Title: Cell Counting of Dilution Series Experiment Description

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The objectives of this experiment were as follows:

1. To design a system of analyzing the events such that bead events and cell events are accurately identified.

*The identification scheme can be model-based or simple numerical limits in the appropriate channels. The only requirement is that they are robust to some amount of variation in fluorescence, as slight errors in pipetting, or variations in light exposure, cytometer settings or temperature can affect the strength of the signal. NOTE: Those samples labelled with* *propidium iodide (PI) may cause certain populations of cells to appear distinct as it is reported to only label dead/damaged cells, whereas SYBR labels all cells. However, the effectiveness of PI in penetrating cells varies strongly by cell-type, so it is possible that either all cells or no cells are labelled with PI. There also may be an interaction between the two dyes, as they have overlapping emission spectra.*

1. To use this system to count the number of cells and beads in each sample and use this to calculate the cell density in each dilution and then make a calibration curves for (i.e. plots of events counted versus the true cell densities) for the following sets of data:
   1. The filtered SYBR stained cells
   2. The unfiltered SYBR stained cells
   3. The filtered SYBR/PI stained cells
   4. The unfiltered SYBR/PI stained cells

*This will allow us to determine the effect of prefiltering cells on different initial cell densities. It is not clear whether more cells removed when the initial density is high or whether a fixed proportion is removed at all densities. It will also allow us to determine the cell density quantification limits for which using cytometry at our particular bead concentration is appropriate. Too many cells will overload the system (see below) and too few cells per bead will lead to very inaccurate counts on the low end. It is important to determine where these limits are so we can dilute or concentrate our actual samples.*

**Methods:**

Data Location

There are two identically named folders called “2019-11-07 Keith Arora-Williams SYBR PI Dilution Series” in both the “Experiment” and “FCS” folders in the “Rock Creek Project > Data > FlowCytometryData” subfolder in Box. The data is the same, but different programs may require one or the other.

Sample Preparation

Right after fixing the bacterial culture used for this experiment, dilutions were plated and the optical densities were taken. Two colonies were taken from the same plate and grown separately. The optical densities were as follows:

|  |  |  |
| --- | --- | --- |
| Dilution (Volume Media: Volume Cells) | Culture 1 (OD600) | Culture 2 (OD600) |
| 0 mL : 1 mL | 1.58 | 1.526 |
| 900 uL : 100 uL | 0.257 | 0.253 |
| 999 uL : 1 uL | 0.028 | 0.028 |
| 999.9 uL : 0.1 uL | 0.003 | 0.003 |
| 999.99 uL : 10 nL | 0.001 | 0.001 |

These two cultures were then pooled, diluted for plate counts, and fixed. Two series of dilutions were plated and the plates that had counts near the acceptable range are as follows:

|  |  |  |
| --- | --- | --- |
| Approx. Volume of Cells Plated (mL) | Plate 1 (Colonies) | Plate 2 (Colonies) |
| 1e-7 | 81 | 152 |
| 1e-8 | 4 | 23 |

Based on those counts, the expected cell density in culture is ~1.17E+9 cells/mL but is likely higher. The culture was grown overnight and had reached peak density and likely contains non-viable cells and detritus. These dead cells will not create countable colonies may appear in the cytometry data, and will appear in our sequencing data, so I think using a baseline estimate of ~1.5 billion cells /mL (i.e. the higher plate count) is justifiable. There are also cells that take longer to form full colonies and thus may not have been visible at the time of counting.

For processing on the cytometer, a portion of culture was mixed with an equal volume of fixative. Two overlapping sets of 10-fold dilutions were made and half of each of those were passed through a 5 micron prefilter. The following chart describes the dilutions in PBS as *expected cell densities*, assuming that the “true” unfixed cell density is 1.5e9 cells/mL. Note that for tubes 3 and 4, fresh media and the contamination control (respectively) culture were fixed and used, undiluted. For these samples, 50 uL of CountBright beads were added to 500 uL of stained culture. More details about the staining process are in the Growth Curve Protocol Box Note.

**Sample Treatment and Names**

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Tube | Exp. Cell Density | Filtered? | SYBR? | PI? | Notes |
| 1 | 7.5e5 | No | No | No | Unstained Cells control |
| 2 | 0 | NA | No | No | Just Beads Control |
| 3 | 0 | No | Yes | No | Media Control |
| 4 | 0 | No | Yes | No | Contamination Control |
| 5 | 7.5e8 | No | Yes | No |  |
| 6 | 7.5e7 | No | Yes | No |  |
| 7 | 7.5e6 | No | Yes | No |  |
| 8 | 7.5e5 | No | Yes | No |  |
| 9 | 7.5e4 | No | Yes | No |  |
| 10 | 7.5e3 | No | Yes | No |  |
| 11 | 7.5e2 | No | Yes | No |  |
| 12 | 75 | No | Yes | No |  |
| 13 | 7.5e8 | Yes | Yes | No |  |
| 14 | 7.5e7 | Yes | Yes | No |  |
| 15 | 7.5e6 | Yes | Yes | No |  |
| 16 | 7.5e5 | Yes | Yes | No |  |
| 17 | 7.5e4 | Yes | Yes | No |  |
| 18 | 7.5e3 | Yes | Yes | No |  |
| 19 | 7.5e2 | Yes | Yes | No |  |
| 20 | 75 | Yes | Yes | No |  |
| 21 | 3.75e8 | No | Yes | Yes |  |
| 22 | 3.75e7 | No | Yes | Yes |  |
| 23 | 3.75e6 | No | Yes | Yes |  |
| 24 | 3.75e5 | No | Yes | Yes |  |
| 25 | 3.75e4 | No | Yes | Yes |  |
| 26 | 3.75e3 | No | Yes | Yes |  |
| 27 | 3.75e2 | No | Yes | Yes |  |
| 28 | 38 | No | Yes | Yes |  |
| 29 | 3.75e8 | Yes | Yes | Yes |  |
| 30 | 3.75e7 | Yes | Yes | Yes |  |
| 31 | 3.75e6 | Yes | Yes | Yes |  |
| 32 | 3.75e5 | Yes | Yes | Yes |  |
| 33 | 3.75e4 | Yes | Yes | Yes |  |
| 34 | 3.75e3 | Yes | Yes | Yes |  |
| 35 | 3.75e2 | Yes | Yes | Yes |  |
| 36 | 38 | Yes | Yes | Yes |  |

Cytometry Data Acquisition and Details

Samples were processed such that either ~1000 beads were counted, or 3 minutes elapsed. It is likely that many of the samples will have fewer than 1000 beads because too much stain was used and there were objects that likely snuck into the gate used to count cells. There were also some samples that were too dense to effectively collect data, for the following reason. In reasonably dilute samples, the cytometer will create droplets containing either 0 or 1 cells each and these are readily countable. In the event that a droplet passes the detector containing multiple fluorescent objects, the software triggers an “electronic abort” and does not record the event. This makes data acquisition take MUCH longer and so these samples hit the time limit before reaching 1000 beads. The count ratios for such samples will also be less accurate.

The cytometer is equipped with two lasers that pass through each droplet. The light from these lasers are either scattered or absorbed, but may also be re-emitted as fluorescence. The scattered light is collected by the FSC (forward scatter) and SSC (side scatter) detectors. All particles in solution will generate these signals, however the average amount that goes to one or the other detector depends somewhat on the size and shape of the particles, so discrete populations may be observed.

Fluorescence emitted after excitation with the blue laser (488 nm wavelength) can be detected in 4 channels: from 530/30 nm (500-560 nm), 585/42 (543-627), 670LP (>670), and 780/60 (720-840). Illumination with the red laser (633 nm) can generated data that registers in 660/20 (640-680) and/or 780/60 (720-840) nm channels. Samples stained with SYBR Green register most strongly in 530/30 but will also register in the 585/42. Propidium iodide is only weakly excited by the blue laser (it was not a great choice…) but may register in the 670LP channel. The CountBright beads are excited by both lasers, emit fluorescence in all channels (with differing intensities) and are an exact size and shape (7 micrometers spheres). Therefore they should have a very distinct signal in FSC and SSC and a unique footprint across the fluorescence channels that should be simple to isolate.