

STAINING PROCEDURES

THIRD EDITION

Edited by

Clark

Contributors

Bartholomew

Clark

Coalson

Mohr

Nordquist

Schneider

THE WILLIAMS & WILKINS CO.

STAINING PROCEDURES

**used by the
BIOLOGICAL STAIN COMMISSION**

THIRD EDITION

Edited by George Clark

METHODS FOR ANIMAL TISSUE

George Clark

Robert E. Coalson

Robert E. Nordquist

METHODS FOR BOTANICAL SCIENCES

Henry Schneider

METHODS FOR MICROBIOLOGY

James W. Bartholomew

METHODS FOR PROTOZOANS

John L. Mohr

Published for the
BIOLOGICAL STAIN COMMISSION

By
THE WILLIAMS & WILKINS CO.
BALTIMORE

First Edition Published by
THE BIOLOGICAL STAIN COMMISSION

Second Edition, 1960
The Williams & Wilkins Company

Third Edition
COPYRIGHT ©, 1973
THE WILLIAMS & WILKINS COMPANY
428 E. Preston Street
Baltimore, Md. 21202, U.S.A.

All rights reserved. This book is protected by copyright.
No part of this book may be reproduced in any form or by
any means, including photocopying, or utilized by any
information storage and retrieval system without written
permission from the copyright owner.

Made in the United States of America

Library of Congress
Catalog Card Number 72-92836
SBN 683-01706-3

CONTRIBUTORS

George Clark,	Visiting Professor of Anatomy, Medical University of South Carolina Research Associate, Veterans Administration Hospital, Charleston, South Carolina
James W. Bartholomew,	Professor of Microbiology, University of Southern California
Robert E. Coalson,	Professor of Anatomical Sciences, Assistant Professor of Pathology, University of Oklahoma
John L. Mohr,	Professor of Biology, University of Southern California
R. E. Nordquist,	Assistant Professor of Pathology, University of Oklahoma Assistant Member of Cancer Section, Oklahoma Medical Research Foundation
Henry Schneider,	Plant Pathologist, University of California, Riverside

PREFACE TO SECOND EDITION

This publication is sponsored by the Biological Stain Commission and is intended to represent staining methods used in microtechnic in the laboratories of Commission members. It is not intended to be a complete treatise in regard to staining, nor even to include the only way of getting good results with the various methods here included. Nor are the procedures given intended to be considered as *standard* or *official*. They are all put in a standardized *form*, but only for the convenience of users.

The first edition of this book was published in a loose-leaf form, the first leaflets thereof appearing in 1943. Eventually it consisted of nine leaflets. These leaflets were periodically revised during the period from 1943 to 1955, some of them going through 4 editions. The present publication, however, is the first in which all the material has been gathered together as a "hard-cover" book, and is regarded as only the 2nd edition of the entire contents.

Members of the stain Commission contributing to the first (loose-leaf) edition are listed in its introductory leaflet. When the senior author retires several years ago as an officer of the Commission, it was anticipated that this publication would be allowed to go out of print and that its revision would not be undertaken. However, a continuing steady demand for the material in it has resulted in a decision by the Commission Trustee to sponsor a new and revised edition.

In the first edition the names of 28 collaborators are given on the page of Acknowledgements. On the title page of the current edition only those specially assisting in the revision are named; but acknowledgement is hereby given to the following whose assistance made the first edition possible (but who are not named on the present title page): C. E. Allen, Wanda Brentzel, Clyde Chandler, G. H. Chapman, E. V. Cowdry, A. B. Dawson, John Einset, J. O. Foley, M. F. Guyer, P. H. Hartz, Raphael Isaacs, M. W. Jennison, H. E. Jordan, R. R. Kudo, B. R. Nebel, L. F. Randolph, Ruth Rhines, L. W. Sharp, K. A. Stiles, W. D. Stovall, W. R. Taylor, J. M. Thuringer, W. F. Windle, Isidore Wodinsky, Conway Zirkle. Special mention should also be made of the fact that Chapter 4 (Neurological Methods) has been completely revised for the present edition by H. A. Davenport; while Chapters 6-8 (Plant Microtechnic) owe their new form largely to the valuable suggestions made by Frank H. Smith and Charlotte Pratt.

H. J. Conn, *Geneva, N. Y.*
Mary A. Darrow,
Victor M. Emmel, *Rochester, N. Y.*

PREFACE TO THIRD EDITION

The second edition of this book was published in 1960, and the preface to the second edition is reprinted below. The objectives of the book as stated there remain unchanged.

The authors of the third edition wish to acknowledge their dependence on and their thanks to all those who made possible the earlier editions. Much of the earlier textual material is included sometimes with minor emendations, sometimes with considerable change. The format remains, but in addition some descriptive material has been added. The most noteworthy additions have been in methods for study of the islet cells of the pancreas, a section on fluorescent microscopy, and an entirely new division on methods for protozoans. The divisions on botanical and microbiology techniques have been completely rewritten.

CONTENTS

CHAPTER 1. INTRODUCTION AND GENERAL METHODS

Statement of purpose	1
Preparation of solutions	2
Buffer tables	3
Dyes for staining solutions	8
Dyes certified by Biological Stain Commission	8
General methods	10
Fixing fluids	11
Alcohol	11
Alcohol and acetic acid	12
Formalin mixtures	13
Picro-formalin	15
Chrome-acetic formalin	16
Chrome-acetic fluids	17
Mercuric chloride mixtures	19
Bichromate mixtures, without HgCl ₂	21
Dehydration	23
De-alcoholization (clearing)	23
Paraffin method	24
Celloidin method	26
Other embedding methods	27
Methods for plant microtechnic	27
Tertiary butyl alcohol for dehydration and infiltration	27
Embedding of herbarium material, seeds and woody specimens	28
Softening embedded material	28
Use of water soluble waxes	29

PART I: ANIMAL HISTOLOGY

CHAPTER 2. METHODS FOR GENERAL TISSUE	33
Section A: Nuclear staining with alum hematoxylin	33
Delafield's hematoxylin	34
Ehrlich's hematoxylin	34
Mayer's acid hemalum modified by Lillie	35
Harris' hematoxylin	35
Section B: Counterstains for hematoxylin	36
Eosin Y	36
Sirius red F3BA	36

Congo red	37
Eosin B	37
Section C: Miscellaneous methods	38
Heidenhain's hematoxylin	38
Weigert-Lillie iron chloride hematoxylin	38
Gallocyanine chrome alum	39
Weigert's iron hematoxylin with metachromic dyes	40
Orth's lithium carmine	41
Mallory's phosphotungstic acid hematoxylin	41
Buffered azure eosin method	43
Bismark brown with methyl green	44
Thionin for frozen sections	45
Thionin or Azure A for fixed material	45
Mucicarmine with hematoxylin and metanil yellow	46
Modification of Mayer's mucihematein	47
Crystal violet stain for amyloid	48
Bennehold's congo red for amyloid	49
CHAPTER 3. METHODS FOR CONNECTIVE TISSUE	50
Section A: Van Gieson type stains	50
Van Gieson stain with iron hematoxylin	50
Fast green modification of the Van Gieson stain	51
Biebrich scarlet with picric aniline blue	52
Van Gieson trypan blue	53
Picro amido black 10B	53
Section B: Connective tissue stains with a polyacid step	54
Mallory's aniline blue collagen stain	54
Acid alizarin blue modification	55
Heidenhain's "Azan" modification	56
Masson's trichrome stain	57
Lillie modification of Masson's trichrome	59
Section C: Elastic tissue stains	60
Weigert's resorcin fuchsin	60
Ogilve and Clark's modification of Verhoeff's elastic tissue stain	61
Tänzer-Unna orcein method	62
Darrow's modification with synthetic orcein	63
Frankel's orcein method for elastic tissue	64
Mollier's quadruple stain	64
Kornhauser's "quad" stain	65
Section D: Silver methods	67
Foot's modification of Bielshowsky's method	67
Lillie's modification of Bielshowsky's method	68
Wilder's modification of Bielshowsky's method	70

CHAPTER 4. NEUROLOGICAL STAINING METHODS	74
Section A: Methods for supporting elements	77
Cajal gold-sublimate method for astrocytes	77
Del Rio-Hortega's silver carbonate method for oligodendrocytes	79
Penfield's combined method	80
Del Rio-Hortega's method for microglia	81
Weil-Davenport method for gliomas	82
Stern's method as modified by Weil and Davenport	84
Nassar-Shanklin method for neuroglia in paraffin sections	85
Foot's ammoniated silver carbonate method	86
Section B: Methods for nerve cells, Nissl granules and chromatolysis	87
Nissl staining with cresyl violet acetate, thionin or toluidine blue O	87
Darrow red	89
Section C: Methods for myelin sheaths	89
Pal-Weigert method	90
Luxol fast Blue MBS	91
Weil stain	92
Section D: Methods for nerve fibers and nerve endings	93
Pyridine-Silver method	94
Chloral hydrate method	95
Bielschowsky method	95
Staining nerve fibers in celloidin sections	96
Bodian's protargol method	98
Osmium tetroxide vapor method	100
Concurrent staining of axis cylinders myelin sheaths	101
Gold chloride method for nerve endings	102
Chloral hydrate silver method	102
Section E: Methods for neurofibrils	104
Section F: Methods for degeneration	104
Swank and Davenport modification of Marchi method	105
Lillie's combined myelin and fat stain	107
Polarized light method	108
Nauta and Gygax method for frozen sections	108
Fink-Heimer method for frozen sections	110
Glees' method for normal and degenerating pericellular endings	111
Section G: Golgi methods	113
Rapid Golgi method	113
Golgi-Cox method	115

Recent uses and studies of the Golgi dichromate-silver methods and resulting modifications	116
Golgi-Cox tungstate modification	118
Section H: Supravital staining	119
Methylene blue method	119
CHAPTER 5. MISCELLANEOUS METHODS	122
Section A: Methods for staining blood	122
Wright stain	122
Lillie's modification of Wright stain	123
MacNeal's tetrachrome stain	124
Giemsa stain for thin films	125
Giemsa stain for thick films	126
Combined peroxidase-Wright stain	127
Brilliant cresyl blue for reticulated cells and platelets	128
Brilliant cresyl blue for counting reticulocytes	129
New methylene blue as a reticulocyte stain	130
Prussian blue stain for non-hemoglobin iron	131
Rhodinile blue for Heinz bodies	131
Section B: Methods for staining bone marrow	131
Smears with a May-Grünwald stain preceding Giemsa	131
Custer's method for sections	132
Section C: Supravital staining	133
Section D: Methods for staining bone and calcium	135
Schmorl's method for sections	135
Staining of canaliculi and lacunae in hard tissues	136
Alizarin red S for small vertebrates	136
Alizarin red S for fetal specimens	137
Modification by Crumley, Crow and Griffen	138
Alizarin red S and toluidine blue O	139
Alizarin red S for calcium deposits	140
Silver nitrate method for calcium deposits	141
Glyoxal-bis (2-hydroxyanil) method for calcium	142
Section E: Methods for staining fat	143
Herxheimer's technic	143
Nile blue A	144
Smith and Mair's stain; Dietrich's modification	145
Baker's acid hematein test	146
Baker's pyridine extraction test	147
Proescher's oil red with pyridine	147
Sudan black B stain	148
Fat stains in supersaturated isopropanol	149
Section F: Methods for staining glycogen	150
Best's method	150
Bauer's method	151
Other methods	152

Section G: Cytology	152
Orcein and Feulgen technics	152
Synthetic orcein	154
Section H: Miscellany	155
Methyl green with pyronin	155
Periodic acid leucofuchsin (PAS) method	156
Prussian blue method for hemosiderin	157
Ferric ferricyanide method for reducing groups	158
Alcian blue method for acidic carbohydrates	159
Toluidine blue O for metachromatic substances	159
Aldehyde fuchsin method	160
Methenamine silver method for argentaffin cells	161
Relative basophilia	162
Relative acidophilia	163
Acid phosphatase	163
Alkaline phosphatase	164
Papanicolaou stain	165
Human adenohypophyseal stains	166
AB/PAS/OG stain	169
Permanganate/AB/PAS/OB stain	169
Staining methods for pancreatic islets	170
Mallory-Heidenhain Azan-Gormori's modification for islet cells	171
Mallory-Heidenhain Azan-Permanganate oxidation . . .	172
Phosphotungstic acid hematoxylin (PTAH)	173
Tryptophan: post-coupled p-dimethylaminobenzaldehyde reactions for indoles .	174
Aldehyde-fuchsin	175
Chrome hematoxylin-phloxine	177
Alcoholic silver nitrate	178
Toluidine blue metachromasia	179
Fluorescent technics	181
Introduction	181
Fluorescent Feulgen	181
Fluorescent PAS	182
Acridine orange	183
Fluorescent antibody	183
Fractionation of antiserum	184
Fractionation Procedure	184
Preparing serum globulin-fluorescein isothiocyanate conjugates	185
Fluorescent antibody staining	186
Staining characteristics of selected fluorochromes . .	187

PART II: BOTANICAL SCIENCES**CHAPTER 6: PLANT ANATOMY AND GENERAL BOTANY**

Section A: Mordanted Dyes	192
Premixed Iron Hematoxylin (Weigert's)	193
Iron→Hematoxylin iron (regressive staining) (Heidenhain's)	194
Iron→Hematoxylin (progressive staining) (Heidenhain's)	195
Premixed Aluminum Hematoxylin (Delafield's)	196
Premixed Aluminum Hematoxylin (Harris')	197
Tannic Acid=FeCl ₃ →Safranin→Fast green (Foster's)	198
ZnCl ₂ →Safranin→Orange G-Tannic Acid→NH ₄ Fe(SO ₄) ₂ (Sharman's)	199
Section B: Cationic Stains	201
Bismark Brown Y	201
Crystal violet and Erythrosin B (Jackson's)	201
Periodic Acid, Lecuobasic fuchsin (Schiff's), aniline blue black	202
Safranin	203
Safranin and aniline blue	204
Safranin and fast green	204
Conn's	204
Johansen's	205
Safranin and Picro Aniline Blue	206
Thionin for freehand sections	206
Toluidine blue O for tissues in resins	207
Section C: Anionic Dyes	207
Chlorozol black E	208
Fast Green	209
Light green	209
Orange G	209
Section D: Neutral Dyes	210
Lacmoid counter stain for permanent mounts	210
Section E: Fat Soluble Dyes	212
Sudan IV	212

CHAPTER 7: PLANT CYTOLOGY 213

Aniline blue w.s. and fluorescence microscopy	213
Azure B for differentiation of RNA and DNA	214
Fast green for nuclear histones	214

CHAPTER 8: PLANT CYTOGENETICS 216

Iron→Hematoxylin→Iron (Regressive Staining, Long Schedule) (Heidenhain's)	217
----------------------------------------------------------------------------------------	-----

Iron-Hematoxylin-Picric Acid (Regressive Staining, Short Schedule) (Heidenhain's)	219
Iron Hematoxylin or Crystal Violet with Iodine, Permanent Smear Preparations	219
Acetocarmine (Hyde and Gardella)	221
Iron Acetocarmine (Belling)	222
Method for Making Acetocarmine Smears Permanent	222
Chlorazol Black E with Acetocarmine	224
Crystal Violet→Iodine (Newton)	225
Crystal Violet→Iodine (Randolph)	225
Safranin→Crystal Violet→Orange G (Flemming's Triple Stain)	226
Safranin→ I_2KI →Crystal Violet→ I_2KI →Orange G (Flemming's Triple with Iodine)	227
Lacto-Propionic-Orcein (Dyer)	228
Laco-Propionic-Orcein (Storey <i>et al.</i>)	228
Propionic Acid Orcein (Smith)	230
HCl →Leucobasic Fuchsin→Light Green (De Tomasi's Feulgen Stain)	230
HCl →Leucobasic Fuchsin (Meyer's Feulgen)	232
CHAPTER 9: METHODS FOR POLLEN AND POLLEN TUBES	234
Section A: Pollen	234
Methyl green in glycerin-jelly	234
Section B: Pollen tubes in styles	235
Acetocarmine and basic fuchsin	235
Lacmoid and Martius yellow	235
Section C: Pollen tubes in culture	236
Crystal violet→ I_2KI →Orange II	236
Acetocarmine (Swanson)	237
Section D: Growing pollen tubes on cellophane for easy staining	238
CHAPTER 10. PATHOLOGICAL ANATOMY AND MYCOLOGY OF PLANTS	239
Acid fuchsin—Lactophenol	239
Acridine Orange and Fluorescence Microscopy	240
Aniline blue W.S. (Cotton blue)	241
Giemsa Stain—Azure B type (Lillie)	241
Premixed martius yellow, malachite green and acid fuchsin (Pianese IIIB Stain)	243
Safranin→Picro-aniline Blue (Cartwright)	244
Thionin and Orange G (Stoughton)	244

PART III: MICROBIOLOGY

CHAPTER 11. STAINS FOR MICROORGANISMS IN SMEARS .	247
Section A: The differentiation of microorganisms in smears by the Gram stain procedure	247
Wet & Dry Procedures for the gram staining of micro- organisms in smears	249
Section B: Acid-fast staining procedures	251
Ziehl-Neelsen procedure	252
Fluorescent microscopy acid-fast procedure	254
Acid-fast staining at room temperature.	256
Section C: Stains for bacterial capsules and slime	257
Wet negative capsule stain with India ink	259
Dry negative capsule stain with India ink	259
Dry negative capsule stain using Congo red	260
Dry negative capsule stain using eosin Y or carbol fuchsin	261
The Anthony modification of the Hiss capsule stain	261
Positive capsule stain	262
Section D: Stains for bacterial spores	263
Schaeffer and Fulton modification of the Wirtz spore stain	264
Bartholomew and Mittwer modification	264
Fluorescent spore stain	265
Differential spore stain using inorganic acids and no heat	266
Section E: The staining of lipid	267
Burdon stain for sudanophilic granules	267
Hartman stain for sudanophilic granules	268
Section F: Stains for bacterial cell walls	269
Webb tannic acid-crystal violet stain	269
Dyar cell wall stain	270
Section G: The staining of bacterial nuclear material	271
Cassel and Hutchinson stain	271
Robinow stain	273
Section H: Stains for bacterial flagella	274
Preparation of slides	275
Preparation of cultures	275
Preparation of smears	276
Selection of basic fuchsin	276
Leifson's bacterial flagella stain	277
Casares-Gil flagella stain	278
Gray's modification of Muir flagella stain	279
Section I: Stains for diphtheria organisms, diphtheroids, volutin or metachromatic granules	280

Albert's stain for diphtheria organisms or volutin	281
Gohar's stain for volutin granules	282
Kinyoun's modification of Ponder's stain for volutin and diphtheria organisms	283
Section J: Stains for spirochetes in smears	284
Fontant-Tribondeau silver stain	285
Warthin and Starry silver-agar stain	286
Sodium carbonate basic fuchsin stain	287
Potassium permanganate crystal violet stain	288
Negative stain for spirochetes	288
Section K: Stains for rickettsiae and chlamydiae	289
Macchiavello stain for rickettsia	290
Gienéz stain for rickettsiae and psittacosis agents in yolk-sac cultures	291
Section L: Stains for mycoplasma (pleuropneumonia and PPLO organisms) and L-phase variants of bacteria	293
Dienes stain for colonies of mycoplasma and L-Phase variants of bacteria	294
Klieneberger-Nobel agar-fixation stain for colonies of mycoplasma and L-phase variants	295
Dienes agar-fixation stain for colonies of mycoplasma and L-phase variants	297
Section M: The staining of bacteria in dairy products	299
Levowitz and Weber modification of the Newman (No. 2) single solution stain	299
Acid and water free stain for diary products	301
Aniline methylene blue stain for dairy products	302
Section N: The staining of bacteria in soil	302
Staining of bacteria in soil	303
Fluorescent staining of bacteria in soil	304
Section O: The staining of bacteria on membrane filters . . .	305
Section P: The demonstration of Negri bodies of rabies in smears	307
Sellers' stain for Negri bodies in smears	307
Bond's modification of Mann's Eosin-methylene blue stain for Negri bodies in smears	309
Harris stain for Negri bodies in smears	310
Section Q: The staining of protozoa in smears and wet mounts	311
Direct observation of protozoa in wet mounts	311
Zinc sulfate concentration procedure	312
M.I.F. and M.I.F.C. wet mount technics	312
Concentration procedure for M.I.F. preserved specimens	314

Demonstration of protozoa in fixed and stained smears	315
Iron-hematoxylin stain for protozoa in fixed smears	315
Demonstration of malarial organisms, leishmania, trypanosoma, and microfilariae in blood smears	316
Preparation of thick blood film	316
Preparation of thin film	316
Giemsa stain	317
CHAPTER 12. STAINS FOR MICROORGANISMS IN SECTIONS	319
Section A: Introduction	319
Section B: Gram stain for differentiation of micro- organisms in tissue sections	320
Taylor's modification of Brown and Breen Gram procedure	320
Brown and Hopps Gram stain	322
Section C: Stains for acid-fast bacteria in tissue sections	324
Modified Ziehl-Neelsen procedure	324
Fite II procedure for acid-fast organisms	325
Wade's modification of the Fite I acid-fast procedure	326
Fluorescence microscopy for acid-fast organisms in tissue sections	328
Section D: Stains for spirochetes in tissue blocks and sections	330
Levaditi and Manouélian silver impregnation stain for spirochetes in tissue blocks	330
Modified Warthin-Starry procedure for the silver stain- ing of spirochetes in sections	331
Modified Kraijan silver stain for spirochetes in sections	333
Section E: Stains for viral inclusion bodies in sections	336
Schliefstein stain for Negri bodies in sections	337
Stoval and Black stain for Negri bodies in acetone fixed tissue	339
Mann's method for staining Negri bodies in sections	340
Lendrum stain for inclusion bodies in sections	341
Section F: The staining of rickettsiae, chlamydiae, and mycoplasma in sections	342
Goodburn and Marmion stain for mycoplasma in frozen sections	342
Wolbach's variation of the Giemsa stain for the demonstration of rickettsiae and chlamydiae in sections	344

Section G: The demonstration of fungi in tissue sections	345
Schneidau's gram stain for the demonstration of fungi in sections	345
Gridley's stain for fungi in sections	346
Grocott's modification of the Gomori stain for fungi in sections	348
Section H: Endamoeba in tissue sections	349
Gridley's stain for endamoeba in tissue sections	349
Luthringer and Glenner's modification of Gomori stain	350
 PART IV: METHODS FOR PROTOZOA	
 CHAPTER 13. UNSTAINED MATERIAL AND VITAL STAINS	355
Janus green B	355
Neutral red	356
Inert particles	357
 CHAPTER 14. HANDLING OF PROTOZOA FREE-LIVING OR SEPARABLE FROM HOST CAVITIES OR TISSUES	358
Affixation in albumen smears	358
Individual or mass sections	359
 CHAPTER 15. PROCEDURES WITH KILLED MATERIAL	361
Section A: General methods	361
Rose bengal	361
Cupric picro-formol-acetic fluid as fixative and stain	361
Relief or negative staining	363
Night blue-phloxine relief stain	363
Osmium-toluidine blue temporary stain	364
Iodine-potassium iodide stain	364
Methods for glycogen	365
Section B: Nuclear stains for temporary preparations	365
Iron Aceto+carmine	365
Acidulated methyl green	366
Section C: Permanent nuclear stains	367
Precipitated borax carmine	367
Feulgen nucleal reaction in heat-coagulated albumen	369
Oxidative Schiff-aniline blue-orange G	370