
A MODIFIED TRI-BASIC-DYE TECHNIQUE FOR NEUROGLIA IN AUTONOMIC GANGLIA¹

JAMES L. HALL AND ROBERT C. SCHWYN

Department of Anatomy, The Ohio State University, Columbus, Ohio 43210

ABSTRACT

A specific Tri-Basic-Dye staining technique has been developed for demonstrating neuroglia nuclei in autonomic ganglia. Criteria used for development of this technique was simplicity, specificity, minimum time consumption, paraffin sections, and good photographic qualities. Modifications of a Tri-Basic-Dye procedure, originally reported by Spoerri (1948) for nerve cells and Nissl granules, have elicited a greater differentiation of neuroglia nuclei and greater intensity of their granular pattern. Neuroglia cells are readily distinguishable and well differentiated in the presence of nerve cells and Schwann cells. This technique is recommended for all investigators interested in a simple, rapid, and excellent technique for demonstrating neuroglia nuclei and their relationship to other structures in the autonomic and peripheral nervous system.

The growing realization of the importance of neuroglia, and the interpretation of certain histological phenomena in which they are involved, necessitates newer and different investigative techniques. Most early investigative studies of neuroglia utilized metallic impregnations such as the silver staining methods of del Rio Hortega and the gold sublimate methods of Ramon y Cajal for demonstrating cellular processes. Metallic impregnations are not only difficult to master, but also generally require frozen or celloidin sections cut at approximately 15 to 20 micra. Most staining procedures utilizing paraffin sections are primarily designed for studying the structural appearance of neurons or nerve endings. The staining characteristics of neuroglia are only mentioned in passing. Literature describing specific methods for staining neuroglia in autonomic ganglia is very sparse. Staining procedures obtaining the best results with neuroglia are suggested mainly because neuroglia nuclei contain deoxyribonucleic acid and other unknown substances that will absorb almost any basic dye.

The purpose of this work was to develop a staining procedure specifically for

¹Manuscript received March 2, 1964.

demonstrating neuroglial nuclei in autonomic ganglia and other portions of the peripheral nervous system, and capable of meeting the following requirements.

1. Simplicity: The procedures involved from fixation of tissues to mounting stained sections must be simple and require minimum time consumption. The possibility of artifacts is reduced considerably by reducing the time and steps involved as a tissue is changed from one medium to another.

2. Paraffin sections: This technique is most desirable for obtaining very thin sections in comparison to thicker sections obtained by the freezing method.

3. Specificity: Detailed staining of nuclear material is very important. If the cytoplasmic processes absorb the stain to any extent, the resulting fuzziness in the microscopic field hinders the detailed observation of qualitative and quantitative aspects of neuroglial activity.

4. Good photographic qualities: The capacity of stained tissues to photograph well is necessary for demonstration, clarification, and comparison of neuroglia. Photographic quality also depends largely upon the excellence of the previous three requirements.

Modifications of a Tri Basic neurological stain, originally developed by Spoerri (1948) as a stain for nerve cells and Nissl granules, best meets the above requirements, and has provided us with an excellent staining method for neuroglia in autonomic ganglia.

MATERIALS

Spoerri's original fixation procedure recommended 10 per cent formalin, 95 per cent alcohol, and a combined Bouin and formalin solution. Considering the physicochemical theory of staining tissues, the combined action of pH and reagents of a fixative exerts a complex displacing action on the isoelectric point of protein during fixation which influences the intensity of staining (Lascano, 1958). With this theory in mind, we have employed a formalin sublimate acetic acid fixing solution designed to give more detailed and intensive staining of neuroglial nuclei. The formula used is as follows:

Mercuric chloride	152.4 g
Formaldehyde (conc)	762.0 g
Glacial acetic acid	190.0 g
Distilled water	3048.0 g

The staining solution is the same as Spoerri's original except for the reduction in quantity of Thionin used in the formula:

Cresylecht violet	2.0 g
Toluidine blue	1.0 g
Thionin	0.1 g
30 per cent alcohol	200.0 ml

Differentiating solution is acidified distilled water reduced considerably from the original solution. The solution required for differentiating neuroglia is a mixture of 0.5 ml HNO₃ and 2000 ml distilled water. Since the original procedure was designed for staining nerve cells and Nissl granules, it is conceivable that a stronger solution was needed due to the density of the substance to be differentiated. Such is not the case with the relatively smaller neuroglial nuclei, thus a weaker acidified aqueous solution was necessary.

STAINING PROCEDURE

1. Fix tissues in formalin sublimate acetic acid solution not longer than 24 hr. Tissues of normal thickness allowed to remain in the fixative in excess of 24 hr tend to harden and shrink.

2. Dehydrate in dioxane for 24 hr. This is a softer method than using alcohol for dehydration, and tissues may be left in solution up to 3 days without any ill effects.

3. Transfer to a solution consisting of 50 per cent Dioxane and 50 per cent Bayberry latex-based paraffin imbedding solution at 55 C for at least 1 hr.
4. Transfer to 100 per cent Bayberry latex-based paraffin for at least 2 hr at 55 C.
5. Imbed in fresh Bayberry paraffin and place into previously prepared ice water.
6. Cut sections 4 to 7 micra.
7. Fix sections to slide with gelatin powder dissolved in distilled water and place on a warming table for at least 3 hr.
8. Remove wax with xylene.....5 min
9. Bring to water through a series of alcohols of decreasing strength,

100 per cent.....	2 min
95 per cent.....	2 min
80 per cent.....	2 min
70 per cent.....	2 min
70 per cent plus iodine crystals.....	2 min
(To remove mercuric chloride)	
50 per cent.....	2 min
Distilled water.....	2 min
Sodium thiosulphate solution.....	5 to 10 min
(To remove iodine)	
Running tap water.....	10 min
(To remove sodium thiosulphate)	

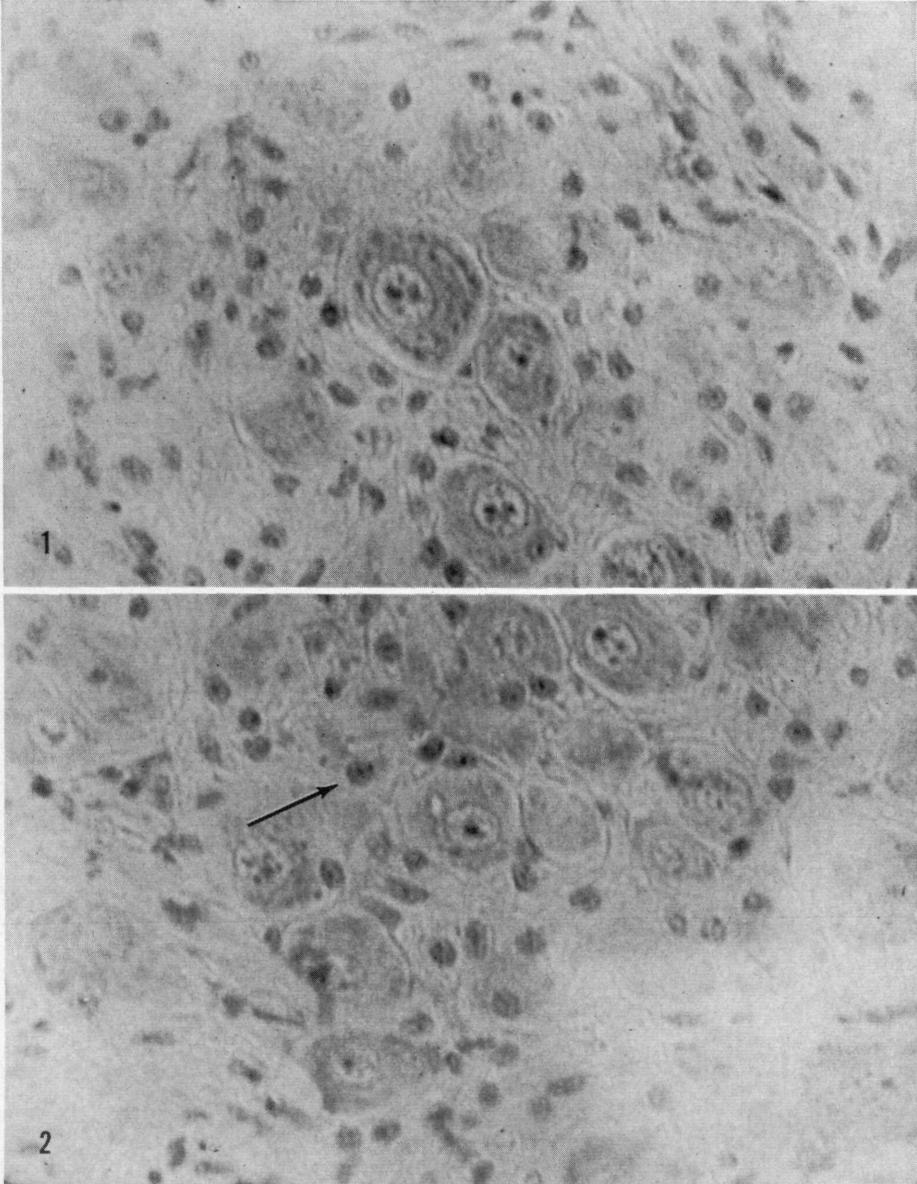
 Tissues may remain in water a reasonable time before staining.
10. Pre-heat stain to 80 to 90 C. Maintain a second solution at approximately 65 C.
11. Stain sections at 80 to 90 C..... 10 sec
12. Pass into differentiating solution.....3-4 sec
13. Thrust immediately into ice cold water and agitate.....5-10 sec
- Check coloring of section; if too light, repeat step 11 for 3 sec and step 12 for 1 sec.
14. Differentiate further in 80, 95, and back to 80 per cent.....2 sec each
15. Dip slide into slightly cooled stain (65 C).....2 sec
16. Return to 80 per cent alcohol.....1 sec
17. Return to cooled stain.....1 sec
18. Stop staining and differentiation process by rinsing in ice-cold distilled water.....5 to 10 sec
19. Dehydrate rapidly, 80, 95, and 100 per cent alcohol.....2 sec each
20. Xylene I.....1 min
21. Xylene II.....3 min
- Colors tend to fade from neuroglia nuclei if sections are allowed to remain in xylene beyond a combined total of 4 min.
22. Dry rapidly and mount in Piccolyte.

DISCUSSION

These procedures elaborate neuroglial nuclei in autonomic ganglia compared to de-emphasis of nerve cells and Nissl granules (see fig. 1, 2). The DNA and other nuclear substances stain deep bluish purple, with excellent differentiation of granular pattern. The nuclear membrane is even darker and very distinguishable. Cytoplasmic processes have a light bluish tint and enhance the nuclei in their light background. Repetition of steps 16 and 17 are only necessary once or twice at the maximum. The acidified fixing solution apparently has increased the nuclear protein affinity for basic dyes so that staining intensity is brought out during fixation more so than during staining procedures. Differentiation during the staining procedure must be performed with a weak acidified distilled water

solution due to the density of the neuroglia nuclei. Spoerri's original solution was designed to differentiate the greater density of nerve cell bodies, thus requiring a stronger acid solution.

This stain also provides a good differentiation between neuroglia and Schwann cells when they are present in the same section. With this stain the Schwann



FIGURES 1 and 2.

Photomicrography of superior cervical ganglion showing larger autonomic neurons with slightly eccentric nuclei and homogeneously distributed Nissl substance. Neuroglial nuclei appear as smaller oval darker staining elements scattered throughout the field.

cells appeared darker, of uniform density, and relatively translucent. In contrast, neuroglia exhibited a lighter, more opaque, and randomly scattered granulation pattern.

It is our intention that this method may be beneficial to all those interested in a simple, rapid, and excellent technique for staining neuroglia in the peripheral nervous system. To those beginning a pursuit of structural relationships of neuroglia, nerve cells, and Schwann cells in autonomic ganglia, this procedure may be of significant value.

REFERENCES

- Lascano, E. F.** 1958. A glycine-HCl-formalin fixative for improved staining of Neuroglia. *Stain Technol.* 33(1): 9-14.
- Spoerri, Rosette.** 1948. A tri-basic-dye stain for nerve cells. *Stain Technol.* 23(3): 133-135.
-