A microflow cytometer for optical analysis of phytoplankton

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ABSTRACT

Analysis of the intrinsic scatter and fluorescence profiles of marine algae can be used for general classification of organisms based on cell size and fluorescence properties. We describe the design and fabrication of a Microflow Cytometer on a chip for characterization of phytoplankton. The Microflow Cytometer measured distinct side-scatter and fluorescence properties of *Synechococcus sp*., *Nitzschia d*., and *Thalassiosira p*. Measurements were confirmed using the benchtop Accuri C6 flow cytometer. The Microflow Cytometer proved sensitive enough to detect and characterize picoplankton with diameter approximately 1 mm and larger phytoplankton of up to 80 mm in length. The wide range in size discrimination coupled with detection of intrinsic fluorescent pigments suggests that this Microflow Cytometer will be able to distinguish different populations of phytoplankton on unmanned underwater vehicles. Reversing the orientation of the grooves in the channel walls returns the sample stream to its original unsheathed position allowing separation of the sample stream from the sheath streams and the recycling of the sheath fluid.

Keywords: flow cytometry, microfluidics, multi-analyte assay, phytoplankton, algae, microflow cytometer

1. INTRODUCTION

Phytoplankton are marine microorganisms that respond very rapidly to changes in their environment, and alterations in phytoplankton assemblies may reflect environmental changes within a few hours or over much longer periods. Changes in phytoplankton populations can be measured in response to introduction of environmental pollutants, alterations in ocean currents, and global climate change [2, 3] and blooms of some species of phytoplankton, can cause illness to mammals, fish, corals, and other marine organisms.[4-6]

A variety of analytical methods have been used to identify changes in populations of marine algae: remote spectrometry from satellites or from airplanes,[7-10] in situ spectrometry, laser-induced fluorescence, and flow cytometry. Grab samples brought to the surface are analyzed using laboratory flow cytometers.[11, 12] Clearly, evaluating algal populations in situ is very appealing, and the size, shape, and intrinsic fluorescence can be measured using flow cytometry without staining or other preprocessing steps.

NRL is developing a Microflow Cytometer for monitoring algal population changes using unmanned underwater vehicles (UUVs). This prototype uses a design similar to that published previously for discriminating fluorescently coded beads in multiplexed immunoassays.[13, 14] Grooves in the top and bottom of the flow channel direct the sheath fluid completely around the sample stream. This design for sheathing the core using passive structures has the advantages of: less chance of clogging, ability to interrogate both large and small objects, and minimal shear stress due to the distance of the core from the wall. The core sample stream is focused in front of the excitation beams for measurement of right angle light scatter and fluorescence. We have also demonstrated the potential for recycling the sheath fluid.[15]

To discriminate populations of marine algae, we set up the system to detect light scatter, as a rough measure of size, and fluorescence at wavelengths appropriate for the intrinsic pigments. We also were interested in comparing data from the Microflow Cytometer to that obtained using the commercial Accuri C6 system, which is being marketed for shipboard

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applications. The Microflow Cytometer prototype was able to distinguish the same algal populations with similar resolution as that of the commercial system.

2. METHODS

2.1 Design and fabrication of Microflow Cytometer

The Microflow Cytometer used sets of chevron-shaped grooves in the top and bottom of a flow channel to create sheath flow, as previously demonstrated.[13, 15, 16] The channels were fabricated in polydimethylsiloxane (PDMS) using standard microfabrication procedures.[15, 16] Channels were 130 μm high throughout and 390 μm wide, and unlike our previous designs, the sample inlet width was chosen to be 390 μm to prevent destruction of the phytoplankton. Optical fibers were cleaved and inserted into their guide channels and carefully aligned with the main channel walls. A schematic of the optical setup is shown in Fig. 1.

Optical excitation at 488 nm, provided by an argon-ion laser (Ion Laser Technologies), was launched into a multimode optical fiber, which delivered excitation light to the interrogation region of the cytometer. A second multimode fiber placed on the far side of the channel served as a beam dump to prevent excess scattered light in the channel. Fluorescence and light scatter were collected using two multimode optical fibers placed at 90 degree angles to the excitation beam. Light emerging from each collection fiber was directed through a fiber splitter, a bandpass filter (Omega Optical Inc., Brattleboro, VT), and then onto a photomultiplier tube (PMT, H9307-02, Hamamatsu, Bridgewater, NJ). The voltage signal from the PMTs was digitized with a 16-bit A/D board (Data Translation Inc.), and sent through a USB cable to a personal computer. The software interface was written using LabWindows (National Instruments). Data was acquired at 200 kilo-samples per second (kSPS). Sample was injected into the channel using a precision syringe pump (CAVRO XE 1000, Tecan Systems Inc., San Jose, CA) at a flow rate of 200 uL/min. A bidirectional peristaltic pump (P625/66.143, Instech Laboratories Inc., Plymouth, PA) was used to introduce the sheath flow into the channel at 800 μL/min. Data from the three PMTs were collected at 488 +/- 5 nm (light scatter), 660 +/- 30 nm (chlorophyll), and 575 +/- 40 nm (phycoerythrin).

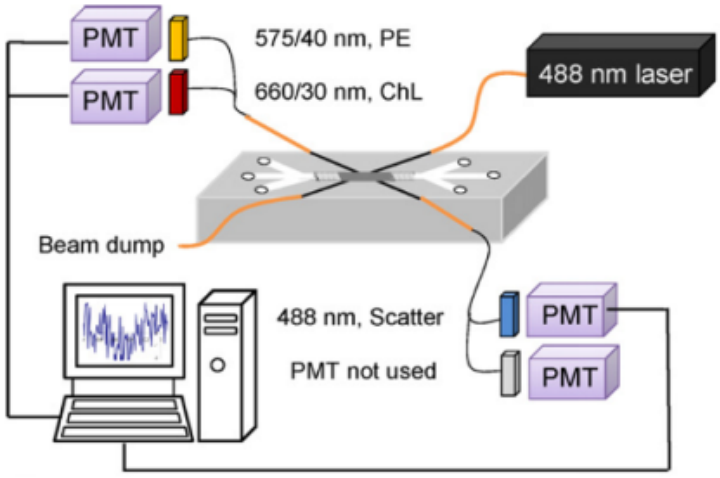

Figure 1. Diagram of optical setup and fluidics for the Microflow cytometer (from Hashemi et. al, [1]).

2.3. Phytoplankton

Cultures of *Synechococcus sp*., *Nitzschia d.*, and *Thalassiosira p.* were purchased from the Culture Collection of Algae (UTEX, Austin, TX),. The three main pigments in these algae are: chlorophylls, phycobilins, and carotenoids. Since chlorophyll is present in all phytoplankton, plants and cyanobacteria, it can be used to discriminate phytoplankton from

most other marine particles. Chlorophyll a absorbs around 675 nm, but also absorbs well at 450 nm. Phycoerythrin absorbs at 495 and between 545–565 nm, and fluoresces at 575 nm. Carotenoids are lipid-soluble pigments that absorb light at approximately 450–490 nm.

3. RESULTS AND DISCUSSION

The cell dimensions measured and found to be 0.5–1.0 mm diameter for *Synechococcus sp*., 16–80 mm in length and 2–5 mm width for *Nitzschia d.*, and 8–38 mm length and 4–29 mm width for *Thalassiosira p.* Each phytoplankton species also produced distinct fluorescent emission spectra as a result of different pigment concentrations. The emission spectra of each of the phytoplankton species was collected using a TECAN Spectrophotometer (Tecan Trading AG, Switzerland) using 488 nm excitation (Fig. 2). When excited with a 488 nm laser, the fluorescence emission spectrum showed that *Synechococcus sp*. emitted strongly in the red region (640–710 nm), and *Nitzschia d.* and *Thalassiosira p.* both had strong peaks at 540–545 nm (green region).

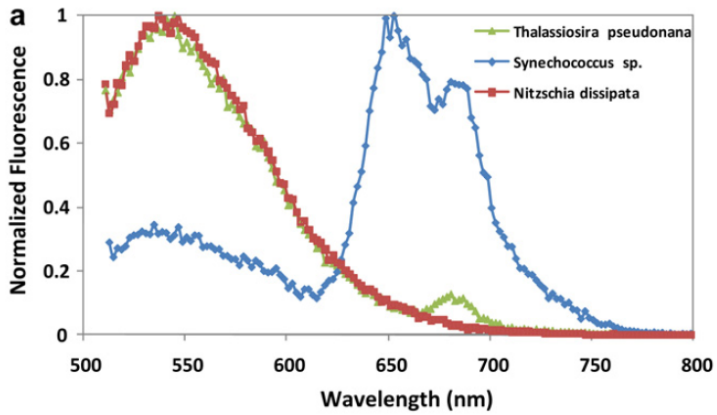

Fig. 2. Fluorescence emission spectra of the three algal species excited with 488 nm light. (from Hashemi et. al, [1]).

3.1 Flow cytometry of phytoplankton

The phytoplankton were analyzed using both the Microflow Cytometer and the Accuri C6 flow cytometer (Ann Arbor, MI). The phytoplankton was flowed through both Microflow Cytometers at concentrations of ~2100 cells/mL for the *Synechococcus sp*., ~50 cells/mL for the *Nitzschia d*., and ~150 cells/mL for the *Thalassiosira p*. The phycoerythrin was excited by the 488 nm laser and detected at ~585 nm. Although the 488 nm excitation was not optimal for exciting the pigments, this wavelength excited both chlorophyll and phycoerythrin, providing a comparison of characterization data from the two instruments. Fig. 3a and b show scattergrams of the phycoerythrin fluorescence (575–585 nm) plotted vs. chlorophyll fluorescence (650–680 nm), where Fig. 3a shows the fluorescence data obtained using the Microflow Cytometer (left column) and Fig. 3b depicts the fluorescence data obtained by the Accuri flow cytometer (right column).

Three different populations can be discriminated based on their fluorescence emission spectrum in both systems. The correlation between the two data sets was found to be very similar statistically. *Nitzschia d.* emits strongly in the orange region (phycoerythrin), *Thalassiosira p.* emits strongly in the red region, and *Synechococcus sp.* emits both in the red and orange spectrum. Scattergrams of chlorophyll fluorescence vs. side scatter (488 nm) and phycoerythrin fluorescence vs. side scatter are shown in Fig. 4c–f.

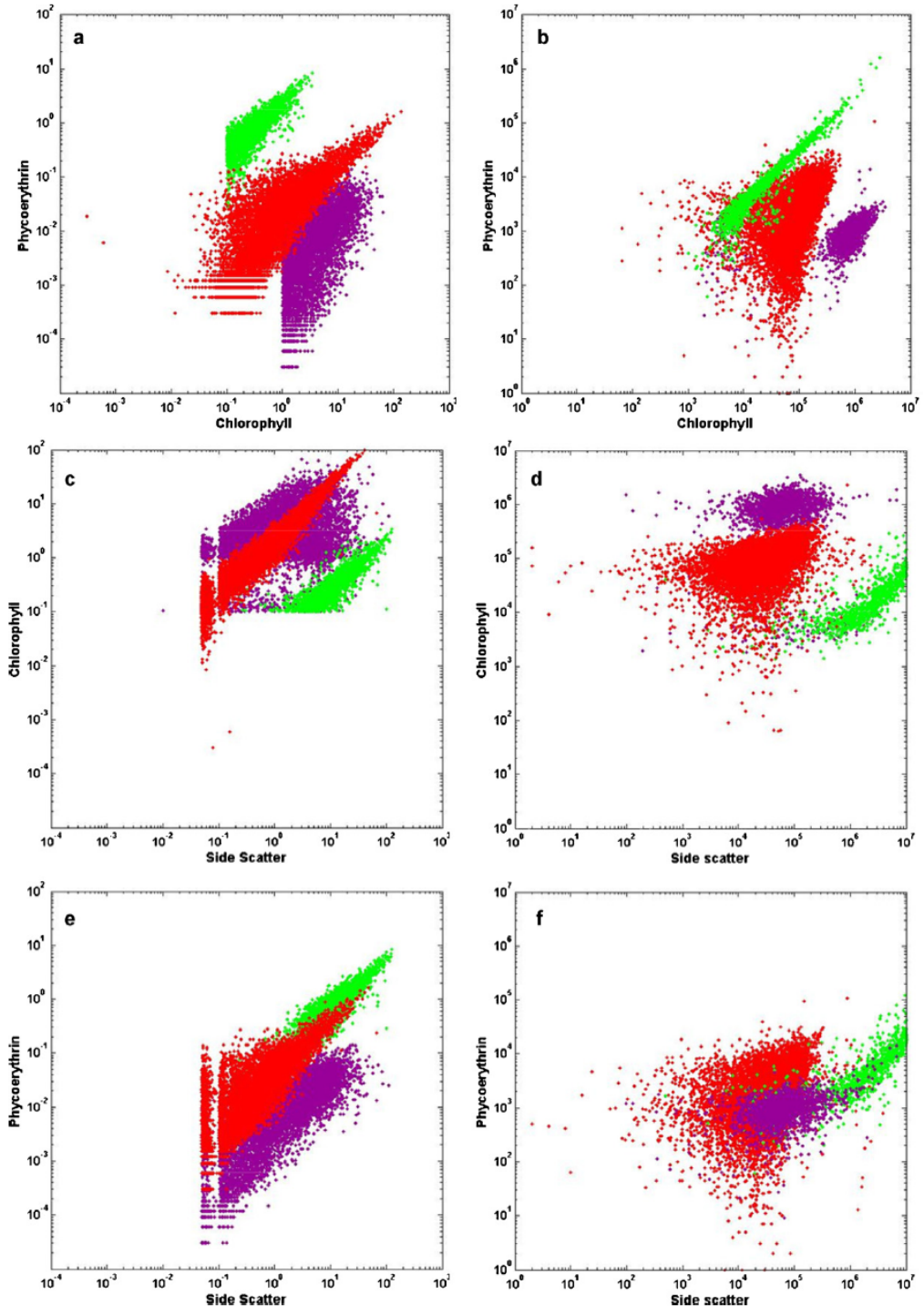

Fig. 3. Scatter plots of the phycoerythrin (y-axis) plotted vs. chlorophyll fluorescence (x-axis) for each individual cell are shown in (a) and (b). Scatter plots of chlorophyll fluorescence vs. side scatter are in (c) and (d), and (e) and (f) represent scatter plots of phycoerythrin fluorescence vs. side scatter. (a, c, and e) are the data obtained using the Microflow Cytometer and (b, d, and f) show the Accuri flow cytometer results. *Synechococcus sp.* is represented by red dots, *Nitzschia d.* by green dots, and *Thalassiosira p.* by purple dots. (from Hashemi et. al, [1]).

4. CONCLUSIONS

We have demonstrated a Microflow Cytometer that measures light scatter and fluorescence properties of microorganisms, suitable for characterizing and discriminating populations of phytoplankton. The phytoplankton were discriminated on the basis of side scatter and multi-wavelength fluorescence. The Microflow Cytometer characterized and detected picoplankton with diameter less than 1 μm (*Synechococcus sp.*) and also measured phytoplankton as long as 80 μm (*Nitzschia d.)*, with results comparable to those of the Accuri C6 flow cytometer. This system has been miniaturized and adapted for deployment on an unmanned underwater vehicle by replacing the large 488 nm argon laser with solid-state lasers that are both small for in situ use and provide excitation light closer to maximum absorbance wavelengths for chlorophyll and phycoerythrin.[1]

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