Enhanced Bryophyte Photomicrography Using Helicon Focus

Why Photograph Bryophytes?

There are several good reasons. One is pure aesthetic. The variety of cellular structure is quite amazing. Capturing this at high magnification can be an end in itself. To see the scope, Malcolm (2006) contains some amazing images. Submitting records of rare and unusual bryophytes to a county recorder or referee for determination requires substantiation. The response can be enhanced by supplying good photographs of the key identification features. A further reason is to add graphic detail to an article submitted to a bryophyte journal for publication. Finally, such images create a digital record of bryophytes which can be consulted in future years and do not take up the space of a slide library.

All of these require good quality images, which are not easy to obtain with the equipment available to most amateur bryologists. Often these images will have only part of the subject in focus. This is due to the small depth of focus at high magnifications (x40 to x600) and the 3-D nature of the subject. Helicon Focus offers the possibility of significantly enhanced results, even with relatively modest equipment. This note describes how to set up the equipment, prepare the slide and take and process images that will be pleasing and of sufficient quality to be accepted for identification or publication.

Optical Equipment Used

The camera used was a Nikon Coolpix 995. This is an older compact digital camera with only 3.3 Megapixels, but it has sufficient control of focus, exposure and white balance to make it suitable for photomicrography. The compound microscope was a Brunel Microscopes Winchester SP60 Trinocular with a plan achromatic x40 objective (infinity corrected) and standard objectives at the other magnifications. This is a near-research standard microscope. The infinity corrected x40 objective gives a wider sweet spot (for a near planar object, 85% or more of the field of view is in focus, compared to about 30% for standard objectives.) Camera and microscope were connected using the Brunel Microscopes Unilink inserted down the third tube, attached to the camera lens by a 28mm to 37mm step up ring. To prepare the bryophytes for imagery, a Brunel Microscopes BMX 4 stereo microscope was used.

A few compact cameras are still available with a threaded lens casing, allowing rigid connection to the Unilink. Brunel Microscopes' current tips on digital photomicrography are based on the Olympus SP350, which offers 8 megapixels. For other compact digital cameras, Brunel provides a Linkarm, which essentially holds the camera lens close to the Unilink and can be locked in position. For further discussion on suitable cameras and adapters (including using digital SLRs) see Brunel's dedicated website http://www.microscopyimaging.co.uk/photomicrography.html.

Equipment Set Up

The camera was set to manual with the focus mode set to infinity. Focus on the subject was achieved by the vernier control of the microscope turret. Focussing is not easy using the monitor alone when the camera is vertical, so the video output was connected to a PC using a video capture device (EZCap) with the live video displayed in full screen mode using ArcSoft ShowBiz 3.5. (Unfortunately the Coolpix is not compatible with Helicon Remote). An alternative was to plug the video lead into a television as an aid to focussing. The video mode of the camera was set to NTSC not

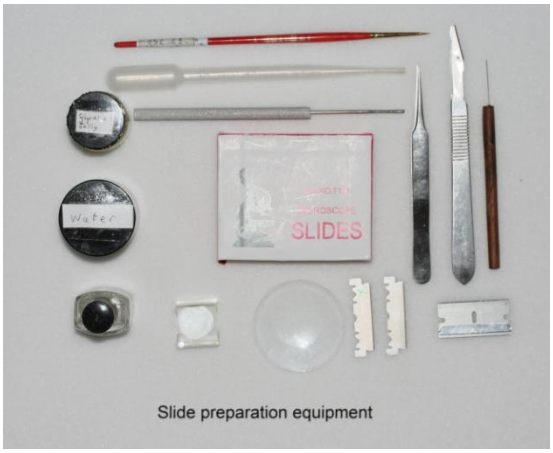
PAL. This is because the remote control for this camera captures some of the information from the monitor window. In PAL mode, this blocks the video from being sent to the PC. The remote control minimises vibration and is recommended. For cameras with a simple remote shutter release, PAL mode is preferable as it has greater resolution. The automatic white balance does not work well with high power microscopes using transmitted illumination. The Winchester has a quartz halogen bulb with rheostat control. The white balance was set to "Incandescent" and then adjusted to give a near life colour (the Coolpix provides seven discrete levels for incandescent light). The process of taking the stack of images is quite long, so the auto power off was set to 30 minutes. In NTSC mode the image is displayed on the monitor as well as being output to the PC. To increase battery life between charges, the monitor was switched off – except when needed for exposure setting. Finally, the zoom level of the camera was set to eliminate vignetting. This gave an equivalent magnification of x28, provided by the camera plus Unilink – compared to x10 provided by the eyepieces. A photo of the set up with the equipment described above is shown below.

Bryophyte Preparation

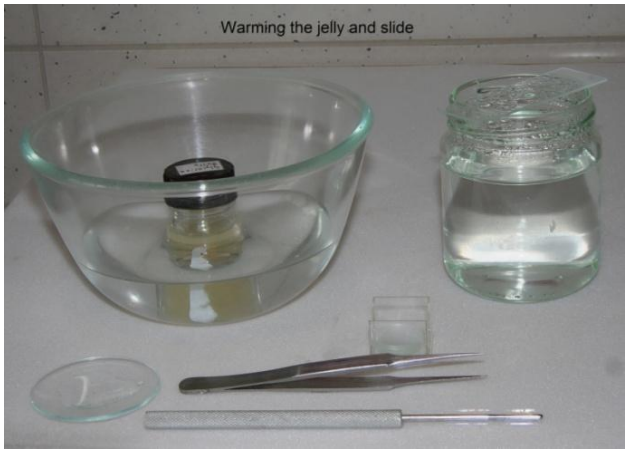
This is perhaps the most critical part. Despite the power of Helicon Focus, a poorly prepared specimen will result in an equally poor final image. There are two parts: cleaning and mounting.

A single bryophyte shoot was separated from the sample (with as little of the substrate attached as practical), put in a small container part filled with water and shaken vigorously to remove most of the dirt. Using the stereo microscope, the selected parts of the shoot were then cleaned using water and a very fine paint brush (size 000). The shoot was then dissected, in a small amount of water, to remove the required part(s).

Bryophytes are generally mounted in a small drop of water between a glass slide and a cover glass. This is a temporary mount, as the water soon dries out – especially under the microscope illumination. This does not allow sufficient time to take the stack of photographs before the bryophyte changes shape as it dries out. There are various mountants available, but one of the simplest, and quickest, is Glycerine jelly. This is firm at room temperature but becomes fluid above 65°C. Glycerine jelly contains water, so the specimen did not need to be dried out (a time consuming process involving several chemicals and required by other mountants, such as Numount). The slide and cover glass were first cleaned using an optical cloth. A small drop of the glycerine fluid was placed on the slide, the bryophyte placed on the drop and a cover slip added. A few minutes in a refrigerator allowed the jelly to harden and the slide was then usable. An assemblage of the equipment used is shown below.



Slide preparation equipment



It was not, of course, as simple as this. The drawback to this mountant is air bubbles forming around the sample. Much of the detail of this part of the procedure is in eliminating, or at least minimising, the formation of air bubbles. Some key tips are:

- The glycerine jelly container was placed in a bowl of boiled water. This kept the jelly fluid for a reasonable time, but the hot water was refreshed from time to time.
- Whilst the jelly was being heated, the specimen was placed in a watch glass containing a small amount of 50% glycerine and 50% water. Care was taken to mix the ingredients without generating air bubbles. The sample was examined using a stereo microscope to ensure it was coated both sides and the fine paint brush was used to remove bubbles. This minimised the risk of bubbles forming when the sample was placed on the warm jelly.
- The slide was warmed by placing it over a small container of boiling water. This ensured that the glycerine flowed when put on the slide and did not cause air bubbles. (Note that, in the picture above right, the condensation is on the underside of the slide. The glycerine is applied to the dry, warm, upper side.)
- When the glycerine was fluid, a small drop was placed on the slide (using a steel rod – a glass rod is also good), the sample was transferred to the glycerine using a pair of very fine (number 5) forceps and the cover glass was added. The slide was examined under the stereo microscope and gentle pressure applied to move any air bubbles away from the sample. This part was as quick as possible, to ensure the glycerine remained fluid until the cover glass was firmly in place.
- The slide was transferred to the refrigerator for several minutes to harden.

This sounds daunting, but with practice it will become routine. If the sample is a whole leaf then it is not necessary to remove all air bubbles – just avoid large ones. Small bubbles will disappear with time. Also, there will be parts of the leaf where there are no bubbles and these can be photographed to show cellular detail. If the whole leaf is to be photographed, and there are a few small bubbles, it is best to wait a day or so for these to permeate through the jelly.

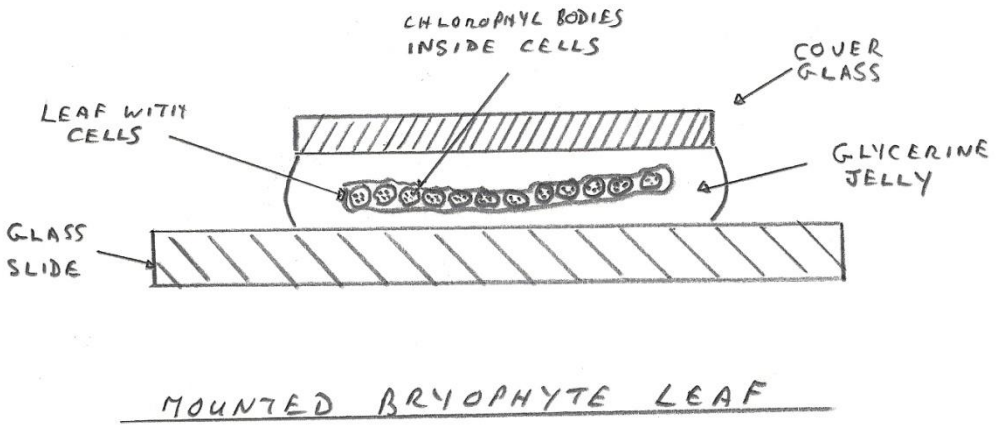
The slide will last a couple of weeks, after which the cover glass may become loose. If it required for longer, the rim of the cover glass can be sealed with clear nail varnish, using a very fine paint brush (not the one in the lid!).

For a detailed account of mounting plant sections using glycerine jelly see booklet 2 of Marson (1983), available through Brunel Microscopes Ltd (http://www.brunelmicroscopes.co.uk/videos.html).

Photographing Bryophytes with Transmitted Illumination

This is not the same as, say, photographing insects, which have hard, opaque cuticular segments that reflect light. A bryophyte leaf is partly transparent, so that as the focus is varied (going down

through the leaf), rather than particular areas becoming unfocussed, the camera focuses on the layers beneath the surface. The figure below shows a cross-section of a microscope slide.



This shows that the leaf will not generally lie flat. A particular "slice" through the moss will have some surface elements in focus and some subsurface elements in focus. Since the leaf is not completely transparent, the subsurface will gradually become unfocussed the deeper the camera "sees" into the leaf. Helicon Focus will try to bring all the areas into focus, with the result that some parts of the image may be a mix of surface structure in good focus and the various bodies within the cells more fuzzy. This needs to be borne in mind when selecting the slices to photograph and when processing the resultant images, because it will affect the choice of processing method – see "Preparing the Composite Image" below.

Another aspect to consider is what each image is intended to portray. For a whole leaf picture, at relatively low magnification, the above factors are almost irrelevant. But to show a particular identification feature will require higher magnification and transparency becomes an issue. This is also discussed in more detail in the section on preparing the composite image.

Taking the Photographs

Brightness variation is a potential issue when photographing at high magnifications. In manual mode, the Coolpix displays the difference between the set exposure and the metered exposure. By cycling through the focus range over which the photographs were to be taken, the exposure was set to minimise the variation over this range. The Coolpix has an aperture range in infinity mode of F4.6 to F10.6. This was set to the widest, except for low magnifications. The rheostat on the lighting was adjusted so that a short exposure time was achieved, that provided reasonably constant exposure over the intended focus range. Exposure times varied from 1/15 to 1/60. Note that selecting different objectives (magnifications) changes the exposure. More light is available at lower magnifications. The exposure settings needed to be changed to compensate for this.

The next stage benefits from practice. It's about getting a feel for adjusting the vernier focus control of the microscope. It was found better to start with taking more individual exposures, downloading all of these and then being selective when running Helicon Focus. The Winchester has a good vernier control, with a scale marked on the controls. Though tedious, it is worth noting at least the start and end points of the focus range – so that it is easy to go back and add more exposures if required.

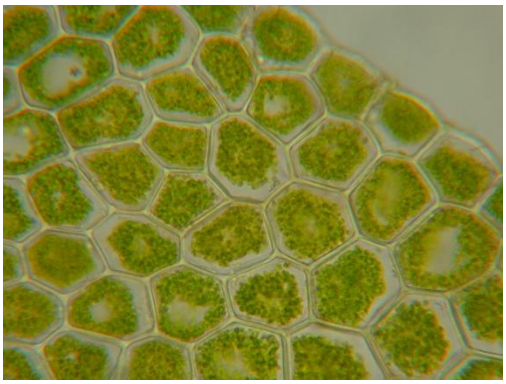
Preparing the Composite Image

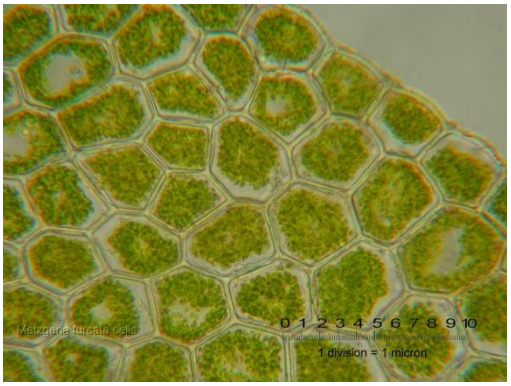
This section uses a number of bryophyte leaves to illustrate how Helicon Focus can be used to achieve good photographic results. In all of these, the left hand image is before applying Helicon Focus (the best from the stack of images). The first image pair is a cross section of a leaf of

Pogonatum urnigerum photographed using the x10 objective (effectively x280). The method of obtaining the cross-section is described later in this article.

The yellow-brown parts represent the leaf lamina and the green parts above are lamellae (linear structures, one cell wide, running most of the length of the leaf) with translucent apical cells. Apart from the leaf margins (the left hand one has a clear tooth), there are only subtle differences between the unprocessed and processed images. In fact, the final image was found to be relatively insensitive to the parameters – though Method B with a large radius (13) and low smoothing (2) did produce better results. The objective of this image is to be able to count the lamellae (the number being a distinguishing feature between two closely related species – there are 43 in this case). It is just easier to do this with the processed image. The reason for the similarity is because most of the image is relatively flat (produced by cutting with a sharp blade). The exception is the marginal tooth, which comes into clearer focus with the processed image. So, for this sort of image (almost two-dimensional) the settings are not critical and relatively few exposures need to be taken to achieve the end result.

A similar situation is seen in the images below, taken with the x40 objective. These show a common Liverwort that grows on trees, *Metzgeria furcata*.



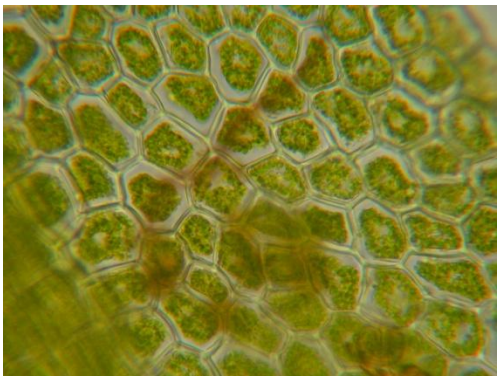


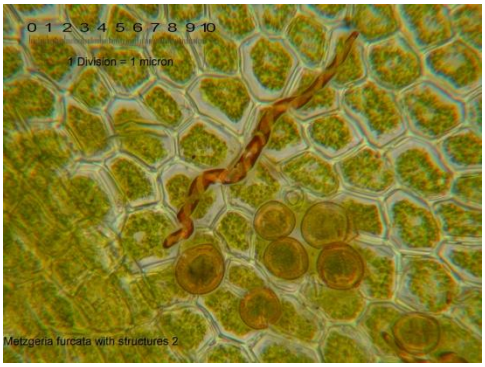
Note that the chlorophyll objects are in sharper focus as a result of processing. There is also greater clarity of cell boundaries in the processed image. Method B again worked best with similar radius and smoothing setting.

Some understanding of the way the algorithms work demonstrates why method B works better for translucent objects. The algorithms measure the contrast of each pixel. Method A produces a weighted average across all layers, based on the contrast of each pixel. Method B essentially selects pixels from different layers (Kozub, 2011). Thus Method A will produce fuzzier chlorophyll bodies, depending how deep into the cells the layers were taken. However, Method B will take the best pixel from whatever layer and thus produce a composite image of all the “best” pixels. This still leaves the distortion introduced by viewing each pixel through the, albeit partly translucent, upper cell wall.

The reason why the unprocessed image is reasonable, though noticeably fuzzier, is the use of the infinity corrected plan achromatic objective at x40. With ordinary achromatic objectives the focal point is not at infinity, resulting in only about 30% of the field of view being in focus, even for a planar subject. Only the central parts of the images were clear with this objective.

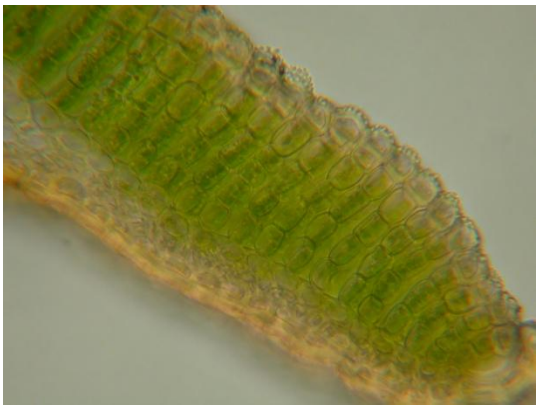
Often, the leaves have other objects resting on them - such as spores or gemmae (vegetative reproductive structures). They also may have hairs at the margin or on the lamina or have papillae (generally single celled protrusions) on the leaf surface. Below is a different area of the same Liverwort, which shows several such structures.





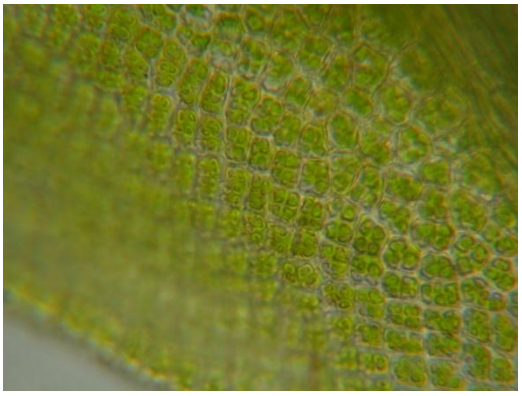
The lower left hand corner is the raised midrib. In the lower middle of the image are several spores (quasi-spherical objects) and in the upper middle is an elator (a helical device for ejecting the spores from the capsule). Without using Helicon Focus, it was not possible to bring both the lamina and the surface bodies into focus at the same time. There are also traces of translucent hairs. However, these do not show well in the composite image because they are colourless and cannot compete with the strongly coloured bodies when the Helicon algorithms are applied (see above discussion on the two methods – these hairs have little contrast and so do not show up well in the processed image). In order to get all in focus, it was helpful to cycle through the area of focus and observe the structures as they came in and out of focus. When the images were captured, the vernier control was adjusted to ensure a reasonable number of exposures of each body around its sharper focus area. Having obtained enough images, the Method B algorithm worked well with settings similar to those above. However, at the first attempt it was noted that the midrib was not in focus, whatever the parameters. It was necessary to go back and extend the range over which the images were taken. The vernier on the Winchester has a graduated scale. By noting the reading at the start and end of the sequence, it was easy to go back to the right area and take some additional images. With a transparent subject such as bryophytes, it was found necessary to extend or re-do a number of sequences in order to achieve good results.

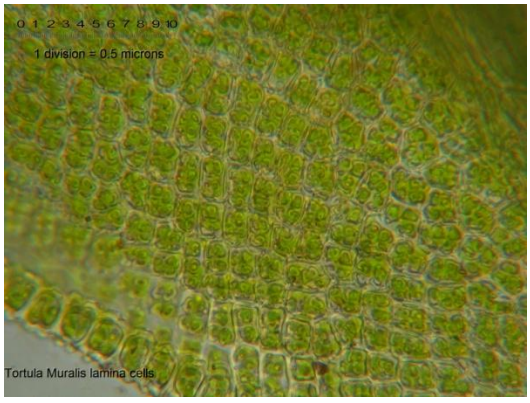
A common identification issue is to produce a narrow cross-section of a leaf (perhaps 20 microns or so wide) and mount this edge on to show the cellular structure of a section of the leaf. The challenge photographically is to try to get the whole length of the cross section in focus (see the first pair of images at the beginning of this section). A leaf cross-section was made from *Pogonatum urnigerum*, a moss with 40 to 50 lamellae running along the length of the leaf, each lamella being 5 or 6 cells high. There are various techniques for making cross sections. The one used was to place several leaves side by side on a slide with a little water and held down by the forefinger so that a small amount of each leaf protruded. A new razor blade was used to cut thin slivers, holding the blade close to the finger (this is less dangerous than it seems!). The finger was gradually eased back to allow more sections to be cut. These were examined under the stereo microscope and the best was mounted on a slide. The results are shown below.



The key identification features are the number of green cells in each lamella and the shape of the final cell. Here there are 5 green cells, plus a roundish final cell, which makes it *Pogonatum urnigerum*. If there were a few more green cells and the upper cell was more strawberry shaped, it would have been *Polytrichastrium alpinum* – a moss growing in a similar habitat to this one and a closely related species. To determine these characteristics, it is not necessary to have so many lamellae in the image. In practice, the parameters were optimised to give more detail in the right hand area. The image would then be cropped if sent to a referee or recorder. Method B was again better, with the radius set to 15 and the smoothing to 2.

A final example is given to show papillae on the leaf surface. These are often only a couple of microns high, but due to the curvature of the leaf surface it is not possible to get a reasonable section of leaf in full focus. The images below show that Helicon Focus can produce quite a large area in focus. The result can be cropped and sent for identification.





The images were taken with the x60 objective (x1680 magnification through the camera). The central part of the processed image shows that the cells are densely packed with C-shaped papillae. To get a reasonably broad leaf section in focus, the radius was set quite large (20) and the smoothing reduced down to 1. As with all other bryophyte images, method B produces a crisper image of the translucent leaf.

Adding Text and a Scale Bar

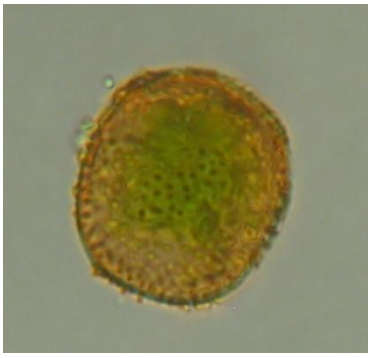
An important parameter to record with the final image was the scale. The magnification of the image differed from that seen through the microscope due to the difference between the magnification of the camera plus Unilink and that of the microscope eyepieces. Helicon Focus provides a superimposed scale. The key is to adjust this to the actual image. This was done by choosing two identifiable points in the image that were a known distance apart (typically 100µm). The microscope's calibrated measurement eyepiece was used to determine this. The scale bar was then sized to equal this distance on the composite image, by reference to the same two points. Note: if

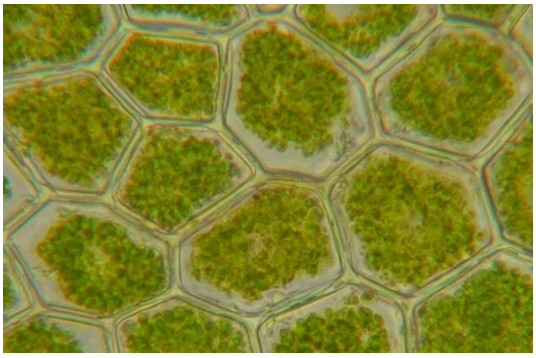
stacks of images are being taken at the same magnification then Helicon focus remembers the scale and it can be applied to subsequent image stacks.

Adding text was relatively straight forward. Two lines were necessary to superimpose the scale calibration and the species name.

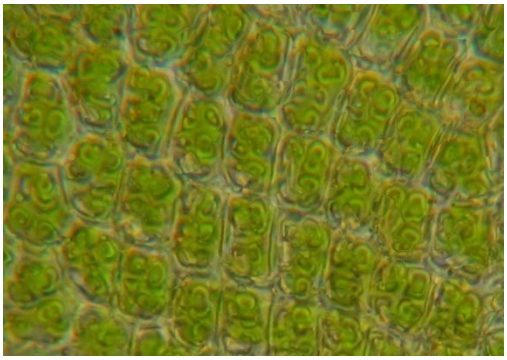
Gallery

Here are some cropped images that would be suitable for submission to a referee or journal:

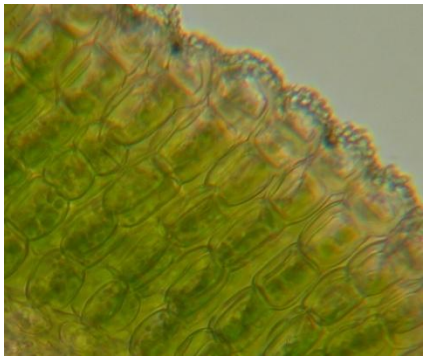




***Metzgeria furcata* spore and leaf cells**



Tortula muralis var. muralis papillae



Pogonatum urnigerum lamellae

Conclusions and Recommendations

The objective of this application note was to describe, to an amateur bryologist, how to produce excellent bryophyte images with relatively inexpensive equipment and the Helicon Focus package. The transparency of most bryophyte material meant this was not as straight forward as for an opaque subject, such as an insect. However, that feature did allow the microscope's transmitted light to be used. The software produces substantially improved final images over those from a single exposure, especially for complex images with several structures protruding beyond / above the leaf surface and when the leaf surface was curved. These are excellent for substantiation of bryophyte identification, in submission to a county recorder or referee. The quality is also suitable for the submission of an illustrated article / paper to a bryophyte journal.

The method B algorithm worked best for all the image stacks used. From discussion with Helicon Soft Ltd (Kozub, 2011) this is due to the way it selects the best individual pixels from the stack of images. It was also the case that relatively high radius and low smoothing optimised the image.

Comparing the image seen through the microscope eyepieces with the individual camera images, it appears that the camera is degrading the image at high magnifications. This may be an optical phenomenon (Kozub, 2011) However, if higher quality is required in the final image, then a camera with a better lens and CCD would be the way to go (e.g. the Olympus SP350). The Winchester is an

excellent microscope for photomicrography, especially when used with Plan objectives. A potential area to improve would be a finer vernier focussing mechanism – giving Helicon Focus more closely separated images to work with. However, the focussing mechanism of the Winchester is very good and improving on this is getting into the range of laboratory microscopes.

One final recommendation would be to try staining the material – so that some of the transparency is reduced, but transmitted illumination is still possible. The procedure, whilst well defined, involves the use of several chemicals – not only for staining but for washing, dehydrating and fixing the resultant stain(s). An addendum to this will be produced if I can master the technique!

References

Kozub, D. (2011) Personal Communication. Helicon Soft Ltd.

Malcolm, B. and N. (2006) **Mosses and Other Bryophytes, an Illustrated Glossary**, second edition. Nelson (New Zealand): Micro-optics Press.

Marson, J.E. (1983) **Practical Microscopy**. Northern Biological Supplies.

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