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Methanol-cellulose Nitrate Embedding Method for Histological Morphometrical Analysis

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Summary

Very recently we developed a new embedding medium, "Shiojirin-E", made chiefly of cellulose nitrate (Kawakami et al. 1995)[1,2]. During the course of its development, we noted that cellulose nitrate would be a very suitable embedding medium for morphometric analysis, especially because of its low and constant rate of contraction. But the high-viscosity of this medium resulted in a very slow rate of infiltration. Therefore we sought to develop a new, better histological embedding medium for morphometric analysis based on information on the methanol-cellulose nitrate method by Seki (1937)[3]. After trial and error, we succeeded in our goal : The composition of the medium is as follows : cellulose nitrate 15.0 g, methanol 78.6 g, and others 6.4 g (total of 100.00 g). When the specimens are not so big, we can easily get 3-5 micron-thick sections like paraffin sections with complete non-contractibility. We believe that this new methanol-cellulose nitrate medium will be useful especially for histological morphometrical analysis.

Introduction

In Japan, the embedding medium "Celloidin (T. Co., Tokyo, Japan)" has been used in the fields of hard tissue research and morphometric analysis. However, this medium is no longer commercially available. Therefore, we developed a new histological embedding medium to replace "Celloidin". This new medium, "Shiojirin-E", is made chiefly from cellulose nitrate (Kawakami et al. 1995)[1,2]. During the development of Shiojirin-E, we noticed that cellulose nitrate was a very suitable medium for morphometric analysis, because of its low and constant rate of contraction. This new medium is useful for preparing sections of various tissues including hard ones. But the high viscosity of the medium results in a low rate of infiltration ; and, therefore, the embedding procedure requires a long time. A low-viscosity embedding medium based on cellulose nitrate is thus desirable. Therefore, using chiefly cellulose nitrate and information from Seki (1937)[3], we decided to develop

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a new low-viscosity embedding medium.

Embedding Medium "Methanol-Cellulose Nitrate"

Cellulose nitrate, the main component of the medium, was from the same source (Daito Industry Co., Ltd. Tokyo, Japan) used for the medium Shiojirin-E. The components per 100 g of the medium were as follows: cellulose nitrate, 15.0 g; methanol, 78.6 g; and others, 6.4 g. The mixture was blended until the cellulose nitrate had completely dissolved in the methanol solution. After that, the blended medium was diluted with methanol to make graded concentrations (2, 4, 6, 8, 10, and 12%) in terms of cellulose nitrate for sequential infiltration of the fixed tissue.

Procedure

The outline of the methanol-cellulose nitrate embedding method is shown in **Figure 1.** First, the fixed materials, with or without decalcification, are dehydrated. In our laboratory, dehydration is usually achieved by passage of the tissue through a graded series of ethanol (50, 70, 80, 90, 95, 100%), enough time in each, with an additional change in the 100% ethanol. Then, the dehydrated material is replaced in 100% methanol. Next, infiltration of the medium "methanol-cellulose nitrate" is begun. In this step the tissue is serially passed through increasing concentrations of cellulose nitrate in methanol solution (2%, 4%, 6%, 8%, 10%, 12%, and finally 15%). Each step of the infiltration requires a few days to 1 week (or enough time) with shaking. Vacuum ventilation is reqired at each step for easier infiltration of the medium. The schedule can be materially shortened or altered according to the wishes of the investigator. The medium-infiltrated material is then embedded in a Petri dish with 15% embedding medium, and the surface is covered with water vapor generated by an ultrasonic humidifier. A membrane gradually forms on the surface of the medium as the medium hardens. The whole Petri dish-embedded material is then steeped in 70% ethanol and chloroform (10 : 3) solution for uniform hardening. The embedding procedure is now complete. After that the embedded block of tissue is trimmed, and sectioned with a sliding microtome wetted with 70% ethanol. The sectioned specimens are finally stained and mounted by ordinary methods.

As examples, we used the above method to prepare bone tissue for staining with hematoxylin-eosin (H-E) or Schmorl's thioneine-picric acid. H-E staining of sections revealed that the relation between bone matrix and soft tissue was well retained with no problem (**Figure 2**), Schmorl's thioneine-picric acid staining of sections clearly revealed bone canaliculi in the bone matrix (**Figure 3**).

Discussion

The chief purpose of the embedding medium in preparation of histological sections is to infiltrate the material and fill in any cavities an to harden the entire specimen so that sectioning can be performed with a microtome. In general, paraffin is used as an embedding medium. On the other hand, for sections of large whole structures (e. g., brain and heart) and sections of hard tissues (e. g., bone and teeth), Celloidin (T. Co., Tokyo, Japan) has been used as an embedding medium in Japan. However, the medium Celloidin is no longer being produced. In Japan, we can obtain the embedding medium "Pro-Celloidin Fluka" (Fluka Chemical Co., N. Y., USA), which is similar to Celloidin (T. Co., Tokyo Japan). But te directions for its use are difficult and troublesome. Therefore, we developed a new embedding medium, "Shiojirin-E", made chiefly of cellulose nitrate (Kawakami et al. 1995)[1,2]. In the course of the development, we noted a most important property of

1. Fixation → decalcification → washing → dehydration by ethanol series
2. Replacement with methanol (some hours～over night)
3. Infiltration (Vacuum ventilation at each step)
   - ① 2 % cellulose nitrate in methanol solution
   - ↓
   - ② 4 % cellulose nitrate in methanol solution
   - ↓
   - ③ 6 % cellulose nitrate in methanol solution
   - ↓
   - ④ 8 % cellulose nitrate in methanol solution
   - ↓
   - ⑤10% cellulose nitrate in methanol solution
   - ↓
   - ⑥12% cellulose nitrate in methanol solution
   - ↓
   - ⑦15% cellulose nitrate in methanol solution (Embedding medium)
4. Embedding in a Petri dish
5. Covering the surface with water vapor from an ultrasonic humidifier
6. Steeping in 70% ethanol and chloroform (10 : 3) solution
7. Trimming
8. Steeping in 70% ethanol for uniform hardening
9. Microtoming → Staining → Mounting

Figure 1: Protocol of the methanol-cellulose nitrate embedding method

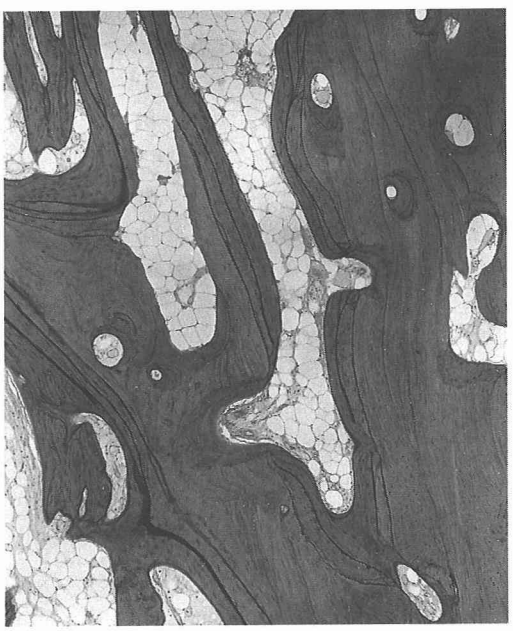

Figure 2: Bone matrix and soft tissue showing no trouble in the H-E stained section (×40)

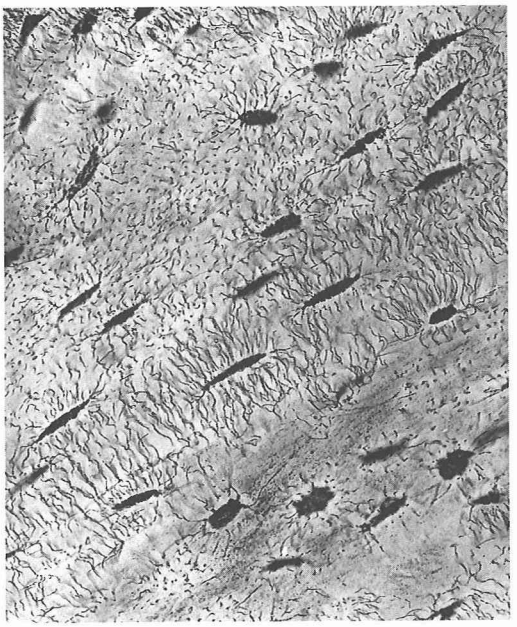

Figure 3: Bone canaliculi in the bone matrix are clearly observed in the Schmorl's thioneine-picric acid stained section (×300).

the cellulose nitrate-medium, that is its low and constant rate of contraction. Therefore, Shiojirin -E is not only a useful embedding medium for sections including hard tissues, but also for histological morphometric analysis (Shibata et al. 1996)[4]. But the embedding procedure takes long time, because of the high viscosity of the medium. A low-viscosity embedding medium based on cellulose nitrate is recommended. Therefore, we planed to develop a new low-viscosity embedding medium with information of the methanol-cellulose nitrate method by Seki (1937)[3].

Long ago Seki (1937)[3] examined the relationship between solvents and cellulose nitrate, and reported that the solubility of cellulose nitrate differed according to the solvent type in the order of ethanol-diethyl ether>methanol. On the contrary, the viscosity order of the solution was methanol-cellulose nitrate > ethanol-diethyl ether-cellulose nitrate. According to his statement, there were no other utility value for embedding medium although he had tested another solvents. When we conducted supplementary studies on the relationship between the solvent and cellulose nitrate, especially regarding hardening of the medium, we obtained nearly the same results as mentioned above. Therefore, we decided to use methanol as a medium solvent. After trial and error, we fixed the component composition as given under embedding medium "methanol-cellulose nitrate". We dissolved cellulose nitrate in methanol up to 15% as embedding medium, although Seki dissolved it up to only 8%. At this higher concentration, our embedded block had the proper hardness for sectioning. According to our examination, the contractibility ratio of embedding mediums varies as shown in the **Table 1**. That is, the contractibility ratio of paraffin, the most common medium, depends upon extending treatment, and Celloidin & Shiojirin-E have the same contractibility : 8-9% measured in two dimensions (Shibata et al. 1995[5] ; Kawakami et al. 1995). The above-mentioned contractibility ratios of Celloidin and Shiojirin were based on two-dimensional measurement, but in general the contractibility ratio for three-dimensional measurement is a much larger value among embedding media examined during the course of hardening. These facts are well known by histological researchers. On the other hand, for histological morphometric analysis in general, the contractibility ratio of the embedding medium is important, and it is especially desirable that the ratio is constant. The most useful medium for such analysis would be one with complete non-contractibility during the course of specimen preparation. According to our results, the "methanol-cellulose nitrate" embedding medium demonstrated complete no-contractibility, not only two dimensionally, but also three-dimensionally. Therefore, we believe that this new embedding medium will prove to be very useful for histological morphometric analysis of hard tissues.

Regarding to the viscosity of the embedding medium, the new developed "methanol-cellulose nitrate medium" showed a much lower of valve infiltration than Celloidin and Shiojirin-E, which we attribute to the solvent methanol. This property allow the medium to infiltrate the material more quickly, and thus the time for preparation is shorter with our new medium than with Celloidin or Shiojirin-E.

Table 1 : Difference of Contractibility Ratio of Varies Embedding Mediums

Embedding Medium	Contractibility Ratio
Paraffin (Merck, Germany)	Depends on Extending Treatment
Celloidin (T. Co., Tokyo, Japan)	8-9% (two-dimensional)
Shiojirin-E (Kawakami et al.)	8-9% (two-dimensional)
Methanol-cellulose nitrate (Kawakami et al.)	Non-Contractible (three-dimensional)

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抄録：形態計測のためのメタノール・硝酸セルロースによる組織包埋法

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我々は先に，硝酸セルロースをベースにした組織包埋剤“シオジリン-E”を開発した．これは包埋に長期間を要するなどの欠点はあるものの，包埋時の組織収縮があまりなく，かつそれが一定していることから，とくに細胞・組織の形態計測のためには極めて有用であると認識した．そこで今回，同じく硝酸セルロースを用いて包埋時間を短縮すること，および形態計測のために包埋時にほとんど収縮しない処方を考案した．Seki (1937)[3]の処方を基に試行錯誤を重ねた結果，メタノール溶液に，Hタイプの硝酸セルロースを15%溶解させ，調製したものが良好な結果を得ることが判った．これによると，三次元的にほとんど組織収縮が起こることなく，小さなものでは3 μ の遊離組織切片を得ることが出来る．したがって，本法は，とくに形態計測を行うための包埋法として有用であると評価された．