Double Embedding in Cellulose Nitrate and Paraffin Wax, an Old and Useful Method that is Easily Misunderstood

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What is Cellulose Nitrate?

Cellulose nitrate is also known as celloidin, collodion, low viscosity nitrocellulose (LVN) necoloidine, nitrocellulose. parlodion, pyroxylin. and soluble gun cotton. (Some of these are trade-names; for even more synonyms, see the Merck Index, under pyroxylin.) It is made by treating cellulose with a mixture of concentrated nitric and sulfuric acids. This converts some of the hydroxyl (-OH) groups of cellulose to nitrate ester (-ONO2) groups. The molecular size and physical form of the original cellulose and the proportion of nitrated hydroxyl groups determine the properties of the product. It is useful in microtechnique because of its physical properties and its unusual responses to solvents and other liquids.

Cellulose nitrate is sold for use in histology either as flat pieces of a transparent solid, or as a viscous 20% (w/v) solution. The most commonly used solvent is a mixture of equal volumes of 100% ethanol and diethyl ether ("ether-alcohol"), but cellulose nitrate is also soluble in methanol, which is less of a fire hazard than ether. Ethyl alcohol (95% or 100%) softens cellulose nitrate but does not dissolve it. A solution of cellulose nitrate is converted into a gel by exposure to 70% ethyl alcohol, chloroform (the liquid or its vapor), or phenol. A 15-20% solution of cellulose nitrate hardens to a translucent material with a firm, rubbery consistency. This offers more resistance to cutting than does paraffin wax, but is not as rigid as polymerized methyl or glycol methacrylate. epoxy resins or other plastics used as embedding media.

Cellulose nitrate can be used as the sole embedding medium in preparing tissues for microtomy, and is valuable for large specimens. The procedure for infiltration and hardening of the block is time consuming, and the techniques for storing, cutting, staining and mounting the sections differ from those used for routine paraffin-embedded material. They are described in all textbooks of microtechnique.

When a solution of cellulose nitrate more dilute than 15% is exposed to a hardening agent such as chloroform, it changes into a flexible, sticky gel. This is not firm enough to be cut into thin sections, but when formed within a biological specimen, it adheres to and supports the extracellular objects, cells and subcellular components of the infiltrated tissues.

What is Double Embedding?

Hardened cellulose nitrate is porous enough to be penetrable by liquids in which it is insoluble: water, some organic solvents, and melted wax. It is therefore possible to infiltrate a piece of tissue with solutions of cellulose nitrate of increasing strength, harden the specimen, and then infiltrate it with paraffin wax. In this process, known as double embedding, interstices of the tissue become filled by two solid materials. The cellulose nitrate probably adheres to the macromolecules that compose the cellular and intercellular structures. The wax replaces the solvents through which the specimen has passed, and fills the space originally occupied by water and water-soluble substances.

The strength and elasticity of cellulose nitrate persist in a double-embedded specimen. When the microtome knife hits a bit of cartilage. bone, lens or wood, the cellulose nitrate adhering to the hard object resists displacement. This prevents damage to soft adjoining structures, and allows the edge of the blade to go on cutting through the specimen without mutilating the softer components.

Some double-embedding procedures are irrational.

Several double embedding procedures can be found in textbooks of microtechnique. In some, the specimens are infiltrated with low concentrations of cellulose nitrate (2% to 4%), and this is not hardened before moving into the intermediate solvent and melted wax. These are quick methods (adding typically 2 days to the processing time) but they miss out on the main advantage of double embedding, which is to deposit within the tissue a three dimensional "skeleton" made of insoluble material that is more elastic but no more resistant to cutting than paraffin wax. To do this, it is necessary to harden the cellulose nitrate within the specimen before infiltrating with melted wax.

A rational double embedding method.

The following procedure is based on the method attributed by Gabe (*Histological Techniques*, Masson, Paris, 1976) to someone called Pfuhl. I have not been able to find the original publication. (Manfred Gabe died before compiling a full bibliography for his 1106-page magnum opus.) My only significant changes are to use methanol and toluene instead of the ether-alcohol and benzene of the original procedure. Pfuhl's method takes longer than some other double embedding schedules, but it allows thorough infiltration with cellulose nitrate, which is then hardened by chloroform and by the action of the phenol dissolved in the clearing agent.

Special solutions needed:

Cellulose nitrate solutions:

2% and 4% cellulose nitrate (nitrocellulose, celloidin, parlodion, LVN or whatever) in absolute methanol. If you start with the solid, allow 48 hours for it to dissolve, with continuous shaking if possible. These solutions are stable indefinitely; keep them in screw-capped glass bottles. If you are not using the procedure regularly, mark the fluid levels on the outsides of the bottles. Any evaporative losses can then be made good by topping up with methanol. **Caution: dangerously flammable.**

Phenolic toluene (clearing agent):

Weigh out 100 g (± 10) of solid phenol into a beaker. Stand it in an oven at 60° C until completely melted, then pour it into 800 mL of toluene in a one litre bottle. Add toluene to make 1000-1100 mL. Note. It is necessary to use solid phenol, which melts at 43° C, not "liquid phenol," which contains 10-20% water. Keeps for 10 years in a dark cupboard. Light brown discoloration does not matter;

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discard when dark brown. **Caution:** Phenol is caustic and poisonous. Don't get it on your skin; if you do, wash it off immediately with plenty of water. Benzene, (which is classified as a carcinogen) was used in the original method. Toluene is considered safer, but don't sniff it or throw it around. Both solvents are highly flammable.

Procedure:

The time for each step is variable: the longest times quoted are appropriate for specimens 1 cm x 2 cm x 2 cm that contain muscle, soft cellular organs, cartilage and decalcified bone.

1) Dehydrate specimens into methanol: 4 to 8 hours.
2) 2% nitrocellulose: 1 to 4 days.
3) 4% nitrocellulose: 2 to 8 days (twice as long as Step 2).
4) Wipe off excess nitrocellulose with absorbent paper, and transfer to chloroform: 1 to 2 days.
5) Clear in phenolic toluene: 12 to 24 hours.
6) Infiltrate with wax (4 changes, each 2 hours), and make blocks.

Double-embedded specimens are sectioned in exactly the same way as ordinary paraffin blocks; ribbons of sections are floated onto slides and dried in the usual way. The wax is removed with xylene and the slides are taken through alcohols to water before staining. Later treatment is determined by the staining method, not by the embedding procedure.

When not to use double embedding.

It could be argued that double embedding is always preferable to simple paraffin processing, because you never know when there might be adjacent hard and soft parts within a particular chunk of tissue. Double embedding cannot be used, however, when results are needed in less than 5 days. Furthermore, a skilled technician can usually obtain a few good sections from an ordinary paraffin block even when it contains "difficult" tissues. For many purposes one section may be all that is needed.

When to use double embedding.

The real need for double-embedding arises when you have to cut many sections of a large specimen (bigger than 0.5 cm^3) that contains both hard and soft materials. I have used the procedures described here for chunks dissected from the heads of laboratory animals (rat, mouse, goldfish) containing such diverse tissues as brain, eye, decalcifled bone, cartllage, and hairy or scaly skin. When a specimen contains hard and soft components, wax alone does not provide enough support. Double embedding makes it easier to obtain great numbers of sections of specimens that would be too difficult to cut serially in paraffin alone. This is not a method for anyone in a hurry, however, so its greatest appeal is probably to research workers. ■

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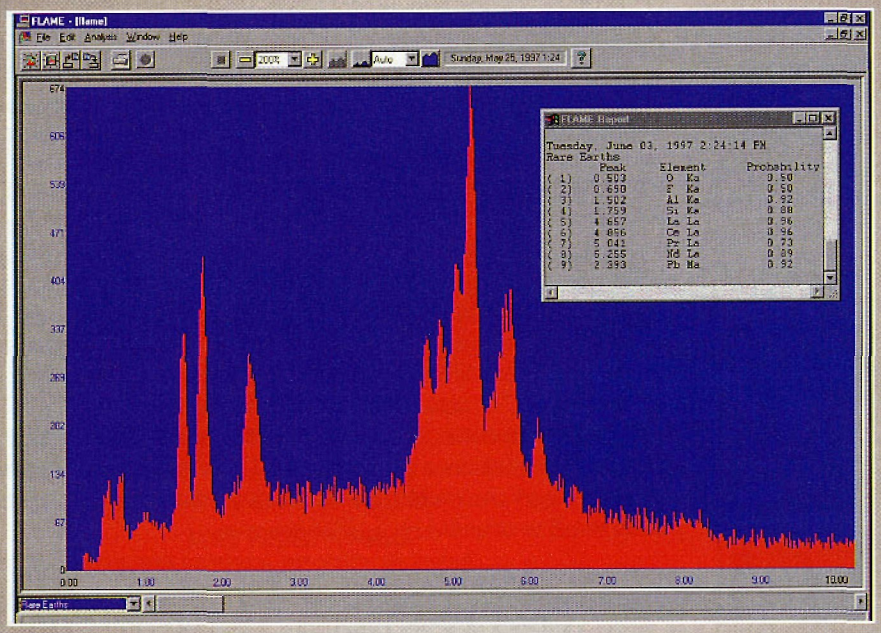

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