**Protocols for fluorescence microscopy**

Download ImageJ here: (it’s free)

<https://imagej.nih.gov/ij/download.html>

Image J makes it easy to process images. It has a *lot* of amazing functions, but we will just be using basic ones.

**Counting zoosporangia**

Quick guide to ImageJ

1. Open up image(s) in ImageJ.
2. If there are multiple images for one field of view, go to the menu at the top of the screen and press “Image > Stacks > Images to stack”. You do not need to “Keep source images”. You will now notice that if you move the sliding bar at the bottom, you can scroll through the images.
3. In the ImageJ toolbar, find the “Multi-point or point” tool. It should be set to “Multi-point”. To switch back and forth between “Multi” and “single” point, right click on the icon.

Double click the “Multi-point” icon; a pop-up should open. Set to the following settings:

A screenshot of a cell phone

Description automatically generated

Note: You can play around with this, but I’ve found that NOT labelling points makes it easier to see. Showing on all slices makes it so you can see your points when you toggle between stacks.

1. Go to “Analyze > Set Measurements” and untick all boxes except “Add to overlay”. This will make your “counting” dots a layer on top of the image.
2. “Count” zoosporangia by click on them. Each click should leave a yellow cross. You can toggle through the stacks to makes sure you get all zoosporangia

To delete a point: Hold down “Command” (or Alt on a PC I think) and click the point again.

1. When you are done counting, press “Command + M” (or, go to the top menu and press “Analyze > Measure”)
2. Scroll down the “Results” window and write down the number of points. This number will also appear on the image itself.
3. Do not save the “Results” file, but save the image itself as a Tiff file.

General tips: You can save an image mid-count and come back to it later. To remove previous “Measure” counts in the overlay, press “Image > Overlay > Remove overlay”. Always close the existing “Results” window BEFORE re-measuring; otherwise ImageJ counts each point twice (it “adds on” all points to the list, regardless whether it’s been counted before).

Counting zoosporangia methods

1. Count all zoosporangia in the field of view (i.e. the photo). If there are multiple stacked images that don’t quite “line up” such that some zoosporangia are visible at the edge of one photo but not the others, choose any single image of the series (e.g. the clearest image) and count only zoosporangia visible in that single image.
2. Overcrowded zoosporangia: even if only part of a zoosporangium is visible because of over-crowding, count it. The goal is to get as close to possible to the “true count” of total zoosporangia in that area.
3. Blurry zoosporangia: I’ve tried my best to include as many focuses as needed to see all zoosporangia, but sometimes I am imperfect. If you see some “blurry” dots that you are fairly certain *are* a cluster of zoosporangia, count them given your best guess of how many there are. (We can always look at ImageJ photos later and remove them if needed). If the blurriness is so bad that you can’t tell whether they are even zoosporangia at all, don’t count them.
4. Edge zoosporangia: only count zoosporangia for which >50% of its area is in the photo (to your best judgement)
5. Each row in your data spreadsheet should be one set of stacked photos.

**Measuring zoosporangia**

Quick guide to ImageJ

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2. If there are multiple images for one field of view, go to the menu at the top of the screen and press “Image > Stacks > Images to stack”. You do not need to “Keep source images”. You will now notice that if you move the sliding bar at the bottom, you can scroll through the images.
3. Go to “Analyze > Set Measurements” and untick all boxes except “Add to overlay”. This will make your “counting” dots a layer on top of the image.
4. Click the \*Straight\* segment icon on the toolbar.
5. Click and draw the distance you want to measure. You can adjust this line by dragging the ends or center. Make sure the line is where you want it before the next step.
6. “Measure” the line—(either Command + M or Analyze > Measure). You can only measure one line at a time. The length will appear in a “Results” window.
7. Repeat for all desired sporangia. \*\* You cannot delete individual measurements from the overlay, so try not to measure anything that you do not want in the “Results” window!\*\*
8. Save the “Results” as a CSV file. Save the image as a Tiff file.

General tips: It is best to do a whole image in one “sitting” because generating the Results table gets complicated when you have to re-open the image. Note that all measurements are in the *single overlay*, which means that once you measure a zoosporangia, you can’t “unmeasured” it without also deleting all your other measurments. Very annoying.

Measuring sporangia methods:

1. Measure the diameter of all zoosporangia in the photo that are in-focus enough to see its outline
2. Save the Results window as a CSV.
3. Each row of your data spreadsheet should be *one zoosporangia****,*** and the zoosporangia ID number (ZS\_ID) should correspond to the ones in the CSV and in the overlay of the image itself.