XIII.—*On the Double and Treble Staining of Animal Tissues for Microscopical Investigations; with a Note on Cleaning Thin Cover-glasses.* By HENEAGE GIBBES, M.B., F.R.M.S.

(Read 10th March, 1880.)

WHILE engaged last year in an examination into the structure of the Vertebrate spermatozoon, I tried the effect of a large number of staining agents, and succeeded at last in staining the head and body of the spermatozoon of *Triton cristatus* different colours, showing thereby a different chemical reaction. This led me to try these stains on sections of animal tissues, and the specimens under the Microscopes will show with what results. It may be interesting to give a few details of the different processes I have used, in the hope that some who have more leisure than myself may work out the subject thoroughly.

The first double stain to be mentioned is the well-known picrocarmine and logwood, which gives very good results in sections of skin and other parts. I have also found it answer better than any other stain in an investigation into the development of spermatozoa, in which I am at present engaged with Dr. Klein.

There is one point which greatly facilitates a good result with this process—that is, after staining the sections in picrocarmine to place them in plain water acidulated with a few drops of acetic or picric acid for an hour before staining with logwood; they take the second stain better and do not fade afterwards.

The next process consists of—

1. A solution of carmine and borax.
2. A mixture of hydrochloric acid and absolute alcohol.
3. A solution of indigo-carmine.

The carmine solution is prepared by mixing

Carmine	..	..	..	..	3ss
Borax	..	..	..	..	3ij
Aqua	..	..	..	..	3iv

and pouring off the clear fluid. It must not be filtered. Three or four drops of this solution are placed in a watch-glass, and the sections immersed for a few minutes; they are then removed to a mixture of hydrochloric acid and absolute alcohol, one part of the acid to twenty parts of alcohol, and allowed to remain there until they take on a bright rose colour; this happens in a few seconds. They must then be washed in methylated spirit several times to remove the acid, when they will be ready for staining with the indigo-carmine, which is prepared in the following manner:—

A saturated solution of indigo-carmine is made in distilled

water and filtered; a portion of this solution is then added to some methylated spirit until it has attained a moderately deep-blue colour; this must then be filtered to remove the colouring matter, a good deal of which is precipitated; it will then be fit for use.

A small quantity is placed in a watch-glass, and the sections allowed to remain until they have a distinctly blue colour.

I have found this process very useful in pathological investigation, especially in the Carcinomata; and in specimens hardened in chromic acid it does away with the tedious process of passing them through a solution of bicarbonate of soda.

This method is modified from one mentioned in the 'American Quarterly Microscopical Journal' by Dr. Seiler.

I next come to the anilines; and of these, after a large number of experiments, I have found roseine, aniline violet, aniline blue, and iodine green, give the most satisfactory results. The principle I worked on was to make a spirituous solution of one colour and a watery solution of another, and in this way I could depend on the result.

I made solutions of roseine and aniline violet in spirit, and of aniline blue and iodine green in distilled water. A few drops of one of the spirit solutions being placed in a watch-glass and diluted with spirit, the sections were immersed for a short time (this will depend on the strength of the solution and the tissue itself, as some stain more quickly than others); they ought to be taken out of the stain and examined in clean spirit, and a little practice will soon show when they are stained enough. They are then washed in methylated spirit until no more colour comes away from them. A little of the aqueous solution is now placed in a watch-glass and diluted, the section is removed from the spirit to it; the spirit causes the section to spread itself out and float on the watery solution, and it may be seen taking on the new colour. For a light stain this is sufficient, and it need not be wholly immersed. It is then well washed in plain water and placed in spirit, when more of the first colour generally comes out. When it is quite clean, it is ready for mounting in the usual way. This is a very good process for double staining, and if the section is of the same thickness throughout, the staining will be perfectly even, and each colour will have picked out those tissues for which it has a special affinity.

To stain with more than two colours is much more difficult, as they so often combine and produce an entirely different colour uniform throughout. I have obtained the best results from chloride of gold or picrocarmine with the anilines just mentioned.

The specimen of rat's tail was first stained with chloride of gold in the usual manner, and then submitted to the aniline process I have already described.

To stain with picrocarmine I make a dilute solution about ten drops to a watch-glass of distilled water, and leave the sections in it for about half an hour; the time will vary with the tissue and the manner in which it has been prepared. They are then removed to plain water acidulated with a few drops of acetic or picric acid, and left in it for an hour, after which they are ready for the aniline process.

This method succeeds very well in the tongue of different animals, as will be seen from the specimens under the Microscope; and I can also say that it does equally well in every other tissue as far as I have tried it. But its great utility consists, I think, in its power to differentiate glandular structures according to their secretions. For instance, in the section of dog's tongue the ordinary mucous gland will be found to have taken on a purple colour, while the serous glands which supply the secretions to the taste-organs stain a totally different colour. Again, in an examination I made lately in a case of Dysidrosis I was able to stain the duct of the sweat-gland an entirely different colour from the surrounding tissues, and so demonstrate its relation to the vesicles. For minute structures, such as the dividing nuclei of germinating epithelium or developing spermatozoa, I think logwood is far above every other stain, and when used with picrocarmine I find its effect is intensified.

The carmine and indigo-carmine process is of great use in demonstrating the blood-vessels in the web of the frog's foot, the tail of the tadpole, and similar structures, as it entirely does away with the necessity of injecting them, and shows the vessels in their natural state, without the bulges in them depicted by some writers, which are caused by the injection mass unduly distending them. It also shows the amyloid substance in amyloid degeneration of the liver to perfection, as the blue stains it alone.

I should like to call attention to the great importance of preparing all tissues properly in the first instance, as unless this is done no good result can be obtained from any staining process. Every specimen properly prepared will bear the highest magnifying power that can be applied to it, and will show plainly the structure of each epithelial cell, muscular fibre, or other element of which it may be composed, and it is utterly impossible to make a good specimen unless it has been first properly hardened.

The following process for cleaning thin cover-glasses has proved very effectual, and has reduced the breakage of $\cdot 003$ to about half a dozen per half ounce:—First place the cover-glasses in strong sulphuric acid for an hour or two; then wash well until the drainings give no acid reaction; then place them in methylated spirit, and as each glass is taken out with a pair of broad pointed forceps dip it in absolute alcohol and wipe carefully with an old silk handkerchief.

I should like to impress practical microscopists with the benefit to be derived from using cover-glasses of a known thickness, and as these can now be procured at a slight advance on the cost of the others, there is really no reason why they should not be universally used. For a long time I only used two thicknesses, $\cdot 006$ and $\cdot 004$; but since I have had a $\frac{1}{25}$ made by Messrs. Powell and Lealand, I have been obliged to use cover-glasses thinner still.

The great advantage of knowing the thickness of the cover-glass will be at once apparent to those accustomed to use glasses with a correction collar, and those glasses without it are generally corrected to a cover-glass $\cdot 006$, so that as there are comparatively few so thin as this in the glass sold as extra thin, the best result is seldom obtained; and, again, in making exchanges of duplicates, how vexing it is to find that your favourite glasses will not work through the cover-glass.