**Fecal Filtrate Flow Protocol 4/11/19**

Materials:

* LIVE/DEAD BacLight Bacterial Viability and Counting Kit (L34856)
  + SYTO-9 nucleic acid stain (component A) in DMSO
  + Propidium Iodide (component B) in DMSO
  + *Microsphere standard (component C) at a concentration of 1.0 x 108 beads/mL in deionized water containing 2mM sodium azide*
* Fecal filtrate
* Flow cytometry analysis tubes with cell strainer top (35µm mesh size)
* 15mL conical tubes
* 1.5mL microcentrifuge tubes
* Phosphate buffered saline (Mg2+ and Ca2+ free)
* Staining buffer (FBS)
* 70% isopropyl alcohol
* Aluminum foil
* Disposable transfer pipettes
* Bucket of ice **\*all prep needs to be done on ice**

**Items to bring to the flow lab in ARC 1214C:**

* Filtered cell suspensions, including single color compensation controls and unstained cells
* Labeled collection tubes containing cell culture medium
* Approx. 10mL of buffer for diluting cells if needed
* 10 disposable, sterile transfer pipettes

Steps:

*\*\*schedule time on the flow cytometer at the Flow Core with a prepared PO\*\**

**Preparing Bacterial Suspensions**

1. Remove LIVE/DEAD components from 4⁰C and allow to reach RT. Before opening the vial for use, briefly centrifuge.
2. Turn the centrifuge on and set it to 4⁰C.
3. Briefly vortex each sample of fecal filtrate (~5 seconds).
4. Dilute each sample of fecal filtrate 3000-fold to reach a cell concentration of approx. 25,000,000 cells/mL.
   1. In four 15mL tubes, aliquot 5uL of fecal filtrate.
   2. Bring the total volume in each tube to 15mL with PBS.
5. Place four 1mL samples of the bacterial suspension in a microcentrifuge at 10,000 x g for 2 minutes to pellet the cells.
6. Remove the supernatants. Resuspend two pellets in 1mL of PBS and the second set of pellets in 1mL of 70% isopropyl alcohol (for the dead cell suspension).
7. Incubate the samples at RT for 30 minutes, mixing every 10 minutes.
8. Pellet all four samples by centrifugation at 10,000 x g for 2 minutes.
9. Wash all four samples in PBS and pellet again via centrifugation at 10,000 x g for 2 minutes.
10. Resuspend all four samples in 1mL of PBS.

**Staining the Bacteria**

1. Aliquot 977µL of staining buffer (FBS) into 8 flow cytometry analysis tubes. *Note: aliquot 987µL if the microsphere standard will not be used.*
2. Add 1.5µL of 3.34 mM SYTO-9 nucleic acid statin (component A) and 1.5µL propidium iodide (component B) to two flow cytometry analysis tubes. Label tubes A1 and A2.
3. Aliquot 1.5µL of 3.34 mM SYTO-9 nucleic acid stain (component A) into two flow cytometry tubes. Label tubes B1 and B2.
4. Aliquot 1.5µL of propidium iodide (component B) into two flow cytometry tubes. Label tubes C1 and C2.
5. Add 10µL of live bacterial suspension to flow cytometry tubes A1, B1 and C1.
6. Add 10µL of dead bacterial suspension to flow cytometry tubes A2, B2 and C2.
7. In the 7th and 8th flow cytometry tubes, add only live 10µL live cells and 10µL dead cells, respectively. These cells will remained unstained.
8. Incubate the samples at RT for 15 minutes protected from light.
9. *Resuspend the microsphere standard (component C) by vortexing the suspension, followed by sonication in a water bath for 5 minutes.*
10. *Add 10µL of the microsphere suspension to the cell sample and mix well.*
11. Arrange samples to be transported to the Abramson building.
    1. The total volume of the sample should be 1000µL for accurate counting.
    2. You should have 8 total samples.

**Analyzing Stained Bacteria by Flow Cytometry**

*Software 🡪 CytExpert*

1. Prior to analyzation, the cells should be passed through the cell strainer using a disposable transfer pipette.
2. Stained bacteria can be assayed in a cytometer equipped with a laser emitting at 488nm. Fluorescence is collected in the green and red channels. Filters used for detecting fluorescein and Texas Red dye, respectively, should be suitable. The forward scatter, side scatter and fluorescence should be collected with logarithmic signal amplification.
3. Use the single color controls to locate bacterial populations and determine compensation settings.

**NOTES:**