Complete Cryostat Protocol

v1.00

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12/06/2009

This protocol uses the Leica CM1850 Cryostat and frozen wheat stem as an example of taking a sample through the necessary steps to acquire a section on a slide for use in a light or FTIR microscope.

An appropriate container for liquid nitrogen (LiN_2) is filled just enough to keep some samples cold during transport. Wheat stem cuttings in Falcon tubes are taken from a -80°C freezer and placed into the LiN_2 container for transport to the Cryostat. The tubes are placed into the Cryostat, and selected cuttings are transferred from the tube into a holding container such as a pre-cooled Petri dish within the Cryostat. The remaining samples are transported back to storage.

The selected samples should be given some time to warm up to the Cryostat's temperature, minus 25°C. If samples are too cold then the mounting medium, TissueTek, will not adhere to the surface properly and likely separate from the tissue as it is cut. If samples are longer than 15mm they should be cut gently (so they don't shatter) with a razor blade, after they have warmed.

All equipment used in the Cryostat should be left to equalise in temperature before coming into contact with samples, mounting media and sections! If equipment is warm it may damage samples and get covered with mounting medium.

An open-topped black cardboard box has a small amount of LiN_2 poured into it, not so much that it covers the plastic lid inside the box. This lid has had three holes cut into it which the mounting stubs fit into. If the LiN_2 is too high in the container then the applied TissueTek will freeze before you have time to put your sample in it. TissueTek should be kept nozzle side down to avoid air bubbles in the media, it also helps to keep it in a warm place for lower viscosity.

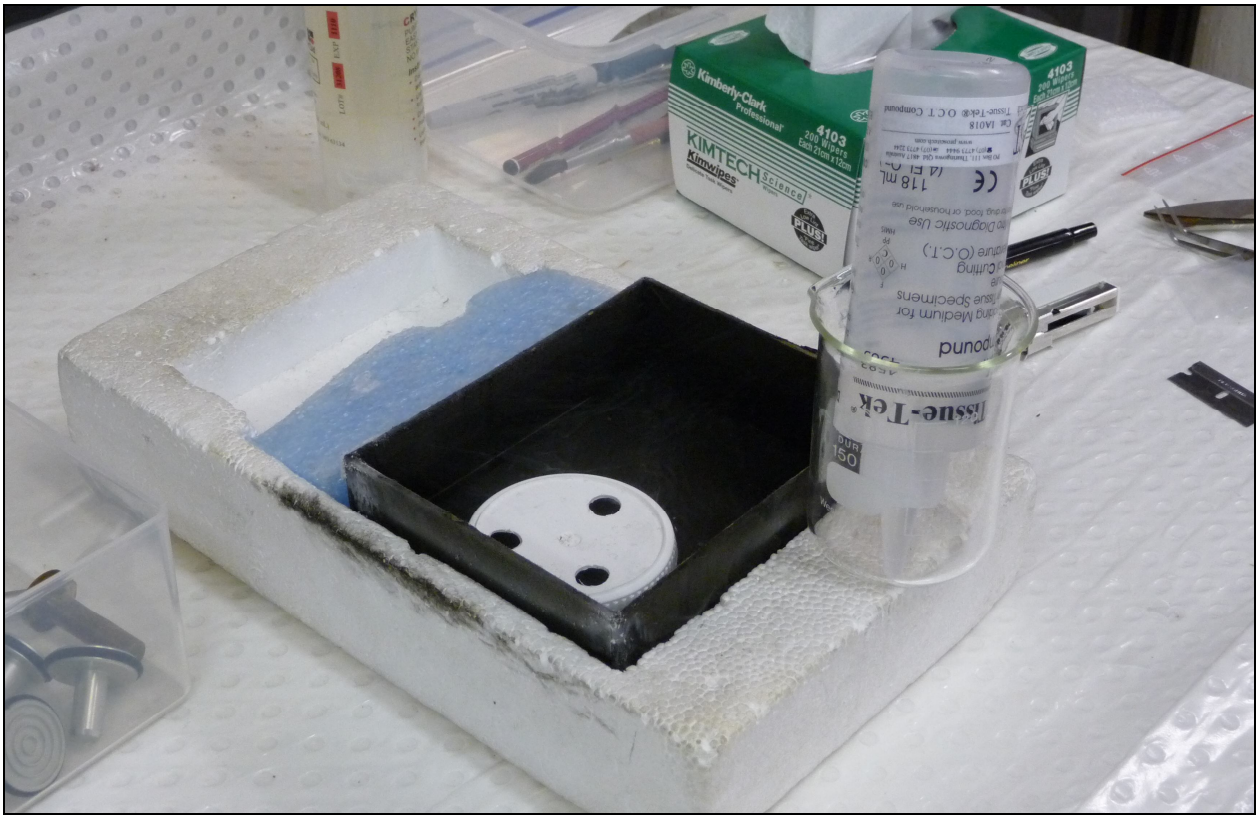

Figure 1. A box of Cryostat stubs (far left). Open container with a small amount of LiN_2 and a makeshift stub holder in it (middle). TissueTek inverted in a beaker (right).

A suitable sized stub is selected and a layer of TissueTek is applied. A medium sized stub is appropriate for the wheat cuttings being used. Try to avoid bubbles, more so toward the top where the sections will be made, as these become holes when solid which can tear the sample.

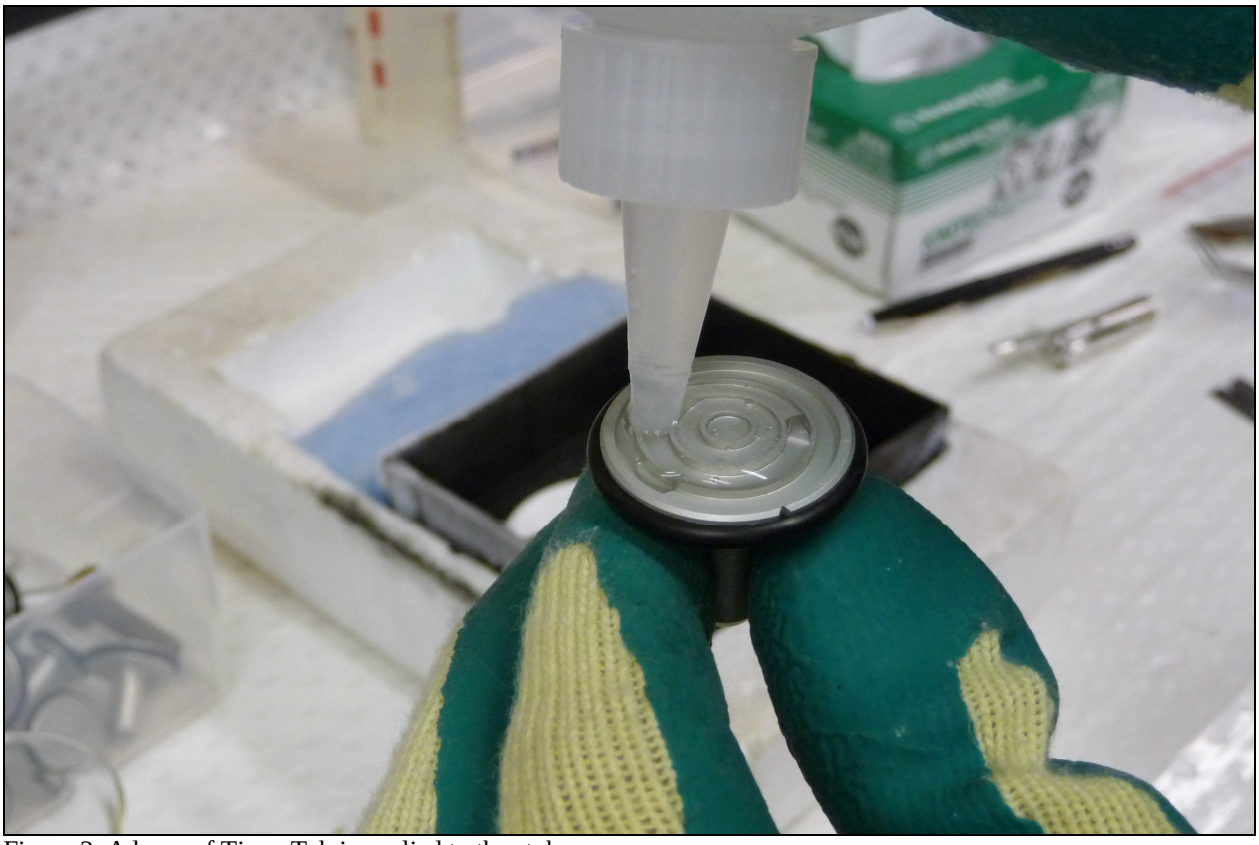

Figure 2. A layer of TissueTek is applied to the stub

An alternative mounting media Cryogel can also be used, with very different properties. I find bubbles impossible to remove if using Cryogel and generally more difficult to make a neat stub.

The stub with a single layer of mounting media on it is placed into the lid in the shallow bath of LiN_2. Quickly taking a single sample from the Cryostat with forceps, the sample is placed into the liquid media on the stub, and held until it stands by itself. As the media hardens it will turn a translucent white, and more TissueTek should be applied before becoming opaque. The media should be applied around the sample first and swirling outwards, to avoid bubbles. Do not apply too quickly as it will droop over the edges and will need to be broken off later. If liquid media is applied on to solid media it will not adhere properly, and will likely come apart under pressure from the knife.

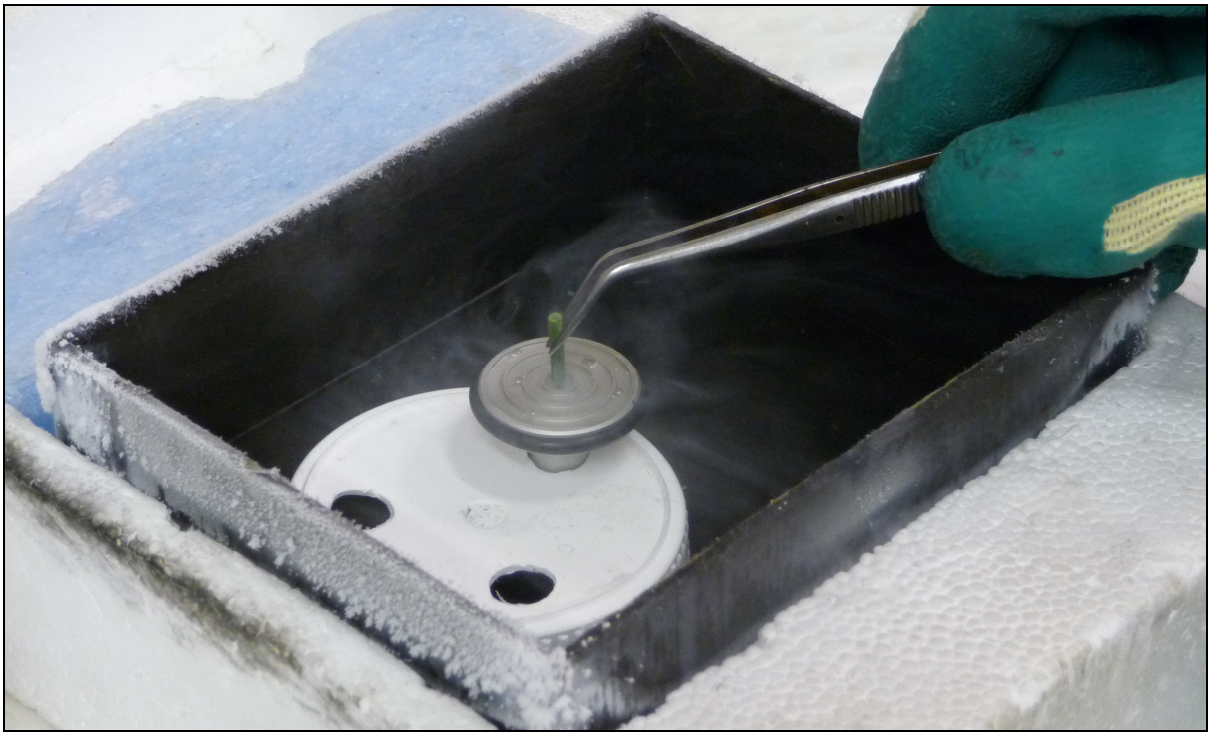

Figure 3. The sample is held with forceps until the media is hard enough to hold it in place.

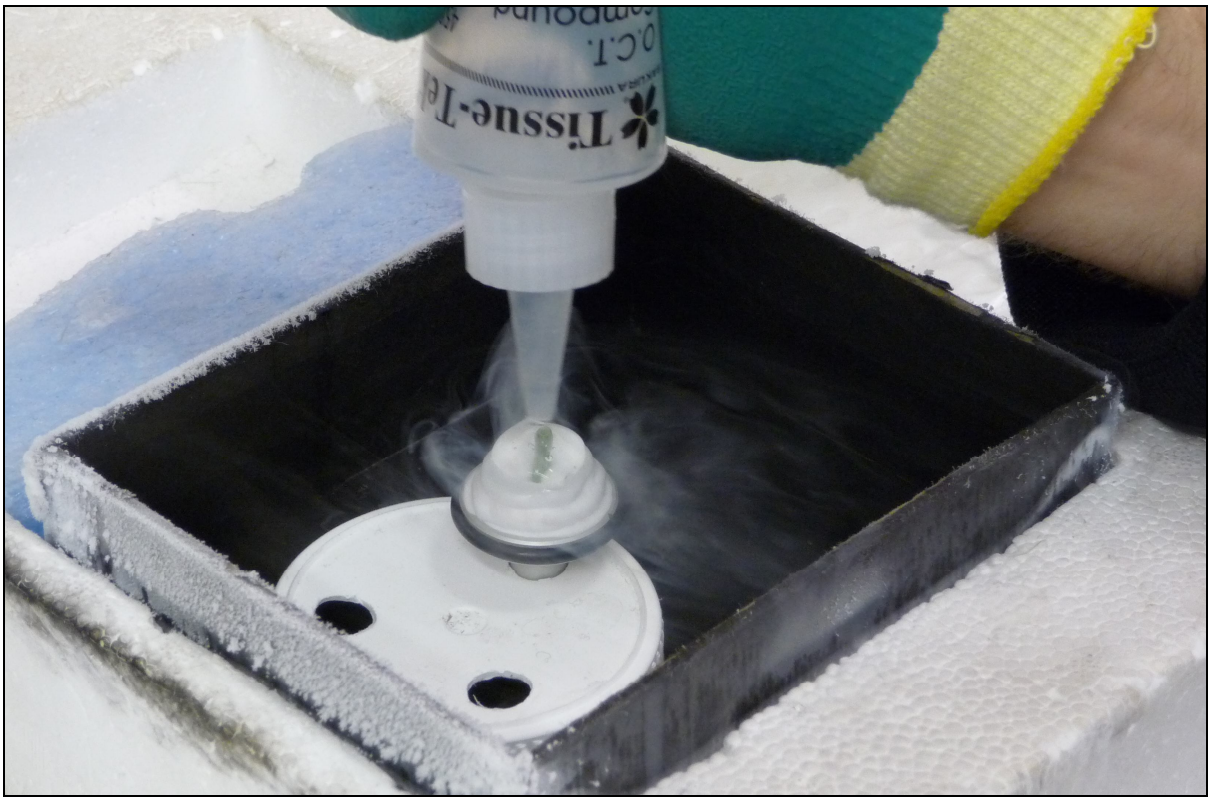

Figure 4. TissueTek is applied gradually until the sample is covered.

Once the sample has been covered with media, the stub is transferred to the Cryostat and given some time for the temperature of the entire stub to equalise with the internal Cryostat temperature, approximately -25°C. If the stub is not given time to come to an even temperature then when sections are made there will be no guarantee that they will be the desired thickness. The temperature of the mounting media also affects its cutting properties, as explained later.

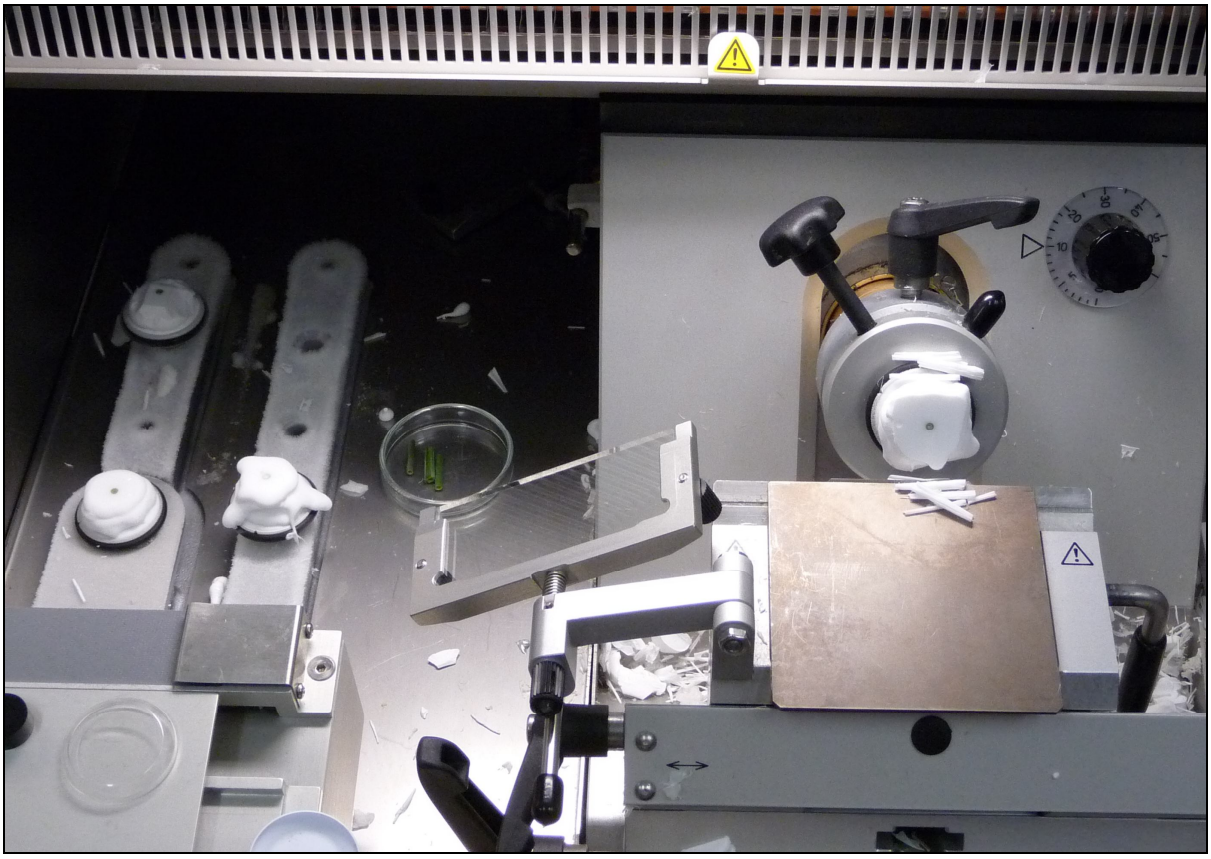

Figure 5. Inside the Cryostat. The prepared stub and previously used stubs sit in the cooled holding rack (left). A stub inserted into the mounting face has been cut down to a desired depth (right). The dial to control section thickness is set to 10µm (top right).

When the stub has come to even temperature, it is placed into the mounting face of the Cryostat. The controls of the side of the machine should be used to move the sample away from the blade, then cuts made until the desired area of the sample is reached. Be wary of how thick you make these initial sections! Much more than 25µm will create a lot of sideways forces which may dislodge the mounting media from the stub's base, or create vibrations which will shatter the ice inside the sample, making future sections brittle or already damaged. When making these initials cuts it is not necessary to use a new blade, but do not use a blunted area for cutting your sample (see Figure 12).

At the desired depth excess mounting media should be cut away from the sample. The mounting media will help prevent the sample from curling, and give something for you to grab with forceps to transfer the sample to a slide. Excess media however will likely bunch up, and take up valuable room on slides. The first bit of media that comes off the blade will probably curl as it touches inside the anti-roll plate so it important to leave an extra lead-in to account for this. Taking a razor blade cut a short distance above the sample, slightly more to the sides, and about double this distance from the sample at the bottom.
You should remove the Cryostat sectioning blade or move the stub elsewhere prior to making these cuts, as you may seriously hurt yourself on the sectioning blade.
The end result should be a rectangle with the sample horizontally in the centre, and 1/3 from the top (Figure 7).

Figure 6. A razor blade is used to remove excess mounting media from the stub.

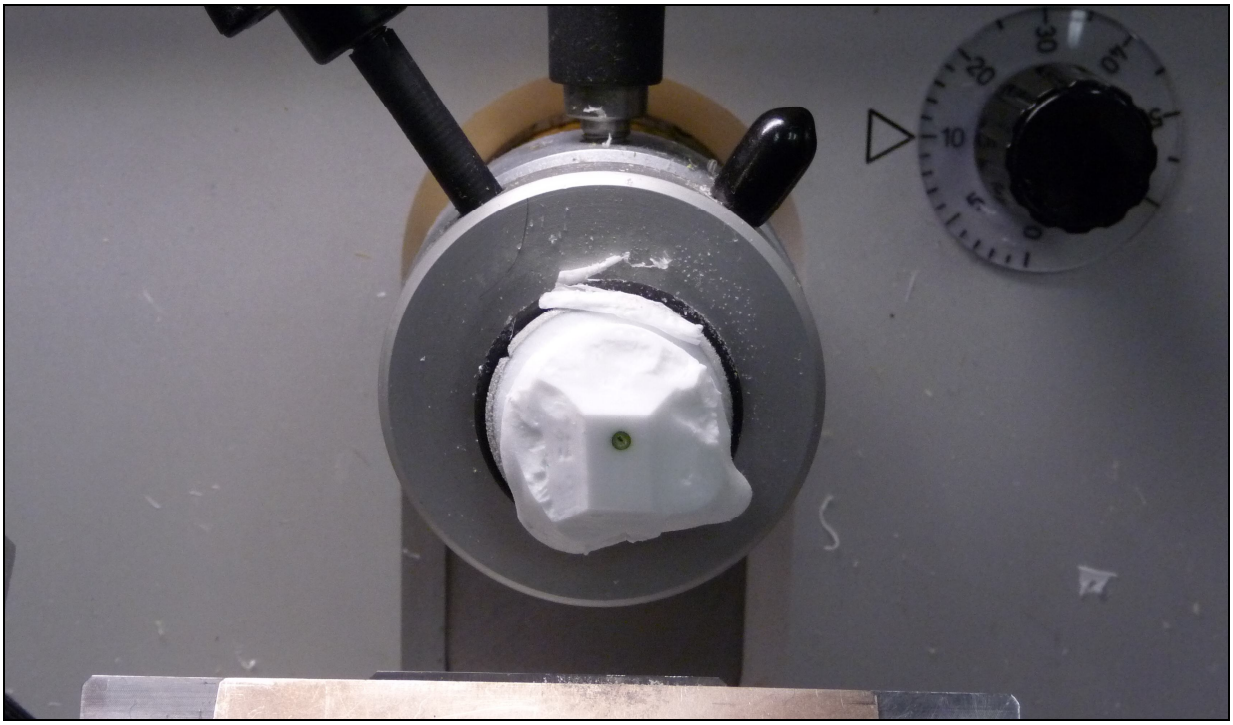

Figure 7. The excess media removed, leaving the sample horizontally central and approximately 1/3 from the top.

The anti-roll plate should be adjusted to catch the section as it comes off the blade. Looking down from directly above the blade, extend the plate out so that it only just hides the blade. If it is too far over it will rub against the media and sample and damage the section and possibly the next. If it is wound to far in then it will not catch the section and it will roll up and fall away.

Note: as sections are made across the blade, the anti-roll plate may need adjusted as it can be slightly on an angle in relation to the blade.

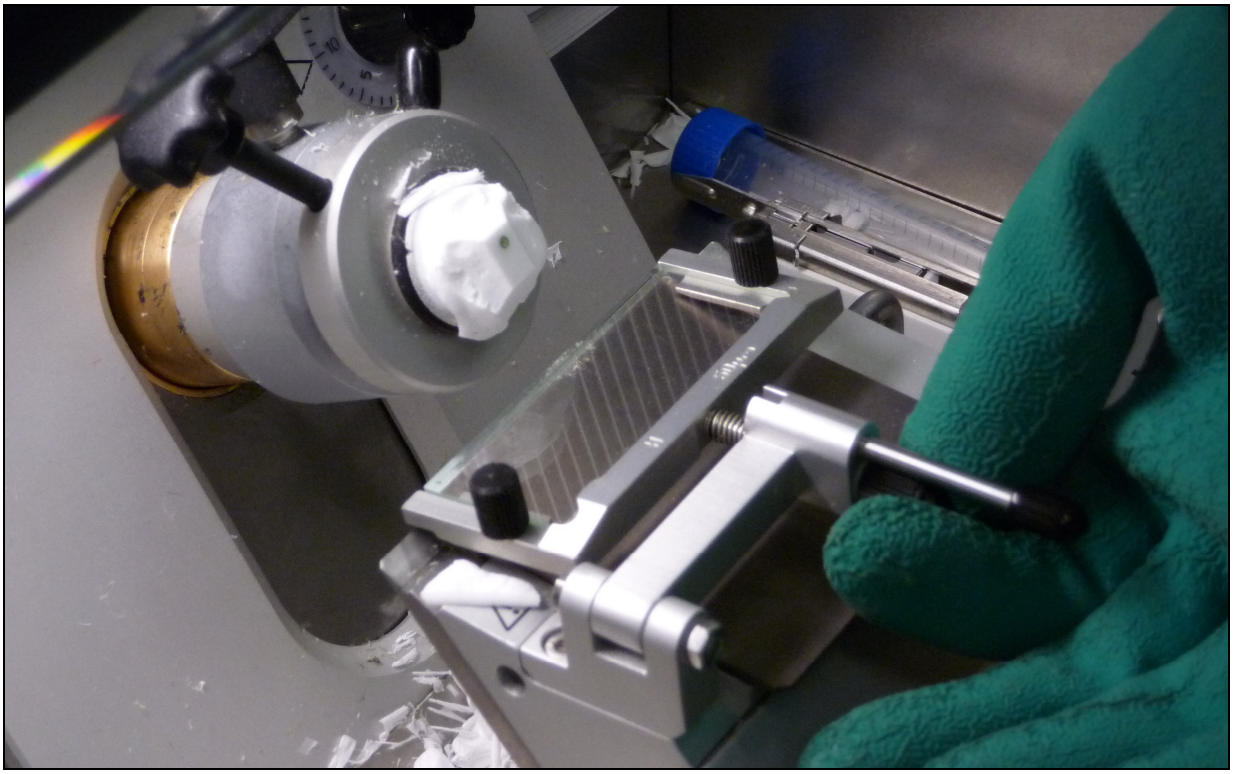

Figure 8. Adjusting the anti-roll plate to catch new sections. The plate should only just hide the blade when viewing from directly above.

As sections are made the blade will quickly become blunt. Rather than moving the blade alone, the entire stage may be moved by first loosening the lever on the upper left, then moving the stage and retightening the lever.

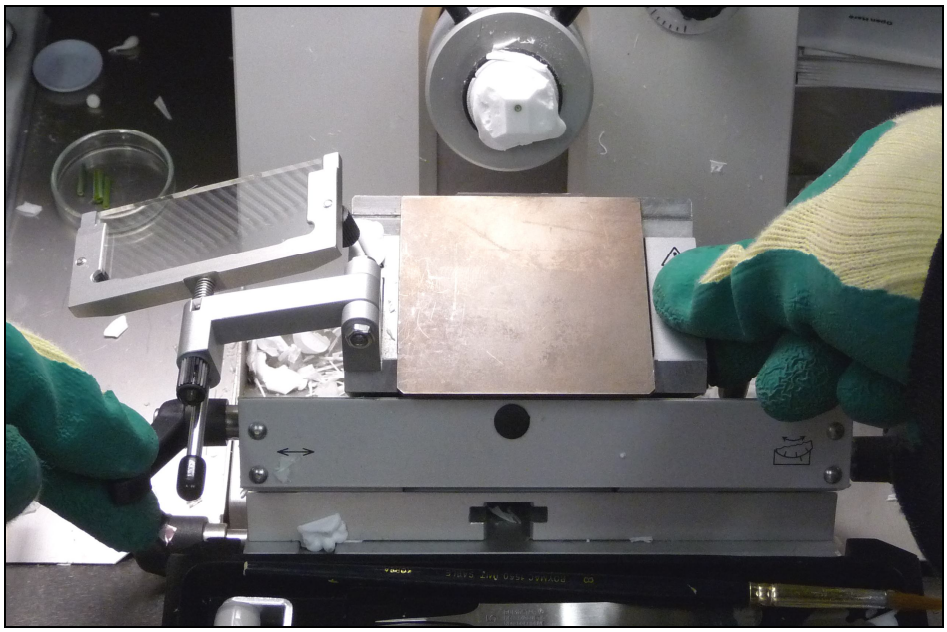

Figure 9. The stage can be moved to the left or right by first loosening the lever on the upper left.

The cutting angle can be set by loosening the lever on the lower right, then tilting the stage forward or back by using both hands, then retightening. I have found that an angle of 6° is best for making sections of wheat stem at 10µm.

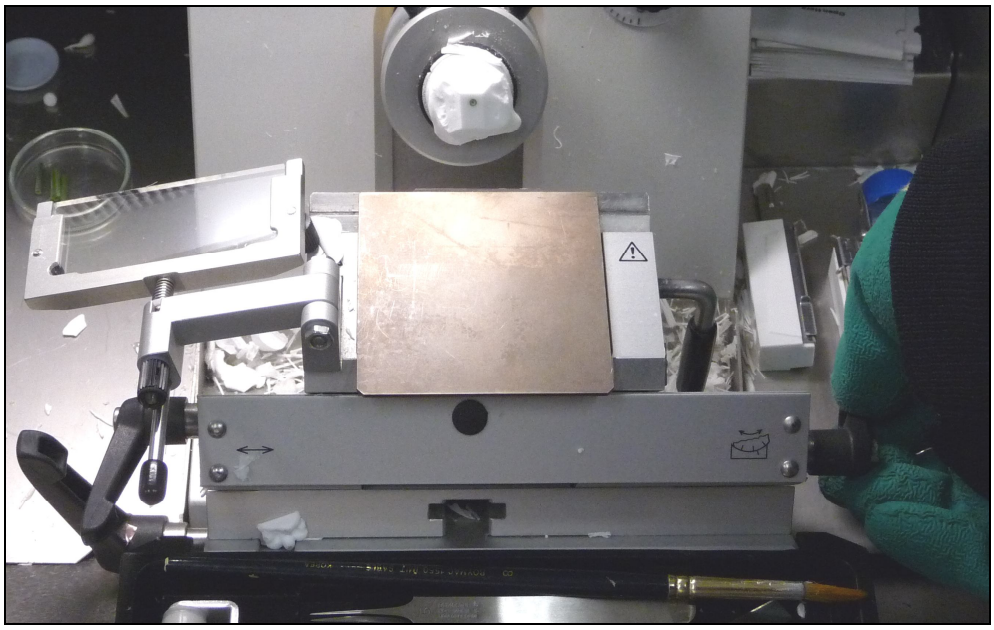

Figure 10. Loosening the lever on the lower right so that the cutting angle can be adjusted.

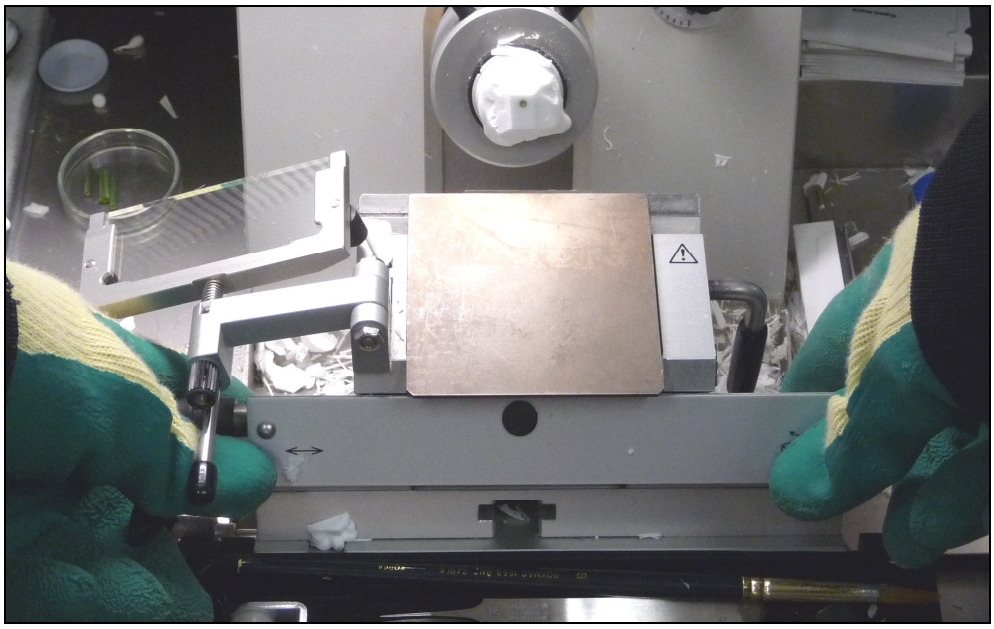

Figure 11. Using both hands, the cutting angle is adjusted by tilting the stage.

The dial to the right of the mounting face sets the thickness of sections. For wheat stems I have found that 10µm is optimal. Too thin and the sample will collapse, too thick and cell walls will cover the contents of cells lowering the quality of viewing.

Once all of the adjustments have been made a few preliminary sections should be made to ensure everything is working. If parts of the blade are unused the stage should be moved appropriately, or a new blade put in (Figures 13 & 14). It takes only 2 or 3 cuts of the sample across the blade to make that area unsuitable for making sections (Figure 12)

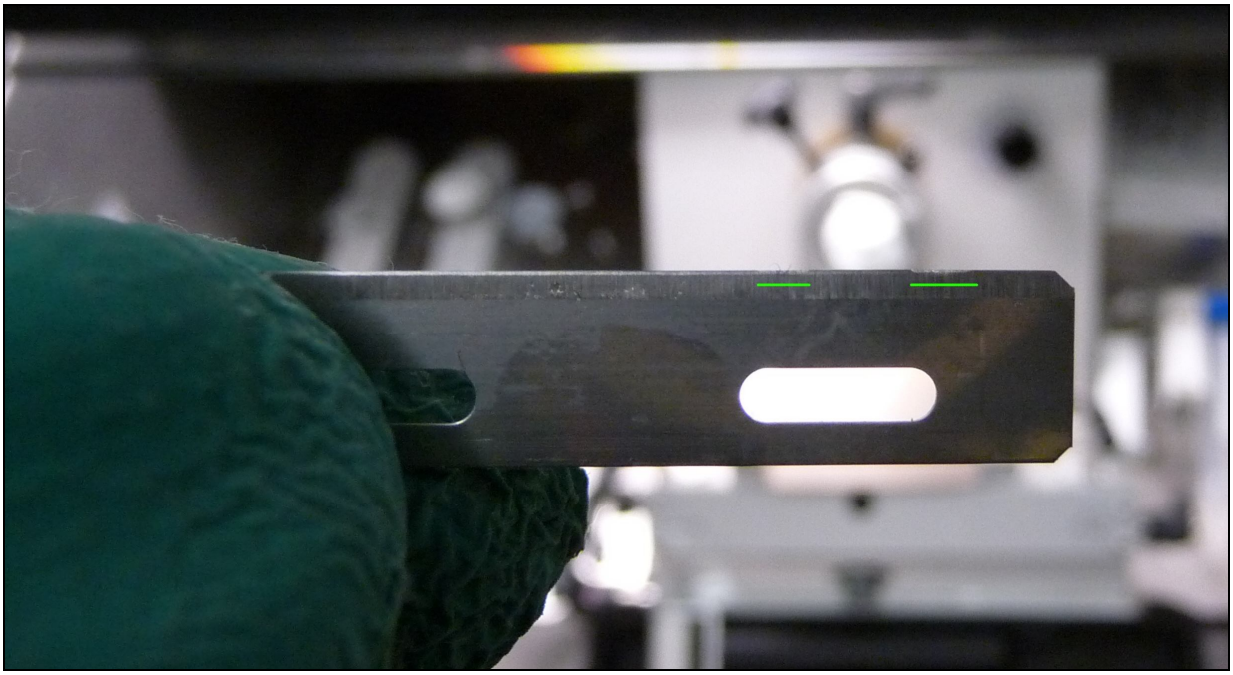

Figure 12. Sectioning blades are quickly blunted. The left side of the blade is unused, while to the right two areas have been highlighted green where the blade is no longer suitable for sectioning.

To replace the blade loosen the upper right lever which will release the blade mounting plate. Either use forceps or thick gloves with care when removing the blade. When replacing with the new blade, be wary of using forceps as even a light touch against the blade may damage it. New blades should be kept inside the Cryostat so they are at the correct temperature.

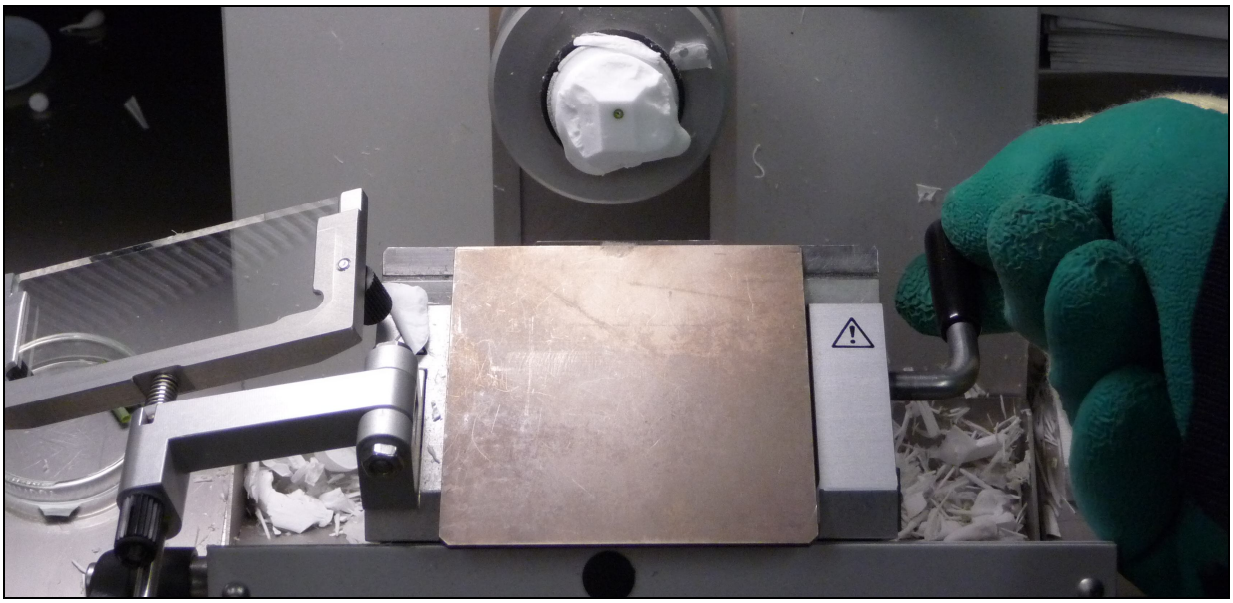

Figure 13. Loosen the upper right level to release the blade mounting plate.

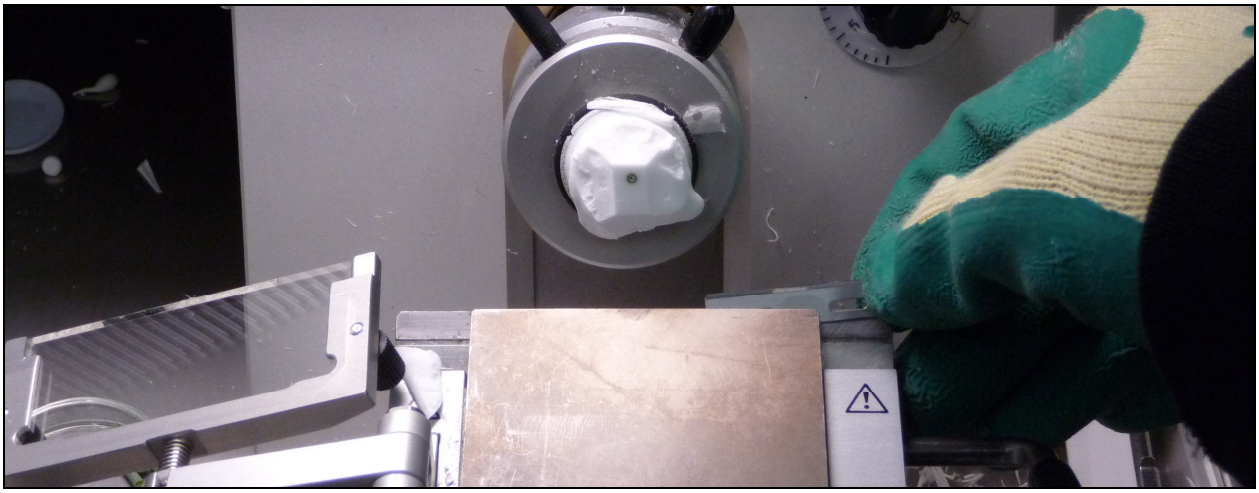

Figure 14. Carefully removing and replacing the blade.

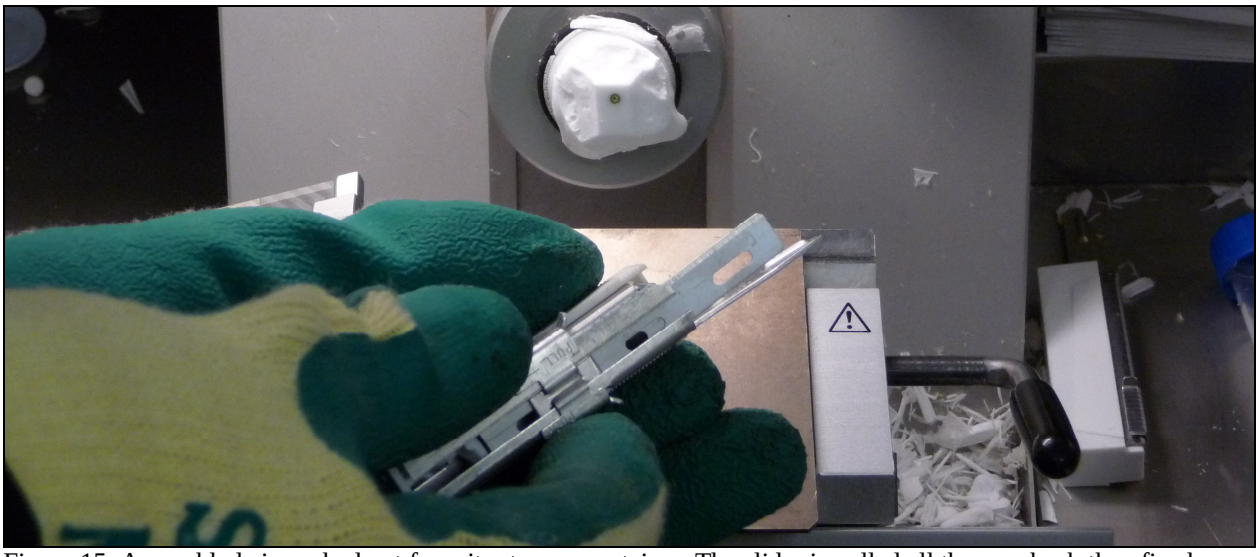

Figure 15. A new blade is pushed out from its storage container. The slider is pulled all the way back then firmly pushed forward. Gloves should be worn and special care made when handling blades.

The mounted sample should be cut once or twice to remove imperfections made by the old blade. You may have noticed that as the blade blunts, vertical scratches are drawn on the freshly cut face of the block which will ruin your sections if not first removed.

It is now time to make proper sections. Lower the anti-roll plate and slowly turn the cutting wheel. The plate should catch the section as previously explained. If caught, several things may now happen: the section may cut quite cleanly but roll excessively, it may bunch up and fold your sample, it may tear, or the tip may roll but the rest remain nice and flat. If your section rolled excessively you may have the temperature set too low on the machine; try a couple more times but if the same thing happens try raising the temperature a couple degree and waiting 20 minutes. Conversely if the sample has bunched up the temperature may be too high, try lowering it a couple degrees. I have found that TissueTek cuts about right at minus 25°C. If the section has torn into 2 or more pieces then the vertical scratches previously mentioned may not have been entirely removed, or the anti-roll plate may have chips in it and need replaced. If the section was flat you can proceed to transfer it to a slide.

Between making sections, old material should be swept away with an acclimatised brush. Sweep toward the blade and never against it, as it will quickly blunt the blade and give it a close shave!

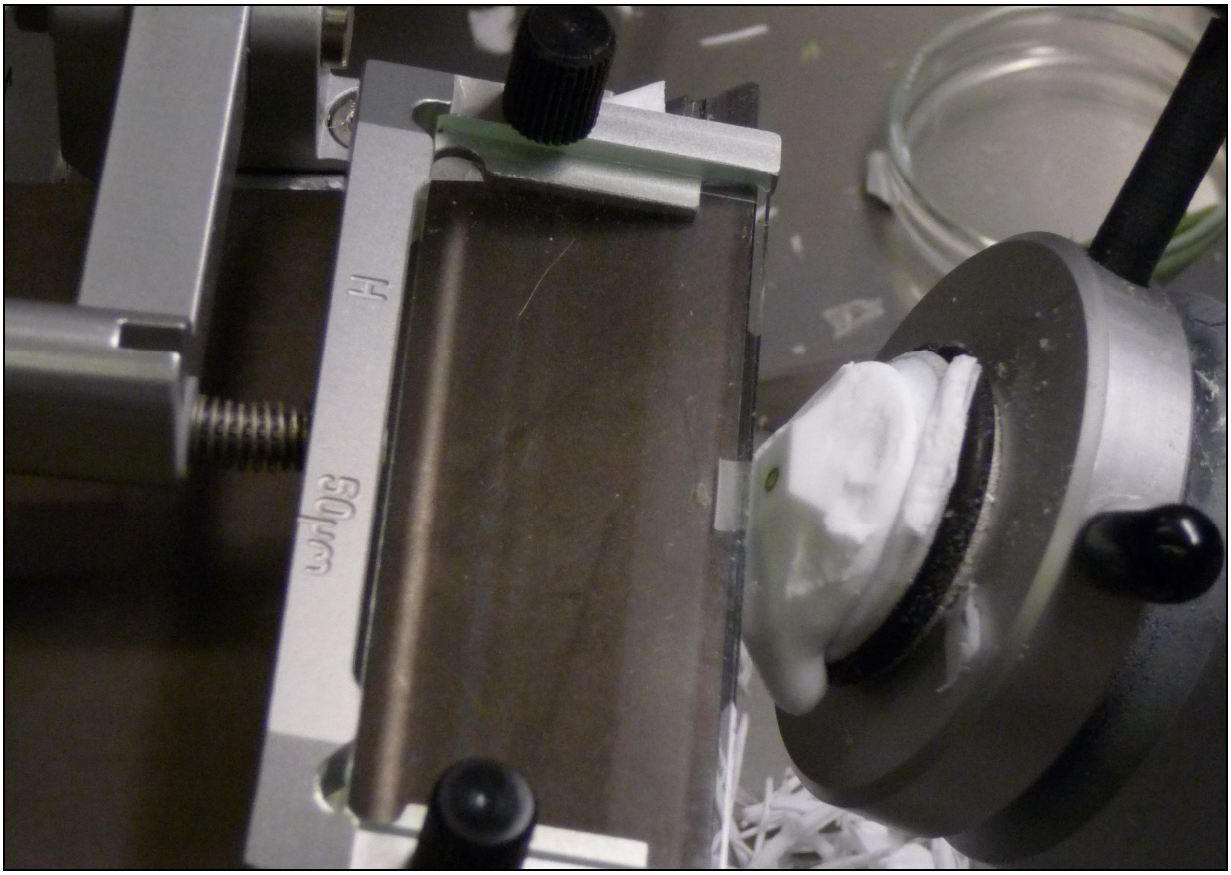

Figure 16. A section coming off cleanly, it has not started to bunch or curl which indicates that the anti-roll plate and the temperature have been set correctly.

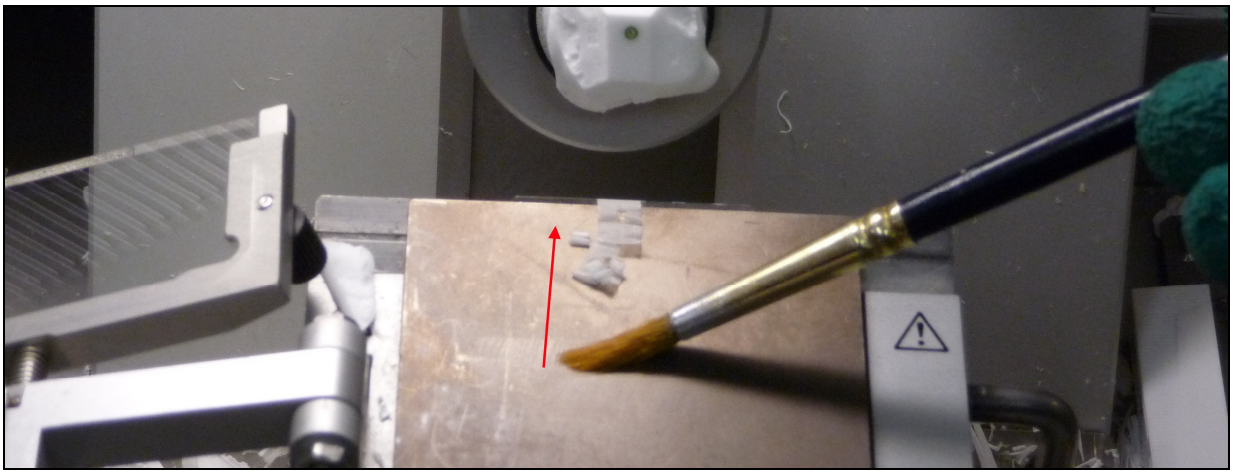

Figure 17. Brushing away old material, toward the blade but never against it.

To transfer the section to a slide, put a room temperature slide onto the blade mounting plate, lift the anti-roll plate up a short distance and use some cold acclimatised forceps to quickly transfer the section to the slide. The section may be stuck to the blade or the anti-roll plate. The section may start to curl up so this must be done quickly, and using the forceps at an angle across the section should help to prevent further rolling. The radiating heat from your hands (even through gloves) will make the sample wrap around the forceps if you've been holding them for too long. As you bring the sample close to the slide the heat radiating upwards from it will also convince the sample to quickly curl up. This is perhaps the most difficult part of making sections using the Cryostat, and will take a lot of practice to make good slides.

If you are making sections for freeze-drying you must use two cold acclimatised slides. As you place the section onto the slide it will have no affinity to it, in fact it will have more affinity to the slightly warmer forceps, and so the second slide will have to be used to keep the sample on the slide, and kept on top to prevent it from rolling until freeze-dried. See freeze drying protocol for more details.

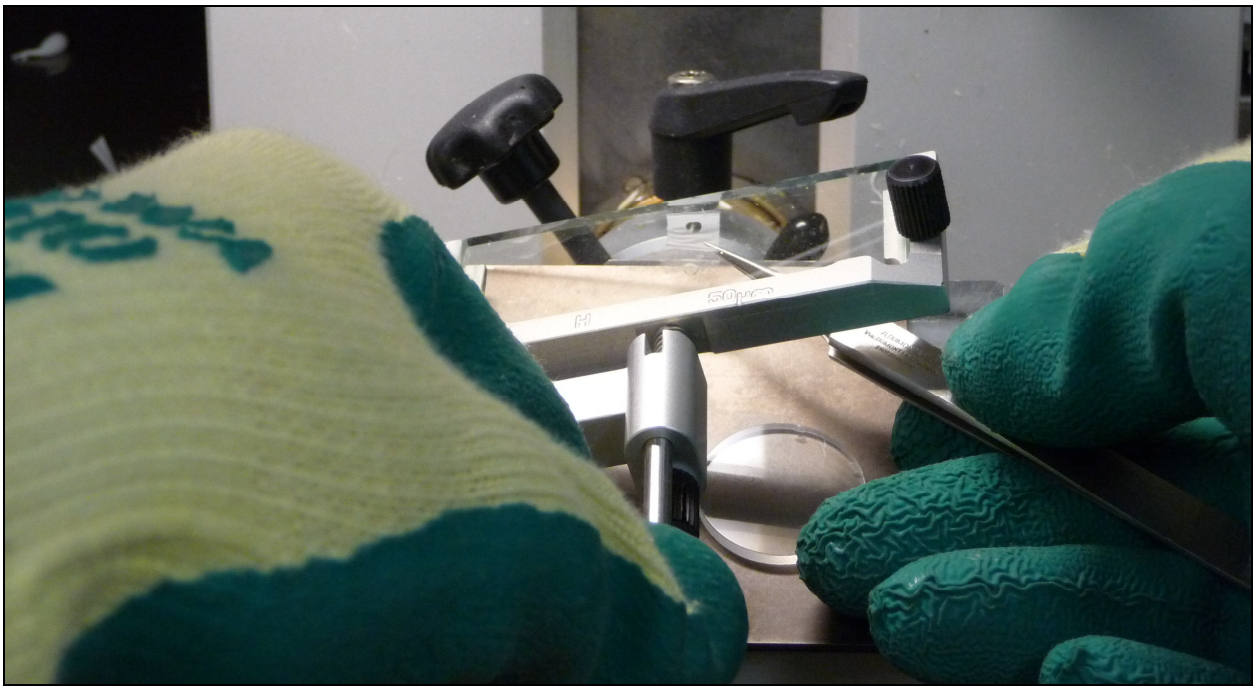

Figure 18. A section is quickly but gently removed from the anti-roll plate with cold forceps, at 45 degrees across the section to help prevent rolling.

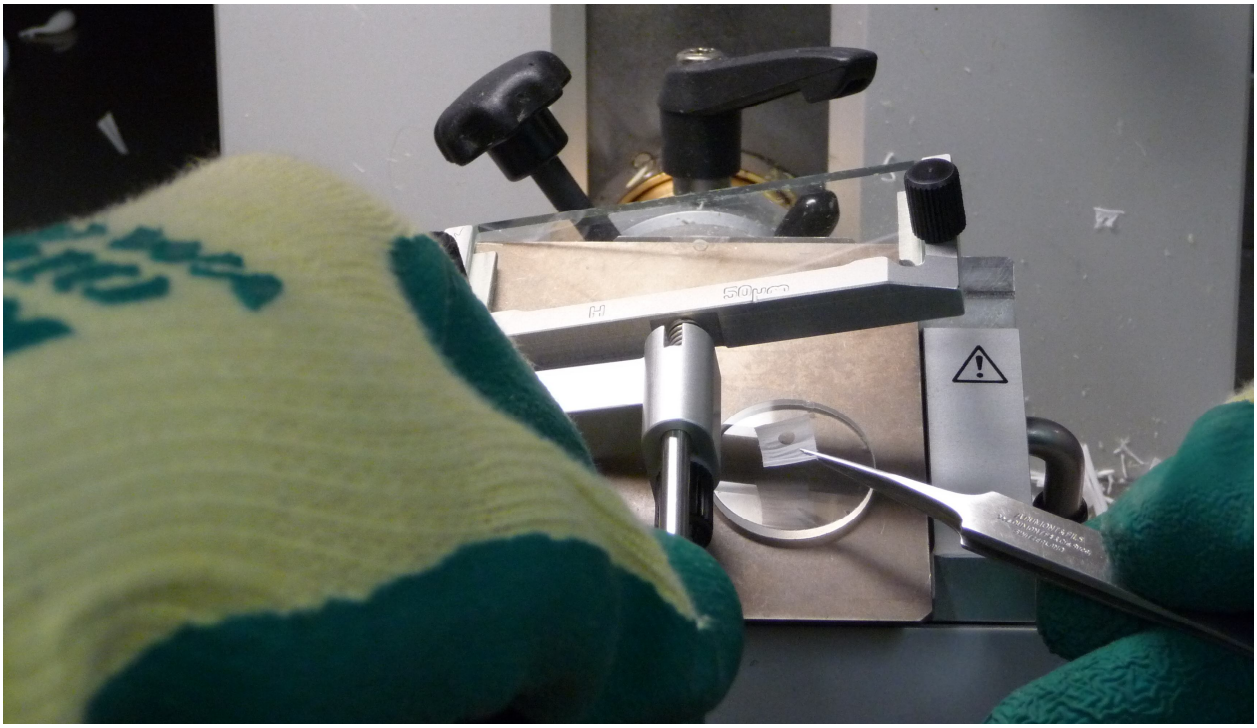

Figure 19. The section is placed quickly down onto the slide so that it sticks evenly across the surface.