PRACTICAL SECTION CUTTING AND STAINING

E. C. CLAYDEN

THIRD EDITION

PRACTICAL SECTION CUTTING AND STAINING

By

E. C. CLAYDEN
F.I.M.L.T.

Senior Technician in the Morbid Histology Department of the Bland-Sutton Institute of Pathology, The Middlesex Hospital, London

THIRD EDITION

With 26 illustrations

CHEMICAL PUBLISHING CO., INC.
212 Fifth Ave. New York 10, N.Y.

To My Wife Lilian
in appreciation of her patience and encouragement whilst this book was being written

FIRST EDITION 1948
SECOND EDITION 1951
THIRD EDITION 1955

Printed in Great Britain
and published in London
by J. & A. Churchill Ltd.

Preface to the Third Edition

The ordinary histological techniques have not altered much over the years, although there have been great advances recently in histo-chemical techniques. No single book on histological techniques can fully meet all the requirements of the technician and this book has been written essentially on basic techniques and methods which have all been proved to give consistent and reliable results. I have described only one method for most techniques because, to the inexperienced, it can be very confusing when referring to one of the larger text books to find three or four alternative methods given for a particular technique.

In the previous editions I purposely omitted certain techniques primarily because I had only a limited experience of them. Methods which have now been added are Mallory's phosphotungstic acid hæmatoxylin for neuroglia fibres, Periodic acid-Schiff reaction (P.A.S.), Feulgen reaction, Diazo method for argentaffin granules, Purpurin for calcium, and Bertorelli and Volpino's method for spirochætes. There is also a more detailed description of the rotary microtome together with an automatic tissue processor.

I am deeply indebted to Professor S. P. Bedson for his most valued help with the methods for the demonstration of inclusion bodies.

I would also like to take this opportunity of thanking the reviewers for their encouraging reception of the previous editions and for certain suggestions they have made to improve this edition.

LONDON E. C. CLAYDEN

Preface to the First Edition

THIS book is written essentially for technicians with little or no experience in the various methods of preparing routine sections. Those who have already had experience may think that too much stress has been laid on small and to them obvious details, but it is these simple essentials that are most important in the preparation of good sections.

Covering the basic principles of histological technique, it is hoped that this book will be found valuable for those intending to sit the examiantions of the Institute of Medical Laboratory Technology.

Whilst not always conforming to those in standard text books, the methods and techniques detailed in this book have all proved successful in routine work and are used in this Institute. It is appreciated that experienced technicians may use other equally successful methods. Providing the techniques are carefully carried out, anyone with average technical ability should be able to prepare reasonably good histological sections. It must be remembered that only by constant practice can really first class sections be obtained.

Most technicians acquire various useful practical "tips" and whenever possible these aids to better technique have been discussed in full detail.

E. C. CLAYDEN.

Acknowledgements

MY thanks are due to the late Professor J. McIntosh and to Professor R. W. Scarff for their valuable criticisms; also to Professor W. D. Newcomb, Dr. A. C. Thackray and Dr. J. B. Walter for their many helpful suggestions.

I am greatly indebted to Dr. C. J. C. Britton for the interest, help and advice he has given me throughout the preparation of this book.

In addition I wish to express my thanks to the Cambridge Instrument Co. Ltd., for their photograph shown as Fig. 7, Ernst Leitz for Fig. 8, R. & J. Beck Ltd., Fig. 9, to Measuring and Scientific Equipment Ltd. for photographs shown as Figs. 21 and 24; also to Elliots, Liverpool Ltd. for the photograph shown as Fig. 10.

I would also like to thank all others who have in any way assisted me.

CONTENTS

PART I

PARAFFIN WAX SECTIONS

Staining techniques:

FIXATION AND HARDENING

FIXATION. The purpose of fixation (usually called fixing) is to act upon the tissue elements, and more particularly the cell nuclei, so that they are preserved most closely to the original form. At the same time, the fixative being used must prevent putrefaction (decomposition of the tissue by micro-organisms) and autolysis (the destruction of tissues or cells by digestion due to the action of enzymes secreted by their own cells).

In order to obtain the best results it is essential that the tissue be put into a large bulk of fixing fluid at the earliest possible moment.

The action of fixing fluids consists of coagulating and rendering insoluble various tissue elements.

Fixation is usually carried out at room temperature as a routine, although penetration can be hastened by placing in a 37°C. incubator, or in cases of extreme urgency in an oven at 50°C.-60°C. Tissue can also be fixed rapidly by boiling in formol saline. This is usually done in fixing tissue when carrying out the frozen section technique in the operating theatre.

When fixing at 37°C. thin pieces of tissue only should be selected, as, although this method favours penetration, it also hastens autolysis.

HARDENING. This is a term used to denote the process which gives the tissues the consistency necessary for the cutting of thin sections.

Hardening takes place with most fixatives during fixation and continues during the subsequent process of dehydration in the alcohols.

FIXATIVES

The length of time for which tissues should be left in fixing fluids depends on the rapidity of penetration of the fixative, and on the thickness of the tissue. In all cases a large bulk of fixative should be used. Do not put several large pieces of tissue into the tube or bottle. A suitable sized tube to use in which to process tissue is a flat bottom 3in. by 1in. corked tube. A word of warning: when dealing with specimens in formol saline, care must be taken not to handle them without wearing rubber gloves, as a very troublesome chronic formalin dermatitis may result from neglect of this precaution. Care must also be taken when using xylol, which is used for removing the paraffin wax from sections before staining, and for clearing sections before mounting in balsam. Slides should be manipulated with forceps, not the fingers, as xylol over a period of time will also produce dermatitis in some workers.

All tissues after fixation in fluids containing potassium dichromate, mercuric chloride or picric acid should be washed for half to one hour before the next procedure. The reason for this washing is to remove the excess of uncombined fixative. Some workers suggest that washing the tissue produces a sharper picture in the stained section.

Washing in running tap-water is usually sufficient for tissues that have been fixed in potassium dichromate or mercuric chloride, although fixatives containing mercuric chloride are perhaps best washed in 70 per cent alcohol.

For tissue that has been in a fixative containing picric acid, washing in water must not be used, as this causes swelling of the collagen fibres. For this also 70 per cent alcohol should be used.

The fixatives about to be described, with their advantages and disadvantages, are the ones more commonly employed.

Formol Saline

Sodium chloride	0.9 g.
Water	90 ml.
*Formalin	10 ml.

Advantages. Comparatively cheap to purchase, penetrates well, causes only little shrinkage, blood and fat are well preserved

*Formalin is a solution of formaldehyde gas in water with a strength usually of 40% of actual gas.

and it permits a large variety of staining methods. It does not make the tissue brittle, and in fixing large specimens the normal colour is preserved better than with most other fixatives. It is particularly useful for frozen sections, which cut very well with this fixative.

Disadvantages. The colour reaction in amyloid tissue is diminished. When specimens are stored in formol saline for many months, formic acid is produced which destroys the staining properties of the tissues ; under these circumstances it is necessary to change the formol saline every six months.

Frequently, when a large quantity of blood is present, a dark brownish-black deposit is formed, which can be removed when the sections have been cut.

Note. Formalin when stored over a long period sometimes decomposes and produces a white deposit of paraformaldehyde. This deposit does not appear to affect the efficiency of the formalin as a fixative. It is advisable that tissue, which is to be stored for any length of time, should be kept in 80 per cent alcohol.

ZENKER'S FLUID

Mercuric chloride	5	g.
Potassium dichromate	2.5	g.
Water	100	ml.

Add 5 ml. of glacial acetic acid immediately before use.

Advantages. The staining of the nuclei and connective tissue is very good, particularly in chicken tumours and certain other animal tissues.

Disadvantages. Only thin pieces of tissue can be fixed. If the tissue is left in the fluid three or four days it becomes brittle. Frozen sections cut badly. Zenker's fluid does not keep well, as the acetic acid produces changes in the chrome salt.

After fixation in Zenker's fluid the tissue should be washed in running tap-water for half to one hour, or in several changes of 70 per cent alcohol for the same period of time.

HELLY'S FLUID

The stock solution is the same as Zenker's ; add 5–10 per cent of formalin immediately before use.

The advantages are similar to Zenker's, indeed the nuclear staining is even more intense.

The disadvantages are also similar, with the addition that, if the tissue is not thin, the surfaces are so hardened that good penetration is prevented. It does not keep well, a heavy brown deposit being formed within 24 hours. The tissue should be washed in running tap water for half to one hour after fixation, or in several changes of 70 per cent alcohol for the same period of time.

ORTH'S FLUID

Potassium dichromate	2.5 g.
Water	100 ml.

Add 10 ml. of formalin at time of use only.

Advantages. Good for mitotic figures, red cells and tissue containing colloid material.

Disadvantages. Should not be used for large pieces of tissue as penetration is slow, so that only very thin pieces of tissue can be fixed. After three to four days a crystalline deposit forms and if fixation is to be carried on beyond this stage, as in the case of bone, the fixative must be changed.

The tissue should be washed in running tap-water for half to one hour after fixation.

CARNOY'S FLUID

Chloroform	30 ml.
Absolute alcohol or 74. O.P. spirit	60 ml.
Glacial acetic acid	10 ml.

Advantages. Rapid fixation, preserves glycogen, nuclear staining is greatly improved. Dehydrates as well as fixes.

Disadvantages. Causes excessive shrinkage, destroys red cells; small pieces of tissue only should be used.

Note. Carnoy's fluid is used principally when dealing with curettings or small pieces of tissue from biopsies, on which reports are required urgently. Fixation seldom takes longer than half-an-hour and the specimen is then transferred direct to absolute alcohol or 74 O.P. spirit.

BOUIN'S FLUID

Picric acid (saturated aqueous solution) (p. 143)	75 ml.
Formalin	25 ml.
Glacial acetic acid	5 ml.

Advantages. Penetrates rapidly; the staining of the nuclei and connective tissue is excellent for most laboratory animal tissues.

Disadvantages. Some tissues become brittle if left in the fixative beyond twenty-four hours, causing difficulty in cutting. If left in beyond three days the nuclei fail to stain properly in alum hæmatoxylin. Red cells are destroyed, and it is a very poor fixative for kidney tissue.

Tissue fixed in Bouin's fluid should not be washed in water, but in 70 per cent alcohol, as the water causes swelling.

Note.—When dealing with very small pieces of tissue, it is advisable to fix in Bouin's fluid, as the yellow colouration enables them to be seen more easily in the subsequent stages of dehydration and embedding.

DEHYDRATION

The term dehydration means "the removal of water from." Dehydration is a most essential step and must be carried out in a thorough manner. Failure to do this properly will prevent the tissue from becoming impregnated successfully later with paraffin wax or celloidin.

The process of dehydration, for all practical purposes, can be carried out by transferring tissue direct from fixative into methylated spirit (64 O.P.) for one to six hours, followed by absolute alcohol one to six hours, and a second change of absolute alcohol one tc twelve hours.

When dealing with delicate tissue such as brain, spinal cord or embryos, dehydration is better carried out in stages using varying grades of alcohol, e.g., 30 per cent alcohol, 50 per cent alcohol, 70 per cent alcohol. The tissue is left in each of the various grades of alcohol for one to six hours before transferring to absolute alcohol for the same length of time. Finally it is put into fresh absolute alcohol for a further one to twelve hours. It is not necessary with all tissues to complete dehydration in absolute alcohol. Thus some clearing agents also dehydrate, e.g., aniline oil clears from 70 per cent alcohol, oil of bergamot from 90 per cent alcohol and cedar wood oil from 95 per cent alcohol.

Before stained sections can be mounted in Canada or other balsams, they must also be dehydrated. In the case of sections

prepared by the paraffin wax technique this is usually done with absolute alcohol except on certain occasions, which will be specifically mentioned in the text; e.g., staining by Weigert's method for fibrin (p. 62). Stained sections that have been prepared by the celloidin technique are not dehydrated with absolute alcohol, because, if this were done, the celloidin would become so soft that manipulation of the sections would be very difficult.

Celloidin sections are dehydrated as follows :—They are transferred to a series of graded alcohols commencing with 50 per cent alcohol, followed by 70 per cent, and finally 95 per cent alcohol. The sections are then transferred to 25 per cent carbol xylol (p. 135) which completes the dehydration. The subject is dealt with more fully under the individual staining technique.

Note. When dehydrating tissue, a large bulk of fluid must always be used A 3in. by 1in. flat bottom corked tube half full (10 ml.) is sufficient for one small piece of tissue $\frac{1}{2}$in. by $\frac{1}{4}$in. by $\frac{1}{16}$in., whilst for two or three such pieces, or a larger piece, the tube should be filled (approx. 25 ml.).

Instead of using the one tube and changing the various reagents, some workers prefer to use a series of large (3in. by 2in.) flat bottom tubes. The tissue is transferred with forceps into the various tubes of reagents. This method is very economical, especially in the use of alcohols. The danger of carrying over too much water and preventing complete dehydration, is guarded against by placing a small gauze bag containing pure, anhydrous copper sulphate in the absolute alcohol or 74 O.P. spirit. Not only does this act as an indicator (by turning blue in the presence of water), but it also dehydrates the alcohol.

Instead of using the rather costly absolute ethyl alcohol, industrial methylated spirit (74 O.P.) which is approximately 99.3 per cent absolute alcohol (methyl and ethyl), can be used with consistently satisfactory results.

When making up the various grades of alcohol, the dilutions can be made from industrial methylated spirit (64 O.P.) which is approximately 95 per cent absolute alcohol.

To economise with the absolute alcohol or 74 O.P. spirit when dehydrating tissue, the second change of absolute alcohol in one series of tissues should be saved and used for the first change in the next series of tissues to be dehydrated.

CLEARING AGENTS

The purpose of clearing is to replace the alcohol in the dehydrated tissue with a solvent of the wax in which the tissue is to be later embedded before it can be cut. Therefore it is obvious that the reagents used in clearing must be freely miscible with alcohol.

Clearing is thus essential in the following instances :—

1. Following the dehydration of tissues which are to be impregnated by the paraffin wax technique.
2. Following dehydration of stained sections prior to their being mounted in Canada or other balsams.
3. To make the gross internal structures of embryos, parasites and certain tissues more readily demonstrated to the naked eye or under a low power lens. Benzene and Oil of Wintergreen are the clearing agents usually used for these purposes. Benzene is the more rapid. Oil of Wintergreen clears slowly and is specially recommended for embryos.

When xylol or benzene is used for the clearing agent, the tissues are rendered more or less transparent, and this effect has led to the use of the term *clearing*. Not all clearing agents cause this transparency of the tissue, so it would be more correct to use the term "*de-alcoholisation*".

Xylol, xylene or benzene are the clearing agents most generally used in routine work.

The length of time in which tissue should remain in the clearing agent depends on :—

The thickness and density of the tissue.

The reagent employed.

Providing the tissue has been dehydrated properly, clearing in xylol or benzene should take from half to three hours. On no account should the specimen be allowed to remain in xylol longer than necessary as this causes brittleness of the tissues with subsequent difficulty in cutting.

Sometimes the xylol or benzene takes on a milky appearance when added to the tube containing the tissue. This indicates that dehydration has not been completely carried out and the tissue must therefore be put into a fresh supply of absolute alcohol, or 74 O.P. spirit, for a further period of time. This indication of incomplete dehydration also applies to stained sections. Thus, if

the xylol when poured over the section goes milky, a further wash with absolute alcohol, or 74 O.P. spirit is necessary.

All tissues from the central nervous system and lymph glands should be cleared in cedar-wood oil or chloroform, as such tissues tend to become brittle and break up when xylol is employed.

In the next few pages the practical advantages and disadvantages of the more common clearing agents will be briefly discussed. The methods of use will be described in full under the various techniques in which they are employed.

Xylol

Advantages. Cheap and rapid in action. Makes the majority of sections transparent. Does not dissolve celloidin and so can be used when dealing with celloidin sections.

Disadvantages. Causes excessive shrinkage of delicate tissues, clears only from absolute alcohol or 74 O.P. spirit.

Toluol

Similar to xylol.

Benzene

Advantages. Similar to xylol and cheaper. Has a less hardening effect on tissue than xylol.

Disadvantages. Similar to xylol but more inflammable and toxic.

Cedar-wood Oil

Advantages. Causes very little shrinkage even when specimens are left in it for a long time.

Clears from 95 per cent alcohol. Can be used as a mounting reagent for sections which have been stained by Leishman's or other Romanowsky stains. It does not affect aniline colours.

Disadvantages. Clears slowly and is difficult to remove when impregnating with molten paraffin wax, usually requiring two or three changes of fresh wax.

Some samples of cedar-wood oil fail to clear tissues even after several days.

When purchasing cedar-wood oil it is advisable to state that it is required for tissue clearing purposes. Such oil is considerably cheaper than the cedar-wood oil which is used in conjunction with a 1/12 oil immersion objective.

After the tissue has been cleared in cedar-wood oil, transfer it to benzene or xylol for half to one hour. This speeds up the

impregnation with molten paraffin wax by helping to remove the oil.

Chloroform

Advantages. Causes only little shrinkage even when specimens are left in it for a long period.

Disadvantages. Expensive. Slow and difficult to remove from the tissue when impregnating with molten paraffin wax, requiring three to four changes of fresh wax.

Even if only a little chloroform is left in the tissue the wax is so softened that sections are impossible to obtain.

Clove Oil

Advantages. Clears quickly from 95 per cent alcohol and causes only little shrinkage.

Disadvantages. Expensive. Dissolves celloidin and extracts aniline colours especially methylene blue. Difficult to remove when impregnating with molten paraffin wax, requiring three or four changes of fresh wax.

Samples of clove oil vary in colour and with age, changing readily from yellow to brown. The paler coloured oil is recommended.

Aniline Oil

Advantages. Clears readily from 70 per cent alcohol and causes only little shrinkage.

Disadvantages. Soon oxidises and requires three or four changes of fresh wax.

Carbon-disulphide

Advantages. Causes only the minimum of shrinkage.

Disadvantages. Highly inflammable, objectionable smell and difficult to remove when impregnating with molten paraffin wax, requiring three or four changes of wax.

EMBEDDING

After dehydration and clearing, the tissue is ready for embedding. This process is to impregnate the tissue completely with molten paraffin wax and is carried out by one of the following techniques :

1. Ordinary paraffin wax oven.
2. Vacuum embedding bath.

Embedding with the Ordinary Paraffin Wax Oven. Impregnation of tissue with wax is carried out in metal pots which