

A light sheet based high throughput 3D-imaging flow cytometer for phytoplankton analysis

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Abstract: This paper reports a light sheet fluorescence imaging flow cytometer for 3D sectioning of phytoplankton. The instrument developed has the inherent advantages of high cell counting throughput and high spatial resolution information derived from flow cytometry and light sheet microscopy. The throughput of the instrument is quantified by the sample volume flow rate of 0.5 $\mu\text{l}/\text{min}$ with a spatial resolution as achieved by light sheet microscopy. Preliminary results from 3D morphology of the internal chlorophyll-*a* structure of two dinoflagellates species show promising application potentials of the method for phytoplankton taxonomy of selected species and species groups.

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1. Introduction

Monitoring the species and species groups of phytoplankton are routine tasks for marine ecological studies and environmental monitoring [1]. Conventional optical microscopy, flow cytometry and confocal microscopy have been used to enumerate and identify phytoplankton; but these techniques have a compromise between spatial information and acquisition speed.

Traditionally, optical microscopy is used to identify and enumerate phytoplankton with the assistance of a trained eye. Even though this technique is most widely used, observing the samples under a microscope is laborious, time consuming and has a low throughput. Flow cytometry is another tool used for phytoplankton analysis. Modern flow cytometers can achieve a sampling speed well exceeding several tens of thousands of cells per second and with 18 colors simultaneously [2]. However, this high throughput has a major drawback in sacrificing the morphological information for speed that is vital for the identification of phytoplankton. The hybrid technology that combines optical microscopy with flow cytometry, known as imaging flow cytometry, significantly improves the throughput of microscopic imaging [3], yet the information obtained is 2-dimensional and lacks morphological data.

As phytoplankton cells are highly structured objects, conventional microscopy is not adequate for taxonomic recognition of many species as it demands constant refocusing to view the whole body especially under high magnifications. Consequently, 3D imaging is one of the best ways to provide detailed morphological information such that automated identification and classification of these microorganisms may be possible [4, 5]. Among those 3D microscopic imaging technologies, laser scanning confocal microscopy (LSCM) is a well-established and widely used technology that could capture profiles of small biological particles. The spatial resolution of confocal microscopy is very good and is close to diffraction limit. LSCM, especially the single-point scanning LSCM, requires raster scanning which suffers from slow performance [6]. For a typical particle scan, it could take up to minutes to get a highly detailed 3D image. This slow data bandwidth hindered the technique to be effective for routine measurements of large collection of particles such as for phytoplankton observation especially with bloom samples.

Light sheet microscopy is a relatively new 3D imaging technology that prospered in life sciences in the recent decade [7–13]. One important feature of light sheet microscopy is its ability to gather consecutive body sections of transparent samples with much higher photon usage efficiency than LSCM. Usually, in light sheet microscopy, the optical arrangement is fixed and the sample is embedded in an immobilizing medium [14]. The sample is then mechanically maneuvered across the light sheet to obtain stack of images that are used to reconstruct the 3D image. This type of sampling system makes light sheet microscopy suitable for situations in long term investigations such as the research of embryonic development where the cells are repeatedly scanned over a length of time. To cope with a large number of samples, the throughput of light sheet microscopy needs to be increased.

To develop an instrument for high volume throughput and high spatial content analysis of phytoplankton, the inadequacy of throughput in 3D microscopic imaging needs to be addressed. In this work, this is tackled with the development of a light sheet based 3D imaging flow cytometer that inherits the benefits from light sheet microscopy and flow cytometry. The light sheet fluorescence microscope is configured in a flow cytometer arrangement such that particles are made to flow normally into the light sheet plane first. As the phytoplankton cells traverse through the light sheet, thin sections are sequentially illuminated and imaged onto a high frame rate camera to realize 3D profile information. Due to the unique scheme used, the instrument presented in this paper highly increases the throughput of 3D microscopic imaging.

2. Experimental setup

Figure 1 shows the schematic diagram of our light sheet 3D imaging flow cytometer. A flow sheath is used to hydro-dynamically focus the particles into the central part of a square capillary to achieve uniform laminar flow [15]. The particles flow orthogonally through the light sheet plane and exit sideways onto the water dipping imaging objective lens. A suction tube (not shown) is then used to collect the waste liquid. An inverted fluorescence microscope is positioned to take the perfectly focused image of the illuminated section. As different layers, or sections, are illuminated when the particles traverse through the beam, a stack of fluorescent images of the phytoplankton cells are obtained. Basically, the 3D imaging flow cytometer comprises 4 parts as follows:

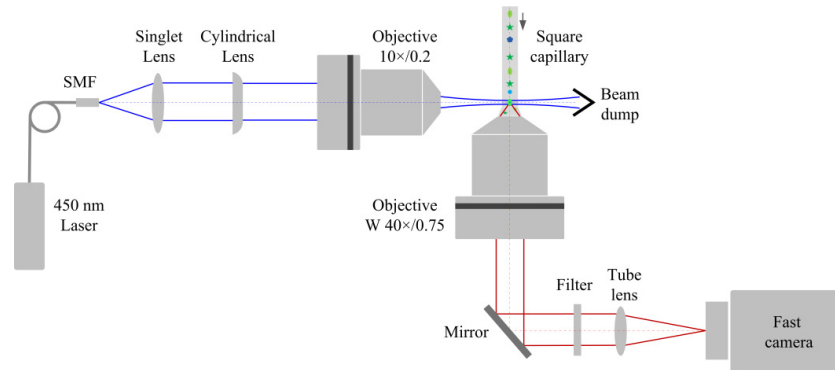


Fig. 1. Schematic setup of the light sheet based 3D imaging flow cytometer.

2.1 Light sheet generation unit

For flexibility and ease of alignment, a single mode fiber (SMF) is used to couple a 25 mW 450 nm laser for generating the light sheet. The wavelength of the laser is chosen to maximize the excitation efficiency of the chlorophyll-*a* in the phytoplankton. The laser is first collimated with a singlet lens (BPX050, Thorlabs) and then produces the light sheet with a cylindrical lens (Cylinder achromat 101.6 mm FL, Melles Griot) in combination with an objective (Epiplan 10 × / 0.2 HD, Carl Zeiss).

The thickness of the beam waist and the Rayleigh range of the light sheet is mainly determined by the effective NA of the illumination objective used [16]. Varying the distance between the cylindrical lens and the illumination objective, the width of the light sheet generated can be changed. The size of the light sheet is optimized to fit the sample core and field of view of the image detection unit.

2.2 Image detection unit

This unit is essentially an inverted fluorescence microscope. The water dipping objective (W N-Achroplan 40 × /0.75, Carl Zeiss) and the tube lens (BPX085, Thorlabs) make up an infinity corrected microscope yielding a total magnification of 48 × . As the chlorophyll-*a* fluorescence has a peak emission around 685 nm, a bandpass filter centered at 684 nm (FF02-684/24, Semrock) is used for laser rejection and for detection of the fluorescence emission. The final image is recorded with a fast camera (PCO, 1200hs with 1280 × 1024 pixels, pixel size 12 × 12 μm²). It has a readout speed of 636 frames per second (fps) at full frame resolution.

2.3 Sample introduction unit

The flow cell is similar to that of a conventional flow cytometer. Samples are injected from the center channel and are hydro-dynamically focused through a square flow capillary. The

square capillary has an inner size of 1mm and it can provide a flat optical surface for laser transmission. The position of the sample core is finely controlled by XYZ translation stages to align with the beam waist of the light sheet. The sample volume flow rate is optimized to be 0.5 $\mu\text{l}/\text{min}$, which corresponds to a flow speed close to 1 mm/s. It is a compromise among axial resolution, throughput and sensitivity of the imaging system. Figure 2(a) is a laser scattering image of the sample core captured with 500 nm fluorescent beads to test the particle confinement ability of the flow tube. It clearly showed the desired diameter of $\sim 100\ \mu\text{m}$.

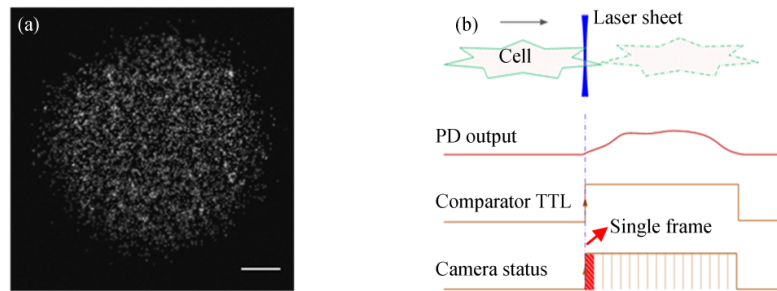


Fig. 2. (a) Laser scattering image of the sample core, 580×580 pixels, scale bar: $20\ \mu\text{m}$, exposure time: 500 ms, (b) Timing chart of the trigger and camera synchronization control.

2.4 Signal synchronization unit

A photodiode (PD) is used to detect the chlorophyll-*a* fluorescence signal as triggers for the camera. Figure 2(b) shows the trigger timing and camera control waveforms. This triggering scheme permits phytoplankton cell identification from untreated samples containing detritus and other inorganic particles. And the axial thickness that single frame covered is determined by the particle velocity and exposure time of the single frame.

3. Results and discussion

3.1 Light sheet characterization

To ascertain the thickness of the light sheet, chlorophyll chemical solution flowing in the inner sheath with a diameter about $35\ \mu\text{m}$ is used as an indicator. Figure 3(a) shows the chlorophyll fluorescence illuminated by the laser sheet. The laser passes through the sample core horizontally and the sample flows vertically. The sample core is imaged with a $5 \times /0.2$ objective lens from the side and recorded with a video camera. Figure 3(b) gives the intensity profile of the light sheet. The intensity profile has FWHM (Full Width at the Half Maximum) of $5.39 \pm 0.13\ \mu\text{m}$.

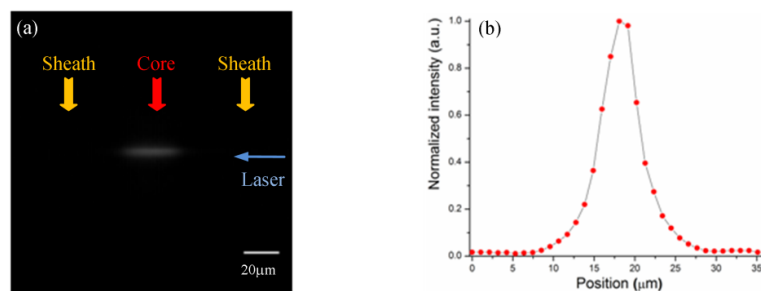


Fig. 3. Characteristics of the light sheet generated. (a) Image of the light sheet, taken with a $5 \times /0.2$ lens from the side with the flowing of chlorophyll solution. (b) Intensity profile of the light sheet, FWHM is $5.39 \pm 0.13\ \mu\text{m}$.

The optimized field of view of the image detection unit should be within the Rayleigh range of the light sheet. With a thickness of $5.39\text{ }\mu\text{m}$ at the beam waist, the Rayleigh range of the light sheet is slightly larger than $50\text{ }\mu\text{m}$. The core diameter is adjusted to $100\text{ }\mu\text{m}$ that guarantees all the cells cross through the light sheet at the uniformly illuminated central area.

3.2 Optical resolution determination

The point spread function (PSF) of the optical system is measured by using 500 nm fluorescent beads. For lateral PSF measurement, fluorescence images of individual beads are acquired. The volume flow rate is $0.5\text{ }\mu\text{l/min}$; the sample flow has a core diameter of $100\text{ }\mu\text{m}$ and a speed of 1 mm/s . A region of interest (ROI) of 580×580 pixels on the camera chip is selected so that it can efficiently cover the sample core as Fig. 2(a) shows. Each bead takes only a few milliseconds to cross the laser sheet plane. The exposure time for each frame is set to 100 ms so that it can collect many beads' fluorescence as they pass through the laser sheet plane. Figure 4(a) illustrates the lateral PSF of the optical imaging system. The Airy disk measured has a FWHM of $0.81 \pm 0.07\text{ }\mu\text{m}$ that covers ~ 9 pixels on the camera. This result shows the beads pass through the light sheet perpendicularly without significant deviation.

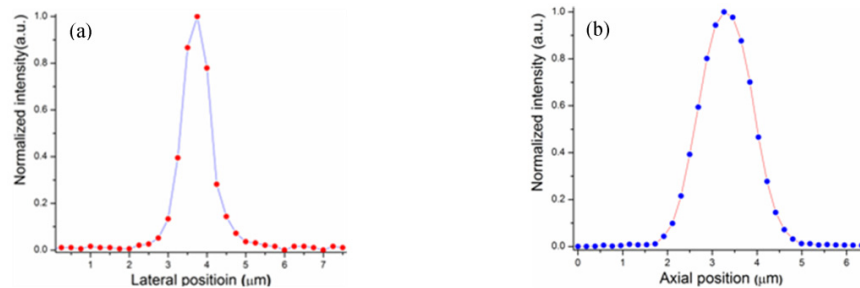


Fig. 4. Experimentally measured PSFs. (a) Lateral PSF measured by fluorescence imaging: the FWHM is $0.81 \pm 0.07\text{ }\mu\text{m}$. (b) Axial PSF measured by laser scattering imaging: the FWHM is $1.42 \pm 0.15\text{ }\mu\text{m}$.

Axial scattering PSF is measured to evaluate the axial resolution of the optical system. The flow conditions for axial PSF determination are the same to that of the lateral PSF measurement. The exposure time for a frame is set to $200\text{ }\mu\text{s}$ such that a stack of images of individual beads could be captured as they flow through the laser sheet plane. A ROI of 130×130 pixels is used to further increase the frame rate. An averaged intensity profile of 15 beads is generated for the axial PSF as shown in Fig. 4(b). The FWHM of the axial scattering PSF is $1.42 \pm 0.15\text{ }\mu\text{m}$. This measured result agrees well with the data published by other researchers [16]. The axial PSF is determined by the thickness of the light sheet plane and the axial PSF of the image detection optics. The theoretical depth of focus of the image detection optics is about $1.5\text{ }\mu\text{m}$ [17]. It should be noted that the slight improvement in axial resolution is originated from enhancing image contrast with light sheet illumination [8]. The results on the illumination tracks of the beads showed small lateral position shift. This shift could be caused by the Brownian motion of the particle in the solution. However, the shift observed is always within one pixel and for large particles this effect will be minimal.

3.3 Phytoplankton samples testing

The instrument constructed could cover a range of sizes from microns to tens of microns which comprise many dinoflagellates, diatoms and potentially harmful algal blooms species that are commonly found in coastal waters. Two lab cultured toxic dinoflagellate species, *Gambierdiscus sp.* and *Procentrum sp.* are used to test the application feasibility of the 3D imaging flow cytometer in phytoplankton measurements. To get high contrast fluorescence images, the frame exposure time is optimized at $750\text{ }\mu\text{s}$. With a flow speed of 1 mm/s , each

frame scans an axial thickness of $0.75\ \mu\text{m}$. Under these conditions, the axial resolution for the system is about $2\ \mu\text{m}$. The number of the images obtained for a single particle is determined by a number of factors including the flow speed, particle size, orientation of the cell, and the preset trigger level.

Figure 5 are 3D projection images generated from the image stacks taken for *Gambierdiscus sp.* and *Procentrum sp.* Fig. 5(a) is a 3D projection of a stack of 30 images of *Procentrum sp.* The total sampling time to acquire the stack is 22.5 ms. Figure 5(b) is a frame out of the stack that reveals a hollow structure of chlorophyll-*a* in *Procentrum sp.* The hollow structure is hard to notice in Fig. 5(a), where the whole corpus is projected onto a 2D plane. In conventional microscopy, the scenario is even worse as the photons from out-of-focus planes would also contribute to the blur of the images. Figures 5(c) and 5(d) are two different *Gambierdiscus sp.* cells. The chlorophyll-*a* structures are slightly different between the two particles with protruding rod-like structures. The small variation between the two cells can be interpreted to be at different stages of cell development which is common for cultured samples. It can be clearly seen from the images that the chlorophyll-*a* structure in phytoplankton cells is very distinct for different species and this information could be very likely to use for taxonomy applications of selected micophytoplankton species. Figure 5(e) illustrates the projections along the lateral directions of the same stack used in Fig. 5(d). The shadowing artifacts, one of the side effects of single beam light sheet illumination, could be easily observed in Fig. 5(e). This could be overcome by illuminating the sample from opposite sides [18].

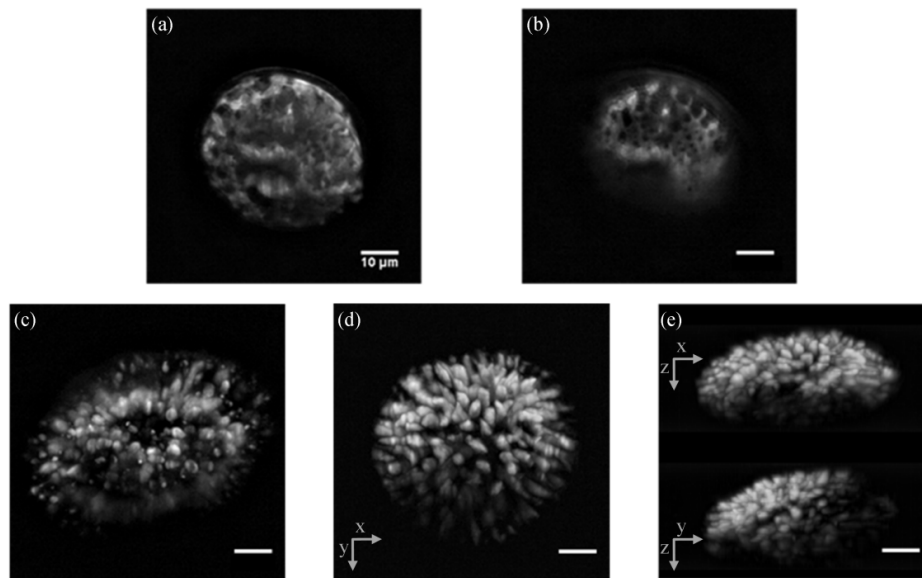


Fig. 5. Images of *Gambierdiscus sp.* and *Procentrum sp.* captured with the 3D imaging flow cytometer. (a) Maximum projection of the stack of 30 images of *Procentrum sp.*; (b) A single image out of the 30 planes; (c) and (d) are maximum projection of two different *Gambierdiscus sp.* cells. (c) has a stack of 35 images and (d) has a stack of 38 images; (e) Projections of the same stack used in Fig. 5(d) along lateral direction. Scale bars are $10\ \mu\text{m}$.

4. Conclusion

We have achieved a new light sheet based 3D fluorescence imaging flow cytometer that could scan a large number of phytoplankton cells with high spatial resolution in a short time. The FWHM of lateral fluorescence PSF achieved is $0.81 \pm 0.07\ \mu\text{m}$ and the FWHM of axial scattering PSF is $1.42 \pm 0.15\ \mu\text{m}$. The throughput of the instrument is quantified by the sample volume flow rate of $0.5\ \mu\text{l/min}$, which benefits from the improvement that particles'

chemical morphology can be acquired without the need of sample immobilization. The intracellular 3D chlorophyll-*a* structure images obtained from lab cultured *Gambierdiscus sp.* and *Procentrum sp.* samples by the method suggest its high potential for phytoplankton identifications.

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