

COUNTING CHLAMYDOMONAS

BACKGROUND

When working with almost any microorganism it is often necessary to get an estimate of the cell density. While this seems like an obvious step, you need to ask what you are looking for. Do you want the number of cells, the number of viable cells, cell mass, or some other criteria? Even if you want living cells: do you count a cell that is dividing as one or two (or four), if the cells have divided, but are still inside the mother cell wall, how do you count them? If you want cell mass, do you measure protein, chlorophyll, total light absorbance *etc.*? Since each of these may be only loosely connected, depending on the health of the culture, its density and whether cell division is synchronous, it is a good idea to have several ways of measuring cell density available. For many experiments the exact number may not be important and you will get used to estimating the cell density from the color of the culture. Generally this is only useful between 10^5 and 10^7 cells/ml.



Appearance of different concentrations of Chlamy. The leftmost tube contains 10^7 cells/ml and the next three are successive tenfold dilutions.

This exercise will introduce students to three different ways that can be used to estimate the density of the cell culture. Each gives a different type of information, as described below.

Direct counting of cell number with a hemocytometer

Advantages: fast; allows user to decide whether to count dividing cells as separate, or as a single unit.

Disadvantages: Can't distinguish living cells from dead; treats all sizes as equal.

Estimate of colony-forming units

Advantages: only counts viable cells

Disadvantages: slow: must use sterile technique; dividing cells always counted as one

Biomass estimate using spectrophotometry

Advantages: fast; can use several criteria

Disadvantages: measures cell mass, rather than number

COUNTING CELLS WITH A HEMOCYTOMETER

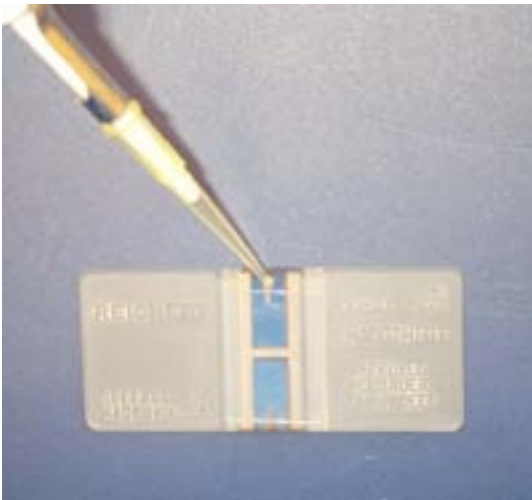
Hemocytometers were developed for counting blood cells, but can also be used to count Chlamydia. A hemocytometer has two chambers and each chamber has a microscopic grid etched on the glass surface. The chambers are overlaid with a glass coverslip that rests on pillars exactly 0.1 mm above the chamber floor. Thus, the volume of fluid above each square of the grid is known with precision.



Procedure

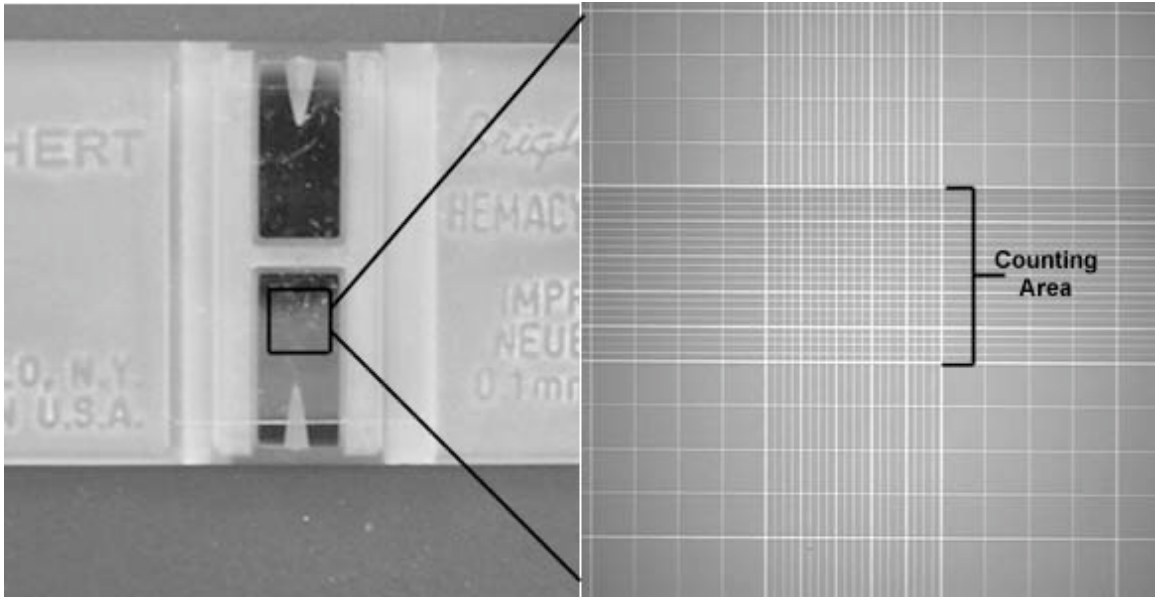
The culture must be killed to prevent movement and diluted before loading into the hemocytometer. This can be done adding 0.1 ml Lugol's iodine to 0.9 ml culture. The dilution factor must be recorded to allow calculating the concentration.

Loading the Hemocytometer: The tip of the pipette is placed in the V-shaped groove on the hemocytometer to load the sample into the chamber (about 15 microliters.) Capillary action will draw the fluid into the chamber. It is important not to overload the chamber, as doing so will give an inaccurate count. The same is true if the cover slip is moved after the sample is loaded.

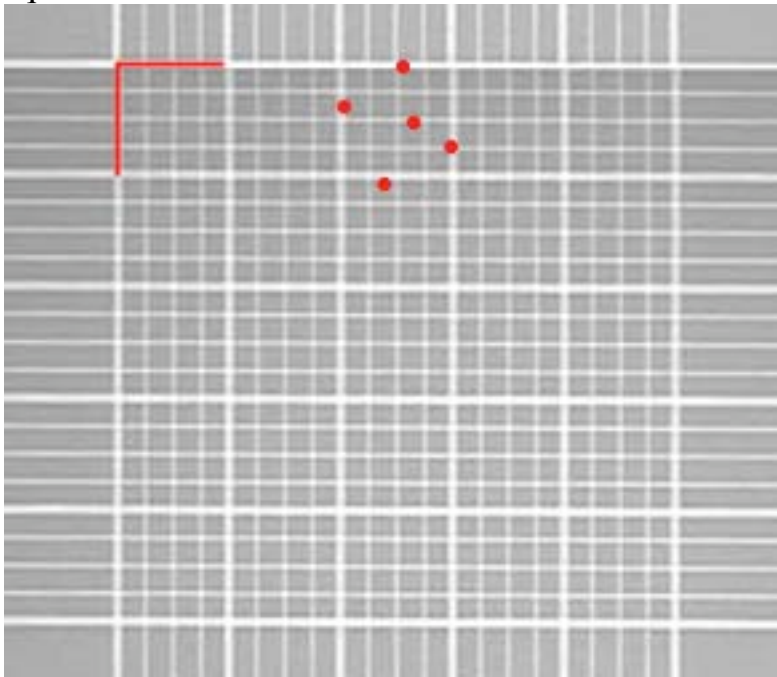


Counting: The full grid on a hemocytometer contains nine squares, each of which is 1 mm square (see figure below). The central counting area of the

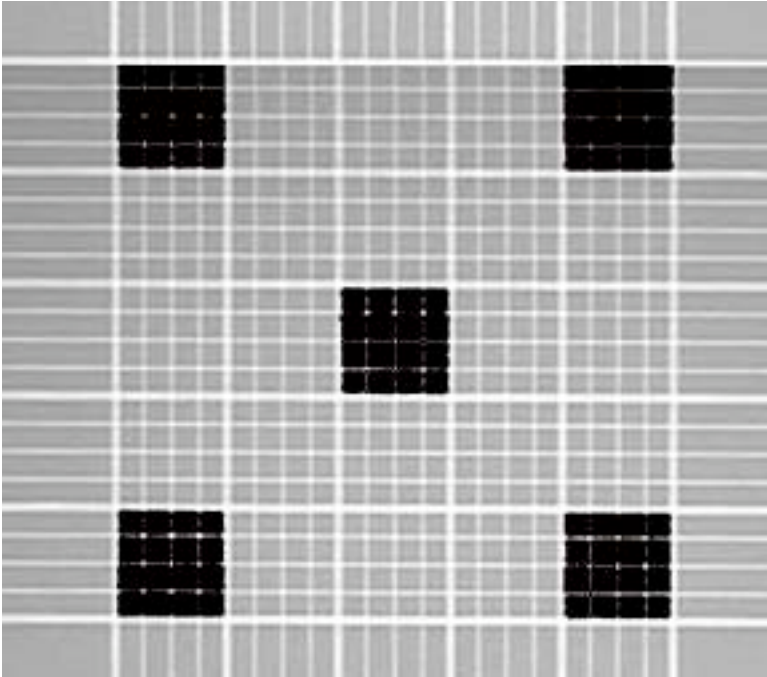
hemocytometer (as it will be called here) contains 25 large squares and each large square has 16 smaller squares. When counting, count only those cells on the lines of two sides of the large square to avoid counting cells twice.



The example below shows red lines where cells on the line would be counted. If red dots represent cells, one would count 3 cells in the top middle large square.



All 25 large squares can be counted, or a counting pattern using fewer squares can be used like the ones below. It is important to distribute the counting areas in a non-biased manner since cells can be more concentrated on one side of the chamber. If you count only 5 of the 25 large squares, then multiply that value by 5 to obtain the number of cells per central counting area



At least two chambers should be counted, including at least 100 cells within each central counting area of each chamber. For higher precision, additional samples can be counted and the average used to calculate cell concentration.

Calculating Concentration

Each of the nine squares on the grid, including the central counting area of 25 large squares, has an area of 1 square mm, and the coverglass rests 0.1 mm above the floor of the chamber. Thus, the volume over the central counting area is 0.1 mm³ or 0.1 microliter. You can thus multiply the average number of Chlamy over each central counting area by 10,000 to obtain the number of Chlamy per ml *of diluted sample*.

In other words, to calculate the number of Chlamy per ml of original sample:

1. Calculate the mean number of Chlamy counted for each chamber (i.e. for each of the central counting areas of each chamber).
2. Multiply the mean obtained in (1) by 10,000 to obtain the number of cells per ml of diluted sample.
3. Multiply the count obtained in (2) by the dilution factor.

Example: Assume that you dilute the original culture sample by adding 0.1 ml of culture to 9.9 ml of diluent (1:100 dilution factor). You then count the number of Chlamy in 5 of the 25 large squares within the central counting

area of two chambers, obtaining counts of 132 and 128 cells.

1. The mean number of Chlamy per chamber is thus 130×5 or 650 cells per counting area (650 cells per 0.1 microliter).
2. Multiply the 650 cells per counting area by 10,000 to obtain the number of cells per ml of diluted sample (answer = 6,500,000)
3. Multiply 6,500,000 cells per ml of diluted sample by 100 (the dilution factor) to obtain 650,000,000 per ml of original culture sample.

The above information was modified, with permission, from a web site created by:

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<http://arbl.cvmbs.colostate.edu/hbooks/pathphys/reprod/semeneval/hemacytometer.html>

VIABLE COUNT

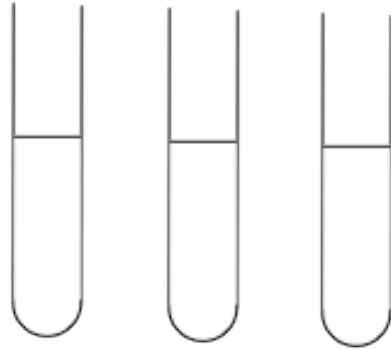
The hemocytometer generally cannot distinguish living from dead cells. If you wish to know the number of living cells, then a viable count needs to be made. This can be done in the hemocytometer using a vital stain but often will not work on cells that are dying due to a treatment such as UV. If a small volume (typically 0.1 ml) of a culture is spread onto a TAP/agar plate, each viable cell will grow into a colony over the course of several days, and can be counted. Note that all cells within a single well will stay together, and so be counted as one. Most cultures contain cell densities that are very high ($>10^5/\text{ml}$), meaning that so many colonies form that they grow together into a confluent 'lawn' that is impossible to count. Consequently, it is necessary to dilute the culture to the point where a reasonable number of colonies grow. The usual aim is to get between 30 and 300 colonies per plate. This is a large enough number to avoid statistical problems, yet be small enough to allow individual colonies to be seen. It is often not clear how much the culture must be diluted to reach this range, so a series of tenfold dilutions are made, and samples plated out at a variety of dilutions. For most purposes it is better to spread out several (3-5) plates at each dilution, to allow statistical analysis.

Original culture

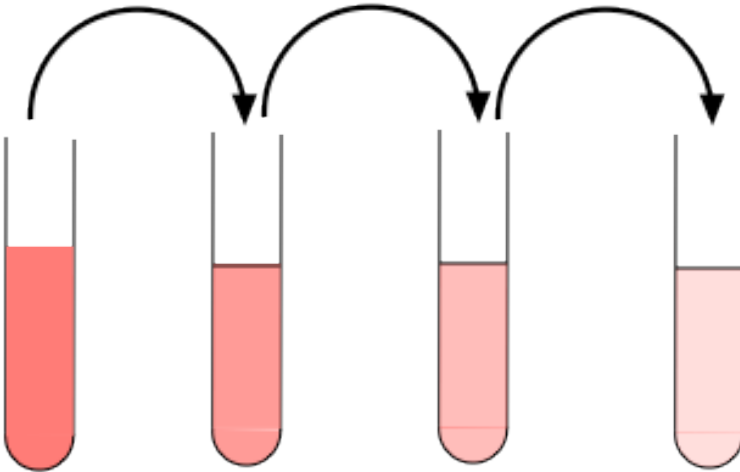


250,000
cells/ml

Dilution tubes, each with 0.9
mls sterile medium



0.1 mls transferred to
next tube and mixed



250,000
cells/ml

25,000
cells/ml

2,500
cells/ml

250
cells/ml

spread 0.1 mls on a plate
and let grown one week



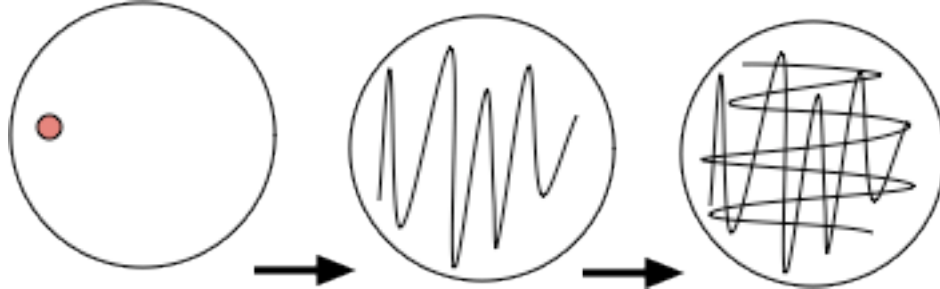
250 colonies



25 colonies

Basic procedure

Inoculate a flask of TAP medium with a small number of cells and place them under whatever growth conditions the experiment calls for. At each time interval, aseptically remove a sample and dilute as shown in the diagram. For convenience the plates should be made a week early and left inverted at room temperature. This allows them to dry out a bit so that the 0.1 ml samples get absorbed quickly. Spread the liquid in a zigzag across the plate and then repeat at right angles.



Streaking out the sample in both directions spreads out the cells, and reduces the chance of two ending up so close that they form a single colony. Store the plates upside down in the growth chamber for a week. The colonies should be large enough to see with the naked eye. If the bottom of the plate is tapped with a Sharpie as each colony is counted it reduces the odds of counting a colony twice.

It is important that all steps be done aseptically. If bacterial or fungal contamination occurs it can make it very difficult to get accurate numbers.

Incidentally, this is also a good (though tedious) way of cleaning up lightly contaminated cultures. The individual colonies can be picked off the agar using a sterile toothpick and spread onto a fresh plate.

SPECTROPHOTOMETRY

The simplest way to do this is to blank the spectrophotometer with TAP and then measure the absorbance of the culture. Because of backscatter the cells absorb quite strongly across the visible spectrum, so you can use almost any wavelength you like, as long as you are consistent over time. Above 10^6 cells/ml the absorbance at many wavelengths may go above 1.5 OD, so the cultures will need to be diluted before reading. The exact figures will vary, depending on the state of the cells, but an absorbance of 1.0 OD at 550nm is typical for about 5×10^6 cells/ml

More complex approaches can be used to estimate total chlorophyll and total protein. For chlorophyll determination: spin down 5 ml of culture and resuspend the pellet in 5 mls of 90% acetone. Spin again, to remove the protein precipitate and measure the absorbance of the supernatant at 652 nm. The result will depend on the total amount of chlorophyll (which may not necessarily be in step with the number of cells) and will be in the region of 1.0-1.2 OD.

POSSIBLE EXPERIMENTS

Do any of the above with a healthy culture. Compare the answer from the hemocytometer and plating.

Do any of the above with an unhealthy culture. This could be starved, exposed to heat, UV, overgrown *etc.* Compare the answer from the hemocytometer and plating. Compare these answers with those from a healthy culture

Do all three on an asynchronous growing culture with samples taken at intervals (once a day; twice a day). Plot the data for all three methods. Have each lab group take their samples from the same culture and look at the variation. Have each group keep a separate culture and look at the variation.

Do all three on a synchronous culture with samples taken at intervals every 3-4 hours). Plot the data for all three methods. Compare the synchronous and asynchronous results.

Measure total chlorophyll in synchronous and asynchronous culture. Look at the effect of prolonged dark growth. Obtain photosynthetic-defective mutants and look at them.