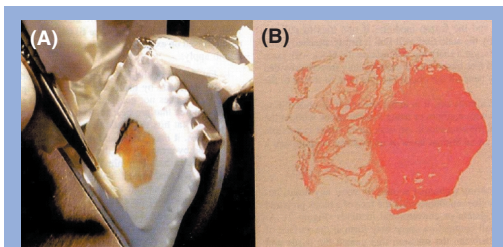


## Face-down cryoembedding in clinical histopathology: The art of embedding tissue for cryosectioning

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To make sure today's pathologist is able to interpret a frozen section under a microscope after cutting and staining, a number of steps must be carefully performed in the laboratory. These include examination and sampling of the tissue, accurate embedding and superior cutting of the tissue, staining on a glass slide and finally, interpreting the slide. At each of these steps, poor preparation can yield unsatisfactory results. This article is about a novel but simple process, developed by Dr Stephen R Peters, USA, for superior embedding of biological tissues for frozen sectioning using the **Leica Microsystems** clinical cryostat models, Leica CM1510 S and CM1850. It addresses the cryoembedding and cutting of specimens that require perfect orientation within the block. Poor orientation of a specimen cannot be corrected easily once the frozen block has been produced. Modern cryostats provide some assistance for orientation of the entire block by making use of the x/y-block or specimen orientation system of the cryostat. However, these kind of orientation systems cannot address a potential requirement to orientate the specimen within the block to make sure the pathologist is presented with a series of stained sections representing the exact areas of interest within the specimen.



**Figure 1** Orientating fat containing tissue to the blade. (A) A trimmed block showing a breast tumour with the inked fatty margin orientated perpendicular to the knife blade. The fatty tissue is cut without interfering with the tumour tissue and the margin is best preserved in this vertical section. (B) Close up of slide cut from block showing an adequate section containing the fatty tissue and clearly visible resection margin at left.

In conventional cryostats, tissue is embedded for cryosectioning by placing it face up on a specimen holder covered with embedding compound. The specimen holder is then placed upon an actively cooled freezing bar inside the cryostat to initiate the block freezing. To speed up the specimen freezing and to flatten the block surface, a heat extraction system may be applied on top of the specimen block at the appropriate time. Of course this method has many shortcomings, which can lead to considerable frustration and, more importantly, unacceptable sections for interpretation. However, this standard procedure works adequately in situations where a large volume of tissue is available and precise orientation is not an issue.

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Unfortunately, this method is inadequate where high precision and predictability of the prepared tissue is essential to the final results. Additional distortion can result from crushing of the tissue by the weighted heat extractor. This method of tissue embedding prepares a rough approximation of what is possible with today's paraffin embedding. The problem is magnified when confronted with minute samples and thin cores. It can be difficult to get all of the desired tissue in a section without trimming away significant portions of the sample. Further difficulties arise in situations where thin and difficult to handle specimens need to be embedded in precise orientation. On top of that, the cryosectioning process is very demanding due to pressure to provide fast results. This is even more of an issue when multiple specimens are delivered to the laboratory by a number of surgeons simultaneously.

By freezing the specimen in the conventional way, it can take 2 minutes or more to freeze a block. During this period the user must monitor the freezing process for the correct time to place the heat

extractor over the freezing block to optimise the freezing speed and ultimately the freezing results. If the results do not meet the user's requirements, the only option is to modify the procedure to a rapid freezing procedure by immersing the tissue into liquid nitrogen or other super-cooled liquids. This fast and effective freezing process leads to fewer freezing artifacts but is limited in precision and often leaves the user with a block too cold to cut smoothly in the cryostat. As a direct consequence, some manipulations have to be applied to bring the block to a temperature that is closer to the cryostat chamber temperature. This article presents a system consisting of a simple apparatus and techniques used to embed tissues for cryosectioning, not losing the requirement of fast freezing for freeze artifact reduction out of sight. The system and methodology favours a tissue face-down, using freezing temperature wells machined into the surface of solid steel bars. The advantage of this method is the physical property that causes tissue to stick to steel is located at freezing temperatures at around  $-20$  to  $-35^{\circ}\text{C}$ . This property facilitates the setting of tissue into wells and is easier than, for example, standing tissue in solidifying paraffin. The advantages include precision, predictability, speed, reduced tissue wastage, comfort and convenience.

### Description of the system

The system consists of five major components, which can easily be inserted into the cryochamber of the Leica CM1510 S, CM1850 or any other Leica Cryostat, except the CM1100 bench top version.

#### Embedding Well Bars

Embedding is performed in wells machined into the face of one-inch thick stainless steel bars. When cooled to the cryostat temperature, these solid mass bodies provide a powerful heat sink for very fast freezing. Storage of the bars inside the cryochamber, at an average temperature of  $-20$  to  $-25^{\circ}\text{C}$ , has proven to be the optimal storage temperature for the bars to assure proper function.

#### Specimen holder (chucks)

The chucks are designed to maximise the gripping power required to hold the embedded tissue block. These special chucks are designed to fit perfectly the chuck holder system of the Leica Cryostat family.

#### Over-Chuck Freezing Block

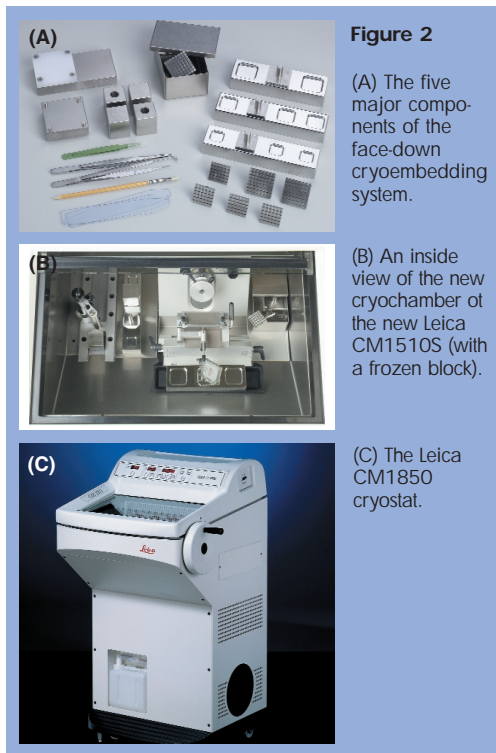
The over-chuck freezing block is designed to function as a powerful heat extractor to speed-up the freezing of the specimen/block. This block may also serve as a convenient flat freezing surface and as a dislodging tool.

#### Dispensing Slides

The thin transparent dispensing slide is made to serve the precise orientation of the tissue into the desired position and as a means to accurately transfer tissue to the embedding well floor.

### Embedding Shelf

The removable embedding shelf is designed to be installed on the front wall below the opening of the cryostat.



**Figure 2**

(A) The five major components of the face-down cryoembedding system.

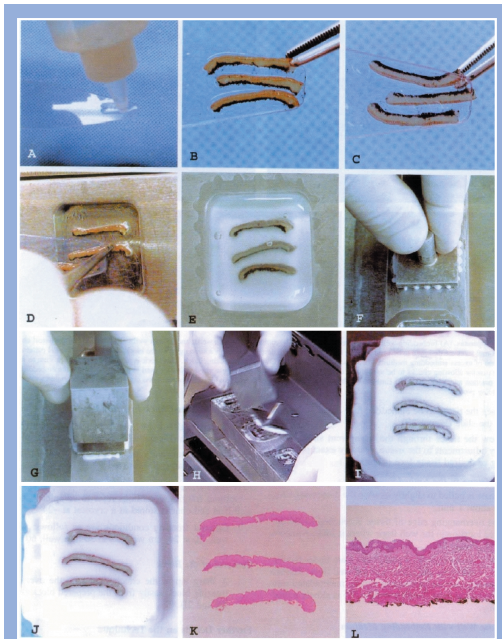
(B) An inside view of the new cryochamber of the new Leica CM1510S (with a frozen block).

(C) The Leica CM1850 cryostat.

### The Technique

1. Wet the dispensing slide with a thin coating of embedding compound. *Figure 3A*.
2. Place the tissue to be embedded face down at the very end of the dispensing slide. *Figure 3B*.
3. Make any adjustments to the tissue to assure exact positioning of the desired tissue face. *Figure 3C*.
4. Pull the tissue to slightly overlap the dispensing slide by about 1 mm. *Figure 3D*.
5. Touch the overhanging edge of the tissue to the desired location in the well floor, where it adheres to the cold steel surface.
6. Pull out the dispensing slide from under the tissue. Tissues requiring precise orientation can be manipulated into exact position as the dispensing slide is slowly pulled away.
7. Fill the well with OCT compound to its maximum capacity. *Figure 3E*.
8. Immediately press the chuck over the well flat to the well bar surface. Any excess medium will be extruded through the channels in the chuck. *Figure 3F*.
9. Place the over-chuck freezing block over the chuck stem. This procedure is a mandatory step when using a warm chuck or using the large 30mm well. *Figure 3G*.

10. Freeze the specimen for approximately 20 sec (18 mm well) to 60 sec (30 mm well). Optimal freezing conditions refer to completely cooled well bars and chucks stored at approximately 25°C.
11. Remove the frozen block from the well with a sharp tap of the chuck stem with the over-chuck freezing block. Figure 3H.
12. Insert the specimen into the specimen orientation system or chuck-holder system of the Leica cryostat. Figure 3J.
13. Start cutting.
14. Replace the chucks and all handling tools inside the cryostat for further use.



**Figure 3** Face down embedding technique.

### **Other cryosectioning considerations**

Ensure the specimen is positioned parallel to the knife before sectioning. The specimen/block can be conveniently orientated using the specimen orientation system of the cryostat. Using the precision cryoembedding system, blocks are all prepared precisely flat, with block faces approximately parallel to the chuck faces. Blocks will be very similar from one to the next and as a result will require considerably less x/y adjustments to achieve a complete section of the block. The orientation of the block to the knife-edge however, does not replace the specimen orientation procedure described before. It is more an additional help for the user to make sure the knife meets the tissue in the required specific orientation. Do not begin cryosectioning using a knife or microtome blade that has been placed outside the cryostat.

Different materials require different sectioning temperatures to achieve good sectioning results. For example, fatty tissues require much lower sectioning temperatures than tissues containing lower levels of fat. In case various tissues are required to be sectioned throughout the day, the investment in a cryostat with an active specimen cooling system, such as the Leica CM1900 and the Leica CM3050S, is recommended. Using freezing sprays to change the temperature of the block prior to cutting is not recommended. Also, the specimen should be allowed to respond to a changed temperature before beginning sectioning, whenever possible. For a temperature change of 5°, a waiting time of 15–20 minutes leads to better sectioning results and fewer thin and thick sections. Trimming off surrounding OCT compound can help to make cryosectioning an easier task. It also helps to trim the block into a V-shaped form, where the short end should meet the knife first.

Fat or specimen parts containing a lot of fat should be the last area of a specimen to hit the blade. Fat does not get hard enough to cut well at temperatures that are the optimum for cutting most other tissues. When fat hits the blade before the other tissue areas, it may smear and ruin the rest of the section. The specimen can be rotated in the specimen/chuck-holder to "avoid" the fatty tissue areas.

A section can be considered as having a beginning, a middle and an end. At the beginning there is a high risk of curling and damage of the tissue by section handling tools (brush, needle etc.). The middle is the area where the tissue passes most smoothly over the knife-edge. The middle part is therefore the part of the section with a very low rate of artifacts and the "cleanest histology".

Epithelial and mucosa lined tissues such as skin and gastrointestinal, bladder, uterus and cervix, should be orientated with the plane of the epithelium perpendicular to the knife. The knife holder angle should always be checked when sections are curling after coming off the knife-edge, or if the sections are coming off very compressed. Sections should not be rejected immediately when longitudinal stripes are seen on the section before staining. The stripes may be a result of the Teflon blade coating and will often disappear during the staining process.

### **Discussion & conclusion**

The procedure of face-down, cryoembedding of biological tissues has various advantages over the currently more common procedures. The distinct advantages are related to the speed of the process to prepare a block for cryosectioning, the precision of accurate embedding, the reduction of tissue wastage, the ease of learning and convenience of using the system described. The ability of this system to provide a flat, highly predictable, embedded tissue face improves the ability to deliver accurate diagnosis and allows the operator to orientate the specimen precisely, with the desired tissue surface in a flat plane. The

### Leica CM1850

The cryostat of choice for routine histology and clinical pathology at a glance:

- Chamber temperature down to -35°C
- Actively cooled quick freeze shelf with optional Peltier unit for freezing down to -60°C
- Power saving refrigeration system
- Splash-protected microtome
- Motorised coarse feed at two speed settings
- Specimen orientation system with automatic centring function
- Automatic and manual chamber defrost cycles
- On demand quick freezing/quick freeze shelf defrosting
- Compact space saving design
- Enclosed drainage system for waste water
- Intuitive control panel

system also avoids crushing by the heat extractor weights, resulting in superior cryosections almost comparable to paraffin sections. Figure 3K & L.

The system has been designed to be easily installed in the Leica Cryostats product range. This makes it a very convenient system for either the attending pathologist to quickly and reliably embed the sample for the resident, or the technologist to cut, knowing that the surface which is placed down is the surface the technologist will see when the block emerges.

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