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Preparation of the Eye for Examination

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The Preparation of the Eye for Histological Examination.

By

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During the last eighteen months I have been endeavouring to prepare satisfactory sections of different portions of the eye, and the following communication, which treats only of method, is based upon the results obtained.

I am especially anxious that no statement in it shall be regarded as being final, since I feel convinced that success in this as in other branches of histology depends as much on the histologist's knowledge of a method as on its intrinsic merits. My results may, however, serve to indicate the direction in which success is to be sought.

The Preparation of Sections of the Entire Eye.

I do not think that sections of an entire eye can be prepared without the aid of embedding and infiltrating materials, and I have been successful with only two, celloidin and paraffin.

1. Preparation of sections of entire eyes by infiltrating and embedding in celloidin.

The eye, removed from the body as soon as possible after death, should be opened by a short incision through the sclerotic, midway between the cornea and the entrance of the optic nerve, and should then be placed in some fixing and hardening agent (Müller's fluid, chromic acid solution). Ulti-

mately, following the usual plan, it should be transferred, first to weak, and subsequently to strong alcohol. Much of the success of the process is dependent on the care taken in fixing and hardening. Although any of the chromic acid preparations harden very well indeed, yet there are two important objections to their indiscriminate use as fixing or hardening agents,—they usually render the lens brittle and unnecessarily hard, and they often render the sections difficult to stain. Therefore, another fixing agent has been employed when I have wished to prepare sections of the lens or to stain the eye thoroughly. This agent is carbolic acid. The eyes after removal from the body should be placed in a 2 per cent. watery solution for a week, and should then be transferred to alcohol and be treated as in the former case. When prepared in this way the eyes stain readily and the lens is not usually brittle.

If a section of the eye without the lens is required it is better to use a chromic acid hardening solution, because that reagent hardens so satisfactorily.

The eye hardened by any of these methods should be stained in bulk; this is not absolutely necessary, for sections may be stained after they have been cut, but staining in bulk enables one to avoid a certain amount of dangerous after-manipulation.

Before placing the eye in the stain four small openings should be made in it, two into the anterior chamber, and two into the vitreous. The openings into the anterior chamber should be situated opposite to one another, and at the periphery; those into the vitreous should also be opposite one another, and should be situated midway between the cornea and the entrance of the optic nerve.

The only stain which was found to be reliable for staining in bulk was borax carmine (alcoholic). Kleinenberg's logwood will not penetrate sufficiently, and (in my hands) often fails to select; and most of the aniline stains (which penetrate admirably) are partially removed during the necessary after-treatment.

The eye should be left in the stain for from two to four days,

according to the rapidity with which it stains; the staining is somewhat diffuse, and it is sometimes preferable to place the eye after staining in alcohol containing a trace of HCl in order to remove the stain from everything but the nuclei.

Formula for Alcoholic Borax Carmine (Woodward's).

Carmine, Nr. 40, gr. xv;
Borax 3j;
Water to 8 oz.

Dissolve by warming and slowly evaporate to 4 oz.; now add 7 oz. of alcohol.

If it is to be used for staining in bulk there is no need to filter it. It should be shaken well from time to time.

After the eye is stained it should be washed and transferred to alcohol, and then to a mixture of alcohol and ether equal parts. In this mixture the eye should be left for twenty-four hours, when it should be transferred to a thin solution of celloidin in equal parts of absolute alcohol and ether; an accurate measurement of the quantities of alcohol and ether is unnecessary, but the quantity of ether should never be greater than the quantity of alcohol.

In this solution the eye may be left for two or three days until the celloidin has fairly penetrated all parts of it.

Embedding.—The infiltrated eye should be placed in a pill-box or paper boat with a perfectly flat floor, and a tolerably thick solution of celloidin should be poured into the box until the eye is completely covered. The box or boat should then be placed on a glass plate and should be covered with a bell jar; the alcohol and ether diffuse into the air beneath the bell jar and the celloidin slowly consolidates. If a bell jar is not used a crust usually forms on the surface of the celloidin and further evaporation is hindered, whilst on the other hand the use of the bell jar permits of an equable removal of the alcohol and ether from all parts of the mass without the formation of bubbles. It should be lifted from time to time to allow of a partial removal of the gaseous alcohol and ether. The use of the bell jar is

particularly indicated in hot weather, and when the mass of celloidin is very large.

When the mass becomes tolerably firm it should be transferred to a mixture of equal parts of commercial alcohol and water, in which the consolidation soon becomes complete. The time the mass must be left under the bell jar depends much on the temperature of the room, and varies from one to six days.

The eye is now infiltrated with and firmly embedded in celloidin, and sections of it may be prepared.

It is almost impossible to cut sections from the block of celloidin in this condition on account of its size, so the whole mass should be cut into slices about a quarter of an inch thick, and one of these pieces should be fixed in the microtome. If the division into slices be made before embedding the lens will be displaced.

When the eyes are exceedingly large, and embedding is consequently difficult, I usually re-embed one of these slices, and so obtain a requisite degree of hardness.

Sections may be cut in three ways:

a. By the freezing microtome.

b. By any "slide microtome," such as Jung's.

c. By a microtome so arranged that the section may be cut under spirit.

(*a*) The mass should be placed in water for from six to twenty-four hours, until the greater part of the spirit has been removed. It should then be dipped in gum for a moment, and may be frozen, the gum serving to attach it to the plate of the microtome.

Sections may next be cut and should be floated off the knife into warm water. If all the spirit has been removed from the mass, the celloidin, when frozen, often becomes intensely hard and difficult to cut. This difficulty may readily be obviated by warming the knife in warm water before cutting the sections.

(*b* or *c*) The mass should be securely fixed to a cork-covered plate. This is always difficult to do unless there is one flat

surface to the celloidin. The most rapid method of fixing is to moisten the cork and the flat surface of celloidin with ether, and to firmly press the moistened surfaces against one another for five to ten minutes; the ether has then evaporated, and the celloidin firmly adheres to the cork.

Another method is to smear some thick solution of celloidin over both surfaces, to press them together for fifteen to thirty minutes, and then to place them in alcohol for twenty-four hours.

There are also other methods of securing the mass, with gelatine or with paraffin, but the two methods described are rather more simple. As regards the relative values of the three methods of section-cutting I think that for the preparation of sections of small pieces of the eye the freezing method answers well, and has the merit of being very convenient, but for the preparation of sections of pieces of any size the method of cutting under spirit is most suitable.

When the sections have been prepared they may be stained or simply mounted; they should always be manipulated between two pieces of tissue paper, since any rough usage causes displacements. They must be thoroughly dehydrated by long immersion in alcohol, and may be then cleared in one of three media:

a. Oil of bergamot.

b. Oil of cedar.

c. Turpentine.

Of these oil of bergamot acts the most rapidly and efficiently; at times, however, samples of oil of bergamot are met with which will dissolve celloidin.

Oil of cedar is very slow in its action; and turpentine often causes a disagreeable shrinkage.

The sections should be mounted in balsam.

I have obtained sections by the freezing method, which are fairly good histological specimens, and which will bear examination with a high power; but by the other method I have rarely obtained sections which serve to illustrate more than the topographical anatomy of the eye—sections which may be examined with a half-inch objective—although, if the

eye be that of a very small animal, the result has been sometimes better.

I have further found that this process almost always produces some histological changes in the tissues; they are, however, sometimes slight.

Infiltration with Paraffin.—This method is exceedingly useful for the preparation of sections of the eyes of embryos, of the eyes of very young animals, and of sections of eyes in those cases where the examination of the lens is not necessary. Its great merit lies in its simplicity.

I have practised two methods of infiltrating. The turpentine process:

a. The eyes hardened, opened, and stained, as before, are transferred from alcohol to oil of cloves, in which they are left until they are cleared; they are then soaked in pure turpentine for several hours, and are finally placed for twelve to forty-eight hours in paraffin, melted at a temperature not exceeding 50° C. The paraffin displaces the turpentine and permeates the crevices of the tissue. The infiltrated eye is then embedded in paraffin, and sections may be cut and sealed to the slide in the usual manner. The cement which has been most serviceable to me is a mixture of oil of cloves and collodion.

It is practically impossible to stain the sections after they have been cut and sealed to the slide. Certain passable results may occasionally be obtained by the use of diffusible stains, but as a rule the result is disappointing.

Unfortunately, this process nearly always ends in the total histological destruction of the lens (in fact too often a section of the lens cannot be prepared, since it instantly crumbles to pieces), and too frequently renders the tissues unfit for very minute examination. I thought that this alteration of the tissues was due to the high temperature of the melted paraffin, and I therefore obtained a paraffin which melted at 35° C., infiltrated the eyes with it, and then embedded in a harder sample. The tissue was, nevertheless, somewhat altered. I have, however, obtained better results by using the paraffin in a different manner.

b. After staining, the eye is placed in a mixture of alcohol and ether, equal parts, for twenty-four hours, and is then submersed in pure chloroform for two days. It is finally placed in melted paraffin for twelve to forty-eight hours, and is treated subsequently as in the former case. By the use of chloroform the treatment with turpentine and oil of cloves is avoided.

Conclusions.—1. Satisfactory sections of a small portion of the eye may be easily obtained by infiltrating and embedding in celloidin, and by cutting sections either with the freezing microtome or under spirit. Such sections may, if necessary, be stained after they have been cut.

2. Sections of parts of the eye without the lens of young or of embryonic eyes may be readily obtained by infiltrating and embedding in paraffin by the chloroform process. The eye must in this case be stained in bulk.

3. Sections of the eye with the lens in sitû may be best procured by infiltrating and embedding in celloidin and cutting under spirit.

If sections of the Classes 1 and 2 are required I believe that it is better to harden the eyes in chromic acid, but if sections of Class 3 are in demand the fixation and hardening should be effected by the use of alcohol and carbolic acid.

Preparation of Retina.

When I first endeavoured to prepare sections of retina I had to determine :—

a. The best fixing and hardening agent.

b. The best staining agent.

c. The best embedding agent.

(*a*) I obtained many eyes from guinea-pigs, fixed and hardened them in different solutions, and prepared sections of the retina. But except in the matter of hardening all were prepared in the same way, so that in the fixing and hardening the only variable factor was consciously introduced.

The sections were prepared in the first set of experiments by infiltrating and embedding in celloidin in the manner already described.

The following fixing and hardening solutions were employed:

1. Müller's Fluid.—The fresh eye, opened in the way already described, was placed in Müller's fluid for two or three weeks, during which time the fluid was changed as often as its altered appearance afforded an indication of the necessity. It was then transferred, after being washed, to strong commercial alcohol, and was completely hardened in about two weeks. Sections of retinas so prepared were serviceable in showing the structure of the inner layers of the retina and the course taken by the blood-vessels (in retinas which contain them), but the rod-and-cone layer and the outer nuclear layer were more or less completely destroyed.

2. Bichloride of Mercury.—A saturated watery solution was employed; the freshly opened eye was placed in this solution for three to six days, and was then hardened in alcohol as before. Some eyes I placed in alcohol containing 2 per cent. of carbolic acid instead of simple alcohol.

The salt "fixed" in a manner much superior to Müller's fluid, but usually permitted or caused shrinkage in the rod layer.

The sections of retinas prepared with the alcoholic solution of carbolic acid were superior histologically to those prepared in alcohol alone, and this I found to hold good for all the fixing agents employed.

It occured to me at this stage of my work that possibly the fixing solution did not gain access to the retina with sufficient rapidity, the opening in the eye not being large enough; yet a very large opening allows the retina to become detached. I therefore procured two cannulæ, and pushed them through the coats of the eye into the vitreous at points a little distant from one another; then I endeavoured to fill the vitreous with the fixing agent by injecting it through one of these cannulæ whilst the intraocular tension remained unaltered.

No good results followed, chiefly because of the firm consistency of the vitreous. A more simple method was then adopted; the length of the incision was made equal to a quarter of the circumference of the eye, and the eye was then placed in the fixing solution. At the end of thirty minutes or less the posterior part of the eye was removed by enlarging the original incision with sharp scissors. By this means the fixing agent obtained access to the retina rapidly, and detachment of the retina was prevented.

3. Picric Acid.—The fresh and opened eye was placed in a saturated watery solution of picric acid for three days, and the hardening was then completed in alcohol and carbolic acid. By this fixing agent everything was rendered intensely hard but rather brittle. Sections of retina prepared in this way were very serviceable in showing the structure of the nerve-layers of the retina, but the outer nuclear layer and the rod layer were profoundly altered. The nuclei (with a twelfth oil immersion lens) showed a remarkable crenation, whilst similar nuclei in another eye prepared with such a reagent as osmic acid showed no such crenation. By the use of picric acid, however, it was possible to trace the Müllerian fibres at all events as far as the outer reticular layer, since the previous immersion of the retina in picric acid seems to intensify the eosinophilous property which those fibres exhibit.

4. Carbolic Acid.—The fresh eye, prepared as before, was placed in a 2 per cent. watery solution of carbolic acid for a week, and was then hardened in alcohol in the usual manner. Carbolic acid itself does not harden. By this means fair specimens of all parts of the retina were occasionally obtained.

5. Zinc Chloride.—The fresh and opened eye was placed for a week in a 1 per cent. watery solution of this salt and was then removed to the alcoholic solution of carbolic acid. The zinc salt did not harden, and seemed to destroy the outer layers of the retina, but its action on the Müllerian fibres was similar to that of picric acid.

6. Permanganate of Potash.—The fresh and opened eye was placed in a 2 per cent. solution of this salt for seven days

and was then hardened in alcohol and carbolic acid. The permanganate salt did not harden, and the sections of retina prepared in this way were unsatisfactory.

7. Chromic Acid.—The fresh and opened eye was placed in a $\frac{1}{6}$ per cent. watery solution of chromic acid and was allowed to remain there for twenty-four to forty-eight hours. The hardening was then completed by the use of the alcohol and carbolic acid solution. If the eye was left more than forty-eight hours in the chromic acid solution difficulty was experienced both in staining and in the preparation of sections (on account of brittleness). Sections so prepared were usually very serviceable in showing the structure of all the layers except the rod layer.

8. Chloral Hydrate.—The fresh and opened eye was placed in a 10 per cent. solution of this salt for two to seven days; the hardening was completed by the alcohol and carbolic solution. Chloral did not harden, and in my hands only yielded first-class results occasionally. It certainly has the merit of preserving the rod layer, and it is quite possible by this method to obtain satisfactory specimens with the rods and cones in sitû.

9. Chloride of Gold.—I have made very many efforts to obtain sections stained with this salt, but they have not been successful.

The following methods have been employed:

a. The fresh freely-opened eye was placed in a solution of 1 per cent. of the salt for fifteen to forty-five minutes and was then transferred to a weak solution of formic or acetic acids, and was left there in the dark till the salt was reduced (usually twenty-four to forty-eight hours).

b. The fresh freely-opened eye was placed for one to three minutes in weak formic acid, and was then treated as before.

c. The fresh freely-opened eye was placed for several days in a $\frac{1}{6}$ per cent. watery solution of chromic acid. When hardened the eye was placed in a neutral or slightly alkaline solution of the gold salt for thirty minutes and was transferred to a solution of weak formic acid kept at a temperature of 30° C. in the

dark. At the end of twenty-four hours the reduction was complete. This process is a modification of that which Mr. Underwood employs with great success in the preparation of sections of teeth.

d. The fresh freely-opened eye was placed in a solution of osmic and chromic acids (afterwards described) for two to five days and was then treated as in *c*. By none of these methods have I been able to procure one satisfactory section.

10. Osmic Acid.—By means of this very reliable reagent I have obtained my best results.

a. The fresh and opened eye was placed for twenty-four to forty-eight hours (not longer) in a watery solution of osmic and chromic acids; $\frac{1}{4}$ per cent. chromic acid, $\frac{1}{10}$ per cent. osmic acid. It was then placed in the mixture of alcohol and carbolic acids for fourteen days or more. By this process the retina was rendered exceedingly hard but not brittle. The sections showed the structure of all parts of the retina, the rods being sharply defined and remaining in sitû. (One of these sections was exhibited at the December meeting of the Physiological Society, 1885.)[1] If the retina was allowed to remain in the solution for more than forty-eight hours brittleness was usually produced.

b. The fresh and opened eye was placed in a ·75 to 1 p. c. solution of osmic acid for from thirty minutes to twelve hours, and was subsequently treated with (*a*) alcohol, glycerine and water, or (*b*) alcohol, or (*c*) alcohol and carbolic acid. The hardening was not usually good and the results were often only passable.

c. In order to obtain very rapid penetration of the retina by the fixing agent, solutions of osmic and chromic acid in alcohol were employed.

They were:

1. Osmic acid . $\frac{1}{10}$ per cent.
   Chromic acid . $\frac{1}{4}$,,
   Commercial alcohol, } Equal parts.
   Water, }

[1] 'Proceedings Physiological Society,' December, 1885.

2. Osmic acid . $\frac{1}{5}$ per cent.
Chromic acid . $\frac{1}{6}$ „
Commercial alcohol, } Equal parts.
Water, }

With these solutions the layers of the retina, exclusive of the rod layer, were very fairly prepared, but in that layer shrinkage was produced.

d. The fresh and opened eye was placed in a solution of

Osmic acid . . $\frac{1}{5}$ per cent.
Chromic acid . $\frac{1}{6}$ „
Water.

for twenty-four to thirty-six hours, and was then transferred to the alcohol and carbolic solution and treated as before. By this method the most uniform and certain results have been obtained. All parts of the retina were fixed and preserved in a manner superior to that produced by any of the other reagents used.

Mode of Staining.—It is quite possible to stain sections of retina if they have been prepared by the celloidin method, but if they are to be prepared by the paraffin or cacao butter method, the retina must be stained in bulk before it is embedded (at least with the nuclear stain). Two nuclear stains, logwood and carmine, have been chiefly used, there being objections to the use of the anilines. Kleinenberg's logwood and the alcoholic borax carmine already described were selected; if thick sections are required (as in searching for blood-vessels) the carmine is preferable because it is a transparent stain, whilst if the thinnest sections are required nothing equals Kleinenberg's logwood.

Retinas should be left in the carmine about two days and in the logwood from twelve to twenty-four hours. The exact time depends much on the hardening agent which has been used, and must vary for each retina. If only very thin sections be cut a moderate amount of overstaining with logwood does no harm whatever.

In order to examine Müllerian fibres or blood-vessels the sections of the retina which have already been stained in bulk

with a nuclear stain should be stained (best on the slide) with either fuchsin or an alcoholic solution of eosin, preferably the latter. The exact method of staining will be described.

Mode of Preparing and Mounting Sections.—Sections may be obtained by :

1. Infiltrating and embedding in celloidin and freezing.
2. Infiltrating and embedding in celloidin and cutting under spirit.
3. Infiltrating and embedding in paraffin.
4. Infiltrating and embedding in cacao butter.

1 and 2. It is difficult to obtain thin sections by the second method, but very fair ones may be obtained by—(1) the whole sclerotic, choroid and retina should be embedded together, and when the celloidin is firm the retina and part of the choroid should be separated with a sharp scalpel; attempts to separate the retina earlier generally end in damage to the rod layer. After the sections have been cut by the method previously described they may be diffusely stained and mounted. The staining may be effected in two ways : (*a*) to the water in which the sections have been placed on removal from the microtome a little eosin is added; in a few minutes they will be sufficiently stained; or (*b*), they may be at once placed on the slide with a section lifter and the staining may be effected there. In either case after staining they should be gently washed and nearly dried with blotting paper, then covered with a few drops of alcohol. On removing this reagent with blotting paper they should be cleared either in oil of cloves or oil of bergamot and may be mounted in balsam.

3. By the paraffin method already described serial sections may be prepared, but I have never yet obtained by this method any sections of very great histological value; they have been at best passable.

4. Infiltrating and embedding in cacao butter. By this method I have been able to prepare the thinnest and best sections of retina with a minimum amount of trouble. A piece of the eye stained with a nuclear stain should be placed first in alcohol until dehydrated, then in oil of cloves till

cleared, and then in cacao butter melted at a temperature of 35° C. for four to six or even twelve hours. At the end of this time it should be embedded in cacao butter in the usual manner. When the butter is quite hard, the sclerotic and part of the choroid should be detached with a sharp scalpel so that the retina and part of the choroid alone remain to be cut into sections, whilst the rod layer has never been tampered with.

The retina should be fixed by pouring over it a little more melted butter which replaces the mass cut away.

The sections may be cut either by hand, or with any accurately constructed "slide microtome," and with care may be made only one nucleus in thickness. Such sections are nearly invisible to the naked eye. If a microtome is used and such sections are prepared, they accumulate on the blade of the knife and look like a little mass of butter. This mass should be swept on to a slide, when the contained sections may be diffusely stained and mounted in the following manner:

A few drops of an alcoholic solution of eosine are poured over the mass and at once soak into it; after a few minutes the mass is partially dried with blotting paper, and the slide is heated to a temperature of 35° C. The melted cacao butter is removed as far as possible with blotting paper, and a drop of oil of cloves is added to remove the remainder. When the sections are cleared a drop of balsam is added and the sections are mounted.

It is very important to remove as much butter as possible before adding the oil, because the oil acts very violently and often destroys a section. In fact the great value of osmic and chromic acids as hardening agents depends largely on the great hardness they give to the retina, the sections of which are therefore not damaged by the oil of cloves.

Conclusion.—I have been able to prepare the best sections of retina by fixing and hardening in the watery solution of osmic and chromic acids in the manner described, staining in bulk with Kleinenberg's logwood and infiltrating and embedding in cacao butter.

Finally, I desire to acknowledge with sincere thanks the

assistance which has been afforded to me by Professor Fuchs, of Vienna (late Liége), by Dr. Bäumler, of Halle, and by Dr. Gade, of Kristiania; also by Mr. Gunn, of Moorfields Eye Hospital, and by Mr. E. F. Herroun, of King's College, London.