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A fluorescence-activated cell sorting subsystem for the Imaging FlowCytobot

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Abstract

Recent advances in plankton ecology have brought to light the importance of variability within populations and have suggested that cell-to-cell differences may influence ecosystem-level processes such as species succession and bloom dynamics. Flow cytometric cell sorting has been used to capture individual plankton cells from natural water samples to investigate variability at the single cell level, but the crude taxonomic resolution afforded by the fluorescence and light scattering measurements of conventional flow cytometers necessitates sorting and analyzing many cells that may not be of interest. Addition of imaging to flow cytometry improves classification capability considerably: Imaging FlowCytobot, which has been deployed at the Martha's Vineyard Coastal Observatory since 2006, allows classification of many kinds of nano- and microplankton to the genus or even species level. We present in this paper a modified bench-top Imaging FlowCytobot (IFCB-Sorter) with the capability to sort both single cells and colonies of phytoplankton and microzooplankton from seawater samples. The cells (or subsets selected based on their images) can then be cultured for further manipulation or processed for analyses such as nucleic acid sequencing. The sorting is carried out in two steps: a fluorescence signal triggers imaging and diversion of the sample flow into a commercially available "catcher tube," and then a solenoid-based flow control system isolates each sorted cell along with 20 μL of fluid.

Flow cytometry has contributed to our understanding of the ecology and biogeography of the world's oceans (see reviews by Olson et al. 1993; Legendre et al. 2001; Sosik et al. 2010 and references within). Perhaps the most striking discovery that can be largely attributed to the application of flow cytometry is the existence of *Prochlorococcus* (Chisholm et al. 1988b). Additionally, important contributions to our understanding of global distributions of marine phytoplankton have come from surveys carried out with flow cytometry as the main measurement technology (e.g., Olson et al. 1985, 1988, 1990*a*,*b*; Veldhuis and Kraay 1990; Johnson et al. 2006). Flow cytometers continue to be essential in advancing understanding of spatio-temporal variability and diversity in the plankton (Anglès et al. 2015; Bonato et al. 2015; Mojica et al. 2015). Along with promoting greater understanding of ocean biogeography and biodiversity, flow cytometry is also an important tool for studies concerning the cell cycle and physiology of marine planktonic organisms (e.g., Armbrust et al. 1989, 1990).

The power of flow cytometry was extended dramatically by the introduction of cell sorting technology (for an overview see Chapter 6 of Shapiro 2005). Cell sorters typically divert cells within a defined flow cytometric parameter space (i.e., defined by fluorescence and/or light scattering measurements) into a holding container; the cells within the holding container can then be analyzed further (Chisholm et al. 1988a). Cell sorting has been used in combination with a variety of chemical analyses to better understand biogeochemical cycling (Fawcett et al. 2011; Lomas et al. 2011), to delve into interactions between bacteria and phytoplankton (Thompson et al. 2012; Baker and Kemp 2014), and to investigate intra-species genetic variability (Kashtan et al. 2014). With advances in single-cell genomics, transcriptomics, and cell sorting technologies, it is now possible to conduct analyses of individual sorted cells that have been isolated from cultures or environmental samples (Baker and Kemp 2014; Kashtan et al. 2014; Luo et al. 2014; Thrash et al. 2014). These studies have revealed dramatic heterogeneity at the single-cell level. With single-cell analytic techniques

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advancing at an impressive rate, cell-sorting flow cytometers will continue to be very valuable for isolating cells from the environment.

The introduction of imaging-in-flow represents another landmark addition to the field of flow cytometry (Sieracki et al. 1998; Kachel and Wietzorrek 2000; Olson and Sosik 2003). Imaging-in-flow cytometry integrated into an automated submersible instrument system enables studies of individual taxa at a greater temporal resolution than previously possible (Sosik and Olson 2007; Sosik et al. 2010). These technologies allow the investigation of numerous processes in situ including cell-cycle progression, prey ingestion, and parasite infection (Campbell et al. 2010; Brosnahan et al. 2014; Peacock et al. 2014). Until now, the power of combining imaging-in-flow cytometry and fluorescence-activated cell sorting has remained untapped.

Here, we bridge this gap with an Imaging FlowCytobot (IFCB; Olson and Sosik 2003) that has been modified to operate as a bench-top instrument capable of sorting individual cells. IFCB is a submersible imaging-in-flow cytometer, which can be used to characterize planktonic cells in the size range of $\sim$ 5–150 μm; it stores images of individual cells, chains, or colonies and records the chlorophyll fluorescence and side angle light scattering associated with each imaged target. The instrument described here is in essence a fluorescence-activated cell sorting (FACS) system, but with several notable differences. Currently, FACS systems utilize fluorescence of various wavelengths and laser light scattering as selection criteria. These criteria cannot be used reliably to distinguish among different species in environmental samples. As a result, detailed investigations of community composition rely on sorting many individuals and then determining the taxonomic identity of each cell by sequencing. The IFCB-Sorter, in contrast, captures an image of a nano- or microplankton cell or colony and then sorts it into a well plate. Since the images can in many cases be used to automatically (or manually) classify the cell to genus or even species (Sosik and Olson 2007), only the sorted cells of interest need be further analyzed. Additionally, the image associated with the sorted cell can contain valuable information about the cell's condition at the time of capture. Images also make it possible to explore questions about relationships between morphology and genotype.

Materials and procedures

IFCB-sorter

The IFCB-Sorter is a bench-top instrument that works in a manner similar to both FlowCytobot (Olson et al. 2003) and Imaging FlowCytobot (Olson and Sosik 2003) in that a seawater sample is drawn into a syringe and subsequently injected into a particle-free sheath stream directed into a quartz flow cell. The main fluidics system of the IFCB-Sorter (Fig. 1) differs from that of the standard IFCB in that it utilizes a

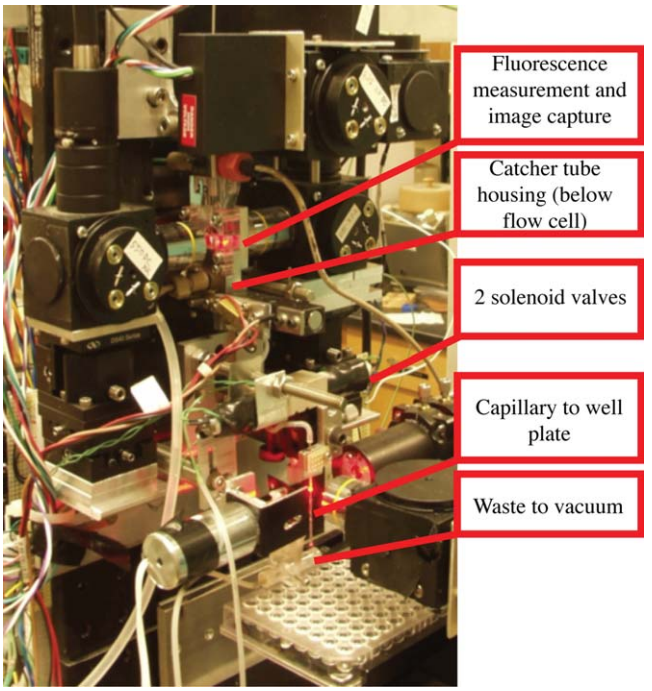

Fig. 1. IFCB-Sorter. Fluorescence measurement and image capture takes place in a BD Biosciences FACSCalibur sorting flow cell. The catcher tube is controlled by FACSCalibur circuitry (not shown), and the solenoids, capillary fluorescence detector, waste catcher (for removing the sheath flow in between sorted cells), and well plate X-Y translator are controlled by a PIC microprocessor (not shown).

FACSCalibur sorting flow cell (BD Biosciences). The programmable syringe pump (VersaPump 6 with 48,000 step resolution) used for sample injection is configured with a 1-mL syringe (Kloehn) (rather than the 5-mL syringe typically used in IFCB, to generate a smaller core stream). In the flow cell, the sample stream is hydrodynamically focused such that particles pass single file through a focused 635 nm laser beam, which excites chlorophyll fluorescence in organisms that contain photosynthetic pigments. If chlorophyll fluorescence exceeds a pre-set threshold, an image is captured by concurrently triggering a 1-μs flash from a xenon lamp and frame capture from a digital camera, which is focused an appropriate distance downstream of the laser beam. Following image acquisition, the sorting flow cell module deploys a catcher tube, which momentarily diverts the sample stream containing the imaged particle into the sorting subsystem (Fig. 2).

The sorting subsystem includes two solenoid valves and a solenoid-operated microinjector (Bio-chem Fluidics) for flow control, a fluorescence-based particle detector, a vacuum-operated waste trap, and a programmable well plate positioner. All components are mounted on an optical breadboard placed underneath the flow cell of the IFCB. The sorting subsystem is controlled by a PIC 16F887 microcontroller (MicroEngineering Labs), which is programmed in PICBASIC (MicroEngineering Labs). The subsystem software

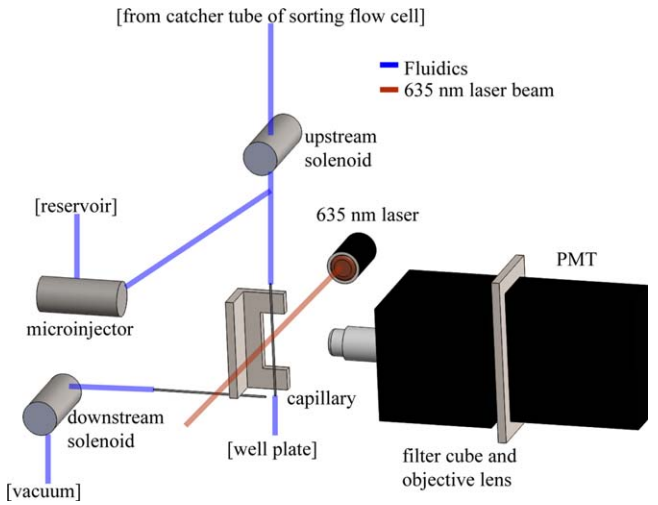

Fig. 2. The sorting subsystem of the IFCB-Sorter. A particle enters the system through the catcher tube located just after the flow cell (top) and passes through the upstream flow control solenoid. The particle then enters a capillary tube where photosynthetic pigments are excited by a 635 nm laser focused on the capillary. Emitted photons are detected by a PMT assembly and when the detected voltage exceeds a comparator threshold the solenoids are activated. The upstream solenoid stops flow in the capillary tube and the downstream solenoid stops vacuum suction. The microinjector then injects 20 μL above the capillary, ejecting the particle into a well plate.

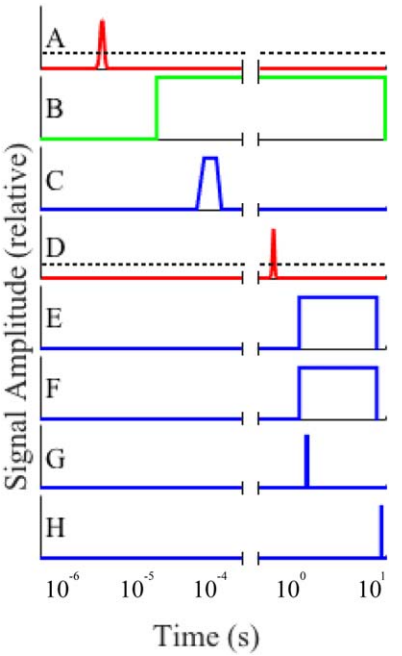

Fig. 3. IFCB-Sorter sorting subsystem signals (red) and controls (inhibition pulses in green, activation pulses in blue). (**A**) Initial signal seen by the IFCB. The comparator threshold is indicated by the dashed line; (**B**) Hold pulse to inhibit triggers until sorting is complete; (**C**) Catcher tube deployment pulse; (**D**) Sorting subsystem PMT signal and comparator threshold (dashed); (**E**) Upstream solenoid closure; (**F**) Downstream solenoid closure; (**G**) Microinjector pulse; (**H**) Pulse to advance well plate.

consists of two main portions: (1) an initialization segment, where subsystem timing parameters are set, microcontroller registers are set to establish pin function, and the comparator parameters are established (channel select, external input, logic not inverted, output is present on the COUT pin, polarity not inverted, comparator is enabled); (2) the main control loop, where the program awaits a trigger pulse from the IFCB. Once the trigger pulse is received, the catcher tube is deployed on the basis of pre-set timing parameters (see below). A sub-loop continuously polls the subsystem for a signal from the comparator, which starts the solenoid flow control sequence. Once the microinjector fires, the system resets and passes flow through the subsystem to flush the contents of the subsystem. The program then awaits the next trigger from the IFCB.

The residence time of particles in the sorting subsystem can vary due to the non-uniform velocity profile of the fluid flow and the absence of hydrodynamic focusing after capture, so a secondary fluorescence system is employed to relocate the sorted particle before deposition (i.e., to ensure that the particle will be in the drop deposited into the well). The fluorescence detection system of the sorting subsystem is similar to that in the upstream IFCB flow cell: a 635 nm diode laser is focused with cylindrical lenses as in IFCB on a glass capillary pipet (OD 1.0 mm, ID 0.5 mm) downstream of the catcher tube, and a 10X microscope objective is placed at a right angle to the incident laser and focused on the capillary. A horizontal bar (3 mm wide) at the entrance to the objective blocks laser light reflected from the capillary. Light collected by the objective is sent to a photomultiplier tube (PMT; HC120-05MOD1, Hamamatsu Photonics, K.K.) through a 680 nm bandpass filter that separates chlorophyll signals from background laser light.

Detection of a suitable particle by the original IFCB activates the sorting subsystem: when the signal detected by the primary PMT exceeds a comparator threshold (Fig. 3A), a deployment pulse is sent to the FACSCalibur catcher tube control board (BD Biosciences), and triggering is disabled (Fig. 3B) so that other cells passing through the IFCB observation window will be ignored while the sorted cell is being processed. The catcher tube pulse ramps from 0 V to 100 V in 140 μs, plateaus, and then returns to 0 V again in 140 μs (Fig. 3C), which causes a piezo element to push the entrance of the catcher tube briefly into the sample core stream. As the captured cell traverses the glass capillary after the catcher tube, it will pass through the subsystem laser beam and emit fluorescence, which is sensed by the subsystem PMT (Fig. 3D). The signal from this PMT is fed to a comparator in the PIC, which generates a pulse if the PMT signal is larger than a preset threshold (Fig. 3D). This pulse initiates another chain of events. First, an upstream solenoid valve (normally open) closes (Fig. 3E) to stop the flow with the sorted cell in the capillary tube. Second, a solenoid valve (normally open)

that controls removal of waste by a vacuum closes (Fig. 3F); this stops suction at the end of the capillary tube. Third, the microinjector is triggered (Fig. 3G) to inject 20 μL of fluid into the capillary upstream of the cell, sending a droplet containing the cell into the well plate. Fourth, a pulse is sent to the Autoclone well plate system (Coulter) (Fig. 3H) shifting the plate to a new well. The system then returns to its base state and awaits another trigger event from the IFCB.

Optimization of timing parameters

To maximize the likelihood of successful sorts and to minimize the potential for coincidental capture of unwanted cells, it is necessary to optimize the timing of catcher tube deployment and duration. We determined capture efficiency (defined as the proportion of initial sorts subsequently sensed by the subsystem PMT) with different timing parameter values. First, we sorted phytoplankton cultures (the diatom *Ditylum brightwellii* and the dinoflagellate *Alexandrium fundyense*) and 9 μm red fluorescent beads (Thermo Fisher Scientific) with no delay between IFCB trigger and catcher tube deployment; by altering the duration of catcher tube deployment we were able to bound the time frame in which capture occurs. In a second experiment, we optimized the delay by varying it while holding constant the sum of delay and catcher tube deployment duration.

The phytoplankton cultures used for optimization of timing parameters were grown in f/2 media (Guillard 1975) at 18°C with a 14 : 10 h light : dark cycle. Cells were transferred regularly to ensure they remained in the exponential growth phase. The sort efficiency was measured indirectly as the proportion of initial sorts that were sensed by the subsystem PMT.

Quantification of capture efficiency for chain-forming cells

Capture of chain-forming cells represents a significant challenge to cell sorters because their large size and complex morphology can cause unpredictable flow behavior. To quantify the capture efficiency of the IFCB-Sorter under these challenges, a culture of the chain-forming diatom *Guinardia delicatula* was used as sample. Prior to sorting, the culture was kept in f/2 media at 11°C with a 14 : 10 h light : dark cycle.

Isolation and culture of *Alexandrium*

Cells can experience high levels of mechanical stress during the process of cell sorting, resulting in a decrease in cell viability (Rivkin et al. 1986). The ability to sort cells that remain viable expands the range of analyses that can be performed once cells are isolated. To assess the viability of sorted cells, *Alexandrium* were cultured as described above and then sorted into a 96-well plate containing f/2 media. The well plate was then returned to the original incubator. Fluorescence readings were taken on a SpectraMax (Molecular Devices, LLC) plate reader to obtain data necessary to construct well-specific growth curves.

Isolation of cells from natural communities

Cells were sorted from seawater collected from Woods Hole Harbor. The cells were sorted onto a microscope slide and then imaged at 40X magnification. Manually identifying cells by microscopy after the final sort event allowed us to verify correspondence with the original target detected and imaged in the upstream flow cell of the main IFCB system.

Assessment

Impact of catcher tube deployment timing on capture efficiency

As described above, a pulse from the PIC microcontroller, generated when a fluorescence signal is detected, deploys the catcher tube. We determined empirically the optimal timing of pulse initiation and duration. Varying the catcher tube deployment duration with no delay showed that most captures occurred between 800 μs and 900 μs regardless of particle type being sorted (Fig. 4A). A second experiment, in which the delay was varied while keeping the sum of delay plus duration equal to 900 μs, showed that high capture efficiency could be maintained with a short-duration deployment pulse that included the period between 800 μs and 900 μs (Fig. 4B). Taken together, these results led us to choose a delay time of 700 μs and deployment pulse of 200 μs as normal operating parameters. Under these conditions, for a range of particle types from beads to cells of different size and shape, capture efficiencies are indistinguishable from the maxima achieved and the pulse duration is no longer than necessary.

Re-isolation of *A. fundyense*

To demonstrate that the IFCB-Sorter can be used to isolate cells for culture and physiological experiments, individual *A. fundyense* cells were sorted into wells, which were then incubated and tracked with a plate reader. In these experiments one row of each well plate was left empty as a control to ensure that no cells were deposited through other means, such as aerosolization. No cell growth was detected in these control wells. Of 168 sorts, 23 were viable and showed consistent growth over a 3-week post-sort period (Fig. 5). The low proportion of viable cells could be due to the health of the culture used to conduct the experiment, abrupt changes in temperature experienced by the cells during the experimental procedure, or mechanical stresses experienced during the sorting procedure. During sorting, the cells encounter several regions of high shear, including capture by the catcher tube and injection into the well-plate. These mechanical stresses are likely much lower in severity than the shear a cell experiences as it enters the flow cell of the IFCB. Notably, the rate of successful isolation in this experiment (12.3%) is considerably higher than values previously reported for isolation of dinoflagellates by manual picking or sorting with a commercial FACS system ($\sim$ 1–2%) (Sinigalliano et al. 2009). Our isolation rate also compares favorably

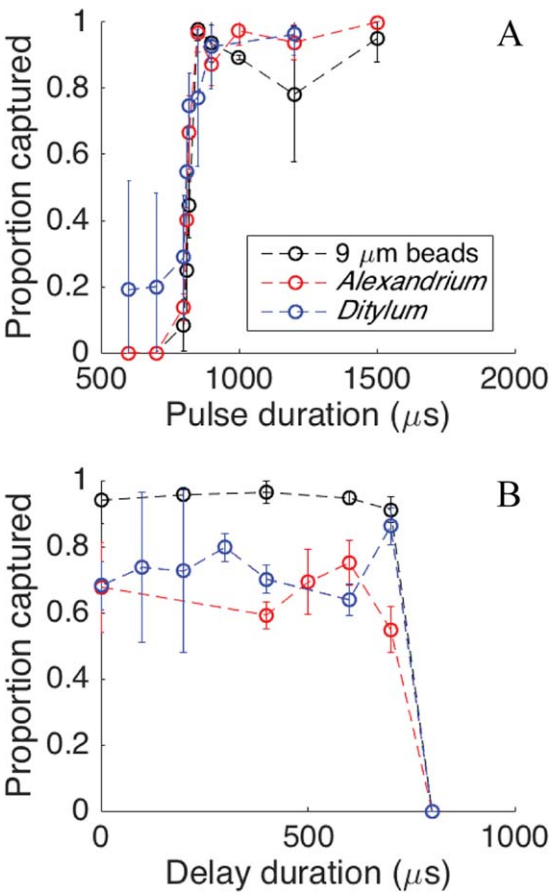

Fig. 4. (**A**) Capture efficiency with varying catcher tube deployment pulses. (**B**) Capture efficiency with differing deployment delays and total time held at 900 μs.

with rates observed for a number of microalgae (Sieracki et al. 2005). This comparison suggests the IFCB-Sorter provides a relatively gentle means of cell isolation.

Capture of cells from the environment

To evaluate the effectiveness of the IFCB-Sorter in isolating cells from natural samples, we used seawater collected from Woods Hole Harbor, Woods Hole, Massachusetts. During the sorting process, we adjusted chlorophyll fluorescence trigger thresholds to select microplankton as the sort targets. Most triggers were ciliates, large dinoflagellates, and diatoms. Post-sort images verify both the integrity of the sorted cells and correspondence with the initial images from IFCB (Fig. 6). During these experiments, a number of ciliates were collected on microscope slides and observed swimming very rapidly. As a result of their swimming speed these cells were very difficult to image and extended time on the microscope stage led to cell lysis, so most ciliates were not documented in post-sort images despite their prevalence and obvious post-sort viability.

It has long been recognized that analysis by flow cytometry can cause physiological damage (Rivkin et al. 1986).

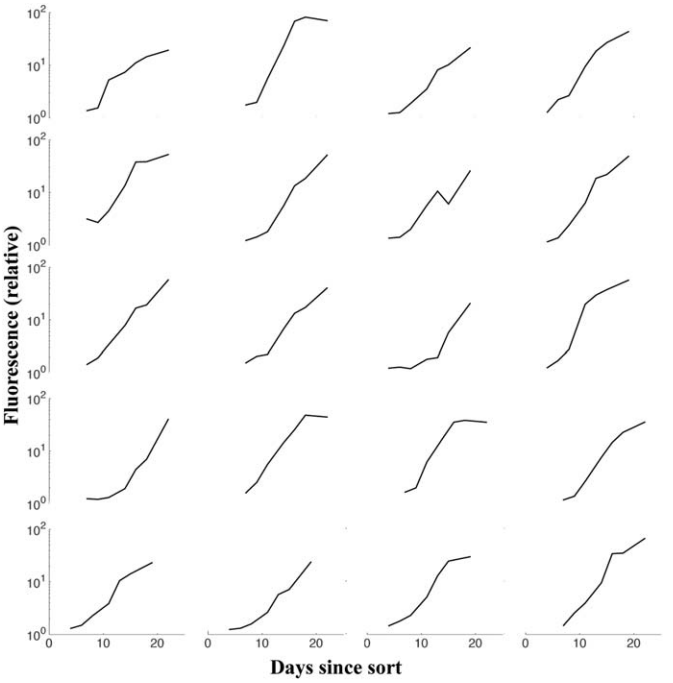

Fig. 5. Growth curves resulting from 20 individual *A. fundyense* cells sorted into wells of a 96-well plate using the IFCB-Sorter. During re-isolation 23 of 168 sorted cells developed into successful subcultures.

While it was initially a concern that sensitive cells would be disrupted during the sorting process, our evaluation here indicates that the IFCB-Sorter can effectively sort cells traditionally thought to be fragile.

Capture of chain-forming cells

The IFCB-Sorter is capable of capturing chain-forming cells, but with lower efficiency than the capture of cells with a simpler morphology. For example, we sorted the chain-forming diatom *G. delicatula* with conservative timing parameters (300 μs delay, 800 μs catcher tube deployment). The result was a capture efficiency of 21.2%. This efficiency may be further decreased if complex morphological features, such as setae, are present.

Capture volume

The probability of coincidental capture of non-targeted cells is an important parameter for sorting systems and in the IFCB-Sorter it is related to the volume captured initially by the catcher tube. To calculate a worst-case estimate of that probability, we assume that during catcher tube deployment (t_d) all the volume captured is sample, and use a conservative catcher tube deployment time of 700 μs. With a sample flow rate (Q_{core}) of 0.05 mL min^{-1}, the captured volume (V_c) can be calculated from

$$V_c = Q_{core} * t_d. \quad (1)$$

With this approach, we find $V_c = 5.8 \times 10^{-7}$ mL.

Fig. 6. Cells isolated from environmental samples. Images on the left were acquired pre-sort by the IFCB and images on the right are the same cells post-sort as seen at 40X magnification with an inverted microscope (Zeiss Axiovert S100) equipped with a Canon EOS Rebel T2i camera. Scale bar indicates 10 μm in the IFCB images. (**A**) Unidentified dinoflagellate, (**B**) unidentified cell, (**C**) *Prorocentrum* spp. (**D**) *G. delicatula*, (**E**) centric diatom, (**F**) *Dinophysis* spp.

The probability of coincidental capture (p) is the product of V_c and the concentration of background contaminant particles (C_{bg})

$$p = V_c * C_{bg} \quad (2)$$

Thus, for a 10% contamination probability, C_{bg} is 1.7×10^5 cells mL^{-1}. This indicates that the IFCB-Sorter should be capable of isolating microalgae with low risk of contamination by other organisms. It should be noted that the captured sample volume will be diluted by mixing with particle-free sheath fluid during passage through the secondary sorting system, which will tend to further purify the sorted particles.

Discussion

Here, we present the first sorting imaging-in-flow cytometer, with the capability to link images, flow cytometric quantities, and downstream physiological or molecular studies of single cells. From our experiments, we have demonstrated a cell sorter with a low probability of coincidental capture and the capability to sort viable cells. The IFCB-Sorter can effectively sort a variety of plankton, which leads to the possibility of investigating cell-to-cell variability in traditionally difficult to capture cell types. The addition of imaging capabilities to a cell-sorting flow cytometer opens up new venues for investigation, especially for microplankton which are difficult to characterize in conventional FACS systems that only measure cell fluorescence and scattering.

The IFCB-Sorter enables new research probing relationships between morphology, genotype, and ecological role. For example, the IFCB-Sorter is capable of sorting both mixotrophic and herbivorous protozoa (depending on gut content), which are very difficult to target with traditional cell-sorting techniques. This capability provides the potential for studying linkages between phytoplankton and their grazers. One understudied group that could benefit greatly from the application of this technology is marine ciliates. Marine ciliates have long been classified according to morphotype. In recent years, it has become apparent that similar morphology does not necessarily coincide with genetic similarity (Snoeyenbos-West et al. 2002). Flow cytometry has supplied crucial information about grazer dynamics in the past (Cucci et al. 1989; Lavin et al. 1990; Taniguchi et al. 2014) and the IFCB-Sorter, along with single-cell genomic methods, could now allow us to investigate these predator-prey interactions in greater detail.

While the IFCB-Sorter is capable of capturing chain-forming cells, it does so with reduced efficiency compared to single cells. Chain-forming cells and those with complex features may traverse the distance between the location of image acquisition and the catcher tube at a slower rate. As a result these cells may not be captured even with conservative timing parameters. It may be possible to increase the capture efficiency by increasing the catcher tube deployment time, but this strategy conflicts with the goal of a low contamination rate. Chain-forming cells may also take longer than our observation time limit to pass through the sorting subsystem and are therefore discarded. Again it may be possible to overcome this by increasing the observation time, but this has the potential to reduce the total number of cells sorted per sample. If the aim of an experiment is to isolate chain-forming cells, the procedure could be modified and the IFCB-Sorter might then be used as an initial step in cell isolation.

Isolation of cells from the environment, along with corresponding images, offers a powerful method to investigate genetic differences in marine planktonic microorganisms, which have been observed to result in the formation of subpopulations during blooms (Koester et al. 2010). Cryptic diversity has been noted in several phytoplankton genera (Montresor et al. 2003; Iglesias-Rodriguez et al. 2006; Kooistra et al. 2010) and the IFCB-Sorter could be used to link morphology to this genetic variability and to environmental conditions. Furthermore, cells from different life cycle stages could be sorted for detailed studies of the factors regulating important processes such as sexual reproduction and cyst or resting-stage production.

Comments and recommendations

The IFCB-Sorter is very slow ($\sim$ 15 s per sort event) compared to conventional fluorescence-activated cell sorters (up

to 10,000 s^{-1}) (Shapiro 2005). The sorting rate could be increased several-fold by miniaturizing the sorting subsystem (thereby decreasing its path length). However, the main limitation to sorting speed is our secondary system for isolating the sorted cell, which requires bulk flow to the storage site followed by re-detection of the cell's position before ejection into a well. In theory sorting speed could be greatly increased by utilizing conventional droplet sorting (with electrostatic deflection of droplets after initial detection rather than a catcher tube). We chose the slower approach because sorting of our large particles of interest would require formation of very large charged droplets, which would be technically more difficult to achieve and maintain than our solenoid-based approach. In addition, compared to a contained-flow system, droplet sorting, which produces aerosols, seems less compatible with our ultimate goal of autonomous in situ operation. Our original storage strategy utilized emulsion microfluidics technology to direct sorted cells to storage chambers in a totally enclosed system, but we found that natural populations of microphytoplankton are not well suited to flow through microchannels (e.g., chain-forming or spine-bearing diatoms caused clogging of channels). Alternative storage methods such as tape reels of self-sealing wells could still enable in situ applications of our sorting approach, but are beyond the scope of this study.

As with any molecular analysis, contamination is a major concern. The ability to run IFCB-Sorter with external sheath fluid and to run regular built-in cleaning programs mitigates this issue. It is also possible to trigger sorting in the subsystem with an external trigger pulse, to serve as a negative DNA control during downstream amplification.

IFCB is commercially available through McLane Research Laboratories. While the development described here involved a prototype design, it should be feasible to alter the commercially available version of IFCB to enable cell sorting in a similar manner. This would require replacing the stock flow cell with the FACSCalibur sorting flow cell used in this study. Additionally, the instrument firmware would need to be altered to incorporate signals from the sorting subsystem.

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Conflict of Interest

None declared.

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