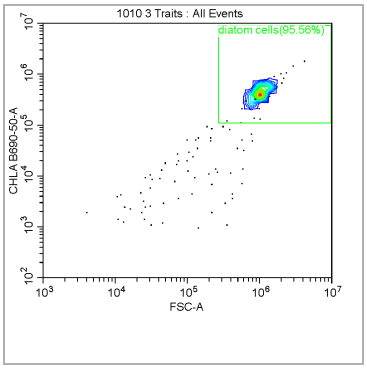

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Flow cytometry trait measurements (size, granularity and chlorophyll-a) of diatoms

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Phoebe Argyle

ABSTRACT

A method and guide for measuring size, relative granularity, and chlorophyll-a content in centric diatom cells. This method was developed on a CytoFlex LX instrument with *Thalassiosira* spp. as the model organism.

This protocol was used in the following publications:

Argyle, P. A., Walworth, N. G., Hinners, J., Collins, S., Levine, N. M., & Doblin, M. A. (2021). Multivariate trait analysis reveals diatom plasticity constrained to a reduced set of biological axes. *ISME Communications*, *1*(1), 59.

Argyle, P. A., Hinners, J., Walworth, N. G., Collins, S., Levine, N. M., & Doblin, M. A. (2021). A high-throughput assay for quantifying phenotypic traits of microalgae. *Frontiers in microbiology*, *12*, 706235.

GUIDELINES

Flow cytometry analyses are most efficient if run in the afternoon of the day of sampling, however these samples can also be snap frozen in liquid nitrogen, stored at −80°C and analysed in delayed mode.

Fluorescence of chlorophyll-a diminishes over time so ensure the time between sampling and measurement is similar for all samples being compared.

Protocol status: Working
We use this protocol and it's working

Created: Apr 22, 2020

Last Modified: Feb 25, 2023

PROTOCOL integer ID: 36013

Keywords: diatom, flow cytometry, trait-based methods, Thalassiosira, high-throughput, microalgae, chlorophyll-a

MATERIALS

Equipment

CytoFLEX LX	NAME
Flow cytometer	TYPE
Beckman Coulter	BRAND
C40312	SKU
https://www.beckman.com	LINK
CytoFLEX LX N3-V5-B3-Y5-R3-I2 Flow Cytometer (21 Detectors, 6 Lasers)	SPECIFICATIONS

Equipment

96 Well TC-Treated Microplates	NAME
Microplate	TYPE
Corning®	BRAND
CLS3799-1EA	SKU
https://www.sigmaaldrich.com	LINK

Equipment	
Parafilm	NAME
lab consumable	TYPE
Parafilm M	BRAND
PM996	SKU
https://www.amazon.com/	LINK
Can be purchased from many vendors	SPECIFICATIONS

Paraformaldehyde 8% EM Grade aqueous solutions **Emgrid Catalog #157-8**

CytoFlex Daily QC Beads **Beckman Coulter Catalog #B53230**

Flow Cytometry Size Calibration Kit (nonfluorescent microspheres) **Thermo Fisher Catalog #F13838**

SAFETY WARNINGS

Paraformaldehyde is toxic so ensure this is added under a fume hood or chemical safety cabinet.

Sample preparation

1h

1 Aliquot 200 µL of live, uniform culture and transfer to a round-bottom tissue culture plate. Agitate culture gently to suspend cells to create a uniform culture.

Equipment	
96 Well TC-Treated Microplates	NAME
Microplate	TYPE
Corning®	BRAND
CLS3799-1EA	SKU
https://www.sigmaaldrich.com	LINK

2 Fix the cells with 20 µL ([M] 10 % volume) paraformaldehyde 8% solution, resulting in a final concentration of 0.8% paraformaldehyde. Use a fume hood as this reagent is toxic.

Paraformaldehyde 8% EM Grade aqueous solutions **Emgrid Catalog #157-8**

3 Secure the lid and wrap plate edges with parafilm to prevent evaporation and store at 4 °C prior to analysis (analyse within 48 hours, ideally later the same day).

Equipment	
Parafilm	NAME
lab consumable	TYPE
Parafilm M	BRAND
PM996	SKU
https://www.amazon.com/	LINK
Can be purchased from many vendors	SPECIFICATIONS

Flow cytometry setup

4 Load plate into the flow cytometer, this protocol was developed using a CytoFLEX LX instrument.

Equipment	
CytoFLEX LX	NAME
Flow cytometer	TYPE
Beckman Coulter	BRAND
C40312	SKU
https://www.beckman.com	LINK
CytoFLEX LX N3-V5-B3-Y5-R3-I2 Flow Cytometer (21 Detectors, 6 Lasers)	SPECIFICATIONS

5 Set up the flow cytometer and run the daily Quality control (QC) protocol as per the instrument instructions.
In this case for the Cytoflex LX, multi-spectra beads
CytoFlex Daily QC Beads **Beckman Coulter Catalog #B53230** are used to ensure the correct functioning of the instrument.
These beads may also be used to ensure consistency across experimental runs, as discussed in step 8.

6 Optimise gain and threshold settings for the detectors of interest in the flow cytometer. A spare aliquot of diatom culture can be used to optimise gain/thresold settings. Culture media obtained from filtering diatom culture (through a 0.22 µm filter) can also be used to identify background scatter and set thresholds.

The lazer and detectors are as follows, however this will depend on the particular instrument so exact numbers may not match if a different instrument is used.

Forward scatter area: Si-photodiode with built-in 488/8 band-pass filter (CytoFLEX Channel name: FSC-A)

Side scatter area: 488nm (50mw), Optical Filter: 488/8 nm, (CytoFLEX Channel name: SSC-A)

Chlorophyll a: Laser: 488nm (50mw), Optical Filter: 690/50 nm, (CytoFLEX Channel name: B690-50)

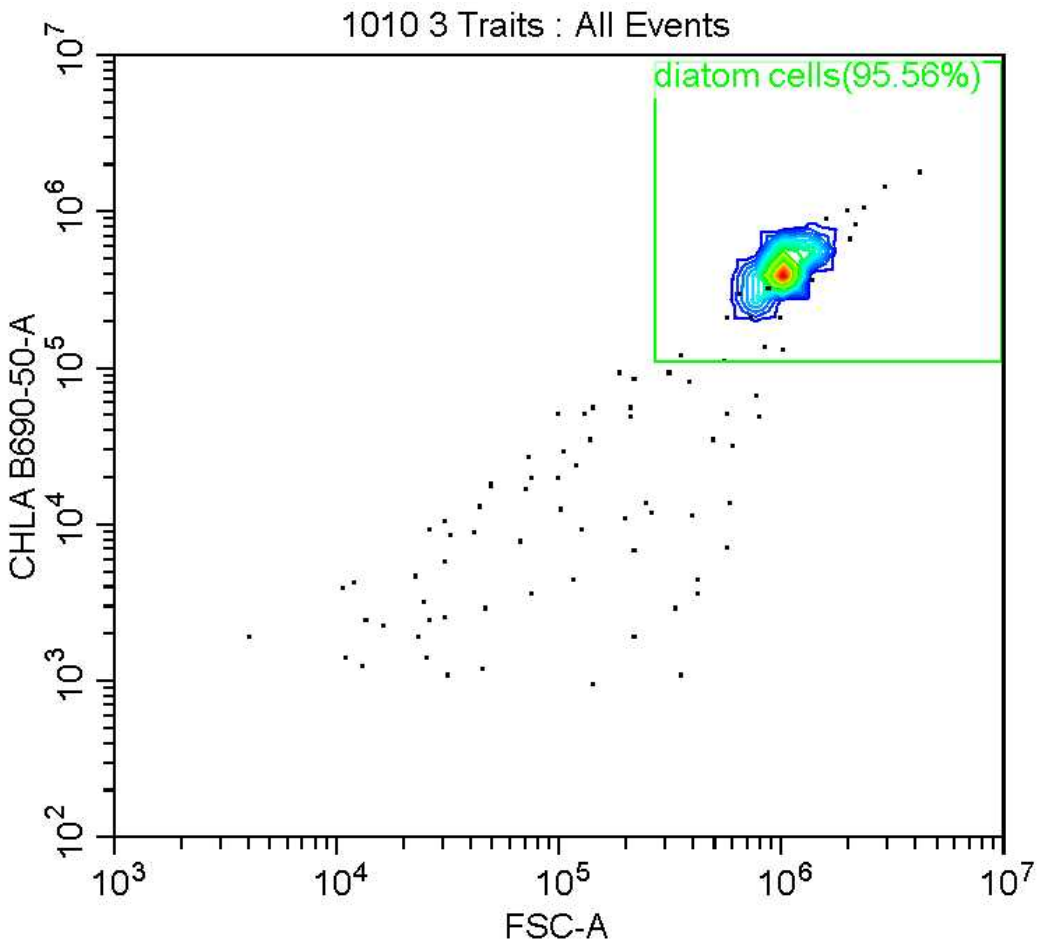
Thresholds were set at 4000 for FSC-A (forward scatter area) and 2000 for B690-50 (chlorophyll-a) to distinguish cells from background debris and particulates.

Flow cytometry analysis

7 Samples are mixed for 6 seconds by the CytoFlex instrument before analysis. 1m

During development samples were generally run at medium speed/flow rate of 30 µL per minute for 00:01:00 . Analysis is done on at least 200 cells, however in most cases it would be at least 2000 cells. The abort rate (%) was <1%. In the case of a highly dense culture, a slower speed maybe prudent to ensure a low abort rate and ensure accuracy of analysis. In the case of a low density culture, a higher flow rate or longer duration of analysis may be used to ensure an adequate number of cells are analyzed.

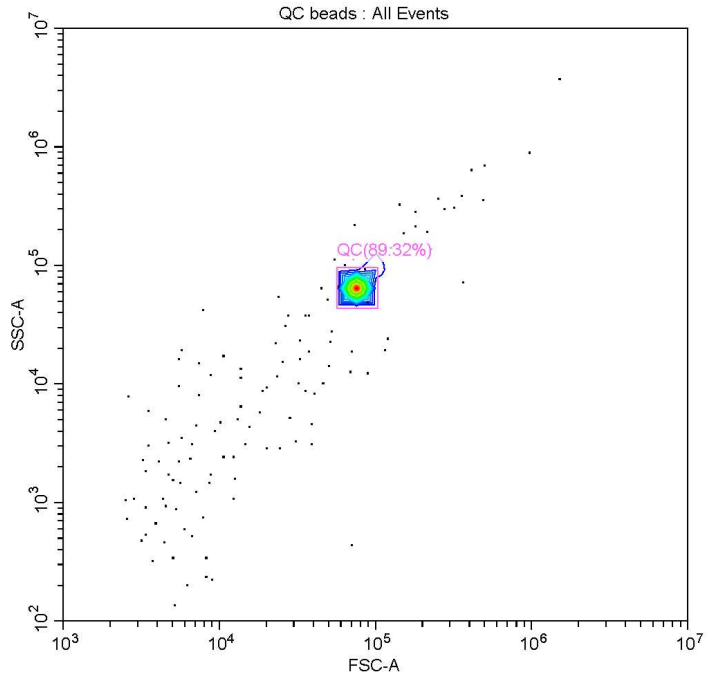
Diatoms were gated visuallt using event density plots of chlorophyll fluorescence and forward scatter (see below).



Calibration using beads

8 The multi-spectra beads CytoFlex Daily QC Beads **Beckman Coulter Catalog #B53230** that are used to calibrate the instrument on startup may also be used to ensure comparability between experimental runs.
It is recommended to analyse samples from one experiment on the same day, however if this is not possible it is necessary to ensure that the instrument is performing similarly on the different days of analysis.

Using the parameters and thresholds established for diatoms, the QC beads may be run as if they were a sample and gated according to their chlorophyll fluorescence, forward scatter and side scatter (see image below). This way, if the beads fall within the same gates on each day of analysis, it may be assumed that samples run on different days may be fairly compared.

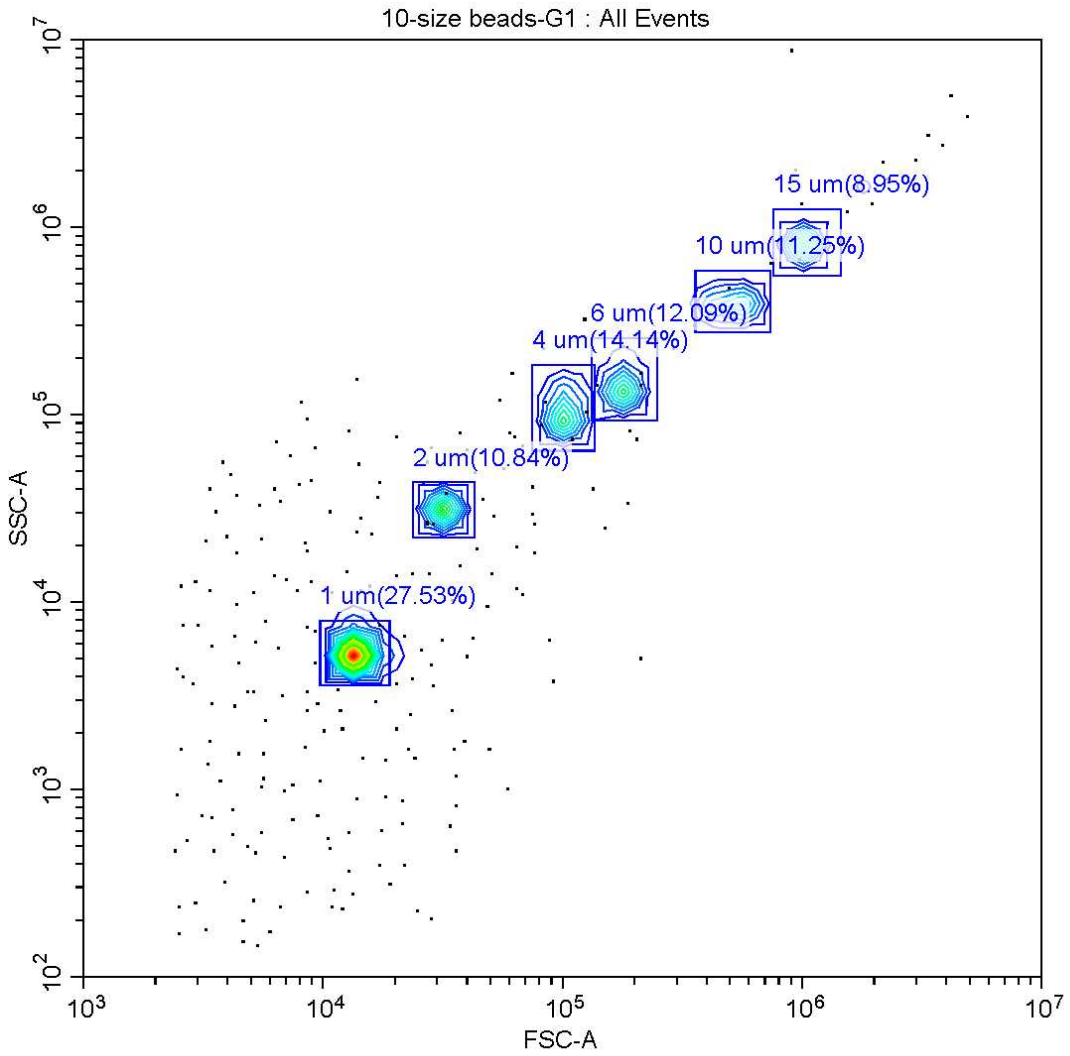


Daily QC beads gated according to forward scatter (FSC-A) and side scatter (SSC-A)

In addition to the QC beads, beads of known diameter were used to create a linear calibration of forward scatter vs. spherical size. For this protocol, beads of 2,4,6,10 and 15µm

Flow Cytometry Size Calibration Kit (nonfluorescent microspheres) **Thermo Fisher Catalog #F13838**

were used. These beads can also be used to ensure the performance of the instrument is consistent across experimental runs for FSC and SSC measures (see image).



Calculating cell size and metrics

9 Using the FSC measure of the size beads and the known diameters, create a linear equation to approximate spherical size of the diatom cells. Use the median FSC value based on a minimum of 200 cells but ideally 2000 or more.
e.g. Spherical size = (FSC +275,549)/83,539

Spherical size may also be considered as "equivalent spherical diameter" or ESD, as the diatom cells are not actually spherical in shape.

10 For other metrics (granularity and chlorophyll-a), units are given as relative fluoresence units (RFU) from the flow cytometer, which can be used for comparative purposes between samples. Again, the median value is used from a minimum of 200 cells.

It is recommended that these values be corrected against cell size (ESD), as these traits are directly correlated with cell size, so it may be useful to assess these traits independent of size.