

A fast fluorescence imaging flow cytometer for phytoplankton analysis

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Abstract: We report a fast fluorescence imaging flow cytometer for phytoplankton analysis that can achieve a volume flow rate up to 1 ml/min. The instrument shows a high immunity to motion blur in image captured with a lateral resolution of $0.75 \pm 0.06 \mu\text{m}$ for a wide size range $\sim 1 \mu\text{m}$ to $\sim 200 \mu\text{m}$. This is made possible by suppressing the out-of-focus light using thin light sheet illumination and image deconvolution, and by precluding the motion-blur with a unique flow configuration. Preliminary results from untreated coastal water samples show the technique has high potential as a practical field instrument for monitoring phytoplankton abundance and species composition.

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OCIS codes: (180.2520) Fluorescence microscopy; (110.2970) Image detection systems; (100.1830) Deconvolution; (010.4450) Oceanic optics.

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1. Introduction

Investigating phytoplankton species composition and their abundance are routine tasks in marine ecological research and environmental monitoring [1]. The phytoplankton species are highly diversified, broad range in size, highly irregular shapes, and most significantly their abundance can change drastically in a very short time. These traits make the tasks in quantifying and monitoring the particles extremely difficult and demanding on the instrumentation [2].

Traditionally, observation using optical microscopes with a trained eye is still the common and reliable technique used to identify and count the phytoplankton species. While invaluable in measuring species composition, observing the sample under an optical microscope is a monotonous and slow process. Moreover, because of cell loss during sample handling and only a small number of particles are usually observed, this method has an inherent inaccuracy in statistical estimation of species abundance [3]. Flow cytometry is another technology that is widely used in phytoplankton analysis due to its high throughput; modern flow cytometers are capable of measuring up to tens of thousands of cells per second [4]. However, as all particles are treated as point-like objects, conventional flow cytometry lacks morphological information that is important for phytoplankton species identification.

Imaging flow cytometry has the potential to integrate the benefit of high spatial resolution from optical microscopy and the advantage of high throughput from flow cytometry [5]. In reality, two intrinsic limitations prevent imaging flow cytometers from obtaining high spatial resolution images with high throughput [6]. One of the limitations is the motion-blur on the images caused by the moving objects [7–10]. The other limitation is the shallow depth of field of optical microscopes, especially when high spatial resolution is needed [11–13]. Numerous work has been done to overcome these limitations with moderate success, but still it always comes out with a tradeoff between throughput and image quality [14].

We have demonstrated a light sheet based high throughput 3D fluorescence imaging flow cytometer that can acquire 3D images of the chlorophylls in phytoplankton particles [15]. The system developed is intrinsically free from the motion-blur because the images are taken from the particles' propagation direction. Furthermore, out-of-focus light is suppressed by the thin light sheet illumination. Thus, the two major obstacles that limit the performance of imaging flow cytometry are well addressed.

In the previous work, using a fast camera, thin consecutive sections are recorded to achieve 3D reconstructions of the particles as they traverse through the thin light sheet plane. Although the speed performance of 0.5 $\mu\text{L}/\text{min}$ is impressive for 3D microscopic imaging, it still lacks practical speeds to tackle natural water samples where diatoms and dinoflagellates usually exist in low abundance. To address this problem, we redesigned our system to capture 2D fluorescence images with a simplified flow system and a slow speed camera. This arrangement achieved an unprecedented flow speed that is over 2000 times faster than the previous 3D fluorescence imaging experiment and over 4 times the speed of the fastest imaging flow cytometer such as the Imaging FlowCytobot [10]. In addition, it is able to work with a large dynamic range of sizes that includes pico-, nano- and microphytoplankton. With these abilities, the instrument developed has high potential as a practical field instrument for monitoring phytoplankton.

2. Materials and methods

The schematic diagram of the 2D fluorescence imaging flow cytometer is shown in Fig. 1. A 450 nm laser is used to excite the chlorophylls in the phytoplankton that emit fluorescence light at wavelengths around 685 nm. The light sheet is formed with a cylindrical lens (Cylinder achromat 101.6 mm FL, Melles Griot) in conjunction with an illumination objective (Epiplan 10 × / 0.2 HD, Carl Zeiss) [16]. The fluorescence images are captured with an inverted fluorescence microscope, which comprises a water dipping lens (W N-Achroplan 40 × / 0.75, Carl Zeiss), a filter (FF02-684/24, Semrock), a tube lens and an electron-multiplying charge-coupled device (EMCCD) camera (PhotonMax: 1024B, Princeton Instruments). Samples are introduced into a 200 × 200 μm² square capillary with a syringe pump and flow directly onto the imaging water dipping objective. The light sheet is focused across the flow capillary near the outlet as shown in Fig. 1(a). The direction of the flow is perpendicular to the light sheet plane and is parallel with the optical axis of the fluorescence microscope. Figure 1(b) shows the beam illumination in the flow capillary taken with fluorescent particles (chlorella). Because of the shear stress, the flow is faster at the center than near the walls of the flow capillary; hence the cells look dimmer at the center of the capillary. Meanwhile, the shear forces drive the cells away from the walls (shown as green square), which reduces the optical vignetting caused by the capillary walls.

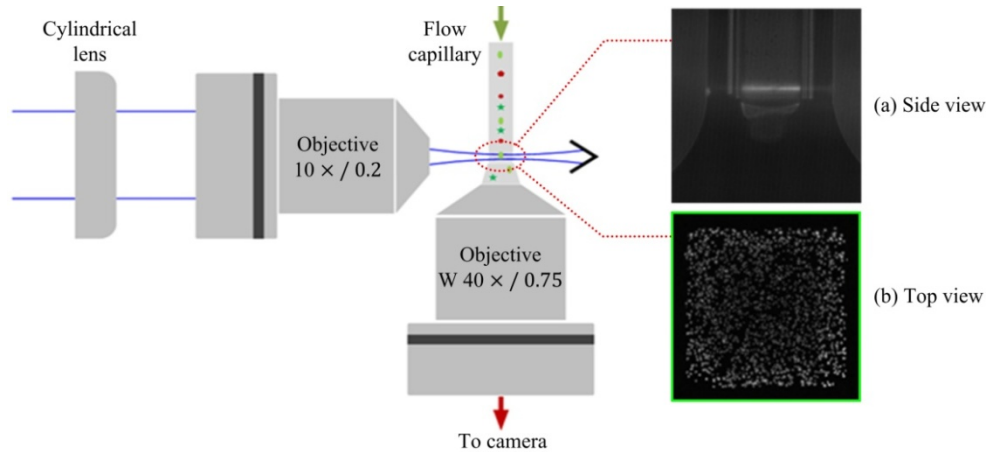


Fig. 1. Schematic diagram of the 2D fluorescence imaging flow cytometer. (a) side view; (b) top view.

Using chlorophyll solution, the bright track of the laser sheet passing through the flow capillary can be observed as shown in Fig. 1(a). The lateral image is taken with a 5 × / 0.2 objective from a video camera. The thickness of the light sheet measured is 5.39 μm [15] at the beam waist and about 10 μm near the walls of the flow capillary. As the theoretically calculated depth of field of the fluorescence microscope is about 2 μm [17], it could be expected that using light sheet illumination can efficiently suppress the fluorescence background. However, there still remains an amount of out-of-focus light, which can be further removed with post image processing using an iterative deconvolution algorithm. The general equation for the intensity integrated 2D images $G(x_i, y_i)$ recorded as the 3D object passing through the focus is given by [18]:

$$G(x_i, y_i) = H(x_i, y_i, x_o, y_o) \otimes S(x_o, y_o).$$

where $H(x_i, y_i, x_o, y_o)$ is the integrated-intensity projection of the 3D point spread function (PSF) of the imaging system; $\otimes$ denotes convolution operation and $S(x_o, y_o)$ is a perfect 2D

projection of the object. Thus, the intensity integrated images obtained can be deconvolved to improve image fidelity with a prior known $H(x_i, y_i, x_o, y_o)$.

Using fluorescent beads with a size of ~ 500 nm, the PSF of the imaging system is measured. The volume flow rate for PSF measurement is $10 \mu\text{l}/\text{min}$. With a cross-section of $200 \times 200 \mu\text{m}^2$, the beads have an average speed of about $4.2 \text{ mm}/\text{s}$. It, therefore, takes about 2.5 ms for the beads to cross the light sheet plane. The exposure time is set to 100 ms per frame such that it integrates the fluorescence during the transit through the light sheet plane. A stack of 50 images is captured with approximately 100 beads per frame. Using randomly selected images, a collection of 200 beads are stacked to generate the airy disk as shown in Fig. 2(a). Figure 2(b) gives the intensity profile of the airy disk which has a full width at the half maximum (FWHM) of $0.75 \pm 0.06 \mu\text{m}$. The FWHM of the PSF occupies less than 9 pixels, which indicates the lateral motions of the beads crossing the light sheet plane, if any, is within the diffraction limit of the imaging optics.

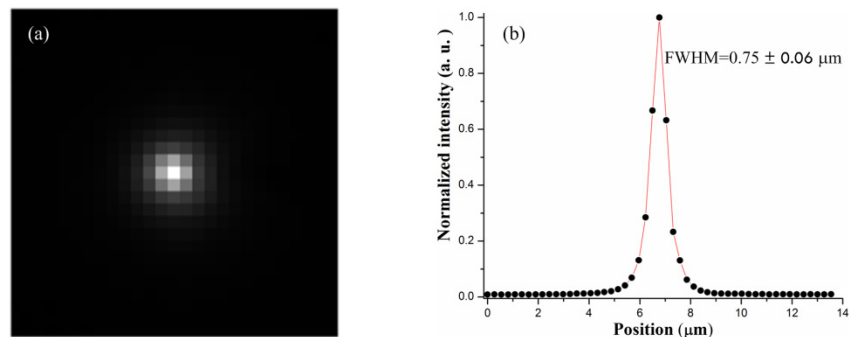


Fig. 2. Experimentally measured lateral PSF. (a) Integrated-intensity projection of the 3D PSF of the imaging optics, 51×51 pixels; (b) Intensity profile of the PSF, FWHM is $0.75 \pm 0.06 \mu\text{m}$.

With the measured PSF, the residual out-of-focus light is further suppressed by image post-processing. After background subtraction, each image was subjected to the Tikhonov-Miller iterative restoration algorithm for reassigning the out-of-focus light to an in focus location. Deconvolution was carried out using the software DeconvolutionLab, ImageJ plugin, a regularization parameter of 0.0001 and an iteration number of 15. The deconvolution process for a frame (700×700 pixels) takes less than one second. All the algorithms were run on a PC with an Intel Core i7 3.6 GHz CPU and 16 GB of RAM.

3. Results and discussion

Figure 3(a) gives an image captured from a fresh untreated coastal water sample with an exposure time of 1 second. The sample flows at a volume rate of $1 \text{ ml}/\text{min}$, which corresponds to an average speed of $0.42 \text{ m}/\text{s}$. Under this flow speed, no motion-blur artifacts were detected in the image because of the special flow configuration used. The large particle in the center is likely to be a dinoflagellate, *Ceratium furca*, which is a frequent visitor in the Hong Kong coasts. It could be seen that the internal structures are clearly visible and detailed enough for possible visual particle identification. The brightness in the small green square area is adjusted to show the occurrence of small picophytoplankton whose size is under the diffraction limit.

As the fluorescence imaging flow cytometer developed is free from motion-blur, the maximum volume rate is mainly limited by the sensitivity of the camera. In this work, the volume rate is set to $1 \text{ ml}/\text{min}$ so that the system is able to sense small picophytoplankton. However, if large phytoplankton particles were targeted, the volume rate could be further increased. The number of particles captured in a frame is determined by the flow speed, exposure time and phytoplankton cell abundance. For the particular untreated water samples,

the exposure time is set to 1 second at flow speed of 1 ml/min such that the camera captures on average some tens of particles in a frame with low occurrences of overlapping.

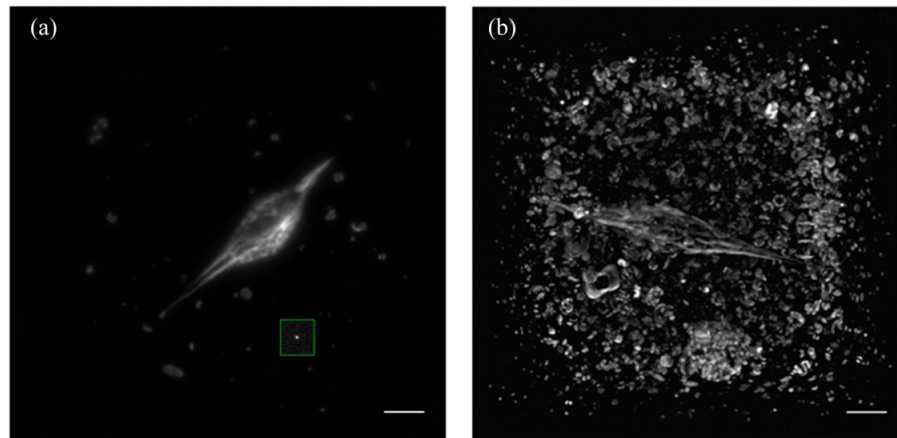


Fig. 3. Images obtained from natural coastal water samples. (a) Single frame; (b) Maximum projection of 60 deconvolved images. Experimental conditions: volume rate is 1 ml/min; exposure time is 1 s. Scale bars: 20 μ m.

Figure 4 gives a qualitative comparison between the original image and the restored image of a randomly selected cell. It shows that deconvolution has a remarkable performance in improving contrast by suppressing the out-of-focus light. The white curves at the bottom of Figs. 4(a) and 4(b) are the pixel intensity profiles along the red horizontal lines in both images. The intensity value is normalized to the maximum value of the two lines.

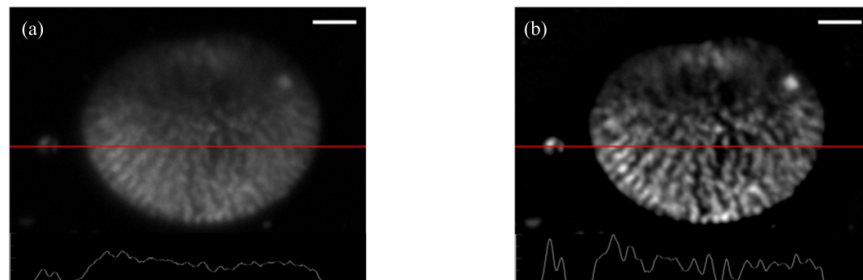


Fig. 4. Comparison between original and deconvolved image. (a) Original image; (b) Deconvolved image. White curves are the intensity profiles along the red lines selected. Scale bars: 10 μ m.

The 2D fluorescence imaging flow cytometer developed can screen a large volume of coastal water samples in a short time and can cover broad range of sizes from small picophytoplankton to large diatoms and dinoflagellates. The cell abundance, therefore, could be determined with much higher confidence than those done by bright-field microscopy, which has difficulties in observing small picophytoplankton. Figure 3(b) presents the combined projection of a stack of 60 deconvolved images representing total particles in 1 ml of the sample. As expected, small cells dominate the abundance with only a few large cells present. The cell abundance measured is ~ 4700 cells/ml, which is considerably denser than the abundance determined by conventional microscope counting technique (Data from Hong Kong marine water quality report: 1500 $\sim$ 4500 cells/ml) [19].

Furthermore, the morphological information of the images of larger cells obtained with our instrument has high potential for taxonomic identification. Figure 5 gives a small collection of images of larger phytoplankton showing different shapes, sizes, and structures captured from natural coastal water samples. For small particles, the instrument cannot be

used to determine species. However, for particles with sizes larger than, say, 5 microns, the detailed images captured may be used to identify some unique species such as those of large diatoms and dinoflagellates.

One possible obstacle of the instrument developed for phytoplankton species identification is that the 2D images obtained depend greatly on the orientation of the phytoplankton particles. To tackle this issue, a 3D image database of the phytoplankton may be needed such that different viewing projections can be correlated with the images. This could be one of our future research directions by using the previous 3D imaging flow cytometer to establish the database and to develop artificial intelligence software for rapid automatic species identification.

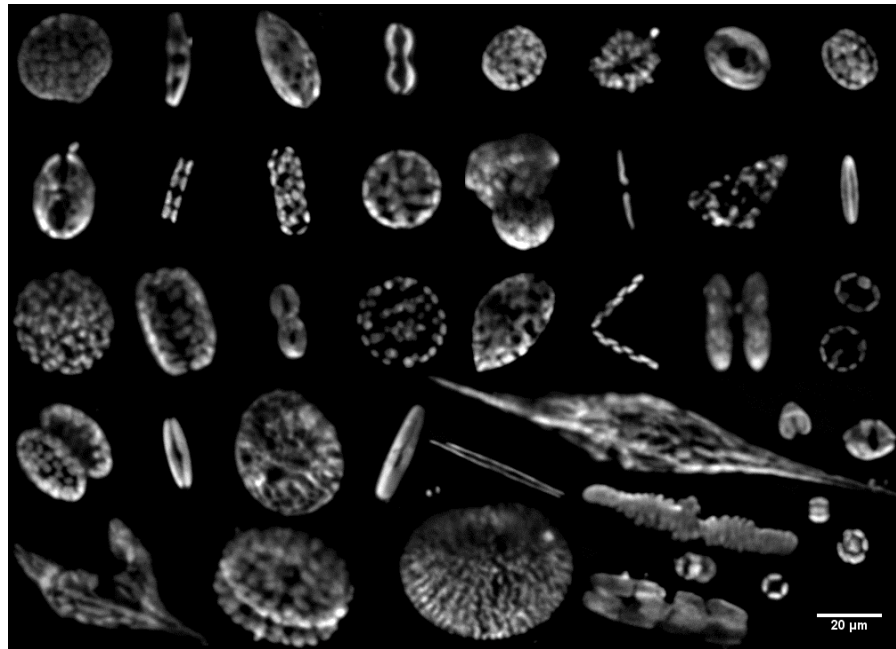


Fig. 5. Imagery of phytoplankton with different morphologies obtained from natural coastal water samples.

4. Conclusion

We have demonstrated a fast fluorescence imaging flow cytometer for taking 2D chlorophyll fluorescence images of phytoplankton from untreated coastal water samples. The instrument reported is free from the shallow depth-of-field issue and motion-blur effect. This is achieved by using a unique flow configuration, thin light sheet illumination and image deconvolution. The instrument developed measures water samples at a volume rate up to 1 ml/min with a lateral resolution less than one micron and covers a broad range of sizes from $\sim 1 \mu\text{m}$ to $\sim 200 \mu\text{m}$. Images taken from the coastal water samples showed detailed morphological information that is unique to different phytoplankton species which can be used as a characteristic signature for phytoplankton species identification.

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