Fluorescence Properties of Chlorella sp. Algae

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Abstract. *Water quality and its fast and reliable monitoring is the challenge of the future. Design of appropriate biosensors that would be capable of non-invasive identification of water pollution is an important prerequisite for such challenge. Chlorophylls are pigments, naturally presented in all plants that absorb light. The main forms of chlorophyll in algae are chlorophyll a and chlorophyll b, other pigments include xantophylls and beta-carotenes. Our aim was to characterize endogenous fluorescence of the Chlorella sp. algae, present naturally in drinking water. We recorded spatial, spectral and lifetime fluorescence distribution in the native algae. We noted that the fluorescence was evenly distributed in the algae cytosol, but lacked in the nucleus and reached maximum at 680–690 nm. Fluorescence decay of chlorella sp. was double-exponential, and clearly shorter than that of its isolated pigments. For the first time, fluorescence lifetime image of the algae is presented. Study of the fluorescence properties of algae is aimed at the improvement of water supply contamination detection and cleaning.*

Keywords

Chlorella sp., chlorophylls, confocal microscopy, FLIM fluorescence lifetime spectroscopy.

1. Introduction

Chlorophylls are pigments naturally present in all plants that absorb light. As highly conjugated compounds, they absorb in wide-range, from ultraviolet, via visible to infrared light [1]. Two to three percent of the absorbed sun energy is then re-emitted from the pigment system as the fluorescence. Light energy, captured in the form of radiation, is subsequently - in a series of transfers to other molecules and complexes - converted into chemical energy in the form of ATP. The main forms of chlorophyll in plants, including algae, are chlorophyll a and chlorophyll b, derived from protoporphyrins [2]. In the chemical sense, chlorophylls are tetrapyrol rings of porphyrin, chlorine, or bacteriochlorine, characterized by the fifth isocyclic ring that is biosynthetically derived from the C-13 propionic acidic side chain of protoporphyrin [3], characterized by the presence of a central atom of magnesium Mg^{2+}. Algae exhibit strong autofluorescence from photosynthetic pigments, namely chlorophyll a-d, phycobilins and carotenoids, the emission properties of which vary dependently on metabolic activities and physiological state of algae [4].

Absorption and emission properties of plant natural pigments are well described [1], [2] and [3]. Lately, time-resolved techniques proved to be valuable for evaluation of endogenous fluorophores and their sensitivity to the environment, as fluorescence lifetimes are independent on the fluorophore concentration, but react to changes in local chemical environmental conditions, namely oxygen saturation, or binding [5]. Evaluation of the fluorescence lifetime properties in algae can therefore serve as non-invasive sensor of their physiological state.

Chlorella, as most algae, is famous for removing heavy metal and other synthetic toxins from the body, and/or from its natural source (lake, pond, or swamp). Chlorella sp. can therefore serve for identification of water pollution and its cleaning. Consequently, it can be employed for designing optical biosensors used for monitoring of water pollution, e.g. presence of toxins, herbicides, etc. [6].

The aim of this study is to characterize properties of the endogenous fluorescence in the Chlorella sp. green algae, naturally present in drinking water and compare them to isolated pigments. Advanced microscopy and spectroscopy methods are employed to obtain spatial, spectral and/or lifetime distribution of the endogenous fluorescence in the algae.

2. Material and Methods

2.1. Preparation of the Chlorella sp.

Chlorella sp. was obtained from the Faculty of Natural Sciences, University of SS. Cyril and Methodius in Trnava collection of the green algae. The green algae of genus Chlorella sp. were previously isolated from the main drinking water supply. Green algae were cultivated in Hoagland cultivation medium [7].

2.2. Isolation of Pigments from Chlorella sp.

Pigments were extracted from Chlorella sp. algae. After a centrifugation at 45000 rpm for 15 minutes, pellets were dehydrated, and then crushed with the sea sand. Pigments were extracted with n-hexane, and resulting components were divided using silica gel chromatography in the solution of n-hexane: acetone (7:3) [2]. Resulting bands were separated according to colours (Fig. 1). A Blue-Green (BG), a Yellow-Green (YG) and a Yellow (Y) band, representing the chlorophyll a, the chlorophyll b and the carotenoids respectively [2], were dissolved in the DMSO and used for comparison.

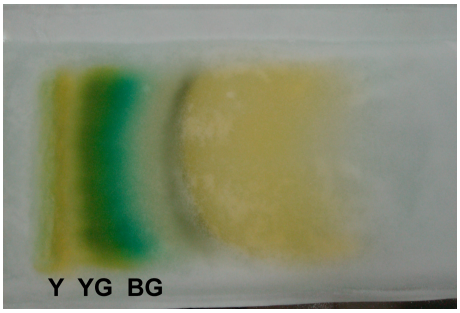


Fig. 1: Extracted Yellow (Y), Yellow-Green (YG) and Blue-Green (BG) bands separated by gel chromatography.

2.3. Confocal Microscopy Imaging and Spectroscopy

Confocal images of the algae autofluorescence were gathered with a laser scanning confocal microscope, equipped with LSM 510 META detector coupled to Axiovert 200 inverted microscope, employing C-Apochromat 40×, 1.2 NA objective (all Carl Zeiss, Germany). Algae were excited with 632 nm laser (Lasos Lasertechnik), using a 16 channel META detector. For spectrally-resolved microscopy measurements, data were recorded by META detector in the range of 650 nm to 740 nm with an 11 nm step.

2.4. Time-Correlated Single Photon Counting (TCSPC) Measurements

Time-Correlated Single Photon Counting (TCSPC) method was applied to measure fluorescence decays of Chlorella sp. and isolated pigments. Fluorescence decays were detected at room temperature in a cuvette after excitation by 635 nm picosecond laser diode BHL-635 (output power < 0.5 mW, pulse width 50 ps, pulse frequency 50 MHz) using SPC-130 TCSPC card (both Becker & Hickl, Germany). Fluorescence was detected by photon counting, using a PMC-100 detector (Becker& Hickl, Germany) after passing through a spectrograph (monochromator PRA B102 Photochemical Research Associates, Canada). To achieve spectral resolution, fluorescence decays were recorded at individual wavelengths from 640 to 740 nm with a 10 nm step.

2.5. Fluorescence Lifetime Imaging Microscopy (FLIM)

FLIM images were recorded using TCSPC technique coupled to the confocal microscope. In these experiments, a 475 nm picoseconds laser diode (BDL-475, Becker& Hickl, Germany) was used. The laser beam was reflected to the sample through the epi-fluorescence path of the LSM 510 META microscope (Zeiss, Germany) with C-Apochromat 40×, 1.2 NA lens. The emitted fluorescence was separated from laser excitation using LP 500 nm filter and detected by HPM 100-40 photomultiplier array (Becker&Hickl, Germany) employing SPC-830 TCSPC imaging board.

2.6. Data Analysis

Confocal data were visualized by ZEN 2011 software (Zeiss, Germany). FLIM images were processed using proprietary software packages SPCImage (Becker & Hickl, Germany), fitted by up to a three-exponential decay to gain $\chi^2 \leq 1.3$. Results were visualized as a map and as a distribution of calculated fluorescence lifetimes.

3. Results

In this work, we aimed to identify fluorescence properties of endogenous fluorescence in Chlorella sp. Although capable of absorbing light from visible up to infrared regions [1], for identification of the algae' endogenous fluorescence we have primarily chosen the 632–635 nm wavelength for excitation, which is harmless for work with living organisms.

3.1. Confocal Microscopy Imaging and Spectroscopy of Endogenous Fluorescence in Chlorella sp.

Our first aim was to identify spatial and spectral distribution of endogenous fluorescence after excitation at 632 nm using confocal microscopy imaging. As expected, we observed that algae had round shape with diameter of around 10–15 μm (Fig. 2), exhibiting bright fluorescence in the red spectral region.

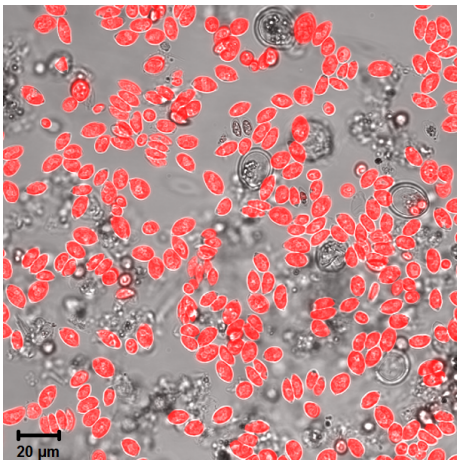
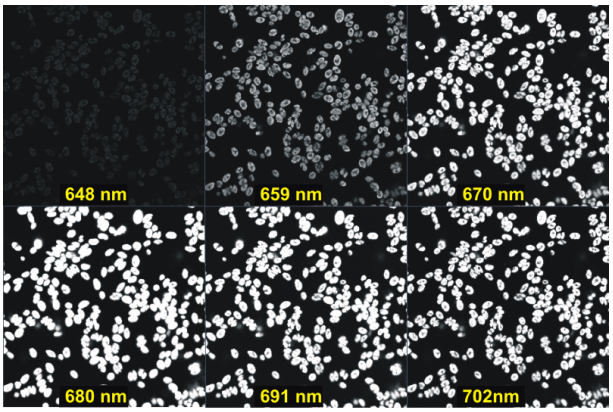
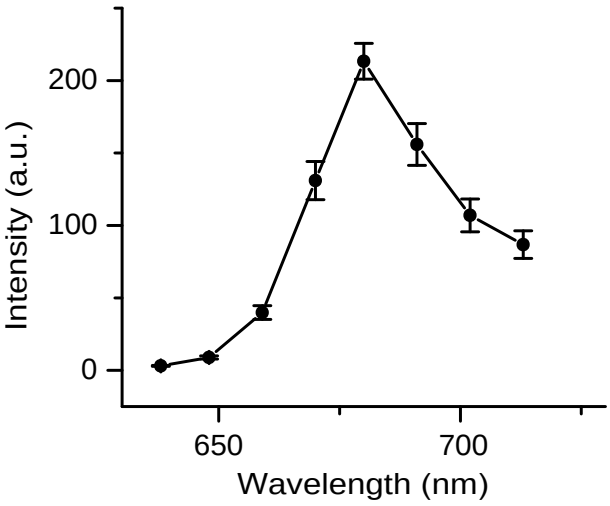


Fig. 2: Transmission and fluorescence image of Chlorella sp., exc. 632 (nm), LP 650 (nm), Scale: 10 (μm).

Spectrally-resolved images were recorded at individual wavelengths (Fig. 3(a)) and uncovered maximum fluorescence at around 680 nm (Fig. 3(b)).



(a)



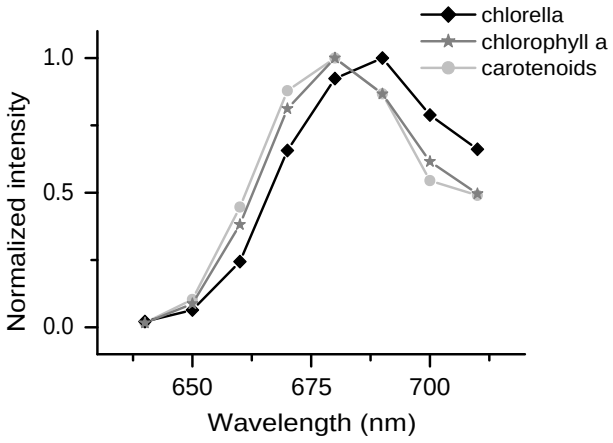
(b)

Fig. 3: Confocal microscopy spectra of Chlorella sp., exc. 632 (nm), emission 648–713 (nm), step 11 (nm). Fluorescence images at individual wavelengths (top, wavelength number in yellow). Fluorescence spectra (bottom), mean ±SEM, $n = 7$.

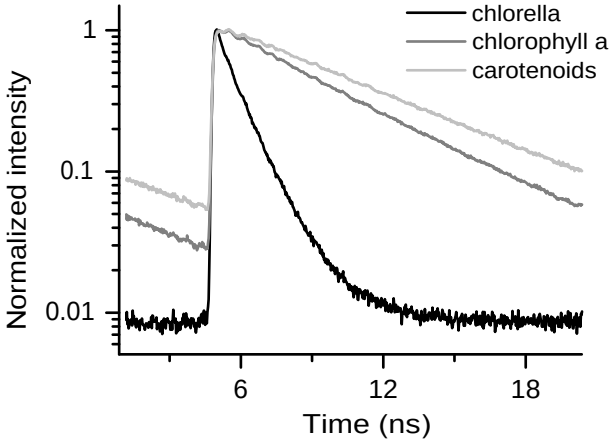
3.2. Fluorescence Lifetimes of Chlorella sp.

Our second aim was to evaluate fluorescence spectra together with fluorescence lifetimes of endogenous fluorescence in Chlorella sp. TCSPC method was applied to measure fluorescence decays of Chlorella sp. Fluorescence signal was recorded at individual wavelengths from 640 to 740 nm. We noted that Chlorella sp. had maximum emission at 690 nm (Fig. 4(a)). Gathered fluorescence decay (Fig. 4(b)) was best fitted with a 2-exponential decay fit. Shorter fluorescence lifetime reached between 500–900 ps, the longer one between 1300–1900 ps (Fig. 4(c)). With increasing wavelength, some decrease in the fluorescence lifetime was noted.

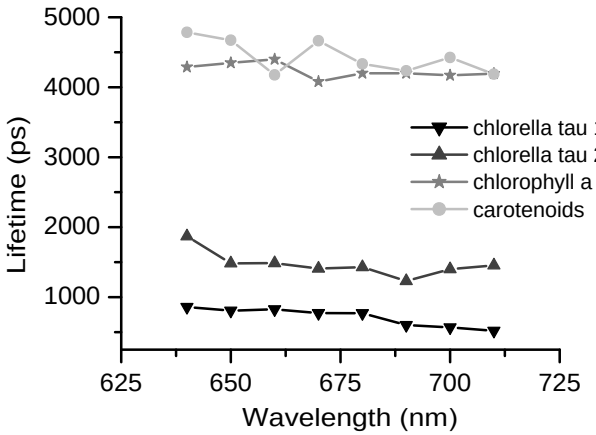
Gathered results were then compared to fluorescence spectra and lifetimes of pigments isolated from Chlorella sp. Isolated pigments showed maximum spectra at 680 nm, which was blue-shifted of about 10 nm when compared to the spectrum of the Chlorella sp. (Fig. 4(a)). Analysis by a mono-exponential decay of the chlorophyll band in DMSO showed fluorescence lifetimes between 4800–5300 ps, while that of the carotenoid band was longer, between 4700–4800 ns (Fig. 4(b) and Fig. 4(c)). Data gathered for the chlorophyll band are in agreement with previously published results for chlorophyll a in ether, which was 4.9 ns [8]. Data showed significantly lower fluorescence lifetime in native Chlorella when compared to isolated pigments.



(a) Emission intensity.



(b) Normalized fluorescence decay at emission 700 (nm).



(c) Fluorescence lifetimes.

Fig. 4: Comparison of total photon counts of the fluorescence of Chlorella sp., chlorophyll a and carotenoids, excited at 635 (nm).

3.3. Fluorescence Lifetime Imaging of Chlorella sp.

Our last goal was to compare distribution of the fluorescence lifetimes in native algae, using FLIM. This unique approach allowed us to gather images of the lifetime distribution in individual algae with spatial resolution. FLIM images were recorded using TCSPC with 475 nm excitation and LP 500 nm emission. Fluorescence lifetimes were recorded from 150 to 2000 ps. Most algae showed short lifetime up to 200 ps, while some also exhibited lifetimes under 500 ps (Fig. 5). At the same time, we noted differences in the lifetime distribution within individual algae.

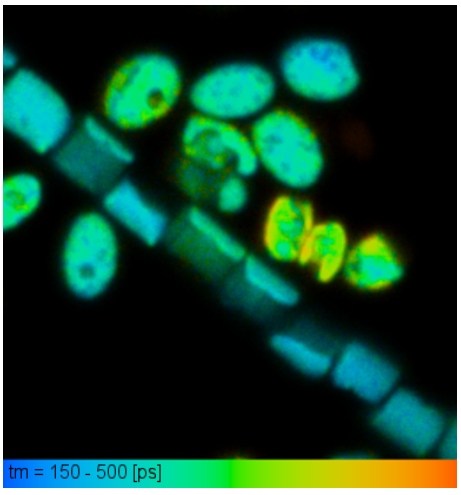


Fig. 5: FLIM image of Chlorella sp., ex. 475 (nm), LP 500 (nm), with mean lifetime 150–450 (ps) blue-red, Scale: 10 (μm).

4. Discussion

The aim of this study is to characterize fluorescence properties of endogenous fluorescence in the Chlorella sp. algae, and compare them to that of their pigments. Gathered data showed endogenous fluorescence of the algae peaking at 680–690 nm. This is in agreement with previously observed maximum value of 683 nm with half-width of about 20 nm [9], and/or the

660–680 nm peak range of fluorescence in green algae [10]. When compared to isolated pigments, fluorescence spectra were red-shifted of about 5–10 nm.

Fluorescence lifetimes of pigments, namely chlorophyll a, gives information about the primary photophysical events in photosynthesis [11]. Fluorescence of Chlorella sp. had double exponential decay with shorter fluorescence lifetime around 500–900 ps and longer one around 1300–1900 ps. Others demonstrated five exponential components in Chlorella, namely 53 ps, 89 ps, 174 ps, 535 ps and 1200 ps [10]. Our data are in agreement with the two longest lifetimes. In addition, FLIM recording uncovered the presence of a lifetime under 200 ps (blue at Fig. 5). However, applied methods did not allow us to record lifetimes at a picoseconds scale.

Importantly, obtained lifetimes in native Chlorella were much shorter than lifetimes of its isolated pigments that reached values longer than 4000 ps. This is expected, taking into consideration differences of the chlorophyll surroundings in live cells vs. in an artificial environment. We previously demonstrated [12], in agreement with others [1], longer fluorescence lifetime of the chlorophyll a compared to the chlorophyll b. Consequently, fluorescence lifetimes of all isolated pigments were clearly longer than endogenous fluorescence of algae. Further experiments are necessary to link these in vitro experiments to the data recorded in cells. Also, in the future, it would be interesting to resolve shortest lifetime components from individual algae using more advanced signal and data processing.

Overall, we can summarize that time-resolved endogenous fluorescence is a useful tool for monitoring the state of living systems and its changes due to modification of the cell environment [13]. In this work, we present, for the first time, the FLIM of the green algae, with distinct lifetimes, suggesting differences in their state. Further work is necessary to understand sensitivity of the recorded lifetimes to changing environment.

5. Conclusion

Understanding fluorescence characteristics of endogenous fluorescence of algae and characterise its changes in different environments can help us to design appropriate monitoring systems, e.g. biosensors, capable of non-invasive identification of the algae presence and its state. This knowledge is valuable for evaluation of water pollution, allowing reliable monitoring of water quality and its cleaning, as well as better comprehension of the efficient solar energy capture mechanisms.

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