# ON THE APPLICATION OF THE SPALTEHOLTZ CLEARING METHOD TO THE STUDY OF THICK SERIAL SECTIONS OF EMBRYOS, WITH DEMONSTRATION OF SPECIMENS

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THE Spalteholtz method of producing transparent anatomical preparations is now well known and widely practised. Originally introduced as a macroscopic method it has been extensively employed, especially in America, in the study of embryonic structure, and more particularly in the clearing, *in toto*, of injected embryos or considerable parts of them. It has also been extensively used in the clearing of embryos whose cartilaginous skeletons have been previously stained by the Van Wijhe method.

At the summer meeting of the Society last year I showed a number of preparations to illustrate the application of the same method to series of quite thick sections of embryos. Since then I have prepared a number of additional serial preparations, several of which I am exhibiting at this meeting. I have found the results of the experiment so instructive that I venture once more to call the attention of the Society to the method as an extremely useful adjunct to microscopical investigation by means of serial sections of the usual type.

Whilst there is nothing really novel in the procedure itself, I have not been able to discover any record of precisely the same application of the method in embryological study. In the systematic study of embryonic structure by means of serial sections it has hitherto been our aim, usually, if not invariably, to obtain complete series of quite thin sections. This is, of course, indispensable for the study of cytological or any minute structural detail; but much embryological work is concerned, not with the most minute structural detail, but with grosser morphological features in the shape of cell-groups, tissue-masses and rudiments of developing organs whose form and relations can be more readily apprehended if studied in thickish blocks than in very thin sections. This the Spalteholtz method enables us to do very simply and conveniently, as I think my demonstration specimens will show.

A 12 mm. embryo cut in transverse series in the usual way into sections of, say, 10 micra thick will involve the study of some twelve hundred sections, each of which furnishes an image of structure practically in two dimensions only. To obtain from such a series anything like a vivid stereognostic conception of structure requires a considerable effort in the way of mental reconstruction and indeed often demands actual graphic reconstruction by diagrams or models. I claim that much of this difficulty is obviated, so far as

the larger features of structure are concerned, by the study of thick serial sectional blocks treated by the Spalteholtz method.

I call your attention, for example, to one of my demonstration specimens in which the entire system of bronchial buds in the lung of a mammalian embryo may be directly seen under the binocular microscope as clearly and stereoscopically as if they were represented in a wax plate model. Although the method I employ cannot in any full sense be called a new one it is perhaps desirable to set forth the small expedients by which such a series is prepared.

Let us suppose there is a 12 mm. mammalian embryo to be studied in transverse series by this method. It is first of all infiltrated with celloidin and a good celloidin block made of it in the usual way. This block is then trimmed into rectangular form, keeping fairly near, though not too close, to the embryo. The elongated block thus obtained has now to be cut into transverse serial sections. Before doing so it is good to cut off obliquely one of the four longitudinal margins of the block so that each cross section will show a cut-off corner for orientation purposes.

I usually cut the sections at anything from ·5 to 1 mm. in thickness, preferably about ·5 mm. The sections may be cut after mounting the block on any microtome which will allow of elevation of the knife for the necessary distance between the sections; but I have cut practically all my sections free hand, laying the celloidin block on a sheet of cork and cutting vertically with a Gillette razor blade held in the hand. I use a pair of magnifying spectacles during the operation. Such free hand operations, of course, involve some irregularity, both in thickness and in plane, but it is not necessary to have the sections of equal thickness and there is sometimes a certain advantage in varying both the thickness and the plane. Thus, for example, with a curved embryo one can often with advantage cut somewhat wedge-shaped slices. Their structural relations can quite easily be correctly interpreted when they are mounted. A 12 mm. embryo will yield, say, 24 half-millimetre sections.

As each section is cut it is transferred to a capsule of alcohol or water. Commonly a dozen capsules will suffice in the case of an object of this size, for it is usually easy to distinguish the second cycle of sections from the first. But, of course, any other expedient for keeping the serial sections in their proper order may be adopted.

During cutting, the block must not be allowed to get dry. After cutting, the sections are transferred to distilled water, if they have not been placed directly in it; or they may be transferred directly to the staining fluid utilizing the same capsules. The staining of the blocks is of the utmost importance. The secret of the method, indeed, is the attainment of an adequate but very light and transparent stain. I have obtained the best results with a very weak haematoxylin stain.

I usually stain in a fluid consisting of 5 per cent. ammonia-alum solution, to which has been added enough haematoxylin stain to give a claret-and-water colour. In this the sections remain all night. It is sometimes advantageous to differentiate for a few hours in pure ammonia-alum solution, 5 per cent. The

sections are then transferred to distilled water in which it is necessary to wash out the alum thoroughly otherwise crystals form during de-hydration. At this stage, of distilled water, they are now to be fixed on a microscope slide. A 10 to 20 per cent. solution of clear gelatin is prepared, to 50 c.c. of which a few drops of absolute phenol are added. I keep a stock of this in a wide test tube and heat it up as required in a beaker of water. A layer of the gelatin is poured on an ordinary microscope slide and with a fine forceps the celloidin sectional blocks are, as quickly as possible, placed in the warm fluid gelatin layer arranged in their proper serial order. The obliquely-cut corner of each section permits of this being done readily and in correct orientation.

In order to avoid premature setting of the gelatin before the manipulation is completed it is well to carry on the operation on a temporary warm stage formed by a glass plate placed over a beaker of hot water. During the operation it is well to affix a descriptive label on the slide in the gelatin of the mount. When the sections are in position the gelatin is allowed to set. Care must be taken prior to setting to get rid of any air bubbles that may form. The setting of the gelatin should be accelerated by placing the slide on a cold surface. Setting should occur before the surfaces of the sections become dry, but if there is any tendency to desiccation of the sections they must be kept moist with a wetted camel hair brush.

When the slide is set it should be placed carefully in formalin solution, 5 or 10 per cent., care being taken to liberate any bubbles that may tend to form. The slide may then be passed through ascending alcohols into absolute, then cleared in benzol and finally transferred to one of the Spalteholtz fluids.

For embryonic tissues I use three parts of artificial oil of wintergreen to one part of benzyl benzoate. The slide is preserved permanently in this fluid. This is obviously one of the limitations of the method, since the slide has to be examined on each occasion in the fluid medium in a Petrie dish. For storage I use grooved glass staining jars. One could, of course, mount in balsam and so obtain permanent solid mounts, but this would be at the expense of just that optimum of transparency which makes the method so valuable; and after all, since it is a laboratory method and not a class method, the inconvenience, except for storage, is not very great. One can easily mount all the sections of even a 20 mm. pig embryo on one 3 in. by 1 in. slide. It is thus possible readily and rapidly to survey the whole structure of such an embryo in the space of one slide with the advantage of visualising the structure in three dimensions.

I confess that I am not yet satisfied with my staining results. The light haematoxylin stain certainly gives exquisite results, and I have latterly used it exclusively, but it tends to fade in time and some of the series shown are not as good as they were some time ago. Paracarmine is permanent but too uniform in tone and lacking in discrimination.

Professor Hill has suggested alum-cochineal to me but I have not had time to try it. I have little doubt that a suitable permanent stain can be devised.

Although I have chosen, as an example of the employment of the method, the preparation of a series of transverse slices, it is obvious that it is applicable to sections made in any direction and in planes that may be either parallel or non-parallel as desired. Sections of some vertebrate embryos in horizontal planes are most instructive as the sections of larval Amia and Cryptobranchus exhibited will show.

The preliminary infiltration of celloidin, and the production of a firm celloidin block, are necessary in order to obtain good clean-cut slices. The mounting in gelatin and not in a layer of celloidin is necessary because the clearing fluid softens and gradually dissolves the celloidin whilst the section itself remains fixed in the gelatin layer. The staining of the celloidin slices before mounting on the slide in the gelatin is necessary since the latter would retain the stain or only imperfectly discharge it. I may point out that quite irregular uncut fragments, or blocks sliced in irregular directions, may be stained and mounted in quite similar fashion, often with excellent results. In fact celloidin blocks may be carved in a variety of ways to bring out special structural features with great advantage, so long as they are not so thick as to become too opaque after even light staining. I may add that whole mounts of chick embryos lightly stained and fixed on the slide by gelatin and then passed into Spalteholtz' fluid are immensely superior to the ordinary balsam mounts of such objects, although, of course, they are not suitable for students' class preparations.

It is not suggested that the adoption of this method will in any way replace the customary systematic study of ordinary serial sections. The method permits of the employment of only quite low power magnification and the finest structural detail is therefore inaccessible by its means.

But I do suggest that a complete exploitation of the structural arrangements of any moderately advanced embryo should now, if possible, include the preparation of one or more Spalteholtz series of thick sections in addition to the usual thin series in the three planes, transverse, sagittal and coronal.

For all forms of study by the method described it is evident that the stereo-binocular microscope is practically indispensable. It is desirable to have an intense illumination of the field, and a convenient source of illumination. I have found the ordinary method of illumination by a substage mirror very troublesome in using the binocular microscope. It is often difficult to get both fields evenly illuminated. The mirrors supplied with the instruments are invariably too small. I have devised a convenient microscope table which permits of constant and adequate direct illumination from below and at the same time enables one to dispense altogether with a special stage; the top of the table forms a stage of far simpler and more effective character.

The table can also be used for work with reflected light by employing any of the excellent electric microscope lamps, for which an alternative switch can be provided.