SEM Diaries - 24

Processing small items, and colouring

Jeremy Poole

Fig. 1: Tardigrade *Dactylobiotus dispar* - a small creature, and a crude attempt at colouring.

It is all a matter of protocol. And, no I do not mean who sits next to whom at some Buckingham Palace banquet, or indeed whether the Ambassador of the European Union has full diplomatic status, or some watered down rights as dictated by a mean-minded Prime Minister. No, I am talking about the "recipes" for preparing specimens for examination under the SEM.

So far the only protocols I have used have been desiccation routines. These basically consist of placing the sample in increasing strengths of alcohol to remove any water from the specimen, and then to dry it using either the low surface tension solvent hexamethyldisilazane (HMDS) [1,2] or the critical point dryer (CPD) [3]. In the absence of further guidance, I have tended to use strengths of alcohol in the following sequence: 70%, 85%, 90%, 95%, 98%, 100%, 100%. When learning slide-making with Ernie Ives we tended to go straight into 100% alcohol, followed by another bath at the same strength. Basically, I have no idea whether or when the intermediate stages are necessary, or, indeed, how long the specimen should be left in each bath. I could experiment by taking short cuts, but only at the risk of losing valuable specimens.

One of the disadvantages of working on one's own, rather than as part of a team at a university or in industry is that one has to learn "from scratch". There is no library of preparation methods to consult, nor more senior members of staff to guide you. I do, at least, have one or two "mentors" and I am grateful to them for their assistance. There is also the Internet, and I have had some success in finding protocols by that means, but often they are behind a paywall so I can only read an abstract, or, if the article is available free to download, the chances are that it will specify the use of chemicals whose names I cannot pronounce, and which are only readily available to individuals associated with industry or a university. (In all honesty I could afford the few dollars most downloads cost, but it does go against the grain to pay!)

Tardigrades

Having seen some rather fine coloured SEM images of tardigrades, and read various articles on these creatures, I thought it would be fun both to find some tardigrades and also to image them on my SEM. Again, I needed a protocol for preparing these. An Internet search came up with two results that were free to download, and one in particular was extremely helpful [4]. There was no special chemical treatment mentioned, other than passing the specimens through a series of alcohols, as described above, but what was interesting was the method used to "contain" the tardigrades so that if you start with, say, 20 tardigrades you arrive at the end of the process with something approaching 20 dry tardigrades.

I have previously described my method of putting spider parts through the various strengths of alcohol [2]. This involves putting the specimen in a steel mesh filter designed to use with pipes (of the kind used for smoking tobacco - Figure 2G). These have a mesh of side 0.4 mm, which is fine for almost everything I have needed to prepare to date. However, when it comes to tardigrades the mesh is too coarse. One particular tardigrade I measured was just 0.4 mm long and less than 100 µm across, so it would easily fall through the mesh.

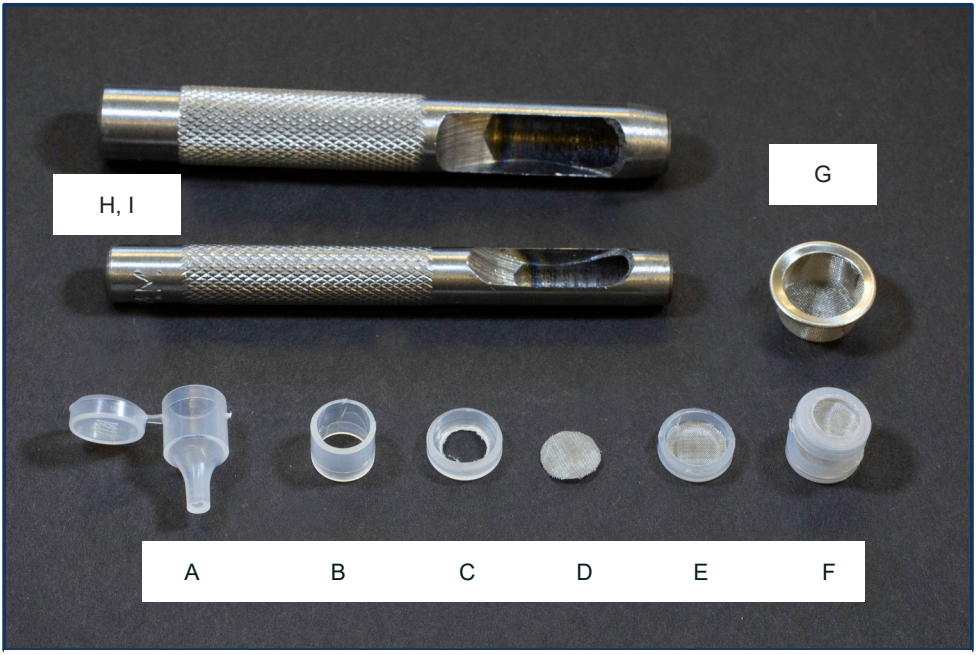

Fig. 2 A-F: Stages in the manufacture of filter tubes. G: Pipe filter.
H, I: 9 mm and 7 mm punches

I shall explain the protocol in some detail, because it would apply equally to preparing tardigrades for mounting in Balsam, and should not therefore be regarded as simply of academic interest to those who do not have access to an SEM.

The method of restraining tardigrades to enable them to be transferred easily between alcohol baths is to construct mini-tubes with fine mesh across the top and bottom. The stages in construction are illustrated in Figure 2 on the previous page. Further details on obtaining the materials can be found in the Annex. The starting point is what is known as a BEEM capsule (A in the figure). These are normally used as disposable moulds for embedding samples in resin prior to sectioning for transmission electron microscopy. The tapering bottom of the capsule is cut off, to leave a cylinder (B), which is 9.4 mm in diameter and 8 mm high. The next stage is to cut the lid from the capsule then punch a 7 mm diameter hole centrally in it (C). We need a second lid for the bottom of the cylinder (taken from a spare capsule), also punched, and two 9 mm diameter circles of fine stainless steel gauze (mesh size 40 µm) punched from a sheet (D). The gauze is laid inside the lids (E) and the lids then placed on the top and bottom of the cylinder to make up a tube with gauze filters on either end (F).

Next find your tardigrades. Well, that is not too difficult, really. A good place to find these is in moss, and you do not need to go further than the roof or gutter of your own home to find some. I am lucky and live in a bungalow, so grabbing a clump of moss from an edge tile of the roof, or even from the gutter, is eminently practical. Those of you who live in homes of several stories are best advised to wander round the base of the house looking for moss that has been flung off the roof by birds, or dislodged by heavy rain. In fact, that was how I found the first piece of moss that I examined.

Well, I said it was not difficult to find tardigrades but in reality it took me about half an hour to find and retrieve a single tardigrade from the squeezings of the moss, and similar lengths of time to find subsequent ones. There must be a better way, I thought. Yes, there is. Simply buy them from Sciento! This little company near Manchester used to sell all sorts of species, both animal and vegetable, for use by universities (and members of the public) at very reasonable prices. I bought three different cultures of at least 100 tardigrades each for £11 per culture plus postage. These were ideal for my purpose, although they were not species native to the British Isles. Sadly, after I had written the first draft of this piece I was advised that they have ceased to trade. A great loss! I guess I shall have to experiment with culturing ones I have found in moss.

Around 20 tardigrades were transferred from a sample of the culture to a water bath at 60 Celsius, and left there for 45 minutes. The purpose of this stage is for the tardigrades to expire "straight" rather than curled up and contracted. (I used the term "expire" in the previous sentence, but given how hardy these creatures are, I am not sure if it is exactly the right word. In reality they go into some sort of "suspended animation" until conditions improve. Suffice it to say that following dehydration the tardigrades processed that way were pretty straight, while ones put directly into alcohol without passing through the hot water stage - including the specimen illustrated in Figure 1 - tended to be curled up.)

The tardigrades from the hot water bath were then transferred to one of the filter tubes and immersed in 70% alcohol for three days. It is necessary to force the alcohol through the gauze to overcome any surface tension. This can be done simply with a Pasteur pipette. When the three days has passed the tube is taken through the successive stages of alcohol to the second 100% bath. The resting time in each bath is 15 minutes, and again a Pasteur pipette is used to force alcohol at the new strength into the tube and force out the alcohol from the previous stage.

Prior to mounting for electron microscopy, the alcohol needs to be removed from the tardigrades without their shrinking or otherwise deforming. This can be done either using the CPD [3] or HMDS [1]. I tried both methods and found that the CPD gave better results.

Mounting can be interesting! The process is to prepare a stub with a sticky carbon tab on it and transfer tardigrades one at a time using a "brush" with a single hair. The hair becomes charged and the dry tardigrade will stick to it. The tardigrade

can then be lowered onto the sticky tab. Previously when mounting specimens I have used a single stereo microscope. I pick up the specimen in tweezers from a raised surface in the field of view of the microscope, then I slide that surface out of the way and slide in the block supporting the stub. This proved all too fiddly with tardigrades so I set up a second stereo microscope. I had the dried tardigrades under the second microscope, which I used to aid locating each specimen. I then moved this on the single hair brush to the stub, which I could observe under my usual stereo as I attempted to lower the specimen onto the sticky disc. It might be possible to intentionally lay it on its back, or on its front, or even on end, but that tends to be more a matter of luck than judgement!

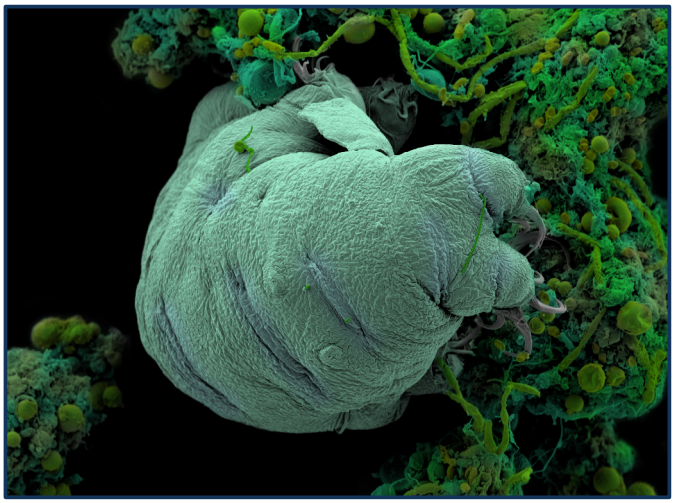

Fig. 3: *Dactylobiotus dispar* image, as coloured by Karl Gaff

The end results of my first foray into preparing and imaging tardigrades can be found on my website [5].

Processing Pollen using BEEM Capsules

Pollen is another small item that I have not yet attempted to process "properly". Interestingly, one of the protocols that I discovered for pollen also involved BEEM capsules, only instead of using a fine steel mesh as the filter medium the hole in the end caps was covered with filter papers. To be honest, I have no idea how one is expected to ensure a complete change of alcohol in a tube sealed with filter papers. I can see myself puncturing the paper every time I apply a pipette to force the new strength of alcohol through the cylinder. I guess one would just have to leave the tubes in each alcohol bath for a much longer period than the 15 minutes I used for the tardigrades.

As it happens, one of my favourite pollens, that of hollyhock, is around 80 µm in diameter, so I might get away with using the steel mesh when I eventually do "proper" preparation of pollens. Roll on the spring!

Simple Colouring

For a long time now I have wanted to hand colour some of my electron micrographs but have never had the courage to sit down and experiment, although I went on a one-day course on the subject several years ago. I think the main reason is that I find Adobe Photoshop rather daunting to use (despite having attended a beginners' and more advanced course). Another reason is that I do quite like the punchy monochrome images that I come up with. In fact, at least one friend says he much prefers monochrome SEM images.

However, from time to time I do consider colouring. In 2019 I was on holiday in Greenland and another member of the party worked for a computer games company. We managed to have half an hour together in front of Photoshop and he passed on a few colouring tips.

Occasionally I exchange emails with another microscopist, Karl Gaff, who produces colour fine art prints of light and electron micrographs [6]. Recently he saw my tardigrade images and asked if I could let him have a file for him to colour in. Later that day he sent me back the results (Figure 3).

Well, I thought, if he can do it then so can I, and a few weeks later I sat down with the same monochrome image and made my own attempt (Figure 1). It probably does not bear close scrutiny, particularly the purple nail varnish, but it was fun to do, and did not take me very long.

References

1. SEM Diaries - 16. Balsam Post 123, pages 44-47, April 2019
2. SEM Diaries - 19. Balsam Post 126 January 2020
3. SEM Diaries - 4. Balsam Post 111, pages 31-35, April 2016
4. Mitchell, C. and Miller, W.R.A Simple SEM Preparation Protocol for Tardigrades. Journal of the Pennsylvania Academy of Science Vol. 81 No. 2/3 February 2008.
5. www.jeremypoolesem.org.uk/gallery.html
6. www.kgaffphotography.com

Note: all issues of SEM Diaries may also be found at:

http://www.jeremypoolesem.org.uk/diaries.html

Annex

Materials for filter cylinders

The type of capsule I used is shown in the front row of Figure 2 at the left (labelled A). This is described as a "Size 00 Hemi-hyperbola capsule", which can be bought from Agar Scientific in packs of 100 (or 1000). The order code for 100 is AGG363-1. The cost is £9.50 +VAT + post.

There is an alternative type of capsule, which has a different design of bottom, but either way the first activity is to cut the bottom off, to leave a polythene cylinder.

There are many suppliers of punches on eBay. My original set did not include a 9 mm punch so I had to purchase a further set that did include this size. When using these punches, back the material being punched on end grain of wood. I found this works much better than using the face of a plank.

Stainless steel mesh (and other materials) is normally specified as a mesh number rather than a size in µm. Search the internet for "Particle Size Conversion Table". A 44 µm mesh is described as No. 325 and a 37µm mesh is No. 400. I bought my (No. 325) mesh from a supplier called Inoxia (www.inoxia.co.uk), who gave good service. I initially found a company called "The Mesh Company" but having read some reviews of their service I decided to look elsewhere!