Single cells diatom Chaetoceros muelleri investigated by homebuilt confocal fluorescence spectro-microscopy

by Tatas Hardo Panintingjati Brotosudarmo

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Single cells diatom *Chaetoceros muelleri* investigated by homebuilt confocal fluorescence spectro-microscopy

Tatas H.P. Brotosudarmo*[a], Arif Agung Wibowo[b], Heriyanto[b], Marcelinus A.S. Adhiwibawa [a]
[a]Ma Chung Research Center for Photosynthetic Pigments (MRCPP), Universitas Ma Chung, Villa Puncak Tidar N01, Malang 65151, Indonesia; [b]Department of Chemistry, Universitas Ma Chung, Villa Puncak Tidar N01, Malang 65151, Indonesia

ABSTRACT

Currently a combination of fluorescence microscopy with spectroscopic analysis in a balance way has played an important role in providing new and exciting information for the *in vivo* study of cell, such as diatoms, photosynthetic marine microorganisms. Diatoms contain photosynthetic pigments, carotenoid and chlorophylls molecules, in their machinery to harvest the sunlight energy for growth. Lacking of these pigments might influence to the overall metabolisms of the cells. Here we reported our work to construct a simple homebuilt confocal fluorescence spectro-microscopy to study the cells of *Chaetoceros muelleri* diatom. The setup was design to get simultaneous data of the image of the single fluorescence cell and the emission spectrum of it.

Keywords: chlorophyll fluorescence, confocal microscopy, emission spectrum, single cells

1. INTRODUCTION

Microscopy is probably one of the most iconic and well-known techniques used in science to elucidate the nature of what surround us. Especially, the confocal microscopy allows one to make thin-slice views in fixed and intact cells as well as to detect interaction that occurs at single molecular level. It has been used as a routine detection technique in biology. Usually, it is using laser to excite the sample causing detectable fluorescence. The light coming from the laser passes through a pinhole, is reflected by a dichroic mirror, and focused by a microscope objective to a small spot in the specimen, in such way confocal microscopy can yield high spatial resolution and reduce out-of-focus blur. This gives advantage to students and researchers to be able to visualise differences at small volume. A combination of fluorescence microscopy with spatially resolved spectroscopic techniques can provide new and exciting tools for functional cell biology in living organisms. The immediate practical application of this technique has driven new studies and discovery in the field of photosynthesis. It has been used to analyse the chlorophyll parameters in an intact leaf tissue from reconstructed 3D images [1] and to reveal structural changes of the grana arrangement and mobility of chlorophyll-binding protein complexes in intact protoplast that was induced by light stress [2]. The morphology of photosystems in thylakoid membrane can also be visualized and one can measure global distribution of photosynthetic pigments in the living cells of photosynthetic microorganisms [3].

Chaetoceros muelleri is a marine diatom that can be found in the Indonesian marine territory. *C. muelleri* is undergoing photosynthetic growth by harvesting light energy to reduce carbon dioxide and produce carbohydrate. *C. muelleri* has diameter between 3 to 10 µm. *C. muelleri* has fucoxanthin-chlorophyll binding proteins (FCP) as its light-harvesting module with fucoxanthin as the dominant molecule [4]. Fucoxanthin is responsible for up to 60% of the energy transfer to chlorophyll *a* in diatoms [5]. When bound to protein, the absorption spectrum of fucoxanthin expands from 450-540 nm to 390-580 nm [6]. Commercially build-in confocal systems are mostly expensive. They can cost about US$ 550,000 or more depending on the configuration. In advanced biological laboratories, the state of the art instrument is usually operated by high-skilled technician and heavily used by researchers that it is impossible to be used as a teaching tool, that allow students to be involved in modification or tuning the optical parts of microscope system. In this work, we constructed a homebuilt confocal fluorescence spectro-microscopy that can be used to study cell physiological characteristics based on the combined information between fluorescence image and online spectrum.

*tatas.brotosudarmo@machung.ac.id; +62-341-550171; https://mrcpp.machung.ac.id

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2. MATERIAL AND METHODS

2.1 Setup of the homebuilt laser confocal microscope

To observe the fluorescence emission, we developed confocal fluorescence microscope using an oil immersion Olympus PLCN 100XO plan achromat objective with a numerical aperture of 1.23. The resulting laser spot size is about 0.5 μm and 0.4 μm for the excitation laser of 532 nm and 402 nm respectively. In this experiment, we used a continuous wavelength collimated laser diodes (Thorlabs) with optical power of 4.5 mW, but in the case of actual measurement it needs to be strongly reduced to prevent photobleaching. The laser power was measured by PM100USB power and energy meter (Thorlabs). By adjusting with neutral density filter, we tuned excitation power to about 110 and 100 μW for the excitation laser at 402 and 532 nm, respectively. The emitted fluorescence of the sample is then captured by the objective lens and transferred to the infinity corrected tube lens after it passes the dichroic mirrors (Figure 1). The image of a fluorescence sample is then formed by a plano convex lens and aspherical lenses. A pinhole (150μm) is used to cut extra-focal signals. The image is captured by a digital 14bit CCD camera (pco.pixelfly usb). Simultaneously, the fluorescence spectra of the captured image are monitored by Ocean Optics USB4000 spectrometer. Camware64 software was used to control the pco.pixelfly usb CCD camera as well as to view and manipulate the image. Overture software was used to control the Ocean Optics USB4000 spectrometer. The advantage of using this homebuilt setup are as follows, i.e. it can observe the image and the fluorescence spectrum simultaneously; when using laser at 532 nm, the resolution is reasonably good, i.e. R_{xy} at 0.17 μm and R_z at 0.48 μm; and the total magnification is about 1500x.

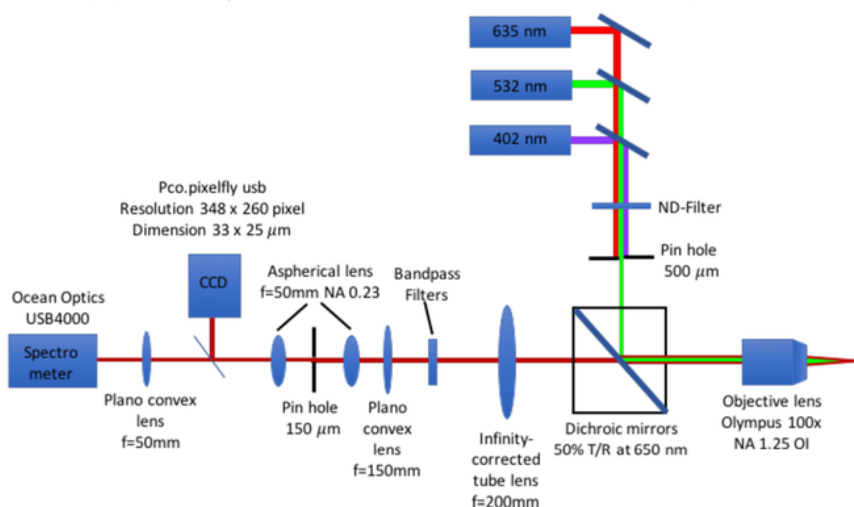

Figure 1. Experimental setup of the homebuilt confocal fluorescence microscope

2.2 Sample preparation

The marine diatom liquid culture of *Chaetoceros muelleri* was obtained from Balai Perikanan Budidaya Air Payau Situbondo (BPBAP), cultured using ESAW medium with slightly modification using fresh sea water. The culture was in the environment condition as follow: 25-26 °C and under 8000-10000 lux. *C. muelleri* was separated from the media by centrifugation (High Speed Refrigerated Centrifuge 6500, Kubota, Tokyo, Japan) at 1[illegible]000 g and 4 °C for 30 min. The pellet immediately stored at -30 °C. The cells were then dissolved and homogenized in 20 mM Tris-HCl buffer pH 8.0 containing 30% glycerol. Glycerol plays a role as a stabilizing matrix for the cells and to immobilize it on the glass. Then the sampel were coated on the glass cover slip using Spin coating with the following parameters, i.e. 1000 rpm for 10 s (1st ramp), 1600 rpm for 10 s (2nd ramp).

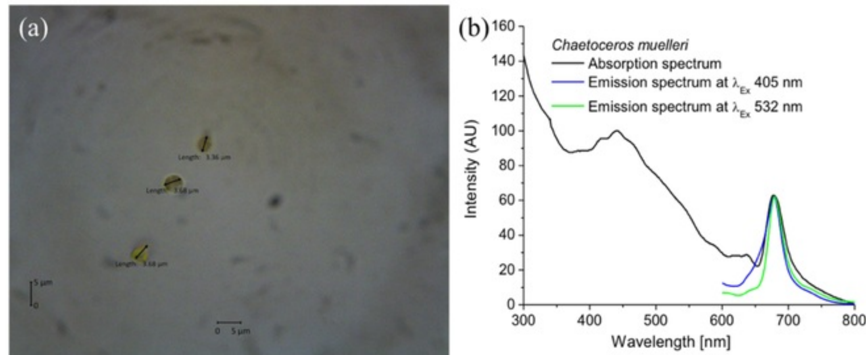

Figure 2. (a) Brightfield microscopy image and (b) optical spectra of *Chaetoceros muelleri* cells. The absorption spectrum was measured by UV-1700 spectrophotometer (Shimadzu).The emission spectra were measured at the excitation wavelength at 405 and 532 nm using FP-8500 spectrofluorometer (Jasco).

3. RESULTS AND DISCUSSION

Figure 2 shows the image of *C. muelleri* cells under a brightfield microscope (Olympus CX41) with an objective 100× NA 1.25 oil immersion. It is shown that the cells are about 3.68 μm in size and have yellow-brown colors. However, the brightfield microscope cannot provide more information about spectral heterogeneity of the cells. To get spectral information, cells were measured by UV-VIS absorption and steady-state fluorescence spectrophotometers. Figure 2 also shown the typical spectrum of *C. muelleri*.

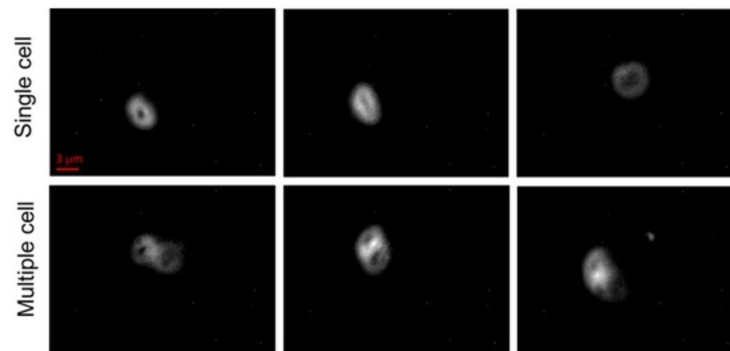

Figure 3. Fluorescence emission images of *C. muelleri* cells constructed by the homebuilt fluorescence confocal microscope

The absorption spectrum of *C. muelleri* shows broad band with pronounce three bands at 400-500 nm, which indicated the presence of carotenoids, and the strong band (650-750 nm), which indicated the Q_y-band of chlorophyll molecules. The emission spectra were recorded at two excitation wavelengths, 405 and 532 nm, respectively. The excitation at 405 nm is due to mainly the excitation of the Soret band of the chlorophyll molecule. While the excitation at 532 nm is corresponding to the excitation of the carotenoid band. Therefore, the emission bands (682 nm), appeared after the excitation at 405 nm, was mainly due to the contribution of chlorophyll molecule emission. The emission band (682 nm), recorded after the excitation at 532 nm, corresponds to the emission of chlorophyll molecule and with mainly from the contribution of the energy transfer of carotenoid [5].

The images shown in Figure 3 were images of the *C. muelleri* cells emission formed by the homebuilt setup recorded by the the pco.pixelfly usb CCD camera. Since the recorded image was passing through a dichroic mirror with cut-out wavelength at 650 nm, the bright emission signal indicates the contribution of the chlorophyll autofluorescence, i.e. the Q_y-band emission band (see with Figure 2). The fluorescence images show cells with oval-shaped in agreement to the shape of the cells taken by the brightfield microscope. It is also shown in Figure 3 that the brightest part of the emission signal is not in the middle of the cell. This might indicate that the photosynthetic system, which contain the accumulation of chlorophyll molecules, is mostly located near the cell surface.

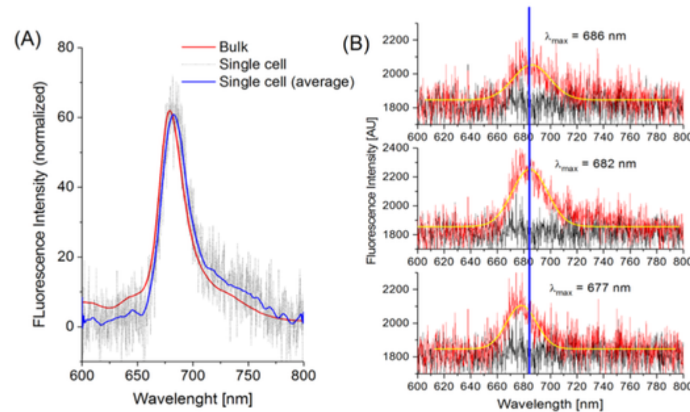

Figure 4. Comparison between bulk and representative of single cell fluorescence spectra (A) of *C. muelleri*, recorded at room temperature. (B) Three representative of single cell fluorescence (λ_{exc} = 532 nm) are compared to show variation occurs. The blue line at 682 nm is drawn to guide the eye.

Figure 4 presents the emission spectra of single *C. muelleri* cells. Here, in Figure 4(A), the bulk emission spectrum (red line) and the emission spectrum of a single cell (gray line) of *C. muelleri* are compared. The bulk spectrum of *C. muelleri* cells were obtained by recording emission of the cells with a steady-state spectrofluorometer (Jasco). While the emission spectrum of a single cell was measured by the homebuilt setup. Figure 4(A) shows that the emission spectrum of the single cell agrees with the spectrum of the bulk measurement. This indicates that the homebuilt setup was working properly to detect the emission signal of a single cell. Figure 4(B) presents typical spectrum from three different individual cells of *C. muelleri*, which showed to have the emission wavelength maximum (λ_{max}) at 686, 682, and 677 nm. The emission spectrum of *C. muelleri* might to have variation from one cell to another cell, however it should be note that the estimated spectral resolution of this setup is about 5.3 nm.

4. CONCLUSION

A homebuilt confocal laser spectro-microscopy has been successfully constructed. Using an objective lens with 100x NA 1.25 oil immersion, the setup could make total magnification more than 1000x and lateral resolution of 0.2 μm with the laser at 532 nm. The spectral resolution was estimated to be 5.3 nm. Using the setup, the emission image and spectrum of the single *C. muelleri* cells (size of 3.68 μm) could be simultaneously recorded and detected. The possible variation in the emission spectrum of the single cells might be detected within the resolution.

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