Setup (Image J for Anatomy)

Part of a CSHL protocol: root anatomical imaging and phenotyping

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Protocol

1. Download ImageJ (https://imagej.net/ij/download.html)
2. Download ObjectJ (https://sils.fnwi.uva.nl/bcb/objectj/download/)
    - Download the latest version of objectj_.jar, put it into ImageJ's plugins folder, and restart ImageJ. (only do this step in the original setup; you do not have to do it every time)
    - On macs: if you can't find the ObjectJ plugin after following instructions, remove the ImageJ.app from the ImageJ folder, put it back in, and then start the program again.
3. In ImageJ choose menu Plugins>ObjectJ. Now, you have an additional menu called ObjectJ.
    - On macs: ImageJ menu is located in the top menu bar of the screen.
4. Put a **copy** of the "Anatomy_Phenotyping.ojj" file in the folder you are currently working in. Please do not put the original file or transfer it from other folders.
5. In the ImageJ menu ObjectJ>Project>Open Project
    - Select the file named "Anatomical_Phenotyping.ojj".
6. In the ImageJ menu ObjectJ>Linked Images>Select **All** Images from Project Folder (only do this step in the original setup; you do not have to do it every time as long as you are working in the same folder. If you move to a different folder you will need to do this again)
7. If the Project Window does not open ObjectJ>Show Project Window (Figure 1)

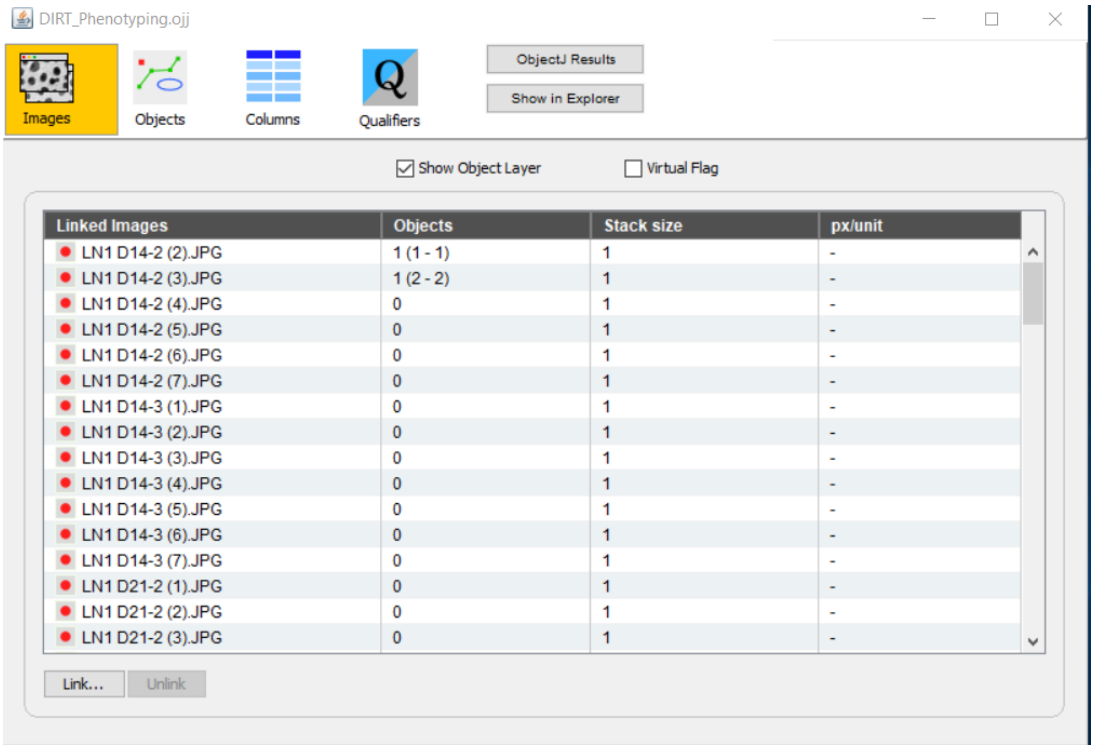

Figure 1: Project Window

8. Click Images on the top left-hand corner
    - A list of linked images should appear
    - If linked images are already phenotyped the "Objects" column will have a value. Images that need to be phenotyped will have a "0" in the "Objects" column.

Phenotyping

1. In the Project Window, click the following image to phenotype (first file with a "0" in the "Objects" column).
    - The image should open in a new window
2. Open the ObjectJ tools menu ObjectJ>Show ObjectJ Tools (Figure 2).

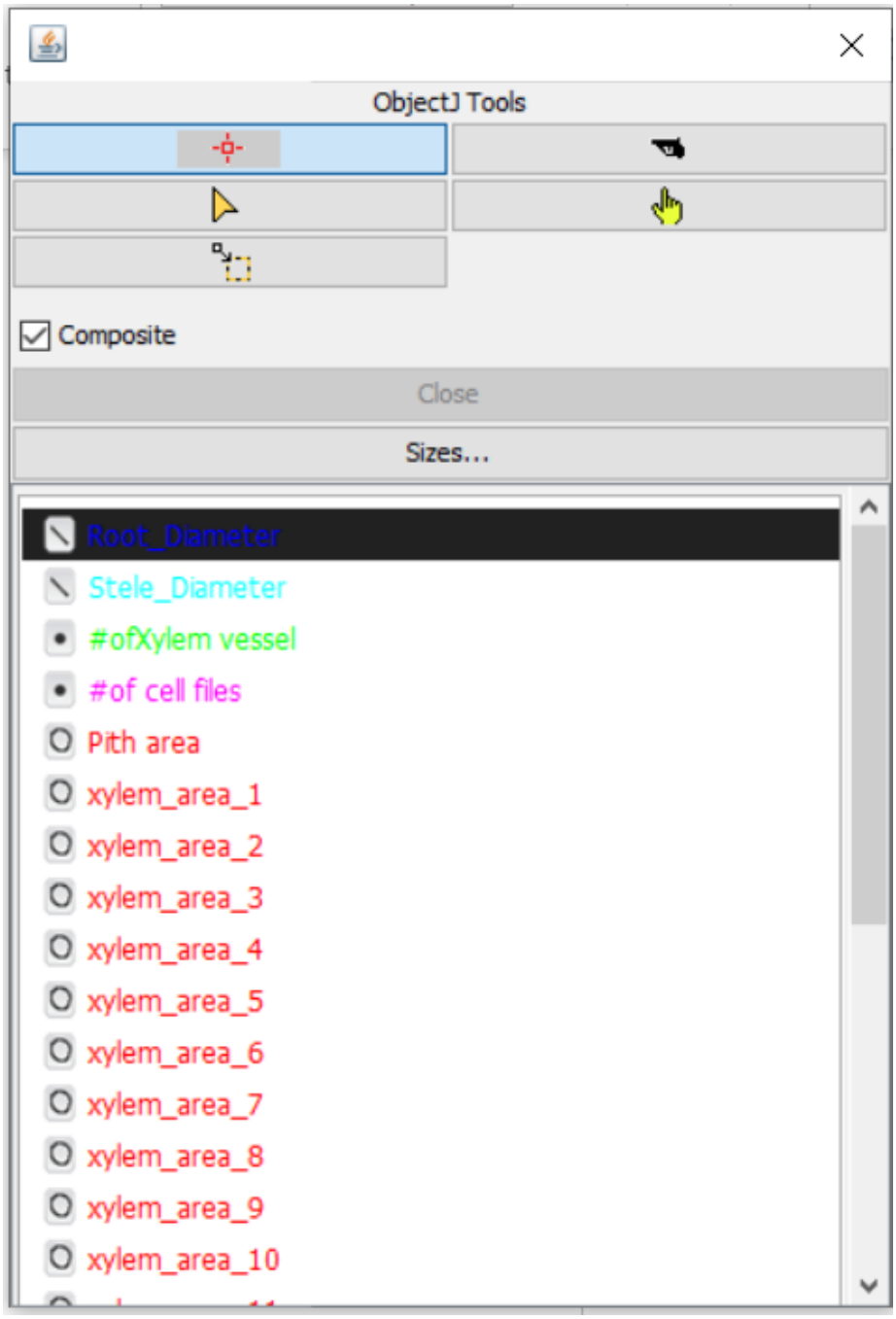

Figure 2: ObjectJ Tools Menu

3. You should have a hemocytometer image in each folder. Ideally, before annotating, select that image, and then from tools, select Root diameter (we will use this as a scale marker) and draw line (Figure 3). This way, we will know how many pixels correspond to my 1 mm. Then close this image and move on to other images.

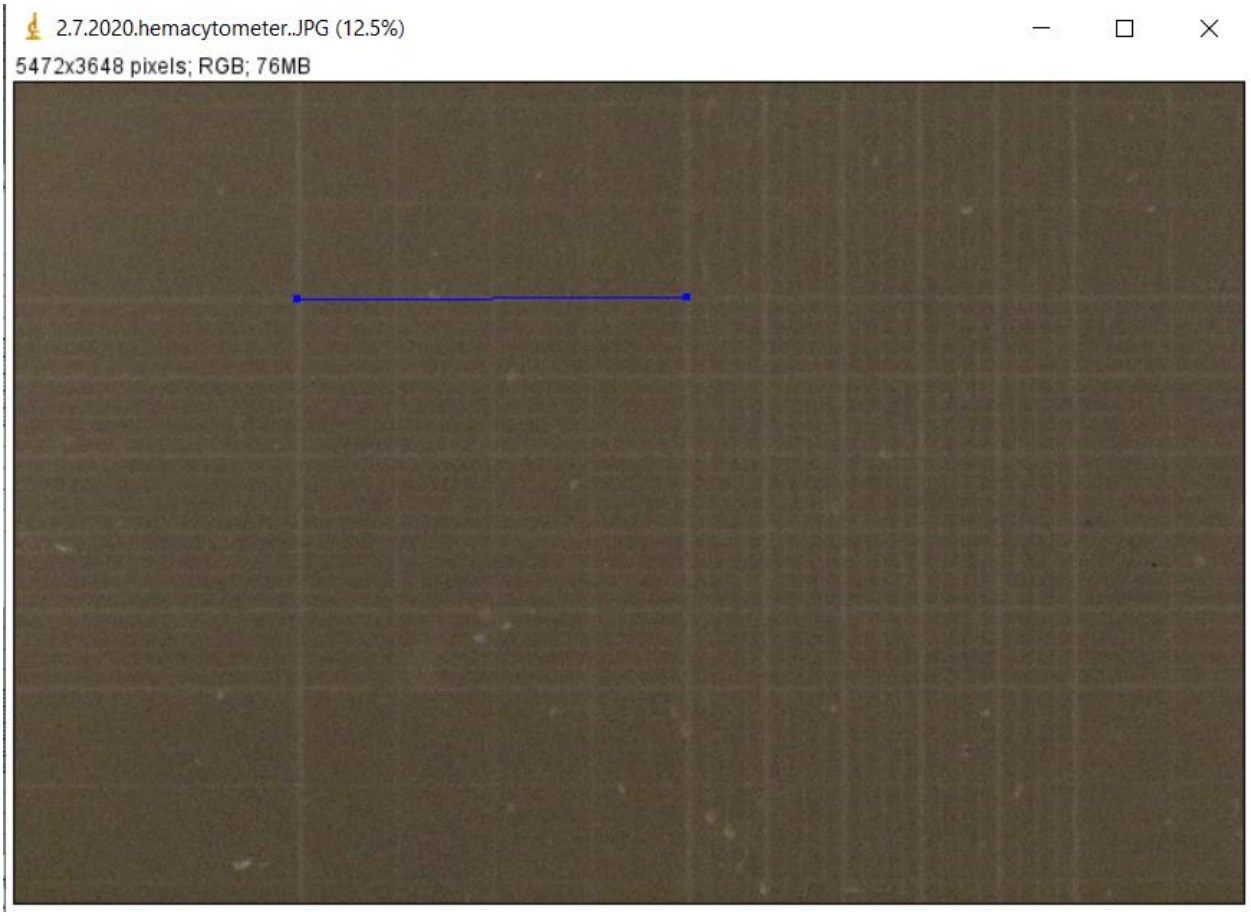

Figure 3: Using a hemacytometer as a scale.

4. Select an image that you like to annotate/measure. Select the first phenotyping tool (bottom half of the tool menu. It should be called "Root_Diameter."
    - Make sure the red box tool always remains highlighted (top, right-hand corner)
    - The steps following this may be out of order, but it doesn't matter what order you go in.
5. Draw a line representing the diameter of the root (the line will be blue, Figure 4), generally the longest possible because roots are sometimes damaged, and we don't want to underestimate them.
    - The line colors will always match the color of the text in the Tools Menu (i.e. blue line always measures root diameter, sky blue line always measures stele diameter, green dots always count number of xylem vessels, pink dots always count the number of cortex cell files, Red line correspond to pith area and other red circles correspond to xylem area. Yellow area circles correspond to aerenchym area.

- If at any point you need to zoom in or out of the image, click image on the ImageJ menu bar >Zoom In/Out or shift + scroll or two fingers on your touchpad.

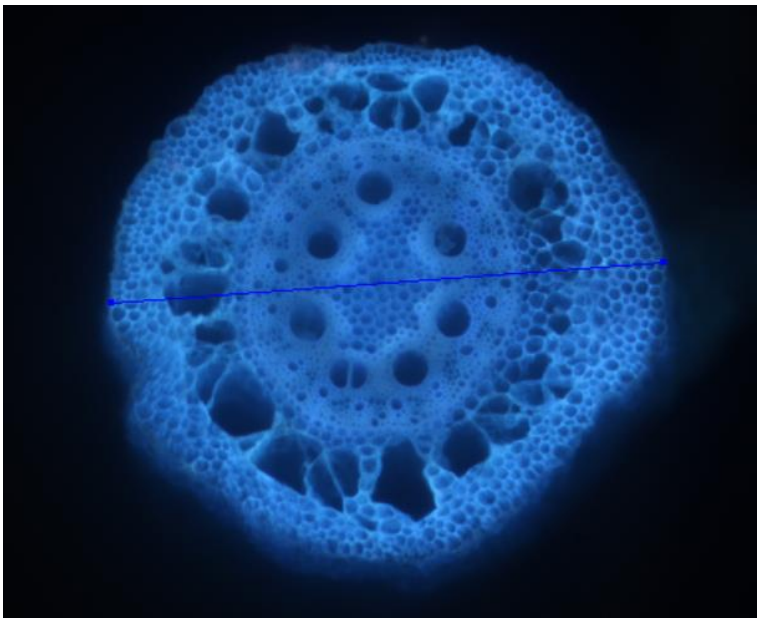

Figure 4: Measuring root diameter

6. After measuring the Root diameter, the tool will automatically switch to a stele diameter color. If it does not, highlight "Stele_Diameter" in the Tools Menu. Measure Stele diameter as shown below (Figure 5).

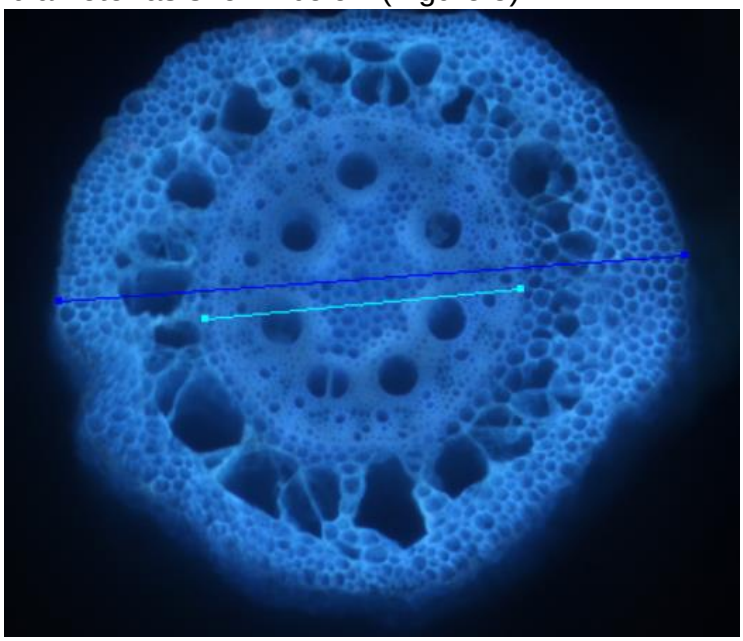

Figure 5; Measuring stele diameter

7. The tool will automatically switch to a green color. This is to count the number of xylem vessels. Just click on each xylem vessel (Figure 6)and once done, select

the following tool, which is cell file counting (# cell files) otherwise you will get stuck at just counting the xylem # and of course we don't want that, right?

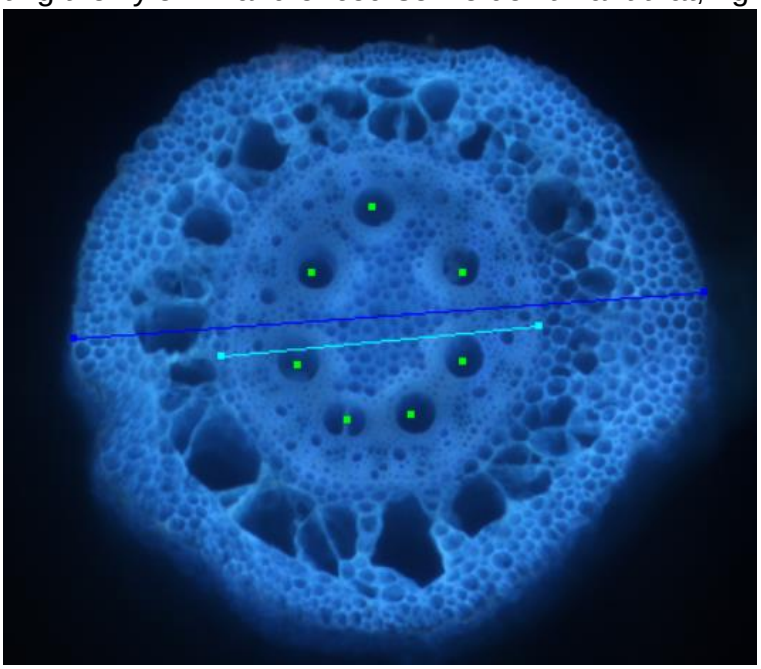

Figure 6. Counting metaxylem vessels

8. Select the next tool "# cell files" and then count the cortical cell files as shown below (red dots, Figure 7).

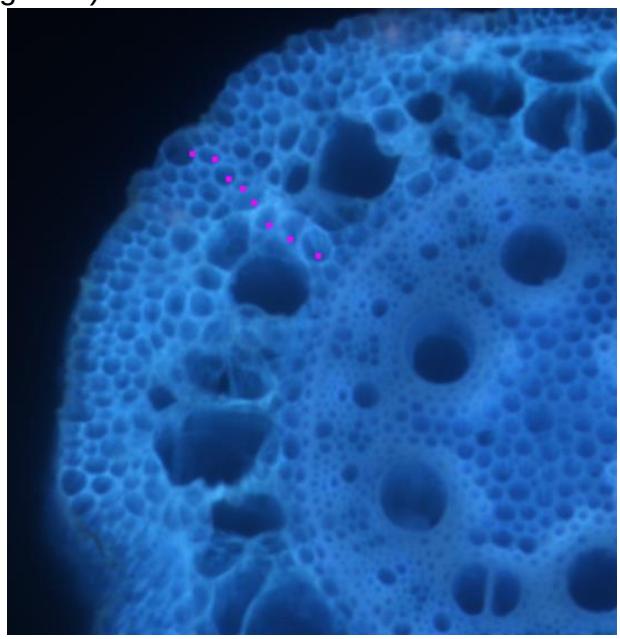

Figure 7. Counting the number of cortical cell files

9. Since the cell file number tool was also a counter, you have to manually select the following tool, which is the pith area, and then measure the pith area as

shown below (Figure 8), make sure you have pressed left click while measuring this area and complete the circle/area by connecting the starting and ending dot.

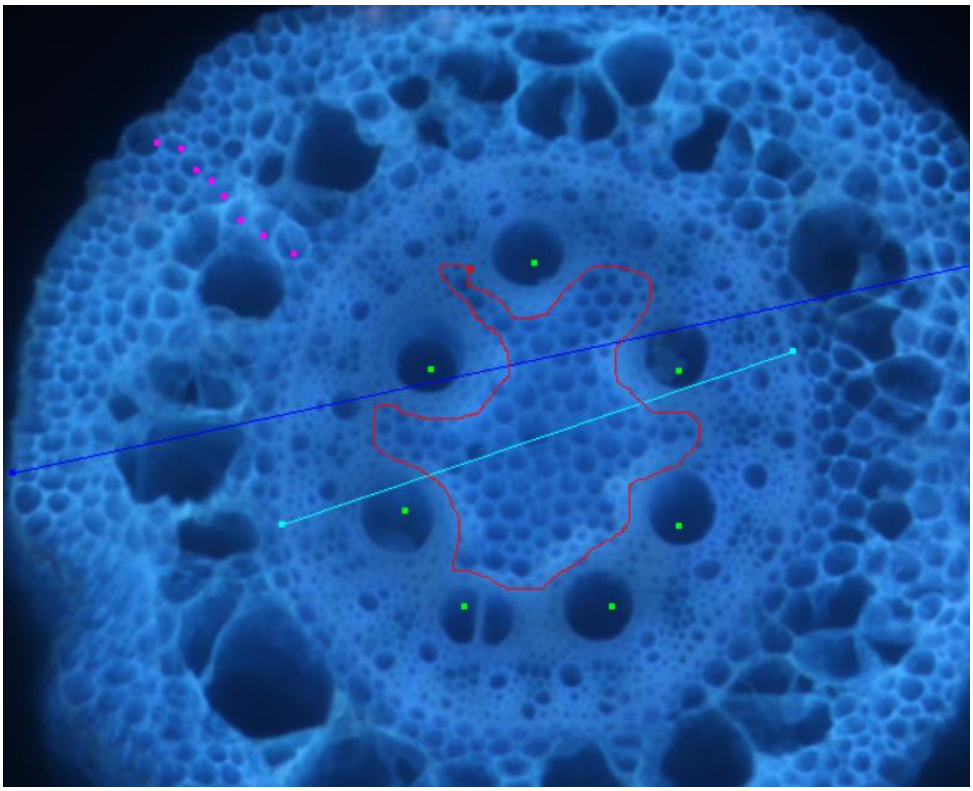

Figure 8. Measuring pith area.

10. Next, measure the area of each xylem vessel with the ROI tool (Figure 9), which operates similarly to the pith area measurement tool.

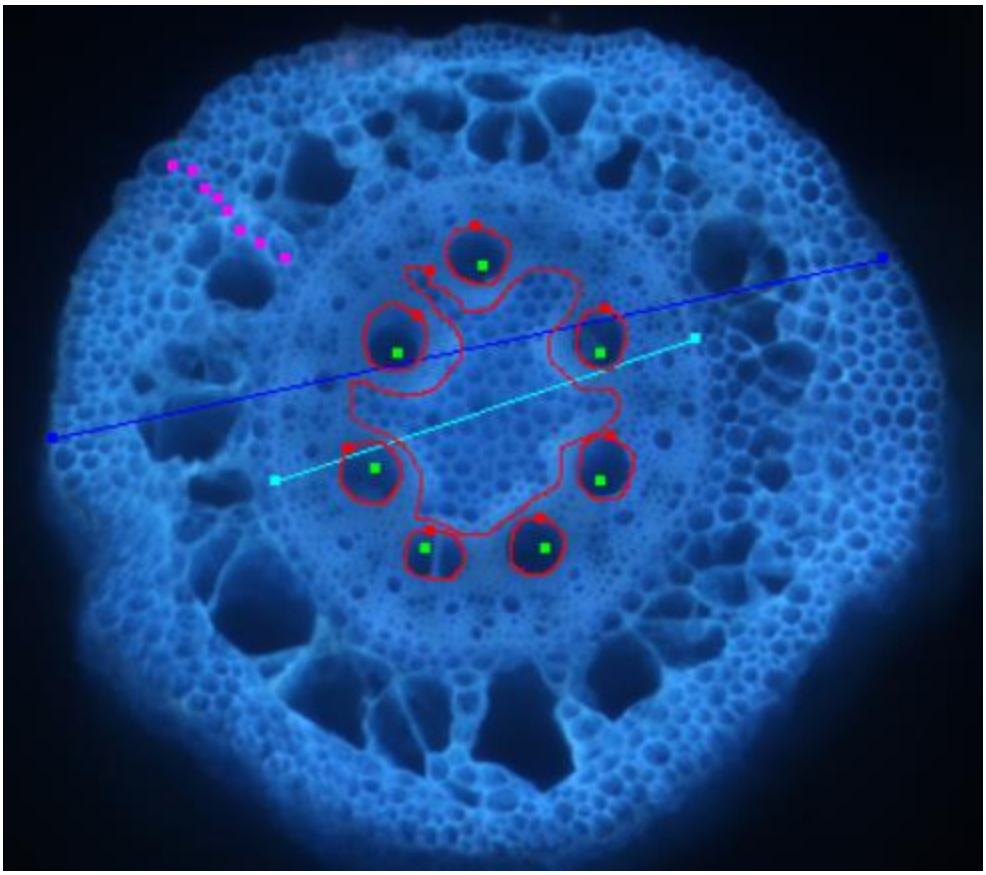

Figure 9. Measuring metaxylem vessel area.

11. Once the xylem vessel area is measured, select the aerenchym_area tool and measure all the aerenchyma pockets one by one (Figure 10).

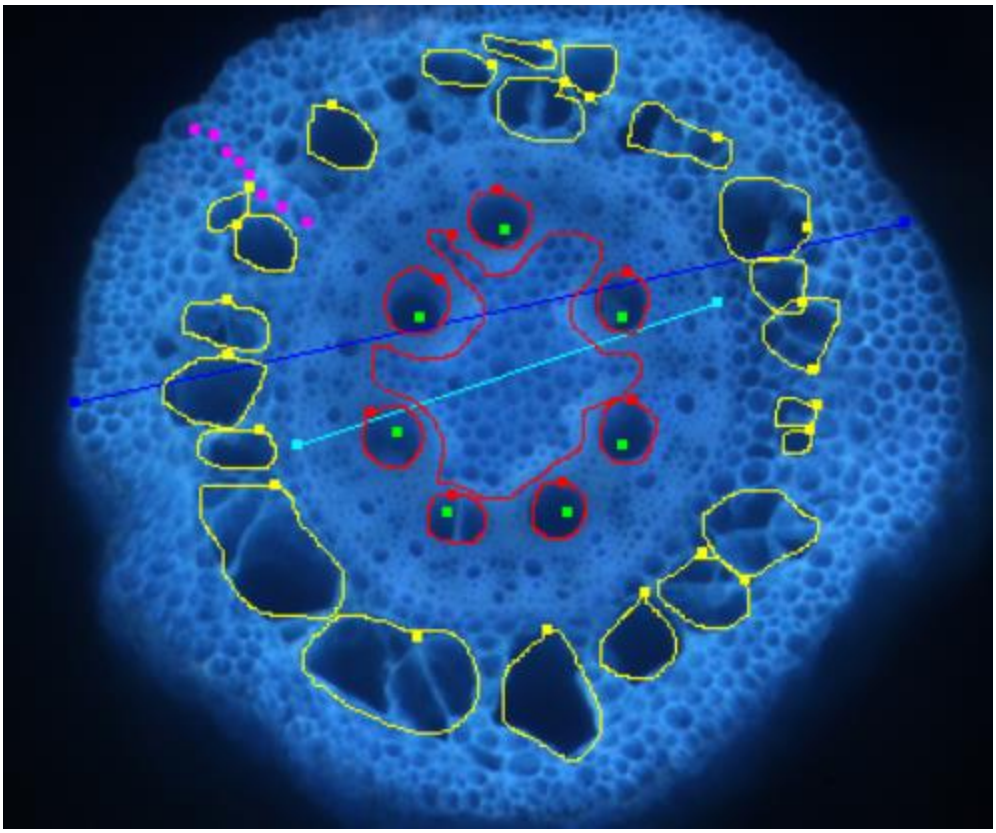

Figure 10. Measuring aerenchyma area.

12. If you accidentally phenotype an image incorrectly, you can erase the phenotype by selecting the "pistol" icon in the ObjectJ tools menu and then clicking on the phenotype you want to remeasure. After the line disappears, select the red square from the top of the menu and select the phenotype you need to remeasure from the bottom of the ObjectJ tools menu and remeasure. If you want to correct only one measurement and don't want to delete the whole measurement, then select the pistol, press ALT, and shoot the measurement you want to delete.
13. Repeat until all images are phenotyped. If you must stop before all images are phenotyped, click "ObjectJ Results" in the Project Window. In the upper Right-hand side click copy/export, select "all columns", click "export", rename the file to "1" or "2", … and click "save". A text file with the results will be saved in your "phenotyping" folder on the hard drive.