Silver Impregnation for the Nervous System Being Used in Our Laboratory.

By

Hachiro Seto.

(From the Anatomical Laboratory of Prof. H. Seto, Medical Faculty, Tohoku University, Sendai.)

(Received for publication, September 11, 1950)

To be most regarded as a matter of grave affair in the study of histology of nervous system is that the studying method should not be incomplete. Because any natural and real views could never be effected, if there was a fault in the staining. As our method ought to be compared with the diagnosis before the treatment of the clinical authority, it is beyond dispute how important it is.

That the histology of nervous tissue has fallen too much behind that of other tissues in development depends upon such a fact, that discovery of its successful ideal staining has not appeared because of its extreme difficulty. In order to clear the definitive signification of the nervous tissue one must try in earnest to represent not only its essential elements, "Neurofibrils", positively, but also at the same time its accessory elements and the other various tissues surrounding so specially and beautifully, that the evident difference between both of them might be easily examined through the microscope. The so-called ideal staining is namely such a method, that all kinds of tissues in every section can be stained clearly at the same time. It is therefore considerably difficult to originate such a brilliant one.

The silver impregnation of Bielschowsky which followed Cajal's one was the very first step to the ideal staining. Bielschowsky's impregnation has since then given birth to the various modifications by many authors. The impregnation modified by Stöhr since 1928 has been above all extraordinarily so excellent, that he has succeeded with his pupils in discovering many new views. His great definitive contribution to the histology of nervous system has been commended universally by the world.

I had in 1932–33 an opportunity to study the nervous histology under Prof. Stöhr at the Anatomy of University Bonn a. Rh. in Germany, learned his silver impregnation directly from him and was impressed greatly by its superiority. After returning to Japan I have still now tried so earnestly
to make the Stöhr's method more ideal, and succeeded before several years in finding out such an excellent one, as follows. Its excellency became already the object of the extraordinary admiration of Prof. Wasano, Kyushu University, and I am also confident of the best one of the staining formulas for the nervous system, which self and others together should recognize.

1. **Operation.** To fix use 10 per cent neutral formol. For this purpose one needs in the first place to neutralize the commercial formol solution by adding calcium carbonicum precipitatum in proportion of 1/12. At the moment of using it, dilute this mother solution to 10 per cent with distilled water, taking its upper solution already cleared. The materials remain almost more than half an year in this fixer, being sectioned with the freezing microtome afterwards. The sections remain further in the fixer and when desired to stain only such a few pieces as desired are taken out from the formol solution.

When one will hurry in making preparations, section the material with the freezing microtome after fixation for 1 to 2 weeks and continue further to fix, one could then acquire good results after 2 or 3 months. 30μ to 40μ is suitable in thickness. If the sections are too much thin, the cubic observation of the pictures of tissues is not effective.

2. **Operation.** For the operation of the sections from now, use a glass stick bended ahead hook-like. The number of sections to be stained are suitable for the beginner about 7. The sections taken out from the fixer remain now for a short time in distilled water.

3. **Operation.** Then place for 1 to 3 days in 20 per cent silver nitrate fluid filled in a glass plate of good quality. This silver solution is for about 20 days useful.

4. **Operation.** After immersing for 10 to 20 seconds in distilled water one must turn to the 3. operation. So one should prepare the 5. operation before passing to the 4. operation from the 3. operation.

5. **Operation.** One must be ready to make 200 to 300 cc. of 20 per cent neutral formol, which should be made only by deluting the mother neutral formol by all means with running water. This formol solution is then divided into 4 to 3 plates. The sections placed in distilled water in the 4. operation are transferred now by turns from the first plate to the last. Here is reduced a part of silver, producing milky precipitates. This precipitation is carried in the first plate most vigorously, decreases gradually in the 2nd and 3rd plate and disappears almost entirely in the last plate. It is interesting that the disappearance of the precipitation in the last plate is limited only for the materials fixed with neutral formol, while the phenomenon of this kind does not appear on the sections fixed with acid formol, that is to say, the white precipitates do not disappear in the later
instead of renewing water formol solution even more than 10 times, of course without effecting good results in staining afterwards.

6. Operation. Next procedure consists in transfering sections to ammonical silver solution just after washing with running water. As the washing time in the water is very short, one must set about making a ammoniacal silver solution as soon as one has finished the 5. operation.

Formula of ammoniacal silver solution. Set a suitable amount of 20 per cent silver nitrate solution into a glass tube kept clean with distilled water, then drop into it ammonia powerful as possible suitably. The amount of dropping ammonia is not settled constant throughout the four seasons because of having a intimate relation with the chamber temperature, but it is kept in general in 1/5 proportion for silver fluid. In the limiting part between silver fluid and dropped ammonia appears a brown line. Shake the tube up and down after stopping a cork, then this mixture becomes brown. If one could operate, that the precipitation might be colored very poor and weak yellow, such a operation is most convenient, because the mixture becomes immediately clear by adding further only a drop of ammonia. It is the most ideal formula of ammoniacal silver fluid. If the precipitates are produced abundantly and all the fluid has become dark brown, one must continue the dropping of ammonia and try to clear it with a last drop. If, on the contrary, the mixture has become transparent by the first shaking, one can make clear it by means of dropping ammonia after producing brown precipitation of the minimum by adding a little amount of silver nitrate.

It would be especially noticed, that the formula of ammoniacal silver solution must be finished as rapidly as possible. The time the longer, the staining results afterwards the worse.

The ammoniacal silver fluid prepared in now placed in a watch glass. The subject returns here to before. The sections in the last plate filled with 20 per cent neutral formol, which was diluted with running water, are transferred by turns to running water in a plate. This procedure consists really in removing the affinity of connective tissue with silver. It deprives namely the connective tissue of its silver affinity, which appears in general as in the same grade as for the nerve elements, and contributes to make easy very much the distinction between the connective tissue and the nerve elements which stain a dark violet color. This procedure requires about 30 seconds, but the time in running water has to be controlled by various conditions.

Next taking out the sections by turns from the running water and blotting the water with filter paper, transfer to the ammoniacal silver solution prepared in a watch glass, then they are stained gradually a reddish brown color. This staining should be finished by all means just in
about 10 minutes. It is here very important not to hurry the staining but to proceed gradually. When the speed of staining is too fast, silver precipitates stick so strong to the sections, that the results are not good, when it is too slow, they are more effectless.

By the way, the proceeding speed of staining is in proportion with the chamber temperature. When the temperature is high, the sections stain easily, when it is low, they stain hard. Especially in the case of the later therefore, the warming of the watch glass with ammoniacal silver is decidedly necessary.

This warming formula is to be acted with following standard. When the chamber temperature is nearly 0°C, the watch glass is warmed by boiling water filled in a plate, when about 10°C, it is warmed by warm water, then gradually by boiling water, when about 20°C, one needs any warm water. In the middle of summer the staining is possible without warming, but even in this time it effects much better to use lukewarm or warm water.

By Stöhr's method one observes often the sections in the watch glass, with ammoniacal silver fluid through the microscope, in order to examine how they are staining. But it is unnecessary in our method to do it by experience: after observing with naked eyes the best staining tincture of the sections, in other words at the time, when they seemed to have stained most convenient—as it is possible to get the standard of the staining condition by examining the preparations of the sections, which already completed, through the microscope, so we have only some practices before at least 2 or 3 times by using the sections of the same material—we transfer to next operation, merely picking them from the fluid without using microscope.

7. Operation. Next by Stöhr's method the sections are transferred to 2/8 diluted ammoniacal solution with distilled water. But the fluid is still too strong. If we want to see it, it must be more diluted to about 1/3. This procedure consists in removing the tumultuous precipitation of silver. In 20 to 30 seconds the sections are immersed in a dilute solution of acetic acid, in order to acidify just a little. But it was cleared that the operation of this kind does more harm than good, especially acetic acid stains the sections a bluish tinct so often. Therefore instead of using these reagents, we have succeeded in acquiring better result by means of carrying the sections successively in 2 plates filled with distilled water.

8. Operation. Next the sections are transferred to a 1/1000 or 1/5000 solution of gold chloride. Gold chloride truely should be used as a solution as dilute as possible, that is the best showing only a weak yellow tinct. Because if it is too strong, it acts upon the sections so rapidly and vigorously, that not only the nerve elements, but also the other tissues stain univer-
sally a bluish black color. When the sections are stained so convenient in about 3 to 4 hours.

9. **Operation.** One immerses them in 15 to 20 per cent natrium hyposulfite solution, after washing them in distilled water. This fluid is also for about 20 to 30 days useful enough. Natrium hyposulfite removes black brown stained excess of gold from the sections and gives them now a beautiful reddish violet color. After about one minute.

10. **Operation.** Wash the sections with distilled water 2 times, dehydrate gradually in 70, 90, 95 per cent alcohol (often absolute alcohol is used too), transfer to Carbol-Xylol and mount in Canada Balsam at last.

On the preparations thus stained there is no anxiety for changing their color in future, on the contrary it seems that they are increasing their clearness day by day.

By our staining method not only the nervous elements are able to be stained to extremely fine neurofibrils a beautiful black violet tinct, but all the other tissues also take every their special colors. Therefore the nerve innervation of every tissue can be made so clearly.

At the end in the case of trying to use the impregnation for the organs which contain bone tissue, one must decalcify in the first place before silver staining. The decalcifying method in this case, of course, must be somewhat modified from the usual one. The principle of this modification, that is to say, depends upon not to remove the material from the formol fluid. Because if the material is apart from the formol solution the silver staining afterwards never gives us satisfactory results.

This special decalcifying is just carried out as follows. For the purpose of decalcifying is used the mixture of 10 per cent neutral formol 49 cc., 5 per cent nitric acid 49 cc. and acetic acid 2 cc. When one uses the mixture of this formula, the decrease of the impregnations power of silver does never take place. After decalcifying one washes the material with running water for 24 hours, next for 5 to 7 hours in distilled water (several times renewing), then transfers it to 10 per cent neutral formol again and sections it by using the freezing microtome after 2 or 3 days.

**References.**


Wasano, Kyudai Iho, 1940, 14, 362.