# **Standard protocol for algal endosymbiont counts**

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1. Materials
   1. Chemwipes
   2. Glass pasteur pipettes
   3. 70% ethanol
   4. Haemocytometer and cover glass
   5. Light microscope 10x and 40x objective
   6. Ice + container or cooler
   7. Vortex
2. Protocol
   1. After isolating coral tissue + symbionts (methods vary), homogenize, vortex and remove an aliquot of 1 ml and place in microcentrifuge tube. If desiring to store in formalin, z-fix or other, adjust volume accordingly. If sample is in filtered seawater, store in freezer or keep on ice until further processing.  
      Once at microscope…
   2. Place coverslip on slide, and vortex your sample.
   3. Remove a small volume (less than the stem of pipette) and add slowly to haemocytometer “sample introduction point” until counting chamber is completely covered (Fig 1.).
3. Haemocytometer  
   Fig 1. Haemocytometer showing introduction points (where sample is loaded) cover glass, left and right support mounts, and the counting chamber. Note the depth of the sample under slide is 0.1mm thick. Image: Rice University
   1. Expel the remaining sample from pipette back into stock.
   2. Once more remove a small volume and add to the other side of the haemocytometer. Together, this serves as TWO independent loadings (replicates) that you will count.
   3. Place sample back on ice until next loading.
   4. Start with microscope at 10x objective and find the middle 5 x 5 square. Take care not to crack coverslip if moving between objectives. The 25-square internal field has smaller squares that subdivide it. However, you will not likely need to count within these smaller squares. The entire counting chamber (pictured in Fig 2.) is considered a square field, each with a length and width of 1.0 mm. Each of the 9 squares has an area of 1 mm2.
4. Haemocytometer field of view  
   Fig 2. Haemocytometer total field of view. Showing 9 large squares (1 x 1 mm) and an central, middle square divided into smaller fields. Image: Rice University  
   Beginning counts…
   1. You will need to count ~ 100 cells or more in each replicate field (i.e., loading). This may depend on site or any other variability between where samples were collected so make sure to do a trial sample for each. You must count the same # of squares each time you count.
   2. Since some cells may be above or below your current focal field of view, you should use fine-scale adjustment focus on microscope and pan up and down slowly through your sample as you count to bring items into focus or plane of view. You can do counts at 40x or 10x objective, and flipping between objectives can help identify unknown or uncertain objects.
   3. In preliminary counts, if all 25 middle squares are counted, then begin counting the corners (larger square of 4 x 4 smaller squares). If you count new areas (a corner 4 x 4 larger square), you must count the entire area so that you replicate an area of 1 mm2. You will likely have to count 2 - 4 of the larger, 9 squares.
5. Symbiont cells in field of view  
   Fig 3. (A) Field of view of showing 25-square, central field in haemocytometer. A single square is outlined in black in the bottom-right. (B) A close up of a symbiont cells (left, right) and cellular debris (bottom, top). Note symbiont cells will appear tinted brown, green, or yellow, while cellular debris is typically clear or translucent. Some objects may appear to be shriveled or lysed cells or debris (top, panel B). If uncertain of identity, side with caution and do not count these objects. (C) Color image of Symbiodinium (unicellular Dinophycea), note nucleus, organelles are visible; credit: T. LaJeunesse
   1. Once you get an idea of how many squares you need to count, note the # of squares you count in your lab notebook. You will stick to this # and the location of the squares throughout all of your counts for all samples. (ex: 2 squares - only counting top and bottom left corner;3 squares - counting middle, top-right, bottom-left). If you need to add more squares to your counts because of very low densities, make a note of this in your notes and draw a diagram of what was added. Good idea to draw a small diagram of what you counted in lab book notes.
   2. In lab notebook you should record the cell counts, numbers of squares you count, and if any dilution is performed (in the case of very over-concentrated samples)
   3. You will do 6-8 replicate counts for each sample, depending on variance among counts. Your among count CV for each sample should be < 10. To count or not to count…. That is the question
   4. Make rules and stick to them. If you count a cell it must match your criteria.
   5. Record counts in lab notebook and begin with 6 replicate counts. If a count is anomalous (very high or low) relative to other counts, recount and remove this outlier count. If CV is high because all counts show high variance (potentially due to low count #), then add more counts: up to 8, then 10 if necessary. Compare CV and determine whether replicate counts reduced variance or not.
   6. To calculate cell densities use the excel spreadsheet formulas. In short, # of squares counted = (area of count field in mm2) x width of chamber (0.1 mm) = mm3 = μl. From μl can calculate to ml and scale up to # cells/blastate volume (ml). Divide total cell counts by preferred denominator (tissue biomass or skeletal surface area).
6. References
   1. Wall, C. 2016. Symbiodinium counts in coral tissue protocol. Dr. Ruth Gates’ Laboratory Hawaii Institute of Marine Biology, University of Hawaii.