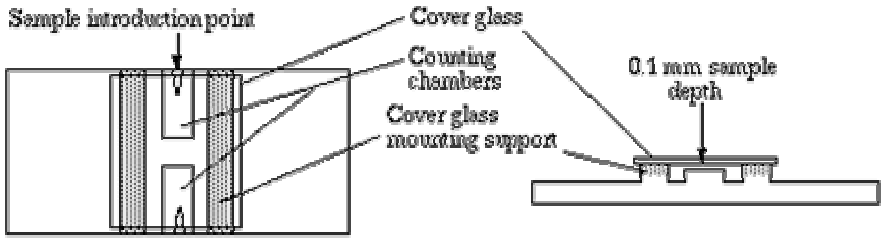
Cell counts using Improved Neubauer haemocytometer

Prepared by Santiago Perez; 3/22/2006

Preparing the sample. Your sample tubes should have a random number label if they come from an experimental treatment group and they should be processed for counting at random!!! The counter should have no idea what treatment the sample comes from to avoid bias. Do not loose your key!

- Optional: Add a drop or more of Lugol's reagent (An aqueous solution of iodine and potassium iodide in water) to the homogenate before spinning down pellet. Note: if the algae you are counting are motile (e.g. live cultured algae) you must immobilize them first, Lugol's does this as well. Lugol stains starch granules and makes cells darker and also heavier. They are therefore easier to visualize and sink faster onto your counting chamber surface and plane of focus.
- Make sure your pellets are dry, then you can freeze these.
- Add an exact (known) but minimum volume of filtered sea water (0.45um filter; FSW) to the pellet and resuspend well by vigorous shaking or vortexing until no visible clumps remain.
- Take an aliquot of the resuspension to dilute if necessary.
- I recommend using a dilution that will give a concentration of 25 cells/square. I try to count the 4 corner squares, so that in total I count about 100 cells per loading of the chamber. If the sample is very low in cells, count the whole chamber (9 squares). If you count less than 4 squares, always count the same ones, e.g. the top left and bottom right. If you count only one always count the center square. Keep track of your dilution factor (DF=final vol/initial vol).

Filling the chamber:



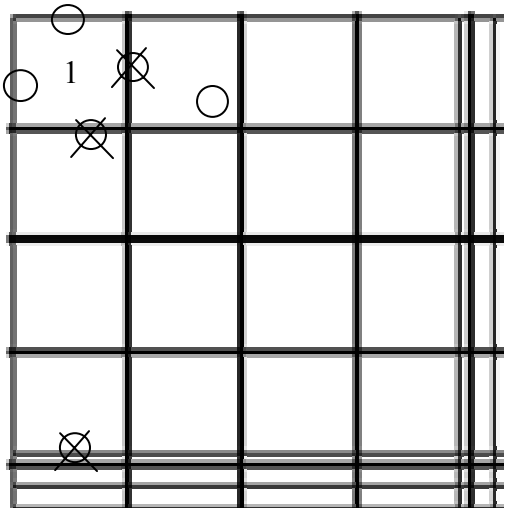
NOTE: CONSISTENCY IN FILLING TECHNIQUE IS PARAMOUNT TO SUCCESSFUL COUNTS!!!!! (Practice first with mock samples and not with your precious samples!)

- This is so critical that it is recommended that one person do the counts, or at least does the counts of one type of samples. For example one person counts the expelled algae and the other counts the expelled algae for the measurement of bleaching.

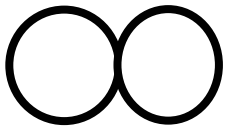
- Clean chamber and coverslip with lens paper with a little ethanol to remove any grease.
- **Always** use the correct coverslip (rectangular glass provided with chamber), a regular coverslip will **not** give you accurate values. Make sure you have extra coverslips!
- Use a glass Pasteur pipette to mix your sample well. I recommend that you use the same pipette to load all samples, just have a little beaker with water to rinse the pipette between samples. Do not use plastic pipettes since they can bind significant amounts of debris and algae. The surface of a fresh glass pipette will also bind some algae but it quickly coats and equilibrates. The initial mixing will help achieve this.
- With the coverslip on the chamber quickly touch the chamber inlet groove with the tip of the pipette making sure there is liquid at the tip. Hold the pipette with one hand and use the upper surface of the index finger of your other hand to guide the tip; the pipette should be held at 45° angle to the chamber. The chamber should fill very quickly (almost instantaneously) with liquid by capillary action. Not much pipette bulb pressure is required. If the is any slow filling or if the liquid front is not even (e.g. when it forms a bubble) start over. If it takes too long to fill (I count three or five seconds) while you are filling, redo.
- Each haemocytometer has two chambers. Remix your sample and draw a new sample for every chamber. i.e. do not fill both chambers with liquid from the same draw.
- The part of the haemocytometer that is filled is just the plateau, polished glass surfaces that has the grid etched onto it, not the whole slide (i.e. not the deep grooves around the plateaus. Quickly draw any excess liquid from the filling port.
- Mount slide onto microscope stage, being careful to have secure mounting. Allow time (about 0.5 -1.0 minute) for the algae to settle. Tip: Have two (or three) haemocytometers so that you can fill them at the same time and while you count one the other will be ready.
- Count away… (a counting clicker or two helps as well as some techno music)

Counting

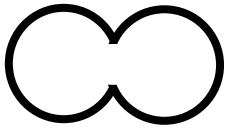
- Visually check to make sure your dilution and mixing was adequate (see sample prep above). You do not want too many overlapping algae and you do not want clumps.
- Use 450x magnification for zooxanthellae.
- Scan square subdivisions left to right, up-down and count algaeCount algae if they are **touching** the left or top line of each square (doesn't matter which ones –it can be bottom and right line but be consistent!) too make sure algae touching lines are not counted twice. In the example shown, do not count cells with an X for subdivision 1. Do not count the cell in the bottom row either.



- Decide what a dividing algae looks (versus what a doublet -or two algae in host symbiosome, looks like) like and how you are going to count it. I count a dividing algae as two.



Doublet
Look for cell wall material between cells



Dividing Cells

- What to do when you run into an arbitrary clump…as mentioned if you see too many of these better vortex your sample some more. If you count a clump, count some number of at least 1 but don't count all of them. If you have a "feel" for what the average density of cells is throughout the chamber, use that as an approximation. Whatever you decide to do be consistent within your own technique as well as among people counting.
- Keep track of how many squares you count. The whole chamber has 9 squares (See last figure). The 4 corner squares have 4X4 subdivisions. The center square has 5x5 subdivisions which are further divided into 4x4. Each square is $1mm^2$ and the chamber depth is 0.1mm; therefore the volume overlying each square is $0.1mm^3$ (or 0.0001ml = 0.1µl). Calculate the average number of cells per square (total cells counted/#of squares used) and multiply by 10^4 and the dilution factor to cells per ml. For example, if you resuspended your algal pellet with 250ul of FSW and from this resuspension you took an aliquot of 100ul to dilute with 400ul of FSW (for a total of 500ul), and you counted 100 cells in 4 squares, your sample cell concentration is:

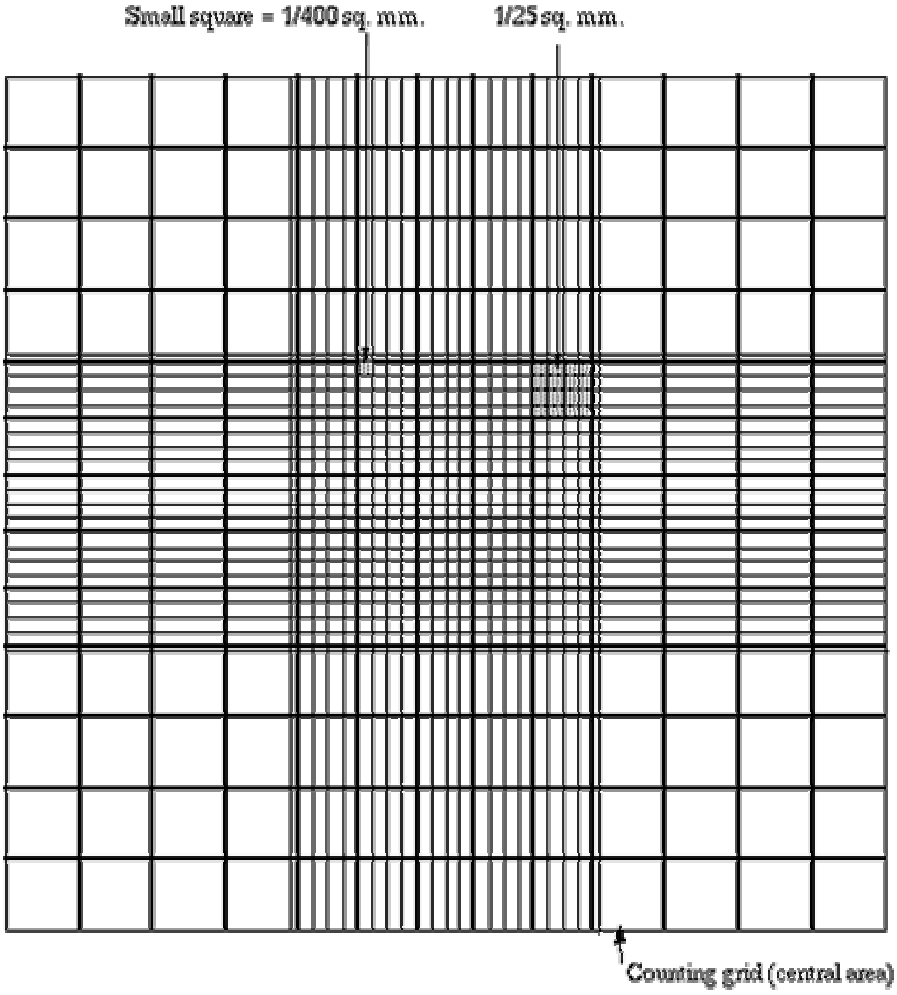
(100 cells /4 squares) X (500/100) X 10000 = 1.25×10^6 cells/ml

To get the total number of cells (your biomass), multiply this measured cell concentration by the original volume. In the example, 250ul = 0.25ml, times your concentration, you get 0.31×10^6 cells.

If you are measuring mitotic index (fancy name for % dividing cells), have a separate clicker to count the number of cells dividing and just divide this by the total number of algae counted (x100%).

About replicate counts, this depends on the amount of variability between counts and the worse the technique, the more replicate chamber counts you will have to perform. It also depends on how critical it is to get an accurate count. In my experience it ranges anywhere from 6 to 20 reps!!! It is helpful to enter the numbers directly onto a spreadsheet using a laptop, that way you can enter a formula to keep track of your coefficient of variation (standard deviation/mean expressed as%) and decide what your maximum should be, e.g. less than 10%. Remember to count your samples at random!!!

HAPPY COUNTING!!!!!!!!!!!!!!!!


Small square = 1/400 sq. mm.
1/25 sq. mm.
Counting grid (central area)