This dilution plan is for our cell counting project which will consist of using multiple cell counting methods. Our reasoning for diluting our samples is to remain consistent throughout the experiments without ruining the cell counting machines, as the samples have varying amounts of cells. We hope with the dilutions, we will be able to rid the samples of cell debris and unmeasurable clumps of cells, so they are easier to measure.

6 counts - what is this telling you? Why do this?

- Goal - to reduce variability, calculate coefficient of variation (CV) = (stdev/mean)*100
- Accept CV less than 5% or 10%

5 samples - why set a sample size? Why measure multiple samples?

- Goal - to quantify variation between samples and to run statistical tests
- ANOVA or t-test to determine if p - value is <0.05
- Regression analysis - look at R2 value between the pairs of methods

Group 1 = undiluted
Group 2 = undiluted but washed
Group 3 = Diluted 1:1 with PBS (0.5ml homogenate + 0.5ml PBS)
Group 4 = Diluted 1:4 with PBS (0.25ml homogenate + 0.75ml PBS)
G1vG2 H_0 = There is no difference in the cell counts *due to washing away of debris*
G1vG3 H_0 = There is no difference in the cell counts due the spatial distance between cells
G2vG3 H_0 = There is no difference in the cell counts due to the spatial distance between cells or washing away of debris
Group 4 hypotheses are the same as for Group 3, just more dilute.

1. ***Dilution Plan***

I will be using *Montipora capitata* as a trial species during the beginning of our dilution plan. I plan to do a variety of dilutions of the samples to compare cell counts. I will be preparing **three** 1mL aliquots for each dilution, using three separate randomized samples. Before every dilution, I will vortex the original sample. The first dilution I will prepare will be the undiluted sample, where I simply vortex the sample, and then pipette the 1 mL sample into another 1.5 mL tube. The second dilution I will prepare will be the resuspension of cells in Phosphate-Buffered Saline (PBS), for a "wash", where I will centrifuge the samples for three to five minutes at 16,000 rpm, pellet out supernatant, and resuspend in 1 ml PBS. The purpose of the resuspension in PBS solution is to dissociate the excess cells and debris from our sample. The third dilution is a 1:1 dilution of 0.500 mL of sample, and 0.500 mL PBS solution. All samples will be vortexed to homogenize the sample before counting on the hemocytometer. Each species will likely be optimized with a different dilution factor, but we will start with *M. capitata* first.

Cell Counting- Hemocytometer

I will be using the hemocytometer for cell counts under the microscope at a 10x magnification setting. We will be referencing the Symbiont Cell Density Counting Protocol found on Dr. Hollie Putnam's GitHub page. I will first clean off the hemocytometer slide with DI water to check that there are no excess cells left on it from previous samplings. I will then vortex each sample prior to counting to ensure homogenization. I will take a glass pipette and mix the sample, and then take a small amount and load one side of the hemacytometer until the sample has completely filled one side, and will repeat the same process on the other side with a newly mixed aliquot from the sample. It is important that a new mixed aliquot is used as you do not want to fill both sides with the same aliquot.
*** Calculate coefficient of variation to ensure low variability in hemocytometer counts.*
*** Compare output to Emma's.*

This dilution and homoganization plan will be used for flow cytometer and cellometer

Cell Counting- Flow Cytometer

Hannah will be operating the Flow Cytometer in the near future, as undergraduate students are not allowed to operate the machine without supervision. We hope I (Sierra) will be able to observe in the coming weeks. Our next steps with the FACs will be using the PMTV settings = FSC laser 212.1; SSC laser 247.7 with the APC-Cy7-A and APC-A filters to identify Symbiodiniaceae cells. 0.5 mL of sample is used for analysis and it is ideal to have 10,000 events (counts) of the target cell population per sample. Hannah and Sierra will work together in verifying whether the desired (aliquoted) number of Symbiodiniaceae is detected by the aforementioned parameters using the FlowJo FACs software (the pharmacy core has a license that undergraduates are allowed to operate).

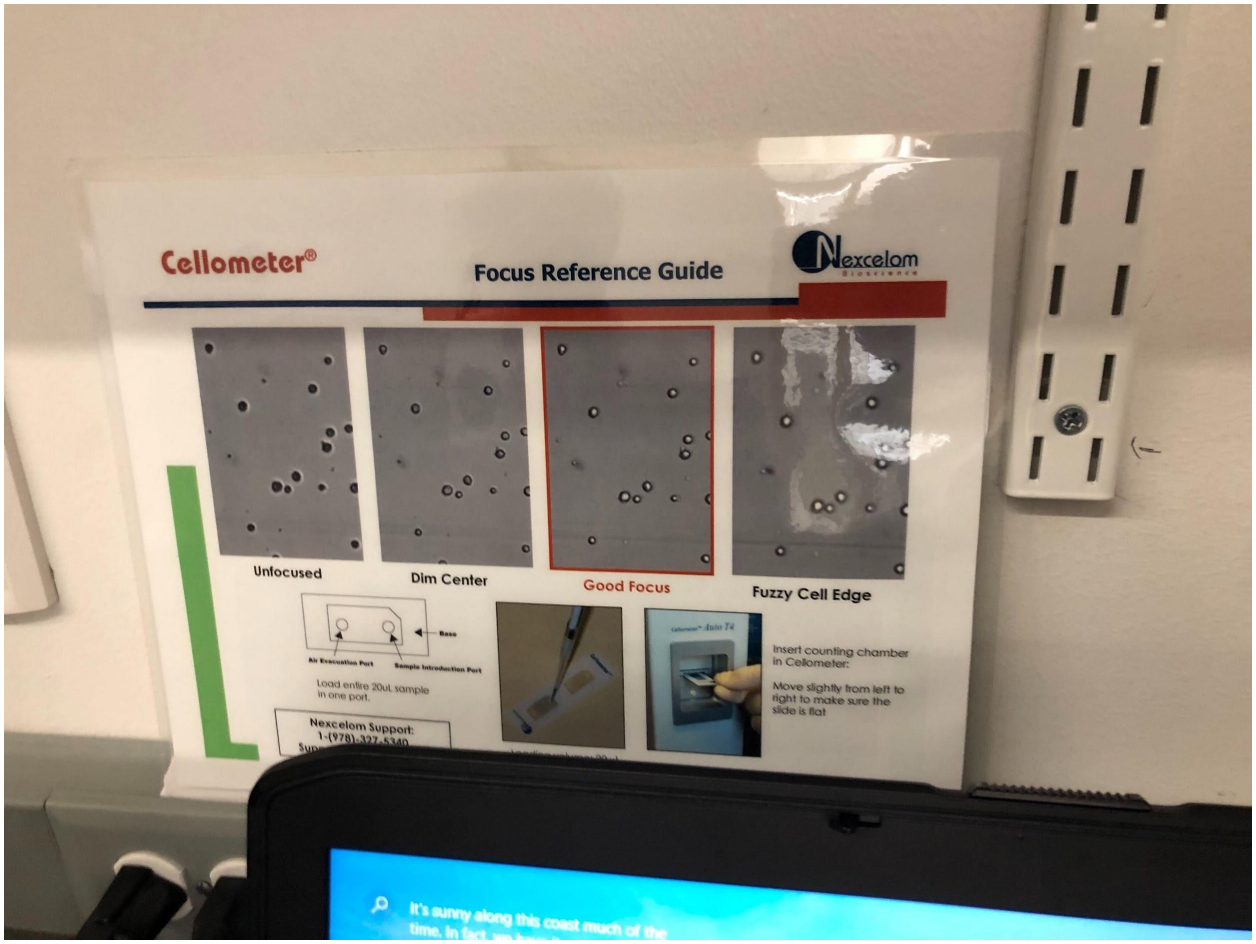
Cell Counting- Nexcelom Bioscience Vision Cellometer

Starting point

- We know that the cells are round and 10µm in diameter
- We know the cells have chlorophyll and autofluoresce red
- So we need to check all the potential software settings (e.g., shape/irregularity, particle size, and fluorescence)

We emailed Kim to ask about training.
We need to look to see if we can examine software on campus or download trial versions to look at settings. We need to know potential settings to ask the rep any questions.

Load 20uL of the sample will be placed in a well. There are two wells per slide. After entering the slide into the machine we make sure that the cellometer is focusing on the right cell populations (a how-to guide is taped on the wall behind the machine).



Then we click “measure” and record the total cells in 20 uL well and the cell density.

Statistical tests of Groups 1, 2, 3, 4

ANOVA of 5 samples per group, Group is the main effects
H_0 = There is no difference in cell counts between groups

Regressions of Groups 2,3,4 against Group 1
R^2 is telling you how much variation is explained by the fit of the line to the data

2. ***<u>Four Species</u>***

Our goal is to test four species commonly used in the Putnam Lab, *Astrangia poculata, Pocillopora acuta, Montipora capitata,* and *Porites astreoides.* We will begin testing dilution samples with *Montipora capitata,* and use this species as a baseline for the other species. I will

have a better understanding of the effects of the dilutions on *Montipora capitata* and have numerical differences between the hemocytometer, flow cytometer, and cellometer.

3. ***Statistical Analysis***

We will be testing the variation between the three methods (ANOVA), and our hope is that the counts are not statistically different. We are hoping for this statistical outcome because we want our cell counts from every method to be similar, and if our counts are statistically different then that indicates that discrepancies in our various methodologies do not portray the correct symbiont densities in our samples.