one individual to keep abreast of and evaluate current staining and related procedures as they appear in the literature. The subject is also too extensive for one text book to cover. For those inclined to pursue the subject further, a list of histological and related textbooks as well as references to the original publications consulted in the preparation of this manual are included.

Much of the content of this manual was formerly available in the form of mimeographed notes. It is hoped that assembling the material in book form will result in a basic and helpful textbook for both the students and technicians for who it is primarily intended.

In describing the various procedures and staining techniques, I have taken into consideration most of the difficulties that I have personally encountered. I have tried to present procedural details clearly and concisely in order to avoid pitfalls. I have also benefited from questions pertinent to a given procedure which have been raised by members of the staff. I wish to express my appreciation to the pathologists, technicians, and students whose stimulating questions and suggestions have been most rewarding.

I am especially grateful to Dr. Charles Carrington for his helpful criticism and advice, and to Dr. Klaus Bensch and Dr. Roy Barnett for their interest and encouragement.

Preface

ix

I am greatly indebted to Mrs. Felicia Naumann for typing the manuscript. I also wish to thank Miss Dorothy Hyatt, Mrs. Judy Daly, and Mrs. Gail Bliss for their technical assistance. A textbook such as this is in great part based on the research and experience of many authors. I acknowledge my indebtedness to authors and publishers who have so kindly allowed me to make use of or extract material from their publications.

New Haven, Conn.
July, 1972

F. A. Putt

CONTENTS

Chapter 1. Smears and Biopsies	1
Cell Blocks, 1	
Cryostat Sectioning, 2	
Mounting, 3	
Storage, 4	
Hematoxylin and Eosin Biopsy Stain (Coplin Jar Setup), 4	
Lipid Stain (Chiffelle and Putt Method), 4	
Reid's Eosin-Methylene Blue Method for Brain Biopsies, 5	
Humason Pinacyanole Method for Frozen Sections, 6	
Humphrey's Brilliant Cresyl Blue Method for Frozen Sections, 6	
Papanicolaou Method: Exfoliative Cytology, 7	
Chapter 2. Fixation	10
Simple Fixatives, 11	
Fixing Mixtures, 14	
Washing, 21	
Storing, 21	
Lendrum's Method to Soften Hard Tissue, 21	
Chapter 3. Frozen-Section Method	22
Cutting Frozen Sections, 22	
Lipid Stains, 23	
Mounting Unstained Sections (Biopsies), 23	
Masson's Gelatin Water Method, 24	
Mounting Unfixed Frozen Sections, 24	
Gelatin Embedding, 25	
Chapter 4. Decalcification	26
Procedure, 26	
Decalcifying Fluids, 27	

Chapter 5. Paraffin Method	30
Dehydration, 30	
(Technicon) Dehydration Schedule, 32	
Embedding, 33	
Paper-Boat Embedding, 35	
Cedarwood Oil Method of Embedding, 36	
Methyl Benzoate Method of Double Embedding, 36	
Masson's Amyl Acetate Method to Embed	
Brittle Tissue, 36	
Barron's Amyl Acetate Paraffin Method to	
Embed Embryos, 37	
Dioxane Procedure for Paraffin Embedding, 37	
Tetrahydrofuran (THF) for Paraffin Embedding, 37	
Rapid Method, 38	
Carbowax Method: Polyethylene Glycol, Water-	
Soluble Wax, 38	
A Method to Embed Cell Colonies, 40	
Paraffin Method to Process Eyes, 40	
Chapter 6. Sharpening and Care of Microtome Knives	43
Hand Honing, 44	
Stropping, 45	
Mechanical Knife Sharpeners, 46	
Sectioning, 47	
Difficulties Commonly Encountered in	
Sectioning, 50	
Mounting Sections on Glass Slides, 52	
Serial Sections, 54	
Care of the Microtome, 56	
Microslides and Cover Glasses, 56	
Hydration of Paraffin Sections, 57	
Chapter 7. Nitrocellulose Method	59
Dehydration and Infiltration, 60	
Embedding, 60	
Mounting, 61	
Section Cutting, 62	
Staining, 63	
Dehydration and Clearing, 63	
Other Methods of Dehydration and Clearing, 64	
Rapid Celloidin Embedding Method for Thin	
Sections Only, 64	
Acetone Celloidin (Rapid Method for Small	
Specimens Only), 65	
Barron's Amyl Acetate Method, 65	

Chapter 8. Accessory Procedures to Staining 66

- Removal of Mercury Crystals, 66
- Removal of Picric Acid Stains, 66
- Removal of Pigments, 67
- Lendrum's Tamp Method for Improving Staining of Inadequately Fixed Tissue, 67
- Postmordanting, 68
- Destaining and Restaining, 68
- Celloidin Coating of Loose Sections, 69
- A Method for Salvaging Histological Sections from Broken Microslides, 70

Chapter 9. Dyes and Staining 71

- Theory of Staining, 73
- Methods of Staining, 74
- Hematoxylin Solutions, 76
- Eosin-Y Counterstaining Solutions, 80
- Red Nuclear Stains, 81
- Biological Stains Commonly Used in Histology, 82
- Dye Houses, 85

Chapter 10. Dehydration, Clearing, and Mounting 86

- Routine Dehydration: Hematoxylin and Eosin, 86
- Cover Glass Mounting, 87
- Mounting in Aqueous Media, 88

Chapter 11. Staining Procedure 89

TO DEMONSTRATE	METHOD
Acid-fast bacilli	Andrala's method Kinyoun's method Putt's method Spengler's method Ziehl-Neelsen method
Actinomycosis	Mallory's hematoxylin-phloxine Periodic acid Schiff (clubs only) Putt's acid fast method
Adrenals	Hematoxylin and eosin Masson's trichrome Verhoeff's elastic stain
Adrenochrome	Schmorl's Giemsa method
Amebae	Best's carmine Periodic acid Schiff
Amniotic fluid	Attwood's Alcian green-phloxine

TO DEMONSTRATE:	METHOD:
Amyloid	Bennhold's Congo red Highman's Congo red Lendrum's Dahlia Lieb's crystal violet Lillie's crystal violet Puchtler, Sweat, and Levine's Congo red Diazo reaction Fontana-Masson method Gomori-Burtner method Schmorl's ferric- ferricyanide reaction Bunting's Prussian blue Mallory's phosphotungstic acid hematoxylin Periodic acid Schiff
Argentaffin reaction	
Asbestos bodies	
Asteroid inclusions	
Bacillus piliformis	Warthin-Starry method Giemsa stain
Bacteria	Brown and Brenn method Glynn's method Gram-Weigert method Lillie-Gram method
Basement membrane	Heidenhain's azan carmine Jones' methenamine silver Mowry's Alcian blue-PAS Periodic acid Schiff
Bile pigment	Stein's iodine stain
Bilirubin	Hall's method
Blood cells	Maximow's hematoxylin azure 11 eosin McNamara's Giemsa method Wolback's Giemsa stain
Blood vessels	Mowry's Alcian blue-PAS Periodic acid Schiff Verhoeff's elastic stain Schmorl's Thionin method
Bone	McNamara's Giemsa stain
Bone marrow	Wolback's Giemsa stain
Calcium	Alizarine red method Grandis and Mainini's purpurin method Schufeninoff's reaction Von Kossa Silver method

TO DEMONSTRATE

METHOD

Cartilage	Bunting's toluidine blue
Ceroid	Mowry's Alcian blue-PAS
	Lillie's performic-peracetic acid method
	Periodic acid Schiff
	Putt's lipid stain
	Putt's acid-fast method
Chlorestero1	Schultz's reaction
Chromatin	Feulgen reaction
	Methyl green-pyronine
Chromaffin granules	Gomori's azocarmine
Collagen fibers	Heidenhain's azan carmine
	Hematoxylin Van Gieson
	Masson's trichrome
	Mallory's phosphotungstic acid hematoxylin
Connective tissue mucins	Mowry's Alcian blue-hematoxylin
	Mowry's Alcian blue-PAS
Corpora amylacea	Best's carmine method
	Periodic acid Schiff
Elastic fibers	Fulmer and Lillie's method
	Gomori's aldehyde fuchsin
	Humason and Lushbaugh's method
	Orcein method
	Pickus' acid orcein-Giemsa method
	Putt and Hukill's elastic-mucin
	Verhoeff's elastic method
	Weigert's elastic method
Enterochromaffin granules	Diazo reaction
	Fontana-Masson method
	Gomori-Burtner method
	Lillie's ferric-ferricyanide reaction
Exfoliative cytology	Papinicolaou's method
Fatty acids	Nile blue sulfate

TO DEMONSTRATE

METHOD

Ferric iron

Bunting's Prussian blue reaction

Hukill and Putt's bath-phenathroline reaction

Ferrous iron

Turnbull blue reaction

Fibrin

Heidenhain's azan

carmine method

Mallory's phosphotungstic acid hematoxylin

Lendrum's picro-Mallory method

Putt's fast fuchsin 6B

Slidder's method

Weigert's method

Fibroblasts

Heidenhain's azan

carmine method

Mallory's phosphotungstic acid hematoxylin

Fungi

Grocott's methenamine

silver method

Gridley's PAS aldehyde

fuchsin method

Giemsa method

Gastric mucin

Maxwell's Alcian green

General survey

Hematoxylin and eosin

Hematoxylin Van Gieson

Mallory's phosphotungstic acid hematoxylin

Masson's trichrome

Glycogen

Bauer-Feulgen method

Best's carmine method

Gold

Elftman's peroxide

reaction

Hemofuchsin

Mallory's fuchsin

granules

method

Hemoglobin

Okajama's alizarine red

Putt's benzidine-thionin

Ralph's benzidine method

Hemosiderin iron

Mallory's hematoxylin

method

Hyalin (alcoholic)

Mallory's hematoxylin-

phloxine method

Masson's trichrome stain

Hyalin droplets

Periodic acid Schiff

TO DEMONSTRATE

METHOD

Inclusion bodies

Feulgen reaction
Lendrum's phloxine-
tartrazine
Mann's methyl blue-
eosin
Maxwell's Alcian yellow

Intestinal mucin

Juxtaglomerular
granules

Bowie's method

Keratin

Gram stain
Lillie's performic-
peracetic acid Schiff
Lendrum's phloxine
tartrazine

Kurloff bodies

Leprosy bacilli
Lipids

Putt's acid-fast method
Chiffelle and Putt's
propylene glycol
Herxheimer's Sudan IV
method
Lillie and Ashburn's
isopropyl alcohol method
Putt's flaming red
method
Nile blue sulfate method
Felton's brilliant cresyl
blue
Lillie's Nile blue A
Periodic acid Schiff
Putt's acid-fast method
Schmorl's method

Lipiodol

Lipofuchsin

Malaria parasites

Mast cell granules

NcNamara's Giemsa stain
Wolbach's Giemsa stain
Allan's neutral red
Bunting's toluidine blue
Maximow's alcoholic
thionin
Maximow's hematoxylin
azure 11 eosin

Melanin

Azure A method
Fontana-Masson method
Laidlaw's Dopa oxidase
reaction
Lillie's ferrous uptake
Lillie's method to

TO DEMONSTRATE

METHOD

Melanin (Cont.)	differentiate melanin and lipofuchsin Schmorl's ferric- ferricyanide
Metachromasia	Azure A method Bunting's toluidine blue Maximow's alcoholic thionin
Mitochondria	Chiffelle and Putt's lipid method Harmond's fast green method Heidenhain's iron hematoxylin Mallory's phosphotungstic acid hematoxylin Heidenhain's iron hematoxylin Mallory's phosphotungstic acid hematoxylin Periodic acid Schiff
Mitotic figures	Attwood's Alcian green method Masson's mucicarmine method Maxwell's method for gastric mucin Maxwell's method for intestinal mucin Mayer's mucicarmine method Mowry's Alcian blue hematoxylin Mowry's Alcian blue-PAS Mowry-Hale colloidal iron method Pioch's Astra blue method Putt and Hukill's Alcian green method Putt's Alcian green- calcium chloride method
Mucins	
Muscle	Mallory's phosphotungstic acid hematoxylin Masson's trichrome stain

TO DEMONSTRATE	METHOD
Negri bodies	Mann's methyl blue-eosin method Schletstein's basic fuchsin-methylene blue method
Nocardia asteroides	Putt's acid-fast method
Nucleic acids	Methyl green-pyronine
Pancreatic islets	Gomori's chrome alum hematoxylin Gomori's chromaffin granule method Heidenhain's azan carmine
Parasites	Feulgen reaction Geimsa-stain
Phosphates	Bunting's molybdate reaction
Phospholipids	Elftman's method
Pituitary cells	Crooke-Russell method McAllister's poirier blue method Paget and Eccleston's method Slidder's method Wilson and Ezran's method
Plasma cells	Mallory's phloxine-methylene blue Methyl green-pyronine method Thomas' phloxine-methylene blue
Plasmasomes	Harmond's fast green method
Pneumocystic carinii	Giesma stain Grocott methanamine silver
Polyvinyl pyrrolidone	Chlorazol fast pink method
Reticulin	Gomori-Bielschowski method Gridley's method Humason and Lushbaugh's method Laidlaw's method Nassar and Shanklin's method