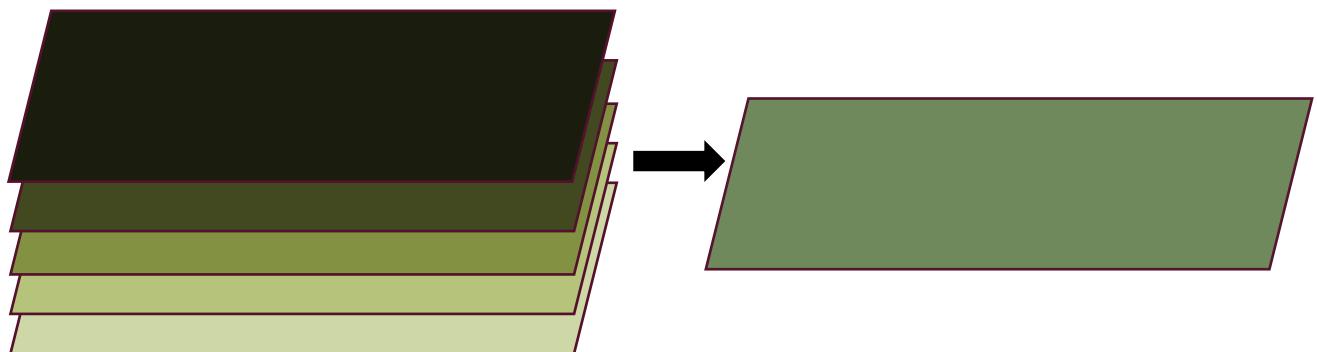


Protocol layer-photography and editing using Adobe Photoshop

Information;

- Written for the visualization of trichomes. Can be used for visualizing up to 5-10 nm, as we also have done for type 4 and 5 trichomes in the example.
- Using the correct setup, the method can be used for large subjects using a DSLR camera with the proper lens setup.

Basics;



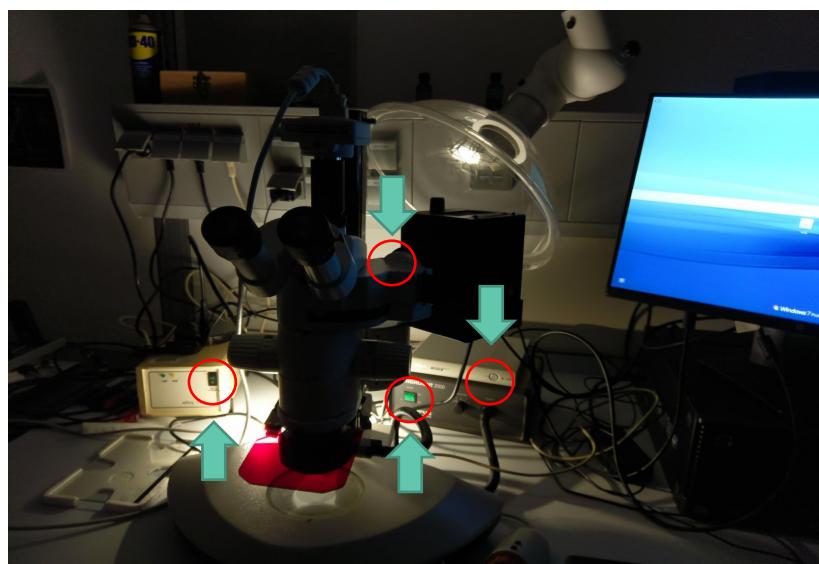
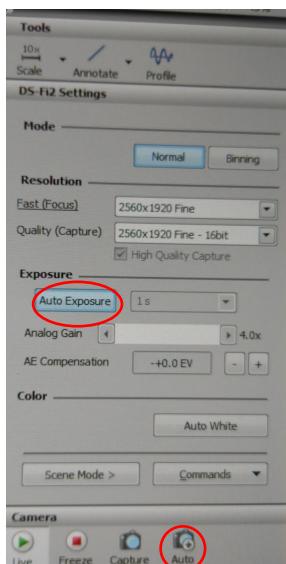
- Multiple pictures (between 20 and 60) are made using a confocal light microscope with an attached camera. The number of pictures is determined by the details of the subject and the amount of zoom. More zoom means that there is less in focus (because of diffraction) and therefore more pictures need to be made. Also, the amount of details in the subject(s) also determines the number of pictures.
- These pictures need to be aligned in programs like Adobe Photoshop (PS), Adobe Illustrator, GIMP or any other photo editing program. In this protocol, PS will be used as it results in the best-looking pictures. Using this protocol in different programs should give similar results.
- This protocol is originally made for the stacking of microscope pictures but with the correct setup it is also possible to perform the same with a DSLR camera and the correct lens for the subject (18-135 or higher) as is described on page 4.

Content

1. Microscopy stacking method;
 Discussion
 Trouble shooting
2. DSLR-camera stacking method;
 Discussion
 Trouble shooting
3. Adobe Photoshop stacking method;
 Discussion
 Trouble shooting

1. Microscopy stacking method:

- 1; Turn on the computer using the 4 on buttons (see red circles in the picture), for the lighting, the microscope, the adjustment tool and camera. Login in on the IMCB account and open NIS-elements.
- 2; Prepare your sample under the microscope, to do so; place it perfectly in the centre when completely zoomed out. Zoom in on the subject and adjust the focus.
- 3; To properly setup the program, click on “auto exposure” on the right side. If the screen turns blue, red, green etc. then slide the color slides to the transparent film, based under the lens of the camera. Make sure the quality is on the highest resolution (2560x1920) as this will prevent a lot of blur in the editing later on.



- 4; Determine a starting point and a end point. Do this by changing the focus and remember these points by a certain detail. Do not zoom in and out when you found your starting point, only use the tool (this small red wheel with the red and black buttons on them) to change the focus.
- 5; Start to take pictures from the start till the end, do this by slowly adjusting the focus using the focustool. Start with a single picture at the start point, then check if it looks good (colors, lighting, zoom etc) and start the series. Use “auto” and **NOT** “capture”. Using auto, the program takes a picture in 1 or 2 seconds and goes back to the live-view again. Go slowly, layer by layer till you reach the end-point. Anything between 20 – 60 pictures will work, but more pictures are better.
- 6; The pictures are saved in a general folder, keep this folder empty by dragging the pictures to your own.
- 7; An example of focussing on the different layers can be found in the PS editing method. In that example 50 pictures are used and it should look like that when moving through the pictures. Try to prevent large gaps as this will most likely end up as a blurred region in the final picture.

Discussion;

- The pictures will be used in PS to adjust and compress them to one final image. Using more pictures results in more preservation of details in the final picture. If using less zoom, fewer pictures need to be taken as there is not as much diffraction of the light.

Trouble shooting:

- The NIS-elements is not working, the lighting does not work good enough etc.
 - You might have missed an “on” button, see step 1.
- The microscope changes focus really fast while the wheel of the focus tool was barely touched.
 - Press either the red or black button to make it go back to the normal mode.
- I can only see through 1 lens, but I can see what happens on the computer screen / I cannot see what happens on the computer screen but I can see through 2 lenses
 - There is a black metal label on the left side of the machine that is used to switch between the camera and the lens. Either pull or push it. It is important to have both vision on the screen and the microscope.
- I can only see through 1 lens AND I cannot see what happens on the computer screen.
 - You should adjust the settings of the program on the computer like the exposure time. Not enough light is hitting the sensor. Click on “auto exposure”.

2. DSLR-camera stacking method;

- 1; A setup need to made for the camera on a tripod that is not able to move.
 - To prevent any movement of the camera or tripod, work with a DSLR-remote to take the pictures and the tripod could be taped to its position.
- 2; On the side of the lens you can find a button with 'AF' (auto focus) and 'MF' (manual focus). The camera itself is not able to make stacks so make sure that the switch is on MF.
- 3; Focus on your subject and if necessary zoom in until you found a good starting point. Check if you are able to focus the whole subject, if not, try to zoom out a bit or create more distance between the lens and subject. From here on you should not touch the camera EXCEPT the focus-ring.
- 4; Using the remote and the focus-ring you can move your focus along your subject.
 - It is important to know that you should not move too fast through the subject as you might miss some details. It is better to make too many pictures than missing a part as it will not be possible to move back.
- 5; Move through the pictures on the camera and check if the pictures move smoothly along the subject as you changed focus.

Discussion;

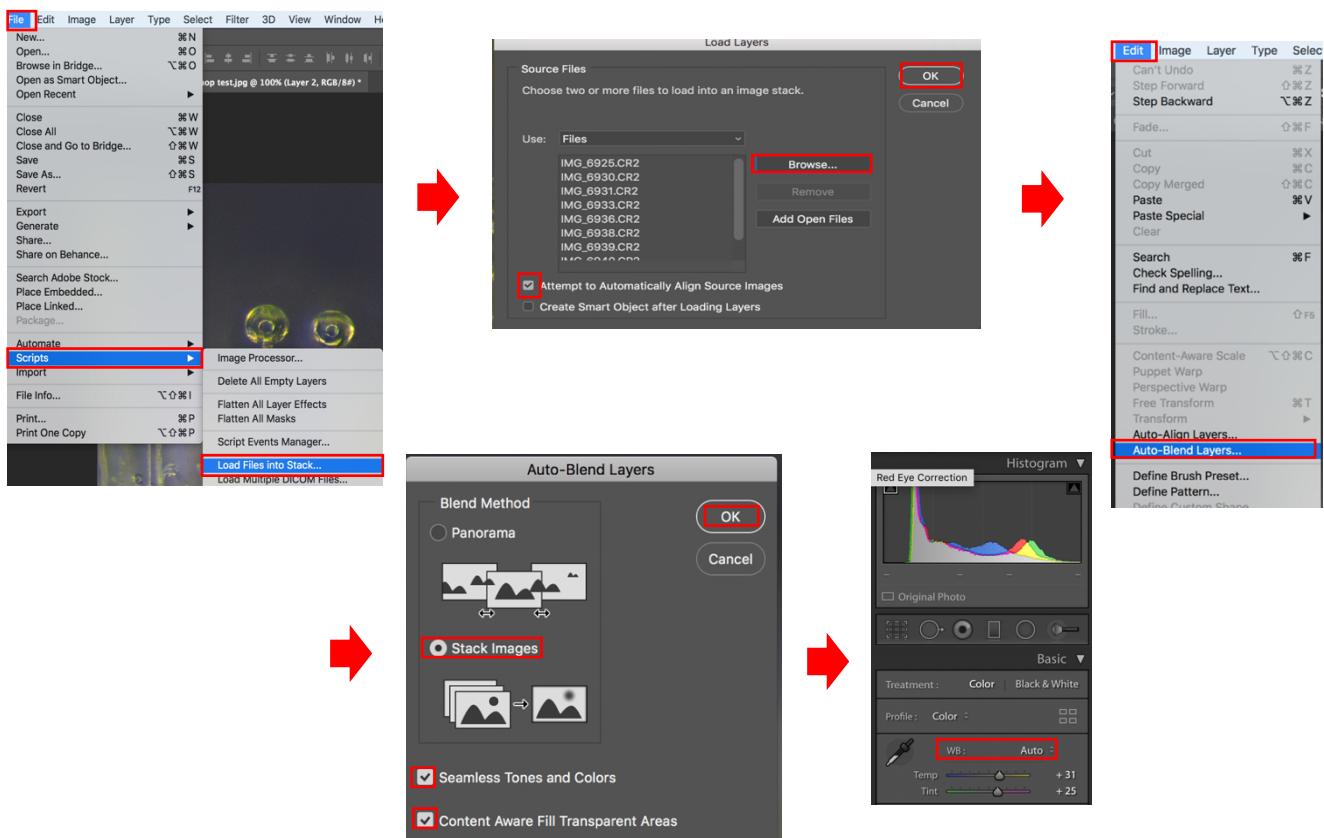
- The pictures will be used to for stacking in Adobe Photoshop. This process can be used for variety of subjects like plants (root hairs, stem, leaf, flowers) and/or larger insects.

Trouble shooting;

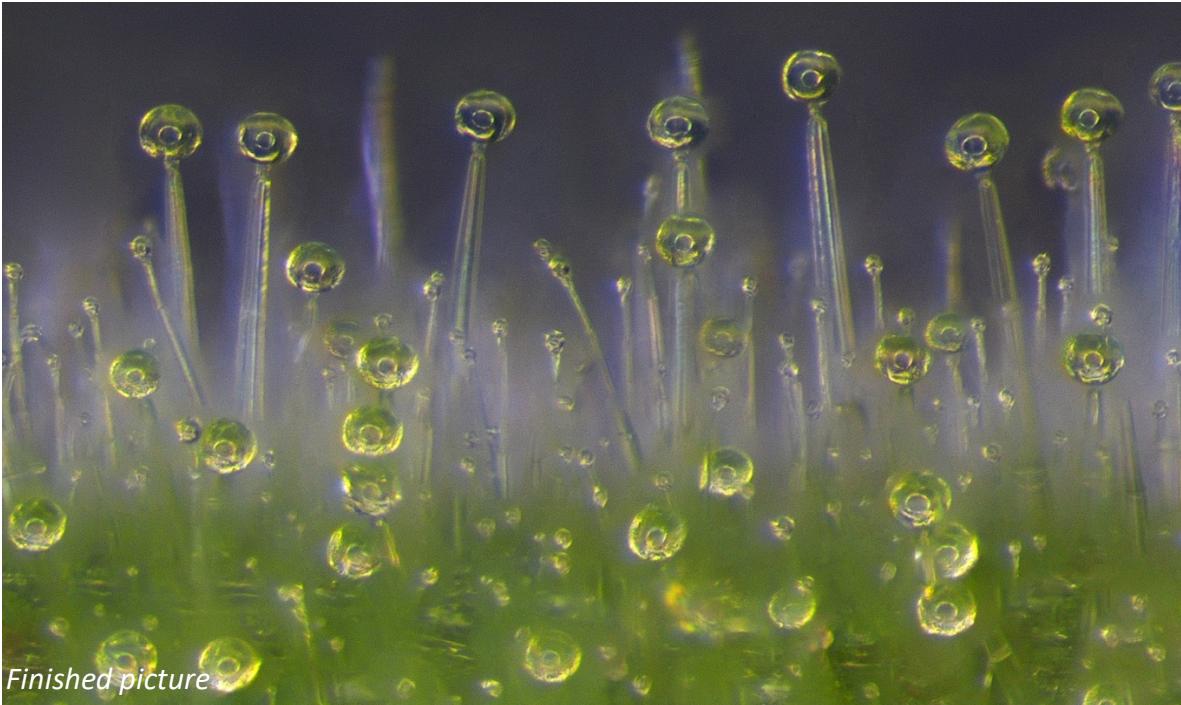
- The subject is small and it is not possible to get it in focus from close-by.
 - This is because there is a certain distance needed for the lens to focus. You can add a macro module between the lens and the camera.
- The camera does not have a focus-ring or is not able to change to MF.
 - In this case you are not using a DSLR as all DSLR-cameras are able to change to MF and have a focus-ring on the lens. The MF might be placed on the camera in some special cases but every lens, even cheap ones, have a switch on the left-side.
- The focus-ring reacts too quickly/ too slow.
 - The focus-ring is made to adjust small movement, but also to quickly change your focus from close-by to far away. The faster you turn the ring, the quicker it changes. This also vice versa as soft and slow movement mean small changes which are sometimes barely noticeable.

3. Adobe Photoshop stacking method:

- 1; Open PS version 2017 or 2018.
- 2; Click on “file” and go to “open”, now select your first image of the stack and press on “ok”.
- 3; Click on “file” and go to “Scripts” and click on “load files into Stack”.
- 4; As a result, you will now get a pop-up where you can choose all layers you want to stack. Click on “browse” and select all the layers, including the one selected before.
- 5; Now check “attempt to automatically align source images” and click on ‘ok’.
- 6; The layers will be stacked and aligned automatically.
- 7; Select all images by clicking on the first stack and click on the last layer while holding the shift-button.
- 8; Go to “edit” and click on “auto-blend layers” and select “stack images” and check both boxes (seamless tones and content aware). Press “ok”. The images will now be stacked into 1 image.
- 9; Click on the layer completely at the bottom called “background”, make this layer invisible.
- 10; Go to “file” and click on “save as”. Now save the picture both as PS-file and as tiff/JPEG/etc.
- 11; The final picture can be fine-tuned using software like Adobe Lightroom (LR) if the colors do not match the reality because of the diffraction of the light and possible interference of the computer software. Do this by loading the picture in LR and change the WB (whitebalance settings) from ‘shot’ to ‘auto’. Make sure to not change any other settings in LR.



- An example of how the finished result of the picture looks like using the microscopy method and a video of the stacks. Click on the right mouse button and select play to see the stacking of the 50 pictures to give a general impression of the distance between the pictures.



Discussion;

- What should be kept in mind, if you use the same amount of zoom under the microscope, then all distances are as good as the same and therefore only 1 scale bar is needed when comparing finished pictures. **BE AWARE**, when you crop the images, stack them on top of each other to make sure that the same cropping is used in every picture. This is to make sure that the proportions and the scale are the same and do not accidentally differ.

Troubleshooting;

- If the stacking does not work, go back to step 5 and uncheck the box to automatically align the pictures. Perform the following steps;
 - Go to “edit” and click on “auto-align layers”.
 - Select Auto and make sure that both boxes are unchecked! Click on ‘Ok’
 - Continue at step 6;
- If in step 5 the “attempt to automatically align source images” cannot be selected, make sure that you opened a new file in step 2 using the first layer.
- If the pictures come out as “smart objects”, then click on “layer” go to “rasterize” and click on “rasterize all layers”.
- When there are transparent gaps or blurry areas, you have either;
 - Made large steps between each picture. This means you miss details that are not captured in the photographs.
 - Or forgot to check the boxes “seamless tones” and “content aware” in step 8.
- If the final result comes out blurry and/or weirdly shaped, keep in mind that;
 - You need enough pictures as there might be too much details in the picture and the stacking-distance was too big, see also the video of step 8 which has about 50 pictures.
 - The new pictures can be taken with less zoom to have more details in focus and therefore need less pictures. The more zoom there is, the less there is in focus.

