SEM Diaries - 7

Ping and it's gone!

The trials and tribulations of specimen preparation, and other topics

Jeremy Poole

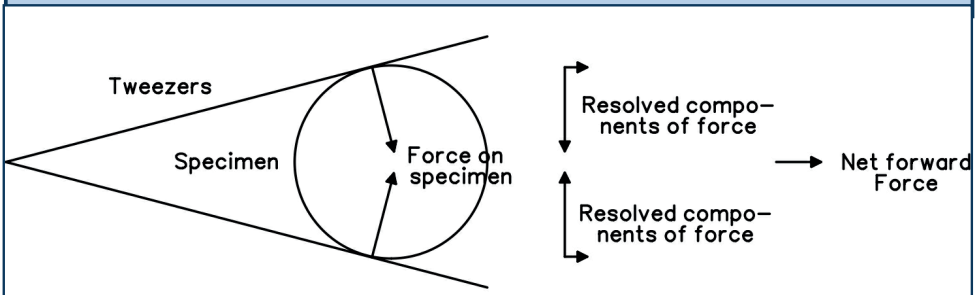


Fig. 1: The physics of projectile specimens

Readers will remember from earlier editions of "Diaries" that specimens for loading into the chamber of the SEM need to be dry. Drying can be carried out using a dehydrating agent such as heat, alcohol, acetone or hexamethylenedisilazane (HMDS), or in the most critical applications with liquid CO_2 in the Critical Point Dryer (CPD).

In preparing to dry the specimen, the first decision that needs to be taken is whether to dissect it prior to drying, or afterwards. Dissection for my applications normally consists of separating out the sexual organs, heads and spinnerets of spiders, and removing their legs. Dissection of a wet specimen is messy but has the advantage that the specimen is pliable, and unwanted tissue can be relatively easily removed. The difficulty with this approach, however, is that it becomes difficult to handle the sub-millimetre scale parts once they are separated from the creature, whether it is putting them in the "thimbles" of the CPD or seeing them through several changes of alcohol or acetone.

An advantage of drying prior to dissection is that the complete spider can be more easily passed through the drying fluids and the CPD, but the trade-off then is that the specimen becomes exceedingly brittle. I have so far mostly adopted this latter

technique, but this has led to some very frustrating moments!

So, one has a small spider, such as a hunting spider (Lycosidae), with a body length of around 5 mm (head to spinnerets). One would like to remove the pedipalps (male genitalia) which are located alongside the head, adjacent to the chelicerae, which also look good under an SEM. The front legs are stiff and sticking out at an arbitrary angle and even my small scalpel blade appears through my stereo microscope like Gulliver's feet moving among the Lilliputian hordes. I finally manage to manipulate the body of the spider so I can sever the palp from the body with a rocking motion of the scalpel - but the severed palp shoots out of the field of view to hide among any debris lurking around in the petri dish. (I carry out my dissection in a plastic petri dish, to help preserve the blade of the scalpel, and also to contain flying appendages.) Having located the palp, I transfer it to a plastic tube for safe keeping.

Having repeated the process for the other pedipalp and the eight legs I turn my attention to the body, of which I require the spinnerets (approximately 1 mm in diameter) and the head and chelicerae (about 3 mm across).

While it is relatively easy to grab a leg with a pair of tweezers, once these have been removed one is left with the body, consisting of head/carapace and abdomen together with some stumps of legs. Again, as the parts are separated they "fly" across the petri dish and, what is worse, when I try and grab a head or other body part with my light tweezers, the scenario illustrated in Figure 1 comes into play.

Imagine trying to pick up a small ball bearing with a pair of fine tweezers. The forces exerted by the tweezers on the ball are in the direction illustrated at the left of the figure, namely normal to the tweezers. These forces can be resolved into a vertical set, which "cancel each other out" and a smaller horizontal pair, which add. The net result is a forward force trying to eject the ball from the tweezers, which will happen unless there is sufficient friction between the tweezers and the ball.

To cut a long rant short, it is common for heads or other parts of spiders to shoot out of the tweezers and become deposited not only elsewhere in the petri dish used for dissection, but also elsewhere on my bench or even on the floor. Sometimes I have managed to locate these; at other times I have lost my specimen and had to start again. Oh for the nice flat specimens, either sections or flattened insects, that I was used to when making conventional slides! I even miss the times when a specimen seemed firmly stuck onto my tweezers with balsam while trying to make deep cell mounts!

In SEM Diaries - 4 (BP111) I mentioned the special "stubs" I manufactured to enable me to rotate items such as legs or pedipalps to obtain a precise angular alignment relative to the electron beam, to illustrate particular features. The object was glued into a small diameter hole in an aluminium rod, which was then slid into my special stub. The rod (1/8" diameter) could then be rotated to achieve the desired orientation. In that same article I mentioned the difficulty encountered in manually inserting the appendage into the hole and holding the specimen steady while the glue dried.

To overcome this problem I called into action various items bought at Microscopium or other Society meetings that I though might come in handy "one day". The arrangement is illustrated in Figure 2. On the right (and along the top) of the picture is a versatile stand with a Bausch and Lomb stereo microscope head attached. In the middle is a Leica micromanipulator, which allows precision movement of a specimen in the X and Y and Z planes, and can also be tilted. On the left is a Lab Jack to bring the steel block that

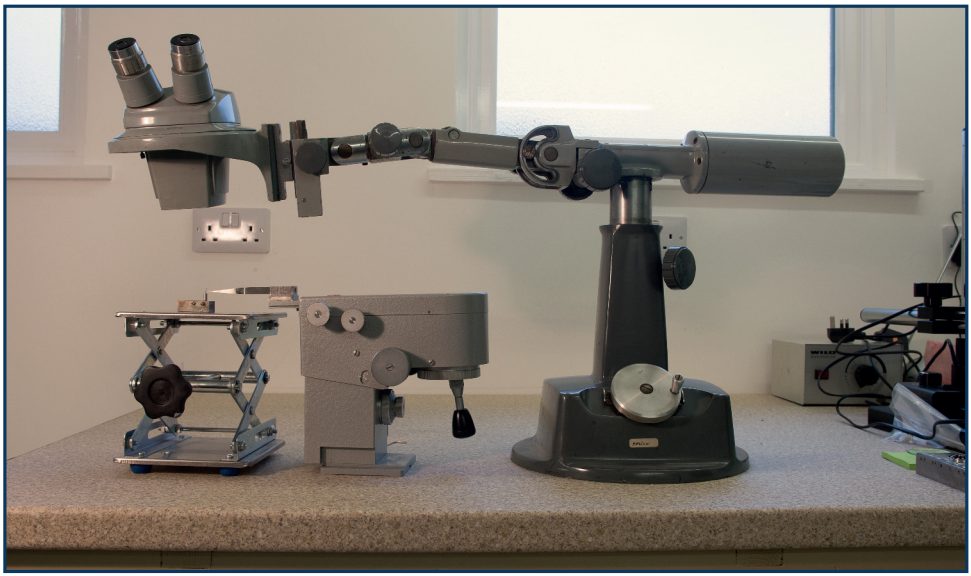


Fig. 2: Arrangement tor inserting a leg or pedipalp into the rod of the rotating stub

I use to support the rod to the correct height for the micro-manipulator. The process for transferring a dissected leg (for example) from the petri dish to the rod is as follows:

- Pick up the leg using a pair of "cross-over" tweezers, grasping the leg approximately in the middle and perpendicular to the leg's axis. (Cross over tweezers are designed to grasp the specimen when pressure on the sides is released rather than applied.)
- Carefully transfer the tweezers to a special block made for the purpose fitted to the micro-manipulator.
- Secure the tweezers using a grub screw in the block, taking care not to release the specimen accidentally.
- Using a combination of the jack and the micro-manipulator, and observing through the stereo microscope, align the leg over the 0.5 mm hole in the end of the rod then raise the jack or lower the micro-manipulator to support the less interesting end of the leg inside the hole (Figure 3).
- Apply glue and leave to dry. (After a failed attempt with superglue I now use special silver-loaded conducting glue designed for mounting onto stubs.)

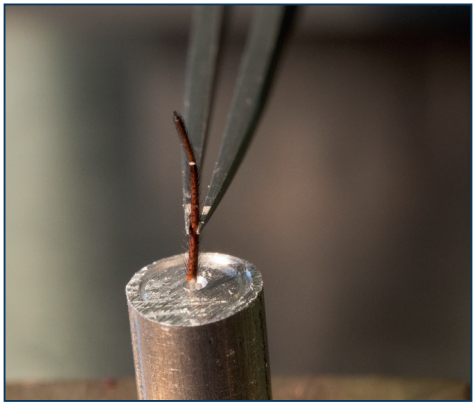


Fig. 3: The leg supported just above the hole in the rod, prior to being lowered into position

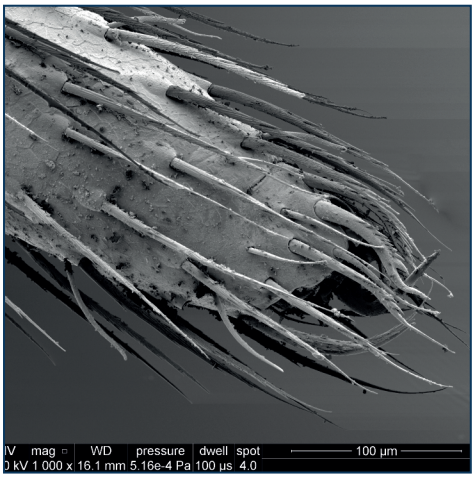


Fig. 4:Tarsus of *Zygiella x-notata* viewed at an arbitrary angle

- Squeeze the tweezers to release their grip on the leg, using a different grub screw on the tweezer support block.
- Swear, or heave a great sigh of relief, depending on the outcome.

My hit rate using this arrangement has been about 30% so far. Lack of success can be attributed to either glue inadvertently remaining on the tweezers that prevents clean separation at the end of the process when the tweezers are opened, or else to jolting the micro-manipulator while inserting or adjusting the tweezers. Assembling the micro-manipulator and lab jack on a common wooden base should increase the success rate significantly.

To illustrate the potential application of the rotating stub technique I have included two micrographs. Figure 4 shows a tarsus (foot) of the common "window frame spider" *Zygiella x-notata*. The orientation of this is arbitrary, with no attempt having been made to align it in any particular orientation. It resembles little else than a hairy stub. Figure 5 shows a similar tarsus viewed from a different angle. Here one can discern the main features of the tarsus of a web-dwelling spider. There are two large combed "claws" (c) with a middle claw (m) and several serrated bristles (b). The middle claw is only present on web-dwelling species of spider, and is used to trap silk from the web against the serrated bristles.

The ability to orientate pedipalps is even more useful. The standard works on British Spiders [1], [2] include sketches of the genitalia of male and female spiders of the majority of the British species.

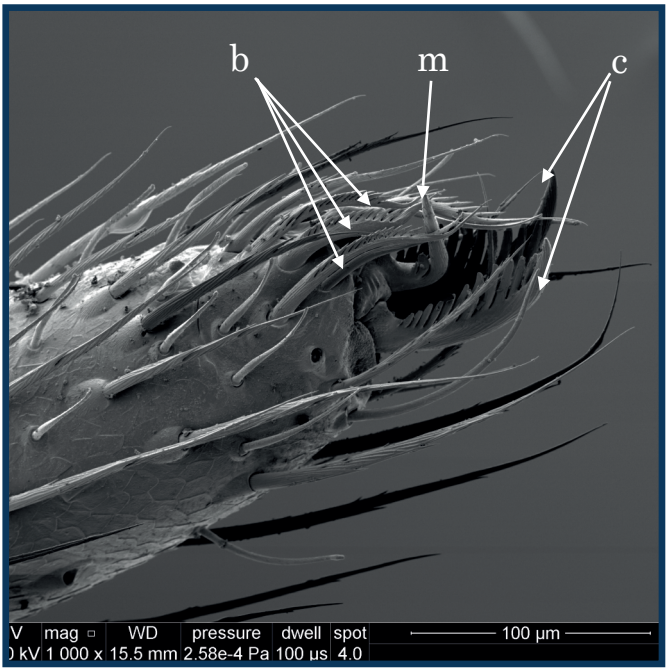


Fig. 5: Tarsus of *Zygiella x-notata* oriented to show outside and middle claws, plus serrated bristles

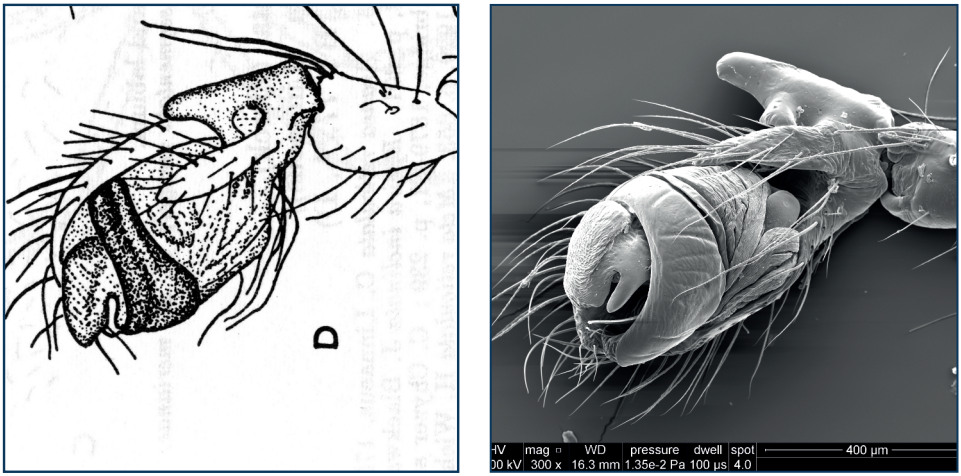


Fig. 6(a) Left, sketch of the left pedipalp from Metellina segmentata taken from Reference 2. Fig. 6(b), right, a micrograph of the pedipalp of the same species.

Figure 6(a) shows a sketch of the pedipalp of the common *Metellina segmentata*, taken from [2] while Figure 6(b) shows a micrograph of an actual pedipalp arranged in the same orientation. I have to own up here to the fact that the micrograph was taken with the pedipalp stuck onto a carbon pad rather than mounted on one of my rotating stubs. As will be seen, the orientation is not exactly the same between Figures 6(a) and 6(b), but is close enough to confirm identification.

You will note evidence of charging to the left of Figure 6(b), exhibited as dark streaks arising from negative charge built up on the hairs interfering with the electron beam. If I were to publish this picture in a book of micrographs I might well spend time in Adobe Photoshop® making selections round the pedipalp, including each individual hair, and paint out the grey streaky background with a black brush. Of course, this technique is not unique to electron microscopists. Many a stunning light micrograph has been improved immeasurably by removing dirty backgrounds, sometimes even by modifying the picture to appear to have been taken with dark field illumination, for example. Another way to eliminate these charging effects would be to use Low Vacuum mode, which I describe in some detail below.

More on Low Vacuum Mode

My ramblings thus far have dwelt on the difficulties of handling specimens that are brittle as a result of the dehydration process, and I have also illustrated the effect of a build up of charge on the specimen. I have not given any column inches this time to the drawn out nature of the dehydration process, nor indeed to the need to sputter coat with gold. Would it not be wonderful if one could take a specimen from the wild, load it into the chamber and image it without having to go through this long-winded, time-consuming and expensive process? Well, with many of the more modern SEMs, including my own, you can, by using what is known as “low vacuum” (LV) or “variable pressure” mode! Indeed, some of the more recent “desktop” SEMs make this their default or only mode of operation.

The principle behind LV mode is that water vapour (or a gas such as nitrogen) is introduced into the specimen chamber, and a fraction (say 30%) of the electrons from the electron beam collide with the water molecules. The act of collision ionises the water molecules and the positive ions (H^+) are attracted towards the uncoated specimen. These positive ions cancel out any build-up of negative charge on the specimen, preventing the unsightly effects of charging. The OH^- ions seek out any positively charged surfaces. This phenomenon is exciting enough on its own, but there is more! Secondary electrons dislodged from the specimen have low energy, which means they collide with gas atoms or molecules before they (the SEs) have travelled more than a few micrometres from the specimen, on their way to a detector at a positive potential. As the SEs (and also negative ions created by collision with the SEs) travel they collide with other gas atoms or molecules, creating yet more ions. This effect is known as "gas amplification" and produces many more negative ions at the detector than there were SEs emitted from the specimen.

This may seem like a win-win situation, but there are negative factors that also come into play. For example, the sophisticated Everhart-Thornley detector for secondary electrons cannot be used, as the high voltage required to accelerate the SEs to the scintillator would cause sparking in the highly ionised atmosphere. The "Large Field Detector" (LFD) in my SEM, used for the LV mode, consists of a simple plate of brass connected to high gain electronics outside of the chamber.

Secondly, the longer the working distance, the fewer electrons from the beam remain un-scattered by the gas in the chamber. It is necessary to maintain a short working distance between the electron gun and the specimen, of around 10mm. This restricts the depth of field obtainable in the final image. (See SEM Diaries 3 in BP110 for an explanation of the depth of field dependencies.)

Thirdly, despite the chamber being at a relatively low vacuum (say 10 to 100 Pa) the column needs to be maintained at a much higher vacuum, typically 10^{-4} Pa for an SEM with a tungsten electron source but at a very much higher vacuum for a field emission source. This differential pressure is maintained by incorporating one or more additional small apertures in the beam path. This limits the lowest magnification available.

Fourthly, the act of collision of some of the electrons in the beam with gas molecules in the chamber imparts a "halo" onto the beam, such that it resembles a headlamp in fog. There is sufficient beam energy in the central part for resolution not to be seriously affected, but the halo part of the beam can lead to a reduction in contrast and an increase in noise.

Fifthly, water vapour is continuously removed from the specimen (assuming it is a moist biological sample) and this can lead to deformation over a short period of time.

Finally, the fact that one can place specimens into the chamber without their having been prepared in any way can lead to the introduction of pollutants which can negatively impact the performance of the SEM.

Overall, though, the availability of a Low Vacuum mode can be a real boon, and most general purpose SEMs are manufactured these days with this capability.

Figure 7 on the following page illustrates the same subject prepared and imaged in different ways. I cut a couple of anthers off a spider plant that adorns the mantelpiece in my kitchen. One of them I dried gently in an oven at 45 Celsius for half an hour, then sputter coated and imaged using the conventional high vacuum mode (Figure 7a). This illustrates the necessity of using a low surface tension drying

technique such as critical point drying, rather than any shortcomings of the high vacuum mode itself.

Figure 7b illustrates a similar anther, simply placed on a stub in the SEM's chamber and imaged using low vacuum mode.

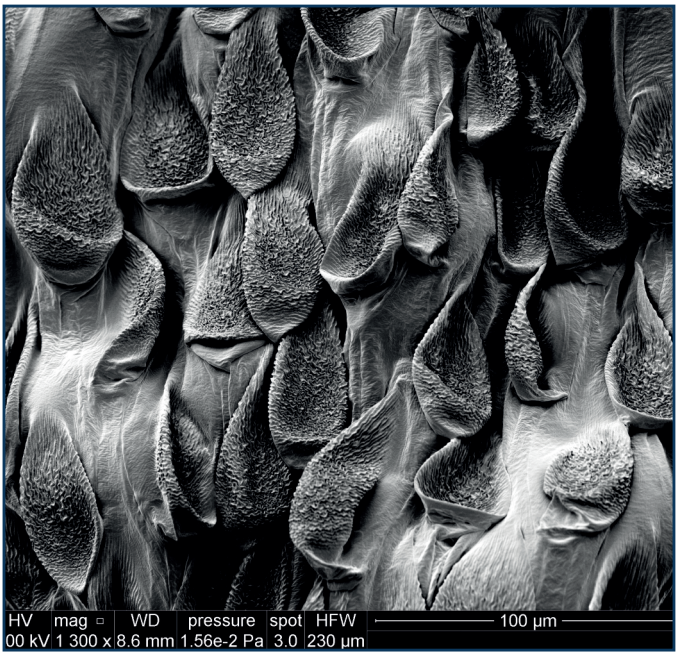
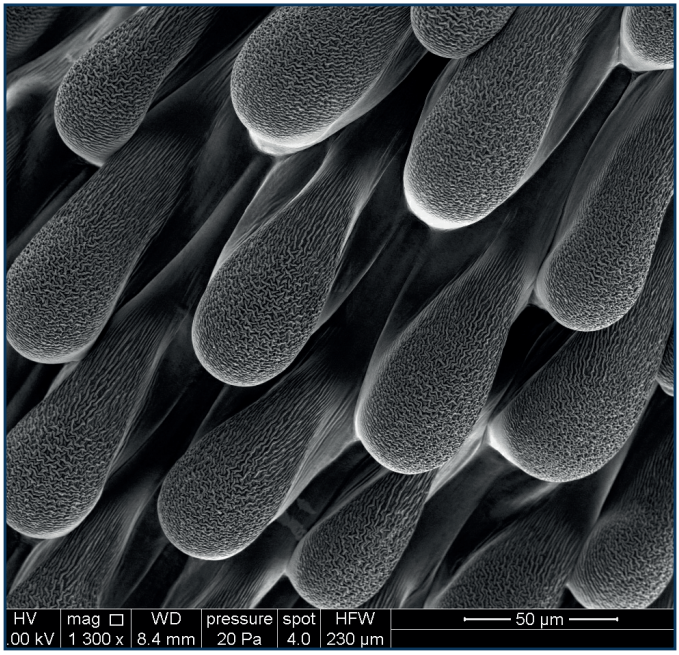


Fig. 7: Two electron micrographs of the anthers of a spider plant. For Figure 7a, above, an anther was air-dried prior to being sputter-coated with gold and imaged in the normal high vacuum mode. Figure 7b, below, was made using low vacuum mode on an untreated sample straight from the plant.



References:

1. Roberts, M. J., Spiders of Britain and Northern Europe. Collins Field Guide
2. Locket, G. H. and Millidge, A. F. British Spiders Vols 1 and 2 1975 The Ray Society