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MODIFICATIONS OF DIFFERENTIAL STAINS WITH SPECIAL REFERENCE TO THE TRICHROMIC STAIN OF CAJAL

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Many pathologists use the silver impregnation methods of Cajal and his pupils for the investigation of the nervous system, but very few know and practice the triple stain, primarily described by Ramon y Cajal[3,4] in 1897, modified later by Calleja,[1] and Gallego.[2]

Without doubt this method is one of the best for staining all kinds of specimens composed of different structural elements, and especially for the study of tumors. Briefly the stain described by Cajal is as follow:

(1) Place sections for five or ten minutes in a saturated or very concentrated solution of basic fuchsin.

(2) Wash rapidly in water to remove the excess stain.

(3) Stain for five or ten minutes in a solution of picro-indigocarmin. (Saturated aqueous solution of picric acid, 100 cc. indigocarmin, 0.25 grams).

(4) Wash rapidly in dilute acetic-picric acid solution (3 drops of glacial acetic acid a few crystals of picric acid and 10 cc. distilled water).

(5) Pass the sections rapidly through water to remove the excess of picric acid and to avoid a green shade in the connective tissue.

(6) Decolorize in absolute alcohol until the sections have lost the excess of fuchsin, which will be known by the general violet color acquired by them. This step is the most delicate moment of the process and has to be performed in abundant alcohol in order to limit its duration to a few seconds. Leaving the sections too long in alcohol more or less decolorizes the nuclei.

(7) Clear in carbol-xylol, xylol and mount in balsam.

The nuclei appear intensely stained red, the cytoplasm takes a clear green or rose-yellow stain, and the connective tissue appears a very intense blue.

This method when properly carried out is without dispute one of the best differential stains, but it is difficult to execute because the alcohol acts as differentiating and dehydrating fluid at the same time. If the dehydration is prolonged too long the color of the nuclei disappears and the section appears with a uniform green-yellowish stain; if the sections are kept in alcohol too short a time the differentiation and dehydration do not take place, and the sections take a mixed purple color without any contrast.

To avoid the difficulty of keeping the stain in the nuclei Calleja[1] modified the technic using lithium carmin or borax carmin and counterstaining with picroindigocarmin. With this substitution the nuclei take a deep red color, and the path through the alcohol can be prolonged without fading the color of the nuclei. The differentiation is perfect, and the stain is permanent. Using this method Calleja has obtained beautiful slides of neoplasms.

I have been using this method for several years in sections of eyes embedded in celloidin, and I have obtained slides which demonstrate beautifully the histological and pathological structures of the eye.

Gallego introduced acetic acid as a differentiating fluid, and formaldehyd as a "viro-fixing" fluid for the sections stained with basic fuchsin, observing that after passing the sections previously stained in fuchsin through a solution of diluted formalin the stain was fixed in the nuclei and was insoluble in alcohol.

He[2] modified the trichromic methods of Cajal and van Gieson, and introduced basic fuchsin associated with eosin, to substitute for the ordinary hematoyhlin and eosin stain.

TRICHROMIC STAIN OF CAJAL MODIFIED BY GALLEGO

(1) Fix in 10 per cent formaldehyde (sections made by freezing microtome or embedded in paraffin or celloidin).

(2) Stain one minute in Ziehl's acetic-fuchsin, (Ziehl's fuchsin 10 drops, acetic acid 1 drop, distilled water 10 cc.

(3) Wash in water.

(4) Differentiation and "viro-fixation" is performed in formalin-acetic solution for five minutes (formalin 2 drops, glacial acetic acid 2 drops, distilled water 10 cc.).

(5) Wash in water.

(6) Picro-indigocarmin one minute (aqueous solution of indigocarmin 1 per cent, one part and aqueous saturated solution of picric acid, two parts).
(7) Wash in water, alcohol, carbol-xylol, et cetera.

The nuclei stain a deep red-violet; the cartilage, mucin and "Mazt-Zellen" in very intense bluish violet; the cytoplasm in clear green or yellowish green; the connective tissue an intense blue; the muscle fibers a clear green, et cetera.

This method has the advantage over the original Cajal method in that the nuclear stain is permanent after the sections are treated by formaldehyd, the differentiation is perfected by the addition of acetic acid to the fuchsin and formalin, and the alcohol completes the differentiation and dehydrates, but does not decolorize the nuclei.

VAN GIESON STAIN MODIFIED BY GALLEGO

The method of van Gieson is one of the most variable methods. Sometimes the nuclei take the stain very lightly, or appear with a red or brown color; at other times there is scarcely any noticeable differentiation between the connective tissue and the muscle fibers, and even the best sections fade in time.

The fundamental principle of the van Gieson stain and modifications of Weigert, Masson, Curtis and others, is the over-staining of the nuclei, and the staining and simultaneous differentiation with picro-fuchsin. In the method of Gallego the nuclear stain is weak, it is fixed with formalin, and the differentiation is very intense before the use of the picro-fuchsin. The technique follows:

(1) Fixation in 10 per cent formalin.
(2) Section by frozen, paraffin or celloidin methods.
(3) Acetic-fuchsin, one minute.
(4) Wash in water.
(5) "Viro-fixation" in acetic-formol, five minutes.
(6) Wash in water.
(7) Stain one minute with picro-fuchsin of van Gieson.
(8) Wash in water. Dehydration, carbol-xylol, xylol, et cetera.

The nuclei take a red-violet stain. The cartilage and mucin bluish-violet; the connective tissue intense red; the cytoplasm,

muscle fibers, red blood cells, yellow, bacteria red-violet. The stain is more constant in its results than the van Gieson, and is also more permanent.

BASIC FUCHSIN AND EOSIN STAIN OF GALLEGO

(1) Fix 10 per cent in formalin.
(2) Section by frozen paraffin or celloidin method.
(3) Stain one minute in acidified Ziehl's fuchsin, (fuchsin of Ziehl, 10 drops, glacial acetic acid 1 drop, distilled water 10 cc.).
(4) Wash in water.
(5) "Viro-fixation" in acetic-formalin.
(6) Wash in water.
(7) Counter stain with aqueous solution of one per cent eosin for one-half minute.
(8) Wash, alcohol, carbol-xylol, xylol, et cetera.

The aspect of the sections obtained by this method is similar to those obtained with hematoxylin and eosin. It has the advantage over the last method of more delicacy and beauty of the nuclear stain; the complete transparency of the sections is due to the double differentiation. The stain is almost specific for the fundamental substance of the cartilage, "Mazt-Zellen" of Ehrlich, some mucins, and bacteria are also stained.

For the study of elastic fibers I refer those interested to the work of Gallego.

AUTHOR'S MODIFICATION

I have been using the technic recommended by Gallego with a slight modification which simplifies the method. Ziehl's fuchsin in a solution of ten drops in 10 cc. of water is not sufficiently strong to stain all kinds of specimens, and many times one has to return the sections to the solution of fuchsin after their "viro-fixation" in formalin because of insufficient nuclear stain. To avoid this inconvenience the solution of fuchsin must be stronger, 15 to 20 drops for each 10 cc. of water, and added with two drops of formaldehyd, and 2 drops of acetic acid for each 10 cc. of water. With this solution of acetic-fuchsin-formalin, the stain, "viro-fixation" and differentiation takes place at the same time, and the degree of stain desired can be followed under the micro-

scope. The nuclear stain is completed in from one to five minutes, depending upon the specimens, and over-staining is impossible due to the presence of acetic acid in the solution.

This mixture of acetic-fuchsin-formalin changes color, gradually becoming darker and opaque, loses its selective properties and becomes useless in a few days. Inasmuch as the amount of fuchsin to be used daily is so small it is preferable to use fresh solution every day.

In order to continue the differentiation in this fluid, and to increase the selective properties of the stain which colors the young and old connective fibers in different grades of blue and green, a drop of acetic acid for each 10 cc. is added to the picro-indigocarmin solution. The details of the technic used by the author are as follows:

(1) Fix in 10 per cent formaldehyd.
(2) Section by frozen, paraffin or celloidin methods.
(3) Acetic-fuchsin-formalin (15 drops of fuchsin of Ziehl, 2 drops of glacial acetic acid, 2 drops of formalin, 10 cc. of water). Variable time as determined by observation.
(4) Wash in water.
(5) Indigocarmin-acetic (10 cc. of the solution of picro-indigocarmin of Cajal, 2 drops of glacial acetic acid).
(6) Wash rapidly in water.
(7) Alcohol, carbol-xylol, xylol, et cetera.

The color of the different structures is the same as in the Gallego stain. In most of the fresh specimens it will not be necessary to use the Ziehl fuchsin, since a 1 per cent solution of basic fuchsin will give the same results, but in older specimens the Ziehl fuchsin is more powerful for nuclear selection.

The first steps of my modification can be used combined with the picro-fuchsin of van Gieson or eosin as in the Gallego technic.

If some sections do not take the nuclear stain well they can be sensitized by keeping them for six to eight hours in a solution of formaldehyde at 45°C.

SUMMARY

For sections of the eyes embedded in celloidin, the method of Callaja will give excellent results.

The use of basic fuchsin associated with formalin and acetic acid and counterstained with picro-indigocarmin, picro-fuchsin or eosin, as recommended by Gallego or in my simplified technic is absolutely constant in its result, has the advantage over the original methods of Cajal and van Gieson of being more permanent, and easier to carry out.

The trichromic method of picro-indigocarmin and fuchsin is one of the best differential stains in use today, especially for the diagnosis of tumors, such as neurofibroma, myofibroma, myosarcoma, fibrosarcoma, et cetera and in tumors which have connective tissue framework associated with epithelial cells. In the squamous cell eqithelioma this method shows very beautifully the different degrees of keratinization and cellular differentiation with different tonalities of specific colors. It is also very useful for the study of blood vessels, demonstrating very well the various structures in pathologic arteries and veins, particularly in lesions of thrombo-angitis obliterans. This stain combines the advantages of hematoxylin and eosin staining and the van Gieson stain.

REFERENCES

(1) Calleja, C.: Método de triple coloración con el carmín litinado y el pirocarmín de índigo. Rev. trimest. microgr., **2**, 101–104. 1897.

(2) Gallego, A.: La fucsina básica y el formol en técnica histológica. Trab. de Labor. de Invest. biol. de la Univers. de Madrid, **17**: 95–110. 1919.

(3) Ramon y Cajal, S.: Manual de histológia normal y técnica micrographica. Valencia, 1897.

(4) Ramon y Cajal, S.: Manual de anatomía patológica general. 6th ed. Madrid, N. Maya, 1918. 616 pp.