Microbiome viability assay

Background

Microbiome studies are limited by the fact that sequencing alone cannot distinguish whether the sequenced DNA originates from a viable or dead bacterial cell. This severely limits our ability to determine which bacteria are metabolically active and produce compounds that interact with host receptors, which can e.g. regulate the differentiation and expansion of different types of T cells. Furthermore, current microbiome assessment methods only provide relative abundance estimates of different bacterial taxa, but no absolute quantification. This SOP describes a novel assay that combines fluorescence-activated cell sorting (FACS) with viability fluorescent markers and shotgun metagenomic sequencing to absolutely quantify at the single cell level the concentration of total and viable bacterial cells and their taxonomic composition. We will apply this technique to quantify and identify viable bacteria that exist in the maternal vagina and that are transferred to the newborns at birth.

SOP

All work is to be done under the biological hood taking the following precautions:

- Cover the working surface with diapers
- Use double gloves
- Use the red biohazard waste plastic bag
- Clean with 10% bleach

1) Pre-treatment of samples (as soon as sample is received in the lab)

- Pre-weigh the 50-ml tubes (blue lid).
- Add a few **0.5** and **1.0 mm** glass beads.
- Suspend the sample in 1X sterile PBS (10 ml of 1X PBS for 1g of sample).
- Homogenize the sample by vortexing for 3 min at high speed
- Transfer at least 10 ml of homogenized sample into a 15-ml tube (blue or orange lid).
- Centrifuge the sample at **700 X g** for **1 min**.
- **Dilution I: 1:10**. Add 2 ml of the supernatant obtained by centrifugation into 18 ml of sterile 1X PBS (50 ml tubes, blue lid).
- **Dilution II:** Dilute **1:50** the sample (50-ml tubes) (1:50; 1ml of sample, 49ml of PBS), (1:25; 1ml of sample, 24ml of PBS). Keep the samples at **4°C**, you will be using those samples for collecting the total cells **SECTION #5: Total cells.**
- Filter **1 ml** of sample into the flow cytometry tube by using Cell Trics red **20 µm** filters. Keep the samples at **4°C** until staining/sorting
- Label 15-ml tubes with the white lid indicating “Viable” and “Dead” as well as the sample ID.

2) Sample staining (before sorting)

The staining should to be done right before you go to the FACS facility (about 20 minutes before).

- Thaw out the dyes (Sybr Green I and Propidium Iodide) at room temperature.
- Add **10 µl** of Sybr Green I, (1:30 dilution in DSMO) and **10 µl** of Propidium Iodide and mix well.
- Add **50 µl** of counting beads and write down bead concentrations.
- Put the flow tubes and the labeled tubes (with the white lid) on ice and go to the Flow Cytometry Core (Icahn Building, 13th Floor)

3) Sorting protocol

Icahn Building 13th Floor, Instrument: IMI3L, you should arrive 5 min before your appointment. Tell the technician we have **4 samples** and the instrument booked **for 2 hours**.

Nozzle 70µm (when you book the instrument be sure to indicate in the instruction: Nozzle 70µm)

- Open the experiment template (dated Nov 2 2017) in the **tambuS01** folder.
- Don't duplicate without data, but overwrite the files.
- Check for each sample: threshold is on **Blue1 200** and PMT values of the **SCC-A**, **FSC-A**, **Blue1** and **Blue4** Channels. The scales should be in log scale.
- Check the shape of the population in the cytograms: First cytogram **FSC-A vs. SCC-A** and second cytogram **Blue1-A vs. Blue-4A** (Figure1).
- Sort the population of viable and the population of dead cells.
- Sort 1 ml of sample.
- Write the number of events of viable and dead cells that are collected.
- Sort a 1X sterile PBS sample as a control (1-2ml are enough)
- Export the file as "**Experiment**" and also as "**FCS files**" (use default parameters).
- Email the FCS files.

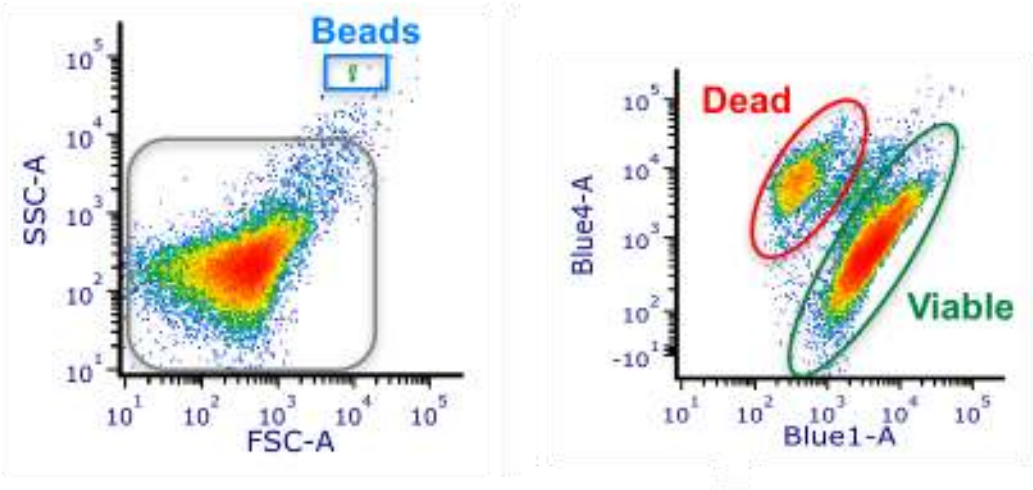

Figure1. Cytograms. The population of beads and the bacteria are indicated in the first Cytogram. In the second cytogram the population of viable and dead cells are indicated.

4) After-sorting

- Label the 50-ml tubes: viable sampleID, dead sampleID, type of sample (donor, recipient, PBS). **Use the tubes with the purple lid, the color of the lid is important!! Those tubes can resist high speeds of centrifugation**.
- Transfer the sorted samples from the 15-ml tube with the white lid to the tube with the purple lid.
- Centrifuge the tubes for **30 min** at **9000 rpm** at **4°C** (use the big centrifuge Sorvall RC6 Plus, you should book the centrifuge by using the eRAP system). Maximum 4 samples at a time.
- Remove the supernatant by inverting the tube with the lid closed and look for the pellet. Work under the biological hood.
- Store the pellet at **-80°C** in a plastic bag.

5) Total cells

- Aliquot **1 ml** of each diluted samples in duplicate (**1:50** for donor, recipient)
- Centrifuge for **30 min** at **10000 rpm** in the micro-centrifuge.
- Remove the supernatant avoiding the pellet.
- Keep the aliquots at **-80°C** in the box for ITN samples