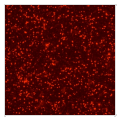

Mar 17, 2020

Symbiodiniaceae Enumeration in Ground Coral Samples Using Countess™ II FL Automated Cell Counter

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1 Works for me dx.doi.org/10.17504/protocols.io.bdc5i2y6

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ABSTRACT

This protocol outlines a method for quantifying the density of Symbiodiniaceae cells in Scleractinian coral samples which have been previously ground into a homogenous paste consisting of aragonite skeleton, coral host tissue and endosymbiotic Symbiodiniaceae cells. There are four parts to quantifying Symbiodiniaceae cell density from ground corals: 1) grind and sub-sample the coral and store at -80 °C until ready to extract, 2) separate Symbiodiniaceae cells from the coral skeleton, 3) image Symbiodiniaceae fluorescence using Countess™ II FL Automated Cell Counter, and 4) quantify cells using the software ImageJ. Note: a CY5 EVOS™ light cube is required for this method. For instructions on installing the CY5 EVOS™ light cube to your Countess™ II FL Cell Counter, see page 40 of the Countess™ II FL Cell Counter user manual.

This method was originally developed by Rowan McLachlan in February 2020. Claire Juracka assisted Rowan McLachlan by conducting tests to compare this method to the traditional method using a hemocytometer and microscope. The traditional cell counting method has been reported in several publications by Grottoli's team (e.g., Rodrigues & Grottoli 2007). This protocol was written by Rowan McLachlan with the guidance of Dr. Andréa Grottoli at The Ohio State University.

Rodrigues LJ & Grottoli AG (2007). Energy reserves and metabolism as indicators of coral recovery from bleaching. Limnology and Oceanography.

MATERIALS TEXT

LEGEND

[1] for Grinding of Coral Fragments

[2] for Separating Symbiodiniaceae Cells and Coral Skeleton

[3] for Imaging Symbiodiniaceae Cells

[4] for Quantifying Cells Using ImageJ

Reusable materials:

- Mortar and pestle [1]
- Plastic spatula [1]
- Metal spatula [1,2]
- Glass graduated cylinder [2]

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- 500 ml glass beakers [2]
- Dropper bulb [2]
- Test tube rack [2]
- 1000 µl capacity pipette [2]
- 200 µl capacity pipette [2]
- 20 µl capacity pipette [3]
- Countess™ white slide carrier [3]
- Countess™ II FL Reusable Slides (one is necessary, two is optional for maximum efficiency) [3]
- Coverslips [3]
- Laboratory squirt bottle [3]

Disposable materials:

- Aluminum weighing pan [1]
- 15 ml polypropylene centrifuge tubes [1]
- 50 ml polypropylene centrifuge tube (two per sample) [1]
- Weighing paper [2]
- Glass Pasteur pipets [2]
- 200 – 1000 µl pipette tips [2]
- 20 - 200 µl pipette tips [2]
- 2 - 20 µl pipette tips [3]
- Cotton swabs [3]
- Kimwipes™ [3]

Equipment:

- -80 °C freezer [1]
- Weighing balance accurate to 4 decimal places [1,2]
- Vortex [2,3]
- Centrifuge [2]
- Refridgerator [2]
- Countess™ II FL Automated Cell Counter and EVOS™ Cy™5 Light Cube [3]
- USB (for use with Countess™ II FL Automated Cell Counter) [3]
- Computer mouse (for use with Countess™ II FL Automated Cell Counter) [3]

Chemicals:

- Instant Ocean® Sea Salt (or sodium chloride powder) [2]
- Buffered Formalin (10%) [2]

Software:

- Microsoft Excel [4]
- ImageJ [4]

SAFETY WARNINGS

Lab Safety

This procedure uses hazardous chemicals:

- Read the MSDS forms for Formalin before starting this procedure.
- Use powder-free nitrile exam gloves throughout the procedure.
- Wear a lab coat and safety glasses throughout the procedure.
- Dispose of all chemical waste in appropriately labeled containers.

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Preparation of ground coral samples

1 **Grind coral fragments.** Grind frozen coral fragments into a homogeneous paste using a chilled mortar and pestle, or within an ice-bath. Attempt to grind the paste into as fine a consistency as possible to get evenly fine skeletal grains.

2 **Weigh and partition ground coral paste.** Place a pre-baked aluminum weighing pan on the balance and tear (i.e. zero). Remove the pan, and using a plastic spatula, transfer all the ground coral material from the mortar into the pan. Place the pan on the balance and record the *total wet weight of the ground coral fragment (grams).* Using a metal spatula, measure ~ 0.5 g of wet paste from the pan into a pre-labeled 15 ml polypropylene centrifuge tube. Record the exact weight of the paste removed as the *wet weight of subsample*. Return the 15 ml tube to the -80 °C freezer as soon as possible for storage until you are ready for the next step. The remaining ground coral material in the pan not allocated for Symbiodiniaceae enumeration can be transferred to a separate 50 ml centrifuge tube and archived in a -80 °C freezer for other future analyses.

Separate Symbiodiniaceae from coral skeleton

3 **Prepare salt water.** Using a glass graduated cylinder, dispense 250 ml of Milli-Q water into a glass beaker. Using weighing paper and a balance, weigh 9 g of Instant Ocean sea salt (or sodium chloride powder) and add to the glass beaker. Using a clean metal spatula, stir the beaker until all salt had dissolved.

4 **Defrost coral samples.** Remove subsamples (0.5 g of ground coral material in 15 ml centrifuge tubes, henceforth referred to as "tube A") from -80 °C freezer, and place in room-temperature tap water bath (Fig. 1A). Leave for ~ 3 minutes, or until fully defrosted (Fig. 1B).

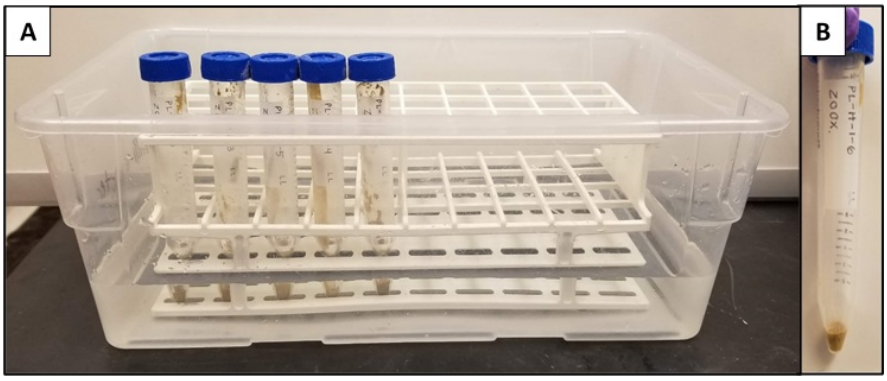

Fig. 1. Image of **(A)** water bath for defrosting ground coral samples in tube A, and **(B)** close-up image of tube A after defrosting.

5 **First seawater dilution & separation.** Using a glass Pasteur pipet and dropper bulb, add saltwater to tube A, up until the 4 ml mark (Fig. 2A). Cap and vortex tube for 30 seconds (Fig. 2B). Make sure that any ground coral material that is spread up the inner walls of tube A is dislodged during the 30-second vortex procedure. A clean spatula can be used to scrape the inner walls of the tube if need be. Shaking the tube by hand can also help, just make sure the cap is on tightly. Centrifuge for 10 minutes at 4000 rpm.

Carefully remove tube A's from the centrifuge and place them in a test tube rack. The supernatant in tube A should now be clear (Fig. 2 C) and ground material in it will have separated into two layers, with the pale skeleton at the base and the dark-colored Symbiodiniaceae cells on top (Fig. 2 D). Depending on the type of centrifuge used (i.e. fixed angle vs. swing bucket), the Symbiodiniaceae layer may spread at an angle up the inner walls of the centrifuge tube (Fig. 2D).

Remove the cap from the second, pre-labeled 15 ml centrifuge tube, henceforth referred to as "tube B" (Fig. 2E). *Note: labeling of tube B can occur during the 10 minute centrifuge period, or prepared before-hand.* Using a glass Pasteur pipet, carefully transfer the supernatant and the non-skeleton layer (Symbiodiniaceae) from tube A into tube B (Fig. 2F). Tube A should now only contain the skeleton layer (Fig. 2G). Do not throw Pasteur pipets away yet - they can be stored in tube B, and reused during the *second separation* step (Fig 2H).

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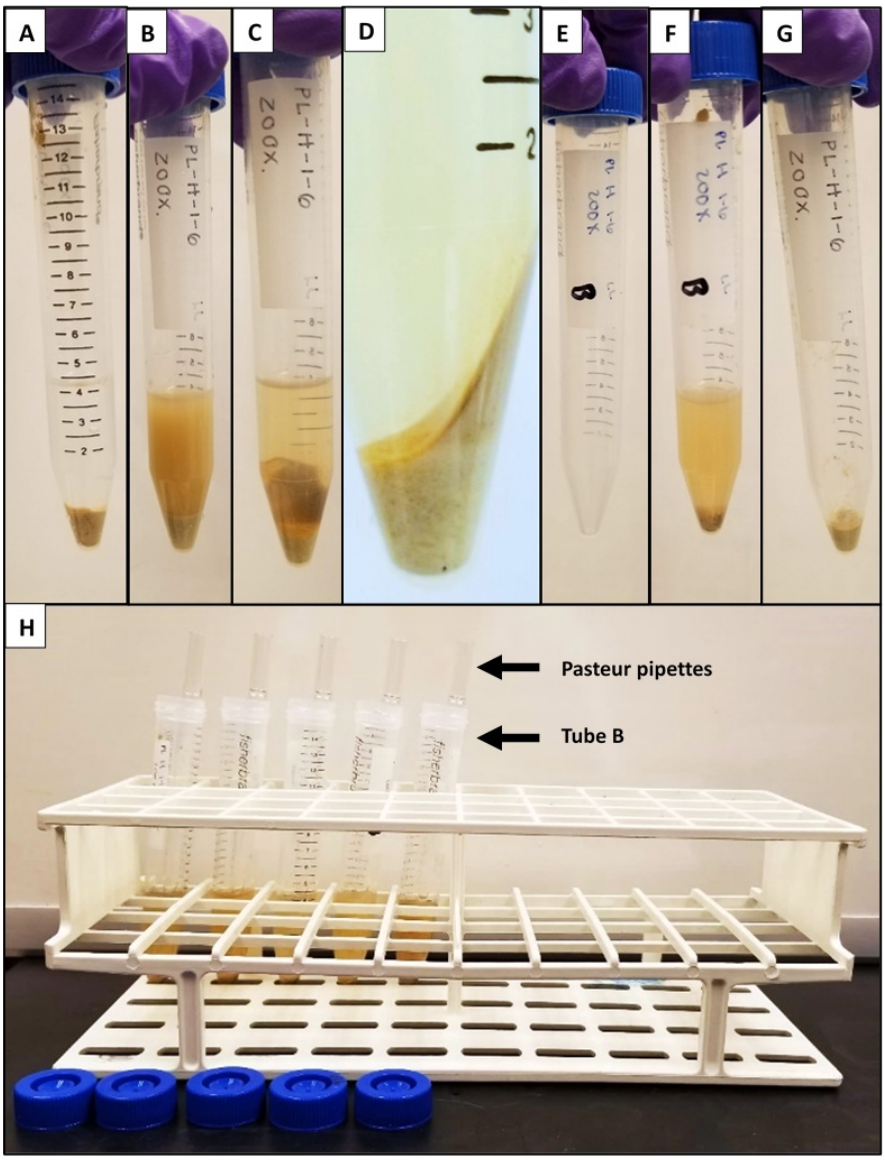

Fig. 2. Photos showing the appearance of **(A)** tube A after adding seawater to 4ml mark, **(B)** tube A after 30-second vortex, **(C)** tube A after10 min centrifuge, **(D)** tube A tissue layer separation close-up, **(E)** pre-labeled tube B, **(F)** tube B with supernatant and Symbiodiniaceae, **(G)** tube A with skeleton layer only, **(H)** storage of Pasteur pipets from the first separation to be used during the second separation.

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6 **Second seawater dilution & separation.** Using a glass Pasteur pipet and dropper bulb, add more salt water to tube A's, up until the 4 ml mark (Fig. 3A). Cap and vortex tube for 30 seconds (Fig. 3B). Centrifuge for 5 minutes at 4000 rpm.

Carefully remove tube A's from the centrifuge and place them in a test tube rack. The supernatant in tube A should now be very clear and coral tissue and endosymbiont layers should be much thinner compared to the *first separation* step (Fig. 3C).

Using the same glass Pasteur pipet used in step # 4, carefully transfer the supernatant and thin tissue layer from tube A into to tube B. Tube A and the skeletal remains (Fig. 3D) can now be discarded.

Note: depending on the make, model, and condition of centrifuge used, a third dilution and separation step may be necessary to ensure that all Symbiodiniaceae cells are separated from the skeleton. This was tested using Hawiiaian coral samples, and it was found that two dilutions and separations retrieved all Symbiodiniaceae cells. Tube B should now contain ~ 8 ml of supernatant, with coral tissue and a Symbiodiniaceae layer at the base (Fig. 3E)

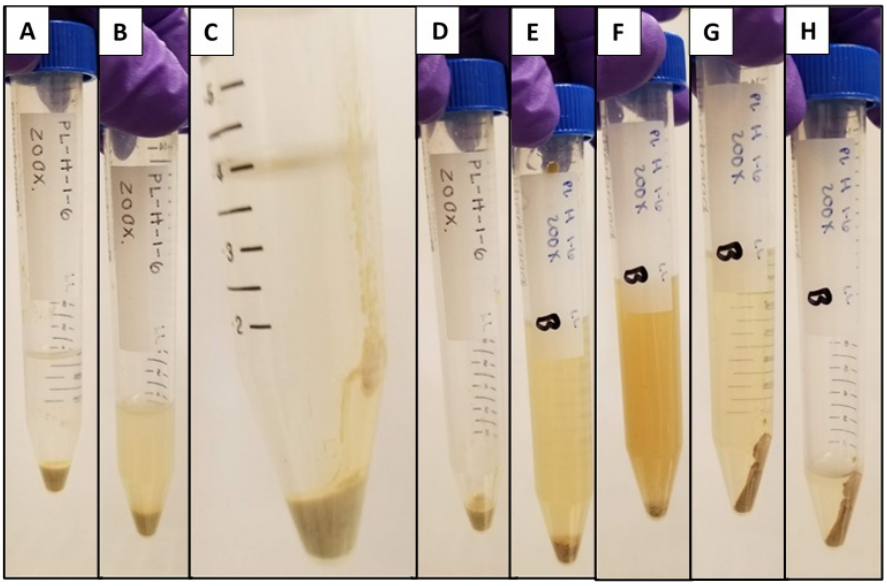

Fig. 3. Photos showing the appearance of **(A)** tube A after adding seawater to 4ml mark during the *second seawater dilution*, **(B)** tube A after 30-second vortex, **(C)** tube A after 5 min centrifuge, **(D)** tube A after *second separation* with only skeleton remaining, **(E)** tube B with ~ 8 ml of supernatant and Symbiodiniaceae, **(F)** tube B after vortex, **(G)** tube B after 5 min centrifuge, **(H)** tube B after supernatant has been removed, and Symbiodiniaceae have been resuspended in 1000 µL seawater and 200 µL formalin.

7 **Resuspension.** Cap and vortex tube B for 30 seconds (Fig. 3F). Centrifuge for 5 minutes at 4000 rpm. Following this, carefully remove tube B's from centrifuge and place in a test tube rack. Tube B should now have a clear supernatant, and a defined coral Symbiodiniaceae layer (Fig. 3G). Using a glass Pasteur pipet, remove all liquid supernatant from tube B, and dispose into the waste beaker. Be careful not to disturb the Symbiodiniaceae layer. Ensure all liquid has been removed. Using a 1000 µL capacity pipet and clean tips per sample, add exactly 1000 µL of saltwater to tube B. Next, using a 200 µL capacity pipet and clean tips per sample, add exactly 200 µL of 10% formalin to tube B (Fig. 3 H). Place tube B's in a refrigerator for storage until you are ready to go to the next step. Preferably count cells on the same day as samples were prepared, however, within two weeks is acceptable if samples are kept refrigerated in the dark.

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8 **Loading the Reusable Slide.** Remove the white slide carrier (Fig. 3A) from the Countess™, and remove the Countess™ II FL Reusable Slide (Fig. 4B) from its box (Fig. 4C). Place a coverslip on the counting chamber of the Countess™ II FL Reusable Slide (Fig. 4D), making sure the coverslip is clean and free of grease. Vortex tube B for 30 seconds, wait 5 seconds and then using a 20 µL capacity pipet and clean tips per sample, remove 10 µL of the concentrated Symbiodiniaceae solution. Gently pipet the 10 µL sample into inlet “side A”, allowing capillary action to draw the solution into the counting chamber (Fig. 4E, F). A properly loaded counting chamber should have a thin, even film of fluid under the coverslip. Cap and vortex tube B again for 30 seconds, wait 5 seconds, and then remove another 10 µL. Load the Reusable Slide inlet “side B”.

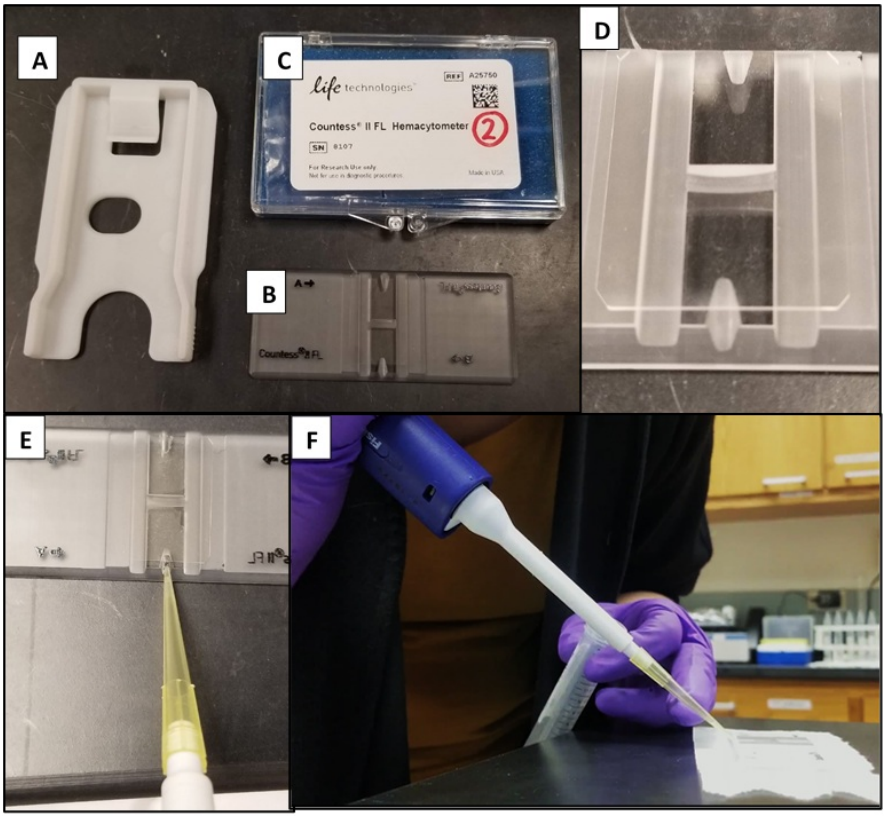

Fig. 4. Equipment required for viewing Symbiodiniaceae cells in Countess™ **(A)** white slide carrier, **(B)** Countess™ II FL Reusable Slide, **(C)** Reusable Slide box, **(D)** coverslip positioned above inlet A and B on the Reusable Slide, and **(E)** carefully loading inlet A with 10 µL of the resuspended coral sample, **(F)** using one hand to steady the pipet whilst loading the slide inlet can be helpful.

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9 **Insert Reusable Slide into Countess™.** Switch on Countess™ using the switch on the back-right side of the instrument, and wait until *Insert Slide* appears on the screen. After both inlets of the Reusable Slide have been loaded, carefully place the loaded slide into the white slide carrier (Fig. 5A). Insert the white slide carrier and Reusable Slide assembly into the slide port (Fig. 5B), and gently push into the instrument until it "clicks" into place (Fig. 5C).

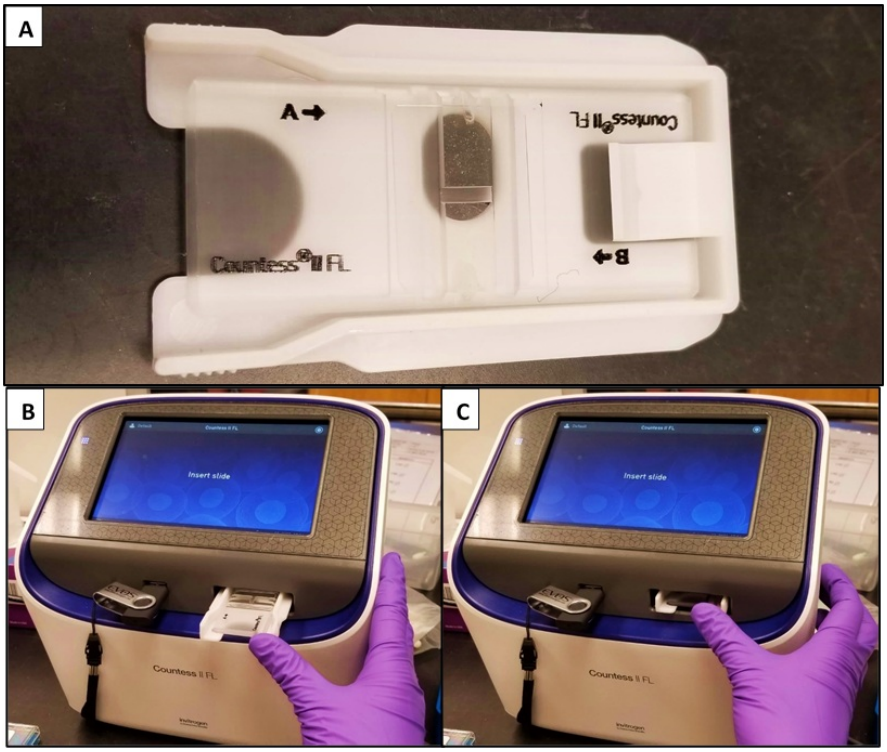

Fig. 5. Loading samples into the Countess™ **(A)** insert the loaded Reusable Slide into the white slide carrier, **(B)** insert the white slide carrier into assembly port on the front of Countess™ II FL cell counter, **(C)** gently push into the instrument until it “clicks” into place.

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10 **Adjust the brightness and capture the fluorescence image.** After inserting the slide, wait a few seconds to allow Countess™ to autofocus. The initial image on the screen is the Brightfield image (Fig. 6A). Change the *Light Source* to CY5 by selecting the red circle in the upper right corner (Fig. 6A). On the fluorescent image screen (Fig. 6B), click *Adjust.* A side panel will appear on the right (Fig. 6C). Use the sliding scale to adjust the brightness so that cells are distinguishable from the background noise and click the *Done* button in the lower right corner (Fig. 6C). The appropriate brightness level will vary depending upon the coral species, the total number of cells in the sample, etc. Note: For *Porites compressa, Porites lobata* and *Montipora capitata*, between 30-60% brightness was sufficient.

Click the *Zoom* button in the lower-left corner (Fig. 6 C), and move the sliding scale upwards to zoom to the maximum value (400%). Next, click the *Focus* button, located to the right of the zoom button (Fig. 6C), and adjust the focus until the cells on the screen are more spherical in shape. Note: the Countess™ tends to over-focus by ~20 units (e.g. autofocus = 2250, but 2230 is better).

After adjusting the brightness, zoom, and focus, ensure the CY5 box is checked with a tick in the *Collect Channels* section on the right-side panel (Fig. 6D). Click the *Capture* button in the lower right corner (Fig. 6D). The screen will display "*Waiting for cell count to finish…*". Wait for ~ 20 seconds.

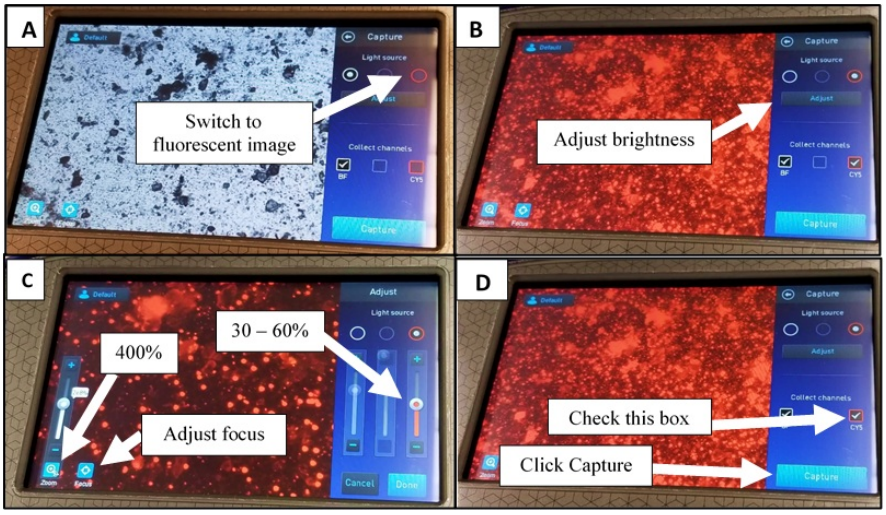

Fig. 6. Countess™ screen display showing **(A)** the brightfield image after inserting the Reusable Slide. Click the red circle in the upper right corner to switch to the **(B)** fluorescent image. Click *Adjust* and use the **(C)** side panel on the right to adjust the brightness (30-60%) and buttons on the lower left to adjust the zoom (400%) and focus before clicking *Done.* On the **(D)** final screen, check the "*collect channels CY5*" box before clicking *Capture.*

11 **Save the image.** When the counting is complete, the Results screen will appear. However, the Countess™ algorithm does not accurately count the Symbiodiniaceae when prepared from ground coral samples (Fig 7). This may be because the Symbiodiniaceae cells are at the lower end of the size spectrum that the Countess™ can measure and because the background coral host tissue particulates (i.e. noise) are similarly sized (Fig. 7).

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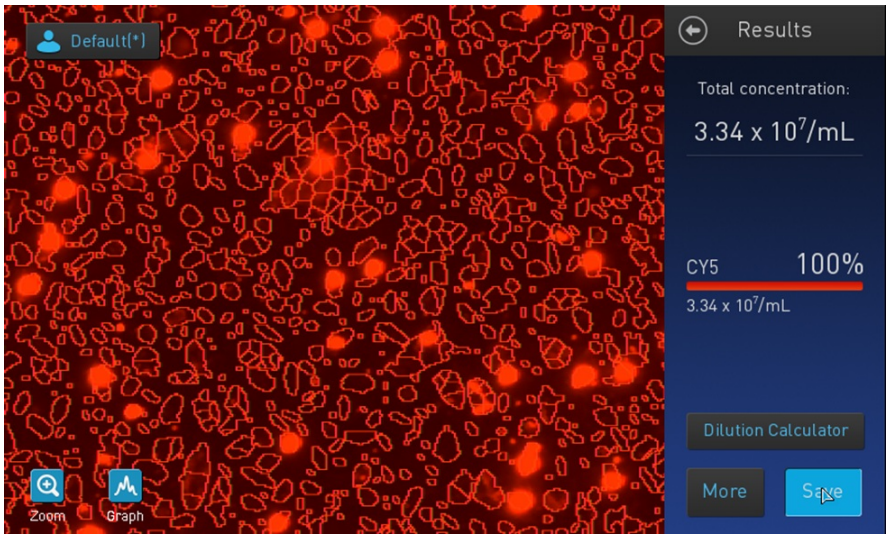

Fig. 7. Results screen display indicated which particles were counted using the Countess™ algorithm.

Instead, accurate and precise cell enumeration can be achieved by capturing the Countess™ fluorescent images, exporting files to a computer, and counting the cells using ImageJ software (described below). The difference between a) Countess™ algorithm values, b) ImageJ algorithm values, and c) hand-counted values using a hemocytometer and light microscope were tested during a pilot study (Fig. 8). It was determined that ImageJ derived concentrations did not differ from the hand-counting method, and that the Countess™ algorithm values over-estimated the number of cells and was more variable due to the erroneous counting of background coral host tissue particulates (Fig. 8).

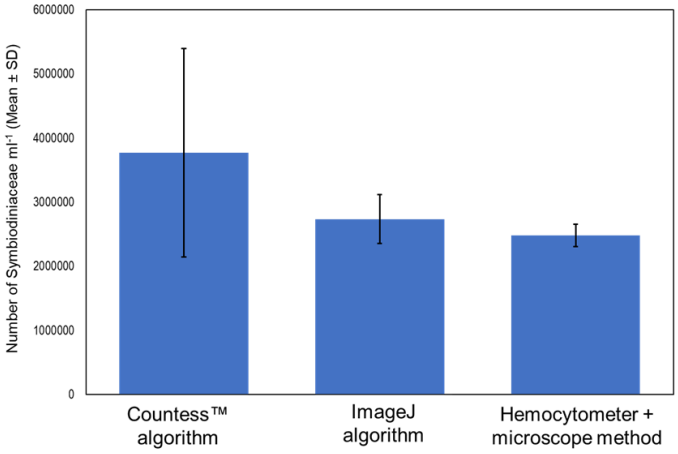

Fig. 8. Results of a pilot study comparing the mean (± SD) number of Symbiodiniaceae cells (ml^{-1}) quantified using the Countess™ algorithm, ImageJ algorithm, and by hand using a hemocytometer and light microscope.

A second advantage to combining Countess™ images with ImageJ for quantification is that the Countess™ Reusable Slide volume is 0.4 µL compared to standard hemocytometers which are only 0.1 µL volume. Therefore, replicate samples quantified with the Countess™ are larger, and thus are more representative of the true number of Symbiodiniaceae cells in the sample than those quantified by hand using a conventional hemocytometer.

Ensure a USB is inserted into a port of the Countess™ II FL cell counter. Click *Save* in the bottom right of the Results screen (Fig. 9A). Click the *File Name* box in the upper left corner of the Save screen (Fig. 9B). Edit the name of the sample using the keypad and click *Enter* (Fig. 9C). Using the dropdown menus, adjust the *Result, Images,* and *Report* file types to PNG (Fig. 9D). Two tips are worth noting: 1) PNG is markedly quicker to save than PDF files, and 2) data automatically saves as .csv file type. The saving process takes ~ 40 seconds. During this time, it is recommended to start loading a second Countess™ II FL Reusable Slide with the next sample to increase efficiency.

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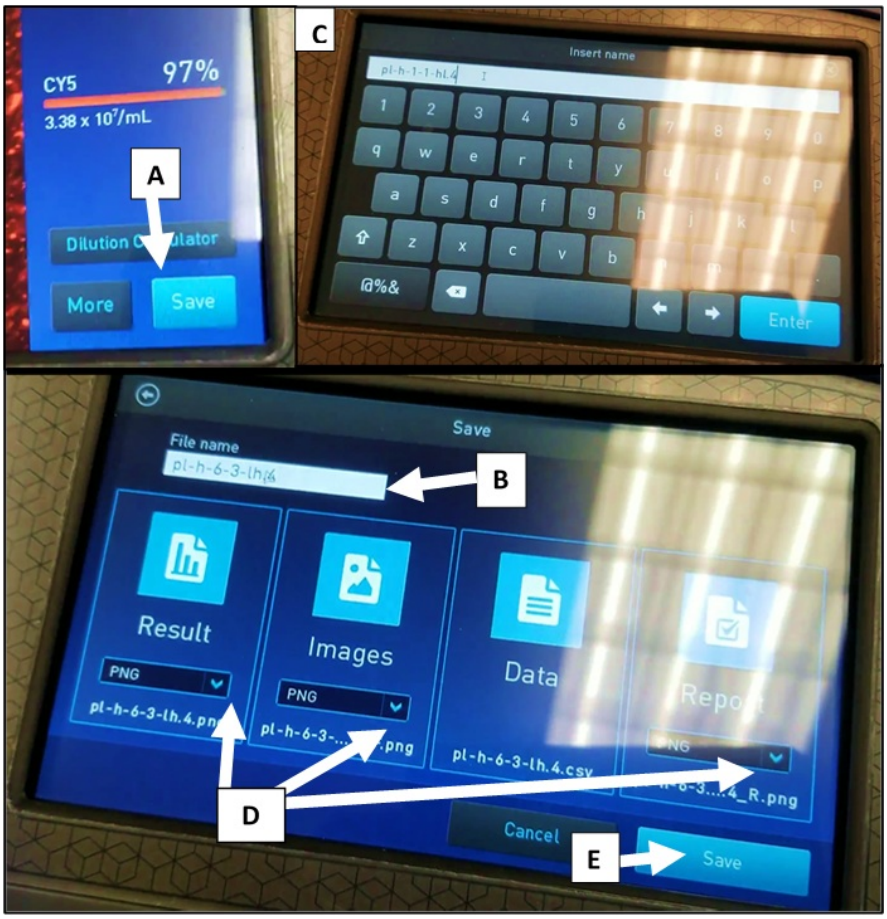

Fig. 9. Countess™ screen display showing **(A)** the location of *Save* button on the Results screen, **(B)** the *File Name* box to click to change file ID, **(C)** the keyboard screen for editing sample name, **(D)** the drop-down menus to change the file type to which data will be saved on USB, **(E)** and the location of final *Save* button on Save screen.

12 **Rotate Reusable Slide.** To remove the slide, push the slide gently into the instrument until it "clicks" and a spring pushes the slide out. Rotate the Reusable Slide in the white slide carrier so that side B is ready to be read. Re-insert slide carrier into the Countess™, and repeat the instructions described in steps 9-11.

13 **Clean Reusable Slide.** Between samples, rinse the glass slide and coverslip of the Countess™ II FL Reusable Slide with Milli-Q water from a squirt bottle, gently rub with a cotton swab, rinse again, and then dry using Kimwipes™ laboratory tissues.

14 **Conduct four replicates per coral sample.** Load a second Reusable Slide (sides A and B) with the same coral sample using the methods described in step 8. Proceed to image both sides A and B using the instructions described in steps 9-11. When four fluorescent images have been saved to the USB, you can move on to the next coral sample. The number of cells quantified in each of these four images using ImageJ will later be averaged.

15 **Export files to a computer using a USB.**

Analyzing Fluorescent Cell Images using ImageJ

16 **Download ImageJ.** ImageJ can be downloaded for free from the NIH website: https://imagej.nih.gov/ij/download.html.

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17 **Open file.** Open the CY5 fluorescence image you saved in step 11 in ImageJ (Fig. 10).

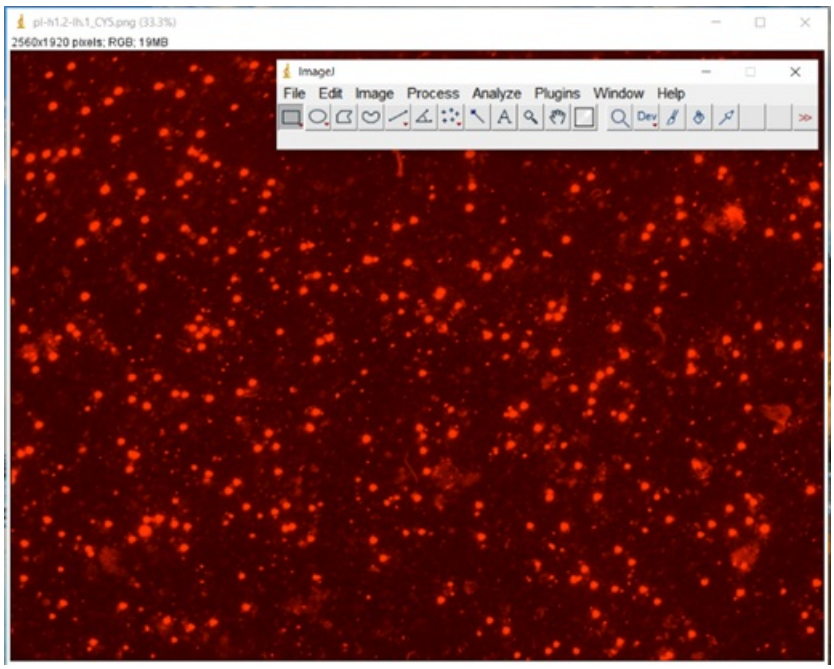

Fig. 10. Fluorescent image of Symbiodiniaceae cells captured by the Countess™ and opened in the software ImageJ.

18 **Convert to greyscale.** Convert the fluorescent image to 8-bit greyscale (Fig. 11).

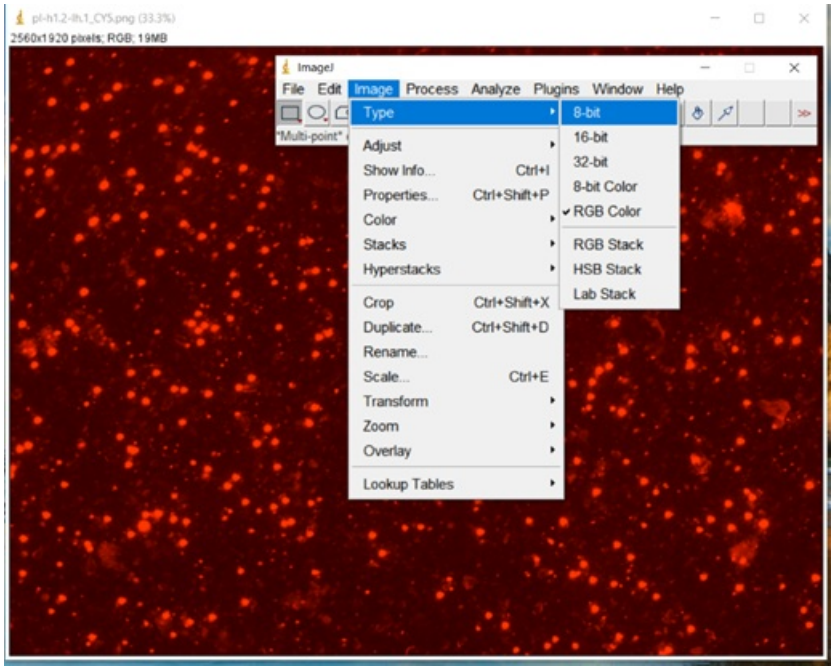

Fig. 11. Convert the original fluorescent images into 8-bit greyscale using the *Image > Type > 8-bit* menu options.

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19 **Subtract background.** Click *Subtract Background* (Fig. 12). This removes the smooth continuous background from the image and makes the cells more visible.

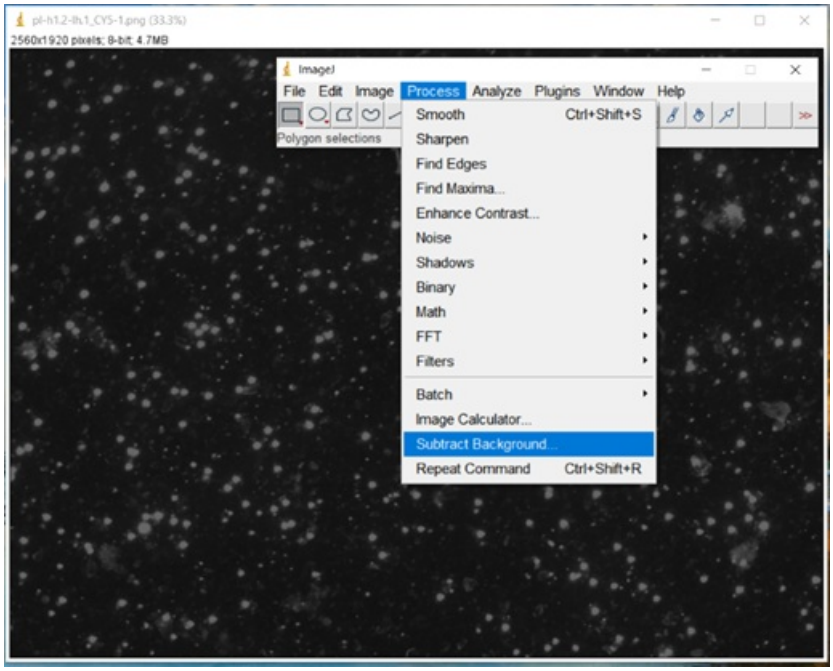

Fig. 12. Subtract background noise using the *Process > Subtract Background* menu options.

20 Set the *Rolling ball radius* to 500 (Fig. 13). Note: This is an arbitrary number that was decided upon after playing around with images in the software for a while. It seemed to subtract enough of the noise, whilst not affecting cell enumeration.

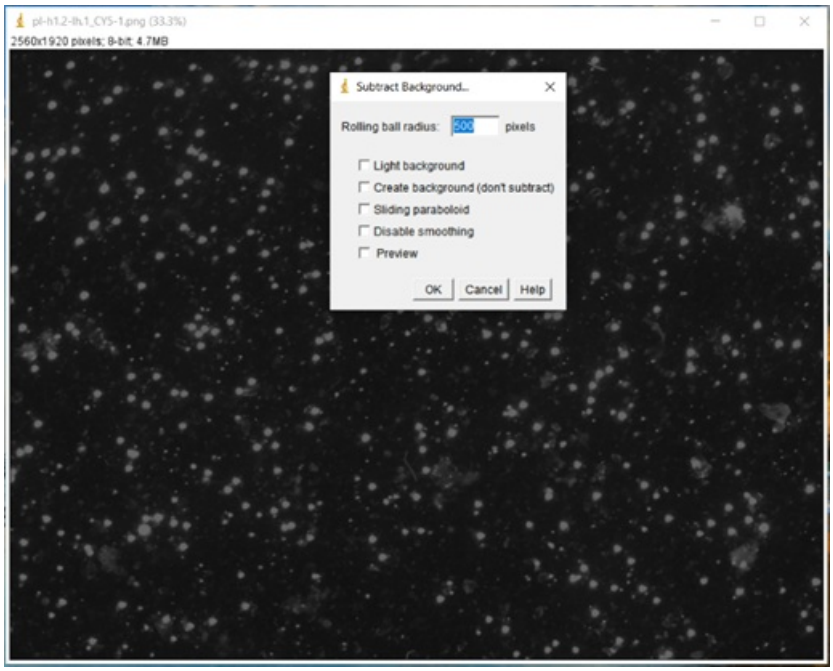

Fig. 13. Set the Rolling ball radius value to 500.

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21 **Adjust the threshold.** Thresholding is a technique for dividing an image into two (or more) classes of pixels, which are typically called "foreground" and "background." Adjust the threshold to the desired level to optimize cell visualization in the foreground (Fig. 14).

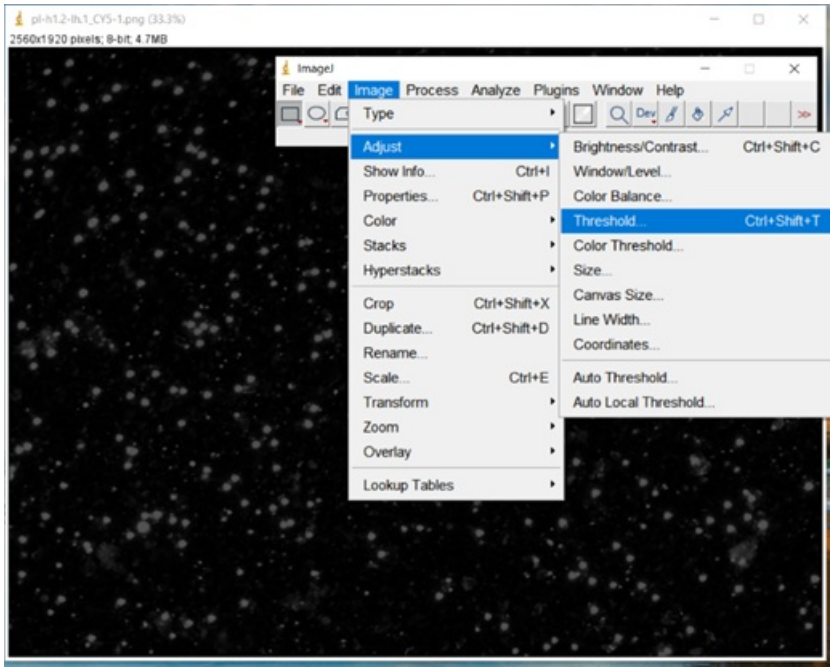

Fig. 14. Adjust the noise threshold using the *Image > Adjust> Threshold* menu options.

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22 Examples of different threshold values in ImageJ, adjusted using the top sliding scale to a threshold value of 10 (Fig. 15) and a threshold value of 30 (Fig. 16). Note: the higher the threshold value, the more noise will be removed from the image. However, if the threshold value is too high, there is also the risk of losing cells from the image.

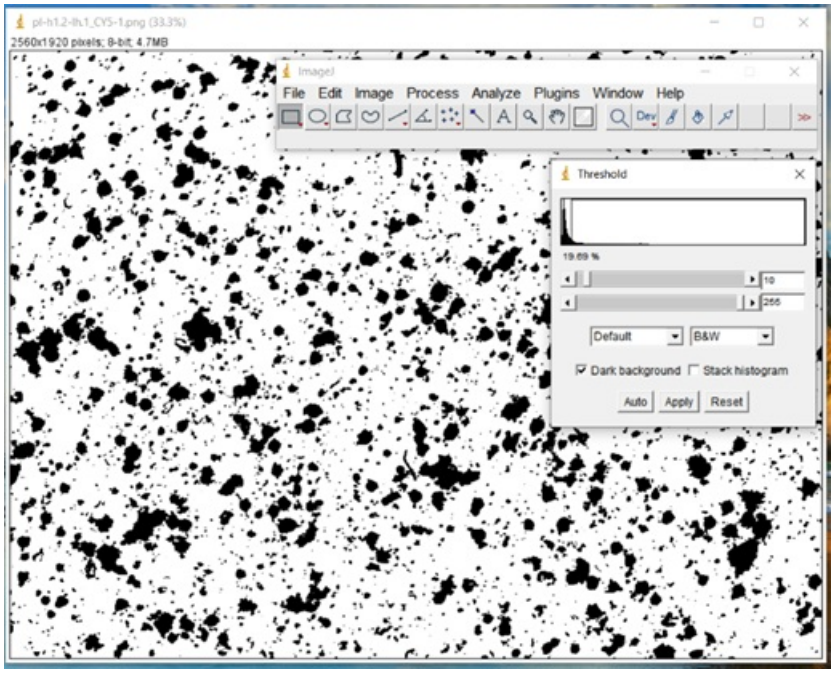

Fig. 15: In this image, the threshold value is set (using the top sliding scale) to 10. Notice how much *more* background noise is remaining compared to Fig 14.

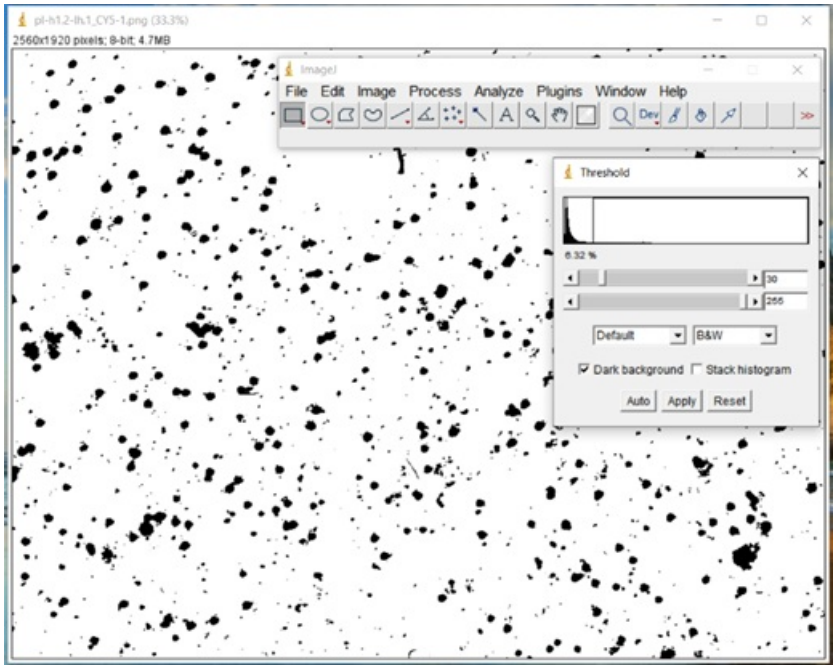

Fig. 16. in this image, the threshold value is set to 30. Notice how much *less* background noise is remaining compared to Fig. 13.

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23 **Convert to mask.** Click *Convert to Mask* (Fig. 17). This step converts images to black and white images based on the current threshold settings.

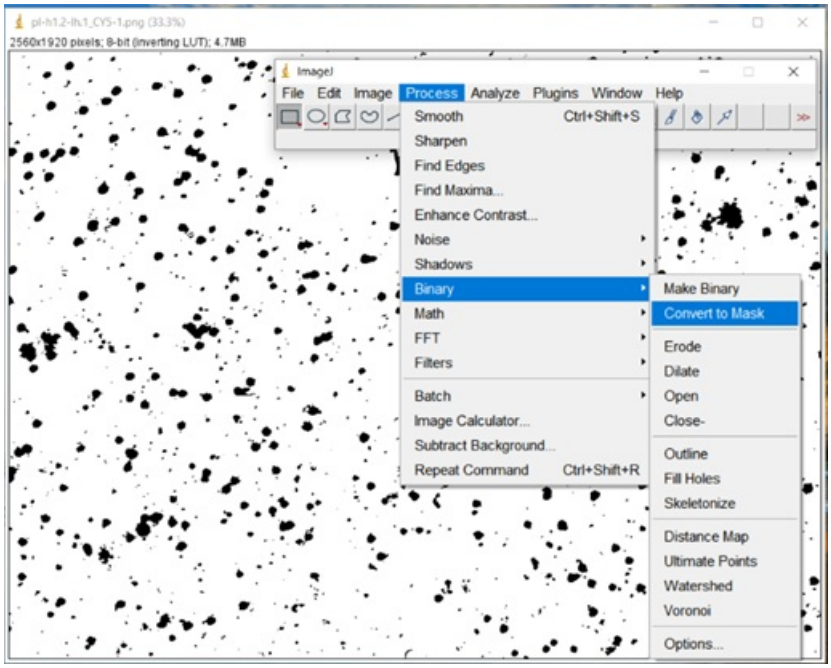

Fig. 17. Convert image to mask using the *Process > Binary > Convert to Mask* menu options.

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24 **Differentiate adjacent cells.** Apply Watershed (Fig. 18). In this step, ImageJ places a one-pixel wide line in between any cells which are side by side, that ImageJ thinks should be two cells (Fig. 19).

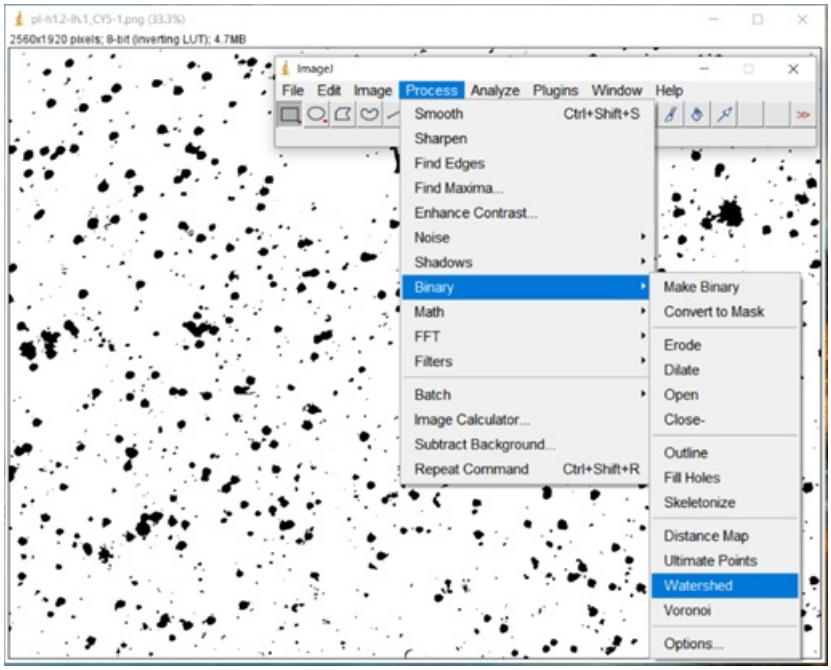

Fig. 18. Apply watershed to the image using the *Process > Binary > Watershed* menu options.

Fig. 19. A magnified portion of Fig. 16 after the Watershed has been applied showing the 1-pixel width line placed between adjacent cells.

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25 **Analyze cells.** Click *Analyze particles* to count the number of cells in the image (Fig. 20).

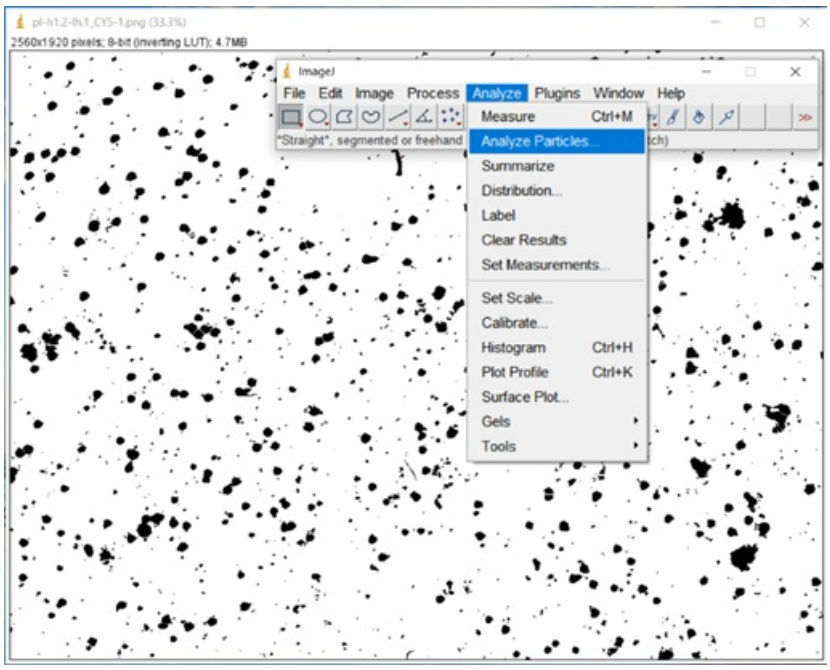

Fig. 20. Analyze the particles using the *Analyze > Analyze Particles* menu options.

26 **Select the size of cell to count.** Set particle parameters to analyze using the **Size (pixel^2)** box (Fig. 21). A 10 - 1000 range is suitable for Hawaiian coral samples, but may need to be adjusted for other sample types. In order to double-check what has been counted, select "Outlines" from the **Show** drop-down menu. You can also select to exclude cells that are on the edge of the inlet chamber from being counted. Click *OK.*

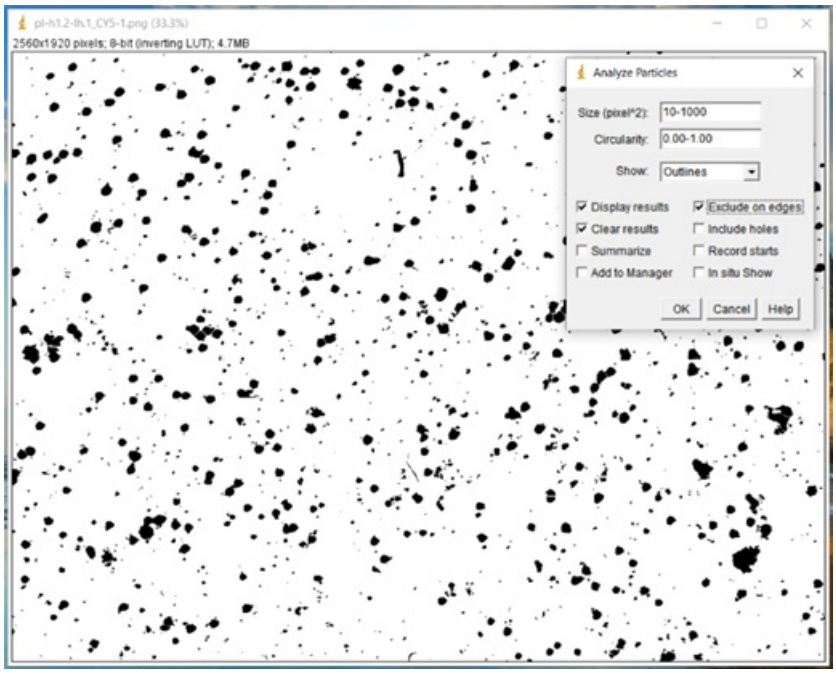

Fig. 21. Set particle parameters to analyze by typing a number range in the *Size (pixel^2)* box. Select *Outlines* from the *Show* dropdown options and check the *Exclude on edges* box, before clicking *OK.*

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27 **Confirm what the ImageJ algorithm has counted.** After clicking *OK,* two new windows will open: (1) the outlines window and (2) the results window (Fig. 22). Double-check what the ImageJ algorithm has counted by zooming in to the same spot on the 8-bit image and outlines image (Fig. 23). To scroll in, click on the image window, then hold **Ctrl** + Scroll with the mouse.

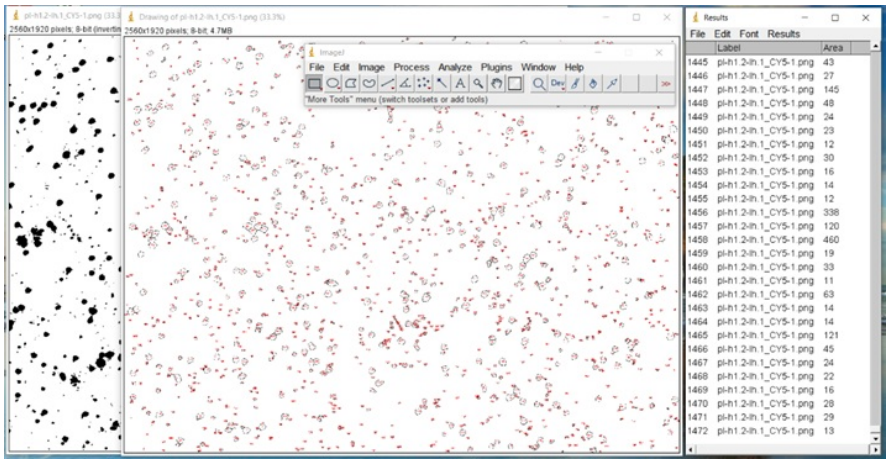

Fig. 22. The middle window shows the outlines of cells that Image J has counted. The window on the right is the results window, and the number at the bottom of that list is the number of cells counted. In this case, there were 1472 cells.

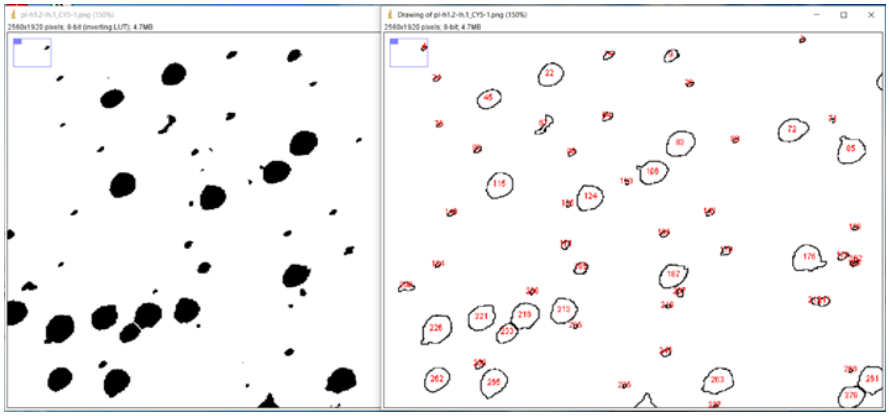

Fig. 23. By zooming into the top left corner of the 8-bit image (left window) and the top left corner of the cell outlines image (right window) you can visually check exactly *which* cells have been counted.

28 **Calculate Symbiodinium cell quantity in tube B.** Average the cell count values of the four replicates per coral sample to obtain the *Average # cells.* Use Equation 1 to calculate the total number of Symbiodiniaceae cell present in tube B:

$$\textit{Total \# cells in tube B} = (\textit{Average \# cells} \div 0.4\ \mu L) \times 1200\ \mu L \qquad \textit{(Equation 1)}$$

where the Countess™ Reusable Slide volume is 0.4 µL and the total volume cells were resuspended in is 1200 µL (1000µL saltwater + 200 µL of Formalin).

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29 **Calculate the surface area of the subsample.** Quantification of the coral surface area is out the scope of this protocol, as there are several methods (foil-wrap, wax-dip, 3d-scan, etc.). This protocol assumes the operator already knows the surface area of the whole coral fragment that was ground into a homogenous paste at the start of this protocol. The surface area (SA) of the subsample used for Symbiodiniaceae enumeration is calculated using the following equation:

$$SA_{subsample}\ (cm^2) =$$
$$(\text{wet weight of subsample (g)} \div \text{total wet weight of the ground coral fragment (g)}) \times SA_{whole\ colony}\ (cm^2)$$

30 **Standardize cell density to the surface area.** The number of Symbiodiniaceae cells (# cells) is standardized to surface area (cm^2) using the following equation:

$$\text{Symbiodiniaceae density (\# cells / } cm^2) = \text{Total \# cells in tube B} \div SA_{subsample}\ (cm^2)$$

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