Photonic tools for evaluating the growth of diatom colonies during long-term batch cultivation

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Abstract. Diatoms are unicellular photosynthetic microalgae with a nanostructured silica cell wall – frustule. Diatoms, considered one of the most abundant and ecologically important phytoplankton groups, can also be easily grown in the laboratory and used for different applications. With long-term cultivation, it is essential to monitor their growth properly; therefore, we proposed new, fast and easy-to-use methods based on the absorption and fluorescence of diatom chromophores. The average radiant efficiency of diatom culture Achnanthidium sibiricum obtained using the IVIS SpectrumCT In vivo Imaging system increased up to 45 days of cultivation, after which we observed steady decrease. The highest photoacoustic signal of diatoms mixed with an agarose gel excited by a 532 nm laser was also recorded after 45 days of cultivation. The results obtained are in good agreement with the obtained spectra during the 100-day cultivation period. Thus, photonic methods have proven to be effective for monitoring the growth of diatoms during long-term cultivation, expanding the possibilities of growing and collecting diatoms for various purposes.

1. Introduction

Diatom algae are among the most abundant and most diverse groups of microorganisms, with more than 100,000 species across all habitats. They provide up to 20% of the oxygen on our planet and contribute to about 40% of marine primary productivity in the World Ocean [1]. Their most important feature is nanostructured highly porous silica cell wall called the frustule, due to which they are widely investigated and applied in various fields, such as bio and nanotechnology, drug delivery, immunodiagnostics, filtration and water purification, biosensing, forensic examinations, oil exploration, environmental indication, toxicity testing, waste degradation, and many others [2, 3]. Moreover, they are multifunctional, biocompatible, thermally stable, easily modified, have good mechanical and optical...
properties [4]. The frustule consists of two halves: the epitheca (the upper part), and the hypotecha (the lower part), which fit into each other, similar to a Petri dish [3]. Diatoms can be divided into two main groups: pennate, with bilateral symmetry, and centric, having multiradial symmetry. The main diatom light-absorbing chromophores are chlorophylls and carotenoids [5]. Chlorophyll $a$ absorbs energy in the violet-blue and orange-red regions of the spectrum, while chlorophyll $c$ mainly absorbs blue and red light. The carotenoid fucoxanthin absorbs from the blue-green to yellow-green part of the spectrum. Chlorophyll can be used as a photosensitizer in photodynamic therapy to treat various infections [6]. Diatoms are also producers of highly valuable bioactive substances, such as omega-3 fatty acids, peptides, etc. Therefore, to facilitate the harvesting and extraction of organic compounds, they need to be adequately grown under optimal conditions and efficiently monitored.

In this study, we monitored the growth of *Achnanthidium sibiricum* diatoms in the incubator using the fluorescence tomography system IVIS and raster scanning optoacoustic mesoscopy (RSOM) approach and collected absorbance and fluorescence spectra to verify the obtained results. We believe that this set of monitoring techniques can be employed in aquaculture and bioreactor practice to facilitate the cultivation of diatom algae and the extraction of bioactive substances for numerous pharmaceutical and biomedical applications.

2. Materials and methods

2.1. *Cultivation of A. sibiricum in the incubator*

In this study, we used *A. sibiricum* pennate diatoms isolated from Lake Baikal as a model and cultivated them in a specially assembled incubator under optimal conditions (temperature – 12 ± 1 °C, 12:12 day-night regime, LED red/blue light, nutritional DM medium [7]). The incubator consists of the control unit, allowing to regulate temperature, the day-night regime and light brightness, and the growth chamber where the cell culture flasks with diatoms are located, as described in [8].

2.2. *Monitoring of the diatom growth using IVIS SpectrumCT In vivo Imaging system*

The IVIS SpectrumCT In Vivo Imaging System (Xenogen Corp., CA, USA) was used to monitor the growth of diatoms over 100 days of cultivation. Cell culture flasks were transferred from the incubator to the imaging chamber, and imaging was performed without any sample preparations. The fluorescence signal was excited with a 465 nm wavelength and registered at 680 nm. The images were obtained and analyzed in Living Image software 4.7.3.

2.3. *Fluorescence and absorbance spectroscopy measurements*

Fluorescence and absorbance spectroscopy measurements were performed with the Infinite M Nano+ dual-mode microplate reader (Tecan Trading AG, Switzerland). The fluorescence excitation wavelength was 465 nm, while the emission was registered in the region 620–750 nm. The absorbance spectra were collected in the wavelength range 400–700 nm with a 2-nm wavelength step at 7, 28, 45, 60, 90, 100 days after the beginning of the cultivation.

2.4. *Photoacoustic imaging*

The photoacoustic imaging was carried out with the RSOM Explorer P50 (iTheraMedical GmbH, Germany) setup. Before each measurement, 3 μL of diatom cell suspension was mixed with 7 μL of 1% agarose gel and pipetted in the center of the Petri dish. After solidification, diatom-agarose drops were covered with deionized water and placed in the RSOM imaging chamber. Measurements were conducted after 7, 28, 45, 60, 90, 100 days of the cultivation. The photoacoustic effect from diatoms was excited with a Wedge HB frequency-doubled flashlamp-pumped Nd:YAG laser (Bright Solutions, Pavia, Italy) at 532 nm wavelength (repetition rate, 1 kHz; pulse energy, 200 μJ; pulse length, 1 ns) and collected with a custom-made spherically focused LiNbO$_3$ detector (center frequency, 50 MHz; bandwidth, 11–99 MHz; focal diameter, 3 mm; focal distance, 3 mm). The raster step size was 20 μm,
and the field of view up to $12 \times 12 \times 3$ mm. Upon reconstruction, images were analyzed in ImageJ software to obtain the mean pixel intensity values.

3. Results and discussion

The results of fluorescence visualization of diatoms in cell culture flasks during 100 days of cultivation using IVIS imaging system are shown in Figure 1a, b. The imaging was performed as reported previously [8, 10].

![Figure 1.](image)

Figure 1. (a) Images of cell culture flasks containing diatoms obtained using the IVIS platform (top view), (b) The average radiant efficiency of diatoms during the cultivation period, (c) Normalized fluorescence spectra of *A. sibiricum* cells, (d) Comparison of fluorescence intensity at 680 nm and average radiant efficiency depending on the cultivation time.

The average radiant efficiency increases with increasing incubation time up to 45 days, followed by a steady decrease. The result indicates that the cell division is slowed down after 45 days, probably due to the lack of necessary nutrients, since batch cultivation implies that nutrients are provided only at the beginning without further addition. However, during 100 days of monitoring, we couldn't observe the final death phase. The fluorescence spectra collected during the cultivation demonstrate an emission band, which is attributable to chlorophyll *a* (Figure 1c). We also compared the fluorescence intensity at 680 nm and IVIS imaging results. According to Figure 1d, the maximum values were obtained after 45 days of incubation, which corresponds well with the results obtained with the IVIS. By using this technique, the growth of diatoms can be evaluated very quickly, without preliminary sample preparation, which would affect the integrity of the cells and sterility flask with culture.

Diatoms exhibit strong photoacoustic signals owing to the presence of chlorophylls and carotenoids that absorb very well the 532 nm wavelength upon illumination [9]. Figure 2a shows the images of diatom-agarose droplets obtained after 7, 28, 45, 60, 90, and 100 days of cultivation using the RSOM system. The dependence of the mean pixel intensity signal on the incubation time is illustrated in Figure 2b, where red color corresponds to a low-frequency signal emitted by larger samples. In contrast, a high-frequency signal, shown with green bars, is emitted by smaller structures. The highest intensity of the photoacoustic response was achieved after 45 days of cultivation. Before mixing with agarose, cells in DM medium have to be properly shaken. However, we can observe that this diatom species tends to aggregate due to secreted mucilage, which explains why the red signal at 11-33 MHz is higher.
Figure 2. (a) RSOM images of *A. sibiricum* diatoms mixed with agarose gel at 11-99 MHz, 11-33 MHz (red), 33-99 MHz (green) after 7, 28, 45, 60, 90, and 100 days of cultivation. Scale bar: x, y-axis – 0.5 mm. (b) The dependence of mean pixel intensity on the cultivation time, (c) Absorbance spectra collected at different periods of cultivation, (d) Comparison of absorbance at 523 nm and mean pixel intensities at 11-33 MHz and 33-99 MHz, depending on the cultivation time.

The absorbance spectra shown in Figure 2c demonstrate peaks attributable to chlorophyll *a* and *c* and the carotenoid fucoxanthin. By comparing the absorbance and photoacoustic signals in two frequency ranges (Figure 2d), we can conclude that they are in good agreement. Thus, the spectroscopic characterization confirmed the results obtained using the IVIS and RSOM imaging systems and proved their capabilities in assessing the growth of diatoms. As shown in our previous study [10], photoacoustic and fluorescence visualization techniques are beneficial for monitoring the life cycle of small, fast-growing cultures that tend to grow clusters and colonies, which interferes with cell counting. The IVIS fluorescence tomograph allowed us to measure the total amount of chlorophyll in the volume without disrupting cell integration, that may be affected in other measurements by selecting aliquots. The cell counting under the microscope is preferable at the initial stage, while for long-term batch culturing, growth can be monitored using the proposed photonic approaches based on chlorophyll absorbance and fluorescence.

4. Conclusions
In this work, we used diatom *A. sibiricum* cultivated in the specially designed incubator and monitored their growth using the IVIS fluorescence imaging system and RSOM technique. The fluorescence and photoacoustic measurements showed that the highest intensity values in both cases were obtained after 45 days, indicating the end of the exponential phase, which was also confirmed by spectroscopic methods. These photonic tools based on the absorption and fluorescence of diatom chromophores allowed us to quickly and successfully evaluate the growth of diatoms, which expands the possibilities of cultivation not only in laboratory conditions but also in aquaculture and bioreactor practice.
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6. References
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