Enumeration of bacteria and cyanobacteria by flow cytometry

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Abstract

Seawater is collected and preserved to be analyzed in lab using an InFlux Flow Cytometer with the Spigot software package. The populations targeted for enumeration are *Prochlorococcus*, *Synechococcus*, unclassied pico-eukaryotes (cells sized around 1µm), and heterotrophic non-fluorescing bacteria. Each sample is divided in two, with one aliquot analyzed for autofluorescence and the other for heterotrophic bacteria stained with SYBR Green I. In both cases, excitation is produced using stacked 488nm and 457nm lasers. The data is analyzed using FlowJo (v10) to determine cell concentrations (cells/mL).

Citation: Eric Shimabukuro Enumeration of bacteria and cyanobacteria by flow cytometry. **protocols.io** dx.doi.org/10.17504/protocols.io.j2wcqfe
Published: 23 Nov 2017

Protocol

Sample Collection

Step 1.

A 15 mL polypropylene centrifuge tube is used to collected 10-12 mL of seawater from the Niskin bottle

Step 2.

A 2.0mL subsample is taken and stored in a 2 mL Corning cryovial (P/N 66021-974 from VWR) that has been pre-loaded with 30 µL (15 µL per 1 mL sample) of 16% paraformaldehyde (PFA).

REAGENTS
Paraformaldehyde Aqueous Solution -16% 15700 by Electron Microscopy Sciences

Step 3.

The sample is mixed well (3x inversions of the cryovial) and stored in the dark at room temperature for 15 minutes, flash frozen in liquid nitrogen, and stored at -80°C.

Instrument Setup

Step 4.

Triple rinse and fill the sheath fluid reservoir with MilliQ Water.

Step 5.

Attach a Millipore Sterivex 0.22 µm In-Line Filter (P/N: SVGPL10RC) to the InFlux intake for the sheath fluid.

Step 6.

The sheath pressure should be set to 20.0 psi, and the sample pressure adjusted to yield a flow rate of approximately 30 µl/min (normally 20.1-20.2psi).

Our setup: InFlux uses a Sensirion SLI-0430 Flow Meter placed in-line between the sample line pinch valve and the nozzle assembly, and the InFlux interfaces with the software Spigot (ver. 6.1.10.0) from Cytopeia.

Laser Alignment

Step 7.

The laser sources are a 488nm 200mW laser (Coherent, 488-200 CRDH) and 457nm 300mW laser (CVI Melles Griot, 85-BLS-601), both operating at 100%.

Initial alignment is done using the 488 nm laser, focusing the beam first on the Red and Orange channels and then on the FSC channel to maximize precision. The 457 nm laser alignment is done second, and is maximized to provide the highest signal on the Red and FSC channels.

Step 8.

Alignment is performed using Spherotech Ultra Rainbow Fluorescent Particles in the 1.0-1.5 µm size (P/N: URFP-10-5). Generally, gains are adjusted such that the 1 µm beads are tightly clustered near the $10^{3:1}$ decade for FSC and Red channels, the $10^{1:5}$ decade for the Orange channel, and the $10^{1:3}$ decade for the Green channel.

The optical pathway filters used for each channels are:

FSC: 488 Blocking (For cleaning the 488 light)

Red: 560LP > 610LP > 692/40BP

Orange: 560LP > (Rfleect) 610LP > 585/40BP

Green: (Reflect) 560LP > 542/27BP

Step 9.

After final alignment, it is best to let the InFlux run for 45 minutes (while back flushing) to stabilize the stream. Moving forward, minor re-alignments may be necessary.

Sample Preparation

Step 10.

Samples are removed from -80°C storage after the InFlux has been fully aligned. The samples are placed on the bench at room temperature, in the dark to allow them to thaw.

Once thawed, the sample is briey mixed by vortex (less than one second), and then split into two 1 mL aliquots placed in 5 mL polypropylene tubes (P/N: 352063 from Corning, henceforth PP tubes), one for unstained autofluorescence counts, and the other for DNA stained heterotroph counts.

Note: vortex used throughout this analysis is the Vortex Genie 2 from Scientic Industries, and the vortex speed is set to 7.

Once aliquoted, unstained samples are placed in a 4°C refrigerator in the dark.

Unstained Samples

Step 11.

2 µL of a URFP-10-5 (1 µm beads) dilution is added to every unstained sample, and mixed by vortex for approximately 2 seconds.

The bead dilution is prepared by adding 2 drops of the URFP-10-5 beads to 3 mL of MilliQ water (in one of the PP tubes), and then briefly mixed by vortexing.

After the beads have been added, store the samples in a 4°C refrigerator in the dark.

Stained Samples

Step 12.

Make a 200x working dilution of SYBR Green I

- Remove the 10,000X SYBR Green I stock from the -20C freezer, and place on ice to thaw.
- Briefly (20 seconds) centrifuge to condense the SYBR Green I stock before removing the aliquot.
- Prepare the 200x working dilution by diluting 20 µL of SYBR Green I to a final volume of 1000 µL using MilliQ water filtered through a 0.1 µm Acrodisc syringe illter (VWR: P/N 28143-309). Mix briefly by vortex.
- SYBR Green I dilutions will be stable at room tempterature for one day, in a 4°C refrigerator for up to five days, and in a -20°C freezer for at least a month.

NOTES

SCOPE Communications 23 Nov 2017

Important Points:

- A working stock of SYBR Green I is made by a 50-fold dilution of the original SYBR concentrate (20µL SYBR Green I to 1000µL final volume, or a 20 SYBR : 980 Water ratio). The working stock is added to the samples at a dilution ratio of 200X (i.e. 5 µL/mL sample).
- When preparing the SYBR working stock, always use sterile or DNA-free consumables.
- The DNA stain used in this analysis is Lonza SYBR Green I Nucleic Acid Gel Stain (P/N 50513). The SYBR Green I stock 10,000X concentrate is stored in a -20°C freezer with the included desiccant packet.

Step 13.

5 µL of the SYBR Green I 200x working dilution is added to each sample aliquot to be used for heterotroph counts, and mixed by vortex for approximately 2 seconds.

Step 14.

Once the stain has been added to all aliquots, place them in a 4°C refrigerator for 20 minutes. Samples are stored in the 4°C refrigerator until analyzed.°

Running Samples

Step 15.

Notes:

- Always check the instrument to make sure the data collected has low noise and few false positives in the areas of interest.
- During the course of a normal run, the unstained sample aliquots will be run before the stained counterparts because the SYBR Green tends to leave a residue in the sample line that must be cleaned out.
- Throughout the run, it may be useful to occasionally take blank samples using MilliQ water (or for stained samples, MilliQ water with 5 µL SYBR Green working dilution added). This will check for noise in the instrument, either electronic or due to deposits in the sample line.

Running Unstained Samples

Step 16.

Ensure that the InFlux is triggering on FSC with a trigger level in the 10-20 range. Place the PP tube on the InFlux sampler, press Run, and equilibrate the sample for 30+ seconds, until the event plots on Spigot are consistent.

Once the sample has equilibrated, stop the run. If you wish to cross compare the SLI-0430 Flow Meter with a mass difference method, remove the sample from the InFlux and get the initial mass.

Step 17.

Begin the list file collection in Spigot, reset the Totalizer on the SLI-0430 software, and press Run on the sample. Continue collecting data until the flow meter reads 100 µL of sample have been run. Stop the run, remove the sample and weigh if desired.

Step 18.

Begin back flushing the InFlux sample line. This should be done for 30+ seconds between each sample.

Running Stained Samples

Step 19.

Change the trigger channel to Green, with a trigger level in the 40-50 range. Prepare the stained samples for the run as described in Step 12-14, ensuring that the samples sit in the refrigerator for a minimum of 20 minutes before beginning the analysis.

Step 20.

Add 5 µL of the SYBR Green I dilution to the remainder of an unstained sample, and run the sample to prepare the InFlux sample line for running stained samples.

Step 21.

Repeat the procedure for running unstained samples (Step 16-17), changing only the analysis volume to 50 µL instead of 100 µL.

Machine Shutdown

Step 22.

During any run where SYBR Green I is used, the InFlux should first be cleaned by running 5% CONTRAD 70 detergent through the sample line, followed by 70% ethanol, followed by MilliQ water until the event rate for FSC detection is less than 20.

Data Analysis

Step 23.

The .fcs files are analyzed using FlowJo (ver. 10.2)

Gates are drawn for the following populations, with sequential gating indicated by the >:

- *Prochlorococcus*: Red vs FSC > Orange vs FSC
- *Synechococcus*: Green vs Orange > Orange vs FSC
- Pico-Eukaryotes: Red vs FSC > Orange vs FSC
- Heterotrophs* (SYBR, stained samples): Green vs FSC (*see below)

The final concentration for heterotroph counts must have the *Prochlorococcus* count subtracted from the SYBR count that was gated in the Green vs FSC plot because both populations take up the DNA stain, and have similar FSC and SSC signal.

Step 24.

Once the raw counts have been extracted from FlowJo, the concentrations are determined by dividing the count by the corrected sample volume and converted to cells/mL. The measured volume (from the Flow Meter) must be corrected to account for dilution by PFA and beads or stain.

- Unstained volumes are divided by 1.017 (15 µL PFA + 2 µL beads) and stained volumes are divided by 1.020 (15 µL PFA + 5 µL stain).
- Conversion to seawater volume using the mass difference method is done using a static coefficient of 0.0009718, attained using the density of 1.026 µg/µL for seawater.