

Injected and Colouring Substances Largely Used in Processing Microscopic Anatomical Structures

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There are many fixing and colouring substances largely used in the anatomy laboratories, for teaching purpose. We have injected 22 adult human hearts, removed from the cadavers belonging to the Anatomy and Embryology Department. On 6 specimens we have followed the embryologic myocardium structures for micro angio architecture studies. We used histological techniques like: haematoxylin-eosin, Van Gieson, and Masson's Trichrome methods. Also we have removed and dissected 14 kidneys and 16 anatomical structures of the peripheral nervous system: coeliac ganglia, aortico renal ganglia and mesenteric ganglia. The least were analyzed using specific techniques of the nervous system: Nissl method and Bielschowsky silver stain method. 8 fetuses (aged between 4 and 8 months of gestation) were dissected and also analysed. Our results were in according to the literature studies.

Keywords: staining methods, anatomical specimens, ganglia, fixation

Microscopic anatomy deals with the very small specimens of human body. For a better preservation, some steps must be followed: dissection of the viscus, fixation, slicing and colouring. There are two important steps: fixation and colouring. Fixation involves substances with special chemical properties, related to proteins amine group, stopping the decay and preserving the tissue shape and structure. Colouring is the last step; highlights the cells and fundamental substance. Special colourings reveal nucleus chromatin, elastic fibres, conjunctive fibres, reticulin, and fat deposits.

Fixing solution properties must be: killing faster the cells, penetrating the tissue, keeping natural the structures, keeping smooth the structures. Such substances are: formalin, Bouin solution. Proper cadaver enzymes destroy the tissue (autolysis), but this process is stopped by fixation, so as faster the fixing solution acts on the tissue, as faster the structures remain intact. Fixing solutions having great power of dispersion are suitable for the preservation techniques: acetone, alcohol and acetone, ethylic alcohol acetic acid, chloroform, methyl alcohol. Some of them like alcohol and chloroform destroy the lipids, others the carbohydrates. The shrinkage of the tissues reaches 40% of the primary volume after alcohol fixation and paraffin inclusion. Using Bouin solution, final shrinkage is up to 1%. Diluted formalin swollen tissue until 5% and then shrinks it up to 20% [1].

The formalin is used largely in the anatomy laboratories, but its harmful action on teguments and *mucosae* avoids its using on large scale. Smaller sections are embedded in formalin (4 - 6 μ), leading to a careful and accurate examination of the structure. This procedure allows one single row of cells examination, but the disadvantage is the long period for inclusion, in order to avoid deformations. For this reason progressive ethylic alcohol solutions are

used, from 70 degrees to 96 degrees, the last treatment is with absolute alcohol, allowing the removal of all the tissue water. The last part is paraffin and chloroform (equal parts) inclusion and/or then only double paraffin inclusion (2 - 24 h in total) [2,3]. The glycerine is considered good preservative, with maintaining appearance, consistency and viewing structures, with no odour, easy handling and exposure of the parts in an anatomy lab. Another benefit to the glycerine use is the possibility of exposure of the outside part of any solution, which facilitates the work, reduces health hazards for those working daily with anatomical specimens [4].

Experimental part

Material and methods

For teaching purpose, we have injected 22 adult human hearts, removed from the cadavers belonging to the Anatomy and Embryology. The hearts were injected *via* Valsalva aortic sinuses, using needle syringe 25 mL of black ink, for the left coronary artery we have injected both, circumflex artery and left anterior descending artery with equal quantities. After that the samples were fixed in formalin. On 6 specimens we have followed the embryologic myocardium structures for micro angio architecture studies. We used histological techniques like: haematoxylin-eosin, Van Gieson, and Masson's Trichrome methods.

Also we have removed and dissected 14 kidneys and 16 anatomical structures of the peripheral nervous system: coeliac ganglia, aortico renal ganglia and mesenteric ganglia. The least were analyzed using specific techniques of the nervous system: Nissl method and Bielschowsky silver stain method. 8 fetuses (aged between 4 and 8 months of gestation) were dissected and also analysed. Specimens were conducted in accordance with the

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Romanian law / procedural norms for medical legal expertises No. 1134/C/25/05.2000 (Ministry of Justice), and 2 55/4.4.2000 of the Health Ministry, Art. 42/ ethics of autopsy practice.

Results and discussions

Our studies have focused both on the identification of microscopic structures in adult anatomical preparations and in ontogenesis. There have been used vital and supra vital colouring substances. A vital colouring is an accumulation of the colloid particles in cells cytoplasm, especially in the reticulo histiocitary system cells. A supra vital colouring is an accumulation of electropositive colouring substances in the cytoplasm organelles. We have introduced the vital colouring substance by intravenous way, subcutaneous or intraperitoneal. Injected quantity must be dosed very well because there is a supra colouring risk (higher when intravenous way is used). Fixation and conservation techniques are necessary to stop the autolysis. Conservation preserves this state of fixation. Common fixation techniques are perfusion, injection, and immersion (Romeis, 1989). Fixation techniques involve formaldehyde, ethanol, or propanol (Wilcke et al., 1987). Phenol, formerly used for anatomical conservation, was abolished due to its toxic, skin-irritating, and possibly mutagenic effects (Murray et al., 2007) and was replaced by formaldehyde; even the last one is also toxic.

Haematoxylin-eosin method

Haematoxylin and eosin (H&E) stains have been used for decades and are still essential for recognizing various tissue types. The stain has been unchanged for many years because it works well with a variety of fixatives and displays a broad range of cytoplasm, nuclear and extracellular matrix features [5,6]. Haematoxylin has a deep blue-purple colour and stains nucleic acids by a complex, incompletely understood reaction. A disadvantage of haematoxylin staining is that it is incompatible with immunofluorescence. It is the fastest laboratory colouring method. Eosin colouring takes 1-2 min, distillate water 1-2 min and xylol: 2-3 min [7-9, 45]. On the formalized kidneys we have performed H&E staining, highlighting the glomeruli and arterial vessels. Other authors also used in their studies the same staining, but with time reducing and no such satisfactory results [10] (fig. 1).

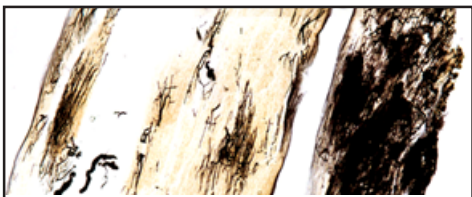


Fig. 1. Left ventricle septal wall, human adult specimen, Black ink method, X40

Masson's Trichrome Stain

Masson's Trichrome Stain requires the following steps: Lugol solution and natrium hyposulfite if there was a previous fixation in the sublimate solution; Weigert ferum haematoxylin (10 min); lithium carbonate 1 minute; fuxin acid + 4 cm³ acetic acid + 300 cm³ distillate water 15 min; finally 1% distillate water and molybdic acid for 4 min [11-15] (fig. 2).

Van-Gieson's Stain

It can be used any universal fixation substance. The slices are immersed 5-10 min in Weigert ferum haematoxylin, then 3-4 min under tap water. The second

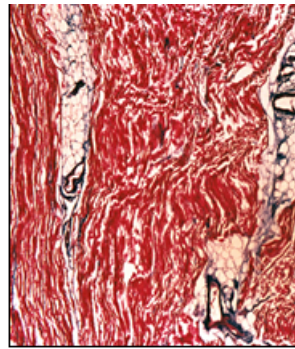


Fig.2. Interventricular wall of the heart - micro arteries contained in the embryonic myocardium, Masson's Trichrome Stain, 10x

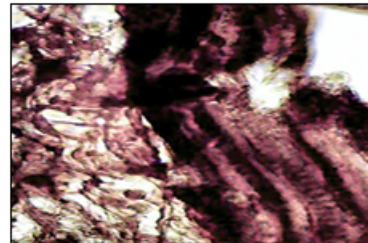


Fig.3. Coeliac ganglion, 4 month- human foetus. Bielschowsky silver staining. 400x; coeliac neuroblasts are seen

step is saturated solution of lithium carbonate until dark blue colouring. Finally, the specimens are immersed in picrofinuxin solution. For micro vascular microscopic study on sinuatral node pathway we have used human adult specimens, either intra coronary black ink injected, either using haematoxylin-eosin, Masson's Trichrome Stain and Van Gieson Method [16-22].

Clinical studies made by some authors showed the importance of the reperfusion areas [23-26,46,47]. They have showed a major importance of knowing the heart structures micro vascularization: moderator band, papillary muscles, and embryonic miocardium. The main threatening is myocardial necrosis and the reperfusion type possibility [27-30]. Haupt et al. (1983) discussed the flowing possibility through a collateral artery, left anterior descending artery and through the moderator band, when a right proximal coronary artery occlusion occurs [31,32]. Kawashima et al. (2003), Berdajs et al. (2003), and Bermanke et al. (2002) [33-35] studied the structure and ultrastructure of the moderator band capillaries on foetal and adult specimens. They consider *trabecula septomarginalis* formed by proper myocardium and a very thin Purkinje cells fascicle peripheral disposed. Cardiac microvascular bed is essential for cardiac pump role. In the heart there is a coronary perfusion increasing, that leads to the oxygen consumption augmentation and the contraction force. Left papillary muscles are important in this pump role [36-38]. The number of papillary muscles is individual variable, could be double (74% of cases) or unique (61%) [39 - 42].

Histological techniques for nervous tissue samples

Histological techniques for nervous system elements identify: nervous cell, its prolongations, myelin sheath, nervous fibres, glial tissue. Firstly, a fixation is necessary. The most used is formalin 10%, then mix of ammonium bromide and formalin; ethylic alcohol; potassium bichrome. Because the nervous system is rich in fat, inclusion technique leads to shrinkage. Neurofibrils are studied using impregnation techniques.

Nissl Method

Original Nissl method requires alcohol 96 degrees fixation. We have used a modified variant of this method, which can be applied even on the structures fixated in formalin. The results from our study were satisfactory. Slides colouring with toluidine blue 0.1%, 12 - 15 h. Next

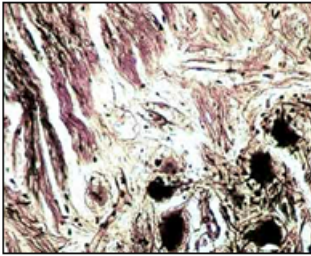


Fig. 4. Adult coeliac ganglion; Coeliac ganglion neurons; well represented dendrite network. Bielschowsky silver staining, 200x

step was embedded in distillate water and a saturated solution of lithium carbonate diluted 10 times. This method is used for the detection of Nissl body in the cytoplasm of neurons on *para* formaldehyde or formalin-fixed, paraffin embedded tissue sections.

Bielschowsky Method

Max Bielschowsky improved this method by incubating formalin-fixed frozen sections in the silver nitrate prior to the ammoniac silver solution [43].

We have used specimens from recently dead bodies fixated in formalin solution 20%, 3 - 4 mm, 5 - 6 days; samples are washed with tap water several hours and distillate water 2 h. Then, silver nitrate 2% solution is used

for 3 - 5 days in the obscure chamber. A short washing in bi distillate water and ammonium silver solution is performed: in an Ehrlenmeyer bottle are mixed 10 cm³ from silver nitrate 10% solution in bi distillate water. Over this, 10 cm³ of aqueous solution of natrium hydroxide is added. The result was a silver oxide precipitate. A rinse in distillate water and a formalin 20% reducing during 12 - 24 h is performed. The slices were immersed in a solution formed of: 5 drops of gold chloride solution 1% + 10 cm³ distillate water + 2 - 3 drops of glacial acetic acid.

Toshiki (2007) used [44] sections in 50 mL 10% silver nitrate in dark at 37°C for 30 min. He washed sections in 0.1% ammonium hydroxide 3X for 2 min at room temperature. Then, he added 350 µL developer solution (0.2 mL 37% formaldehyde, 12 mL dH₂O, 12.5 µL 20% nitric acid and 0.05 g citric acid) to the silver hydroxide solution. He washed in 0.1% ammonium hydroxide 3X for 2 min and dH₂O 2X for 2 min and toned in 0.2% gold chloride for 5 min. Finally, he fixed it in 5% sodium thiosulfate for 1 min. Other modified staining methods have been used during time [42] (figs. 3,4)

Dendritic morphologic evolution and neuronal specialization are highlighted by this method.

Table 1
DIFFERENT STAINING METHODS USED IN LABORATORIES

No.	Staining Method	Structure	Appearance	Disadvantage
1.	H&E	nuclei	stained blue	Incompatible with immunofluorescence
2.	H&E	cytoplasm and extracellular matrix	degrees of pink	Incompatible with immunofluorescence
3.	H&E	Nuclei	heterochromatin condensation	Incompatible with immunofluorescence
4.	H&E	Nucleoli	blue cast	Incompatible with immunofluorescence
5.	H&E	Golgi zone	Absence of staining in a region next to the nucleus	Incompatible with immunofluorescence
6.	Masson's Trichrome	Nuclei , muscle	red	None
7.	Masson's Trichrome	Nucleoli	black	None
8.	Masson's Trichrome	Cytoplasm , amyloid	Pink-purple	None
9.	Masson's Trichrome	Erythrocytes	Orange	None
10.	Masson's Trichrome	Conjunctive tissue, mucus	blue	None
11.	Van-Gieson's	Collagen	Red	None
12.	Van-Gieson's	Smooth and striated muscle	Yellowish to brownish	None
13.	Van-Gieson's	Cornified epithelium, Hyalin, Cytoplasm	Yellow	None
14.	Van-Gieson's	Nuclei	Blue to Black	None
15.	Van-Gieson's	Erythrocytes	Gold	None
16.	Nissl	Neurons	purple-blue grains	Long time
17.	Nissl	Nucleolus, nuclear membrane	Intense coloured	Long time
18.	Bielschowsky silver	Neurofibrils	black	Only nervous tissue
19.	Bielschowsky silver	Conjunctiv tissue	Yellow-grey	Only nervous tissue

Conclusions

Special colourings reveal nucleus chromatin, elastic fibres, conjunctive fibres, reticulin, and fat deposits.

Histological techniques for nervous system elements identify: nervous cell, its prolongations, myelin sheath, nervous fibres, glial tissue.

Bielschowsky's silver stain is a very useful tool to detect nerve fibres. It can be used to stain axons, neurofibrils and senile plaques in the central nervous system. This method is easy to perform in clinic (Alzheimer's disease).

As teaching purpose, these methods are very useful in the anatomy field.

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