

## Preparation and Staining of Tissue for Counting Neurons

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### INTRODUCTION

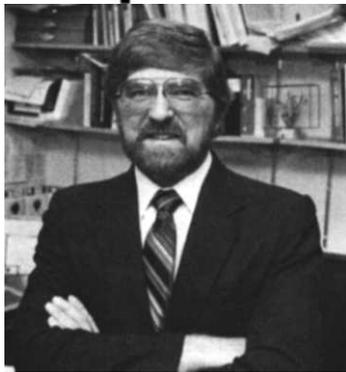
The proper assessment of neuron numbers following various treatments in the nervous system has always been an important part of neuroscience. Because these cells are extremely small, they usually must be determined on histological sections. Although there have been numerous techniques employed for cell counting, a relatively new stereological method, the Disector and Fractionator, has been gaining momentum (5). The Disector technique, which has been described in detail by Gundersen and colleagues (2, 3), is a three-dimensional probe which samples objects with probability that is proportional to their number and not their size. One of the latest improvements has been the use of an Optical Disector. With this technique, one optically sections a relatively thick histological specimen by employing a high power microscopic objective with a high numerical aperture, allowing for a very shallow depth of field. In order to successfully do this, it is important to have histological sections which are extremely transparent and relatively thick (e.g. 40-50um). The objects to be counted need to be spaced such that one object can be easily differentiated from another. This

necessitates that the relative spatial distribution of the objects in the fixed material reflect as close as possible that in the living state. Most regions of the central nervous system have neurons which meet these criteria, even the hippocampal dentate gyrus. Neuroscientists who study the hippocampus would be doubtful of this since histological sections they have seen show a very densely packed granule cell layer. However, if the tissue is prepared properly a very different opinion will soon emerge (see Figure 1).

Many different histological techniques have been employed for the study of brain structure. These include the most common paraffin and frozen or cryostat sectioning. Both of these techniques result in a problematic loss of tissue thickness. It is most obvious with frozen sections (1) which can be cut at 50um but after mounting and drying may only be 6 to 10um thick. Paraffin sections maintain their thickness a little better but can show some drastic reductions ranging from 15 to 50%. The tissue change occurs during the tissue drying and adhesion to the slide. When the thickness compresses, the neurons lose their natural relative distribution and the volume density increases dramatically such that accurate counting is troublesome.

Novel techniques and mounting mediums (e.g. dimethylsulfoxide) have been employed in an attempt to circumvent the dimensional change, but result in poor overall resolution of the tissue. One solution to this problem is to embed the tissue in plastic resins such as glycolmethacrylate. This type of plastic maintains the thickness but has the drawback that tissue must be cut with disposable glass knives using an appropriate microtome such as the LKB Historange. We have found that a reasonable alternative is to embed brains in toto in celloidin. Unfortunately staining relatively thick celloidin sections with cresyl violet or hematoxylin & eosin does not work well. We describe here a method of staining 50um thick celloidin sections with a modified Giemsa stain. This procedure results in a brilliant definition of neuronal bodies with a relatively clear background, creating a section with excellent signal to noise ratio, ideal for cell counting. The celloidin procedure will "shrink" the entire brain the same as the paraffin procedure does. This is not a problem

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## Editor's Column

Here it is almost Christmas. Since this is being prepared in mid-December for publication in January, I can talk about pre-rather than post-Christmas time. We had a

very nice snow early this week and then a few days of cold weather (5 below zero at night). It was great for someone like me who likes snow and winter. It bodes well for the rest of the year, especially since last year we had almost no snow in Kansas City. However, even I might be overwhelmed by 3-4 feet of snow like Buffalo got.

We got up yesterday morning, looked out our bedroom window into the woods behind our house and there were three whitetailed deer bedded down in the snow. It was a beautiful sight, those three deer against the white snow. It was cold though, and I wondered if the deer were really comfortable. How many times do we all see a scene where people seem to be enjoying a beautiful setting but then find out they are suffering and unhappy.

As we go into a new year, there is so much to do in the world to help others become more functional and comfortable. How often do we see a colleague looking "down" and do not greet him or her? How many times has it turned out that the person had a problem and would have welcomed a show of friendship we did not give because we just ~didnT think of" it? Lets all make a New Year's resolution to be more friendly and helpful to our fellow travelers.

The article in this Carrier presents fascinating detail about staining and neuron counting. If you are in this kind of research, it will be very helpful to you.

It was good to see many of you in San Diego at the Neuroscience meetings We wish you all a very

**Happy New Year.**

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since the Disector method utilizes a reference volume and thus uniform shrinkage or swelling of the entire tissue is not an important factor.

## Methods

The entire brain is usually embedded, since the stereology protocols call for a reference volume which necessitates taking sections throughout the entire structure. Frequently the brains are utilized for more than one study and since we do not know what structures may be of interest in the future we save sections from all levels. The only exception that we make is in regards to the cerebellum. We use the area as a "pedestal" which facilitates sectioning throughout the most posterior areas of the hippocampus and cortex. Those interested in the cerebellum can easily use the rostral end of the brain as a pedestal.

Following a post fixation of 24 hours in the fixative we prepare the brains for celloidin embedding. This requires dehydrating the tissue in a graded series of EtOH (50 & 70% - 15 min each; 95 & 100% - 30 min each). The tissue is then placed in a 50:50 mixture of 100% EtOH and ethyl ether for 60 min. The brains are "notched" with a razor blade in order that left and right side can be distinguished after sectioning and staining. It is easier to do this prior to the actual embedding procedure.

The embedding process is begun using a graded series of celloidin dissolved in a 50:50 mixture of EtOH and ethyl ether. Using very small jars with tight fitting lids (celloidin is expensive and you only need enough to cover the tissue), the brains are placed in 2% & 4% celloidin for 24h each followed by placement in 6% and 12% for 48 hours each. These solutions should be made up well in advance since it takes about 10 days for the 12% celloidin to go into solution. The process can be speeded up by placing the bottles on a laboratory shaker. We normally make up 500 ml at a time of each of the stock. You will end up using twice as much 12% since it is also used in the final embedding steps so you might want to have 750 ml.

Although you can make embedding molds out of paper, we have found that the Peel-A-Way® disposable histology embedding molds (22mm sq) work the best. The tissue is placed in these molds with fresh 12% and subsequently exposed to chloroform vapors. Do not let the chloroform touch the celloidin. As the celloidin hardens it has a tendency to shrink, so be sure that the 12% comes to the very top of the mold before exposing to the chloroform fumes. It is also a good idea to have a level surface for this so that the blocks are uniform. To make the identification of the blocks easier, we

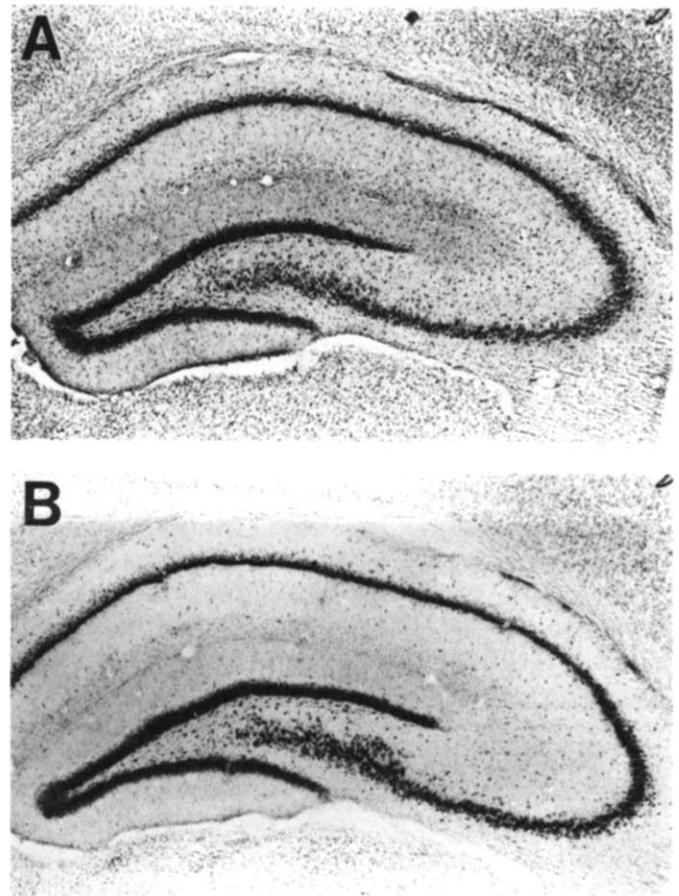
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normally embedded a piece of paper with the identification number written with pencil in the celloidin.

Our laboratory has special jars, which seal very tightly, for the chloroform exposure. Each jar has a small metal platform large enough for about four molds, which sits about an inch off the bottom. The chloroform is placed in the bottom of the jar and the tissue is exposed to the vapors for about 18 hours. The molds are then peeled away. Wooden embedding blocks (25mm sq), (type used for paraffin sectioning) are surrounded by laboratory tape and the celloidin cubes are attached to the wooden blocks with additional fresh 12% celloidin. These are also exposed to chloroform fumes for 18 hours. The tape is removed and the wooden/celloidin block complex is submerged in 70% EtOH until it is convenient to section.

Sections can be cut on a sliding microtome with an ordinary microtome knife. Our laboratory utilizes a Zeiss HM440E sliding microtome, which automatically counts sections. The key to cutting good sections is to keep the block moist with 70% EtOH. This is done by brushing the face of the block between sections. It sometimes helps to partially trim the block with a razor blade such that only a small amount of plastic remains around the brain. This will also help when mounting the sections after staining. Normally the laboratory cuts 50um thick sections for cell counting and these pieces of tissue are stored in 70% EtOH. To keep the sections in order we use plastic Costar cluster wells (#3512) and seal the lids with tape until ready for staining. The sections need to be stained free floating.

We use a modified Giemsa stain (Sigma, St. Louis, #GS-1L), which is a 0.4% w/v solution, in a buffered methanol at pH 6. This stock solution is diluted 1:4 with water and 6 drops of 1% glacial acetic acid is added per 100 ml of this diluted stain. The stain can be made up well in advance and will remain stable for quite some time. The actual staining takes place in Costar cluster wells, with not more than three sections to a well. The sections are stained for approximately 70 minutes at room temperature and then rinsed with 1% glacial acetic acid for 2 minutes. The used stain is thrown away. Sections are then placed in 95% EtOH for 45 minutes, followed by a rinse in 100% EtOH for 2 minutes. The following step is extremely important. The sections soak in cedar wood oil for 5 minutes to reduce background. Since the cedar wood oil can be reused, we normally transfer the sections to special jars containing the oil. Failure to use the cedar wood oil results in sections which are not very transparent and not very useful for cell counting. Finally the sections are transferred to Hemo-D for at least 2 changes of 1 minute each.



**Figure 1.** Coronal sections cut through the dorsal hippocampus and stained with cresyl violet (A) and the Giemsa stain (B). The Giemsa stain has a greater affinity for neurons than it does for glia. This provides for greater ease in neuron counting studies since the background is markedly reduced. Both stains show an excellent delineation of the different neuronal populations in the hippocampus.

Sections are quite pliable until they are placed in the Hemo-D solution. Great care should be taken to flatten out the sections before they reach this step. It is also possible to flatten some of the sections immediately upon placement into the Hemo-D. The sections are placed on slides, covered with permount and cover slipped. Often sections are curled which causes problems with air bubbles after placement of a cover slip. Fishing weights, obtained from any sporting goods store, can be used to hold the coverslip down.

The Giemsa dye solution is alcohol based and contains a mixture of Romanowsky type dyes consisting of methylene blue in combination with eosin Y. It is commonly used in hematology because it stains blood cells polychromatically. Although the Giemsa dye was introduced into neurohistology in the early 70's it has not gained wide acceptance despite the fact that it has been shown to work well in

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conjunction with a wide variety of procedures (4). We feel this procedure provides staining definition superior to many other techniques and is excellent for cell counting studies.

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