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Studying Cyanobacteria by Means of Fluorescence Methods: A Review

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Abstract

Self-fluorescence is a powerful tool for investigation of living photosynthetic microorganisms. Since the physiological state of such microorganisms is closely related to the operation and activity of photosynthetic system; thus, any variations in spectroscopic properties of their self-fluorescence indicate the changes in their physiological state. Cyanobacteria (or blue green alga) are one of the most widespread photosynthetic organisms in nature, and the ecological aspect in their investigation is quite valuable. On the other hand, thousands of strains belonging to different cyanobacterial species are cultivated in biolaboratories all over the world for different biotechnological applications such as biofuel cells, food production, pharmaceuticals, and fertilizers. Thus, the novel noninvasive methods of their investigation are quite important for on-line monitoring of cyanobacterial cultures. In this chapter, several fluorescence techniques are presented for investigation of living cyanobacterial cells and cultures.

Keywords: cyanobacteria, photosynthesis, fluorescence spectroscopy, confocal microscopic spectroscopy, fluorescence induction, flow cytometry, remote sensing

1. Introduction

In the last years, the investigation of cyanobacteria attracts a considerable attention due to their environmental and biotechnological importance. New applications of cyanobacterial communities appear in biotechnology (i.e., production of biofuel, agricultural fertilizers, pharmaceuticals, and food additives ultraviolet protection compounds) [1–5] and biosensing [6–9]. Large-scale industrial production of the cyanobacterial products requires optimization and more detailed control of incubation conditions in order to increase productivity. On the other hand, the ecological problem of harmful bloom monitoring and control makes an important contribution in this rising interest to cyanobacteria, as to one of the main indicator organisms of the environment pollution and to methods of their investigation [10–18]. Obviously, future research in this field will be focused on the improvement of different treatments that inhibit harmful growth; thus, the development of novel noninvasive methods for on-line monitoring of cyanobacterial communities *in vivo* is quite needed. The presence of a photosynthetic apparatus similar to higher plants makes microalgae and cyanobacteria suitable for use in biosensors based on monitoring changes in photosynthesis and other bioenergetic processes in the cell [19–21].

A vast amount of work was done to achieve a present insight into the physiological processes, which rules cyanobacteria life, and their genetic background [10–18]. Nowadays, it was clearly shown that there is a universal genetic response to most abiotic stressors (heat, osmotic, saline, acid stress, strong light, and ultraviolet radiation) [22]. Moreover, this universal response is closely related to the photosynthetic activity of cyanobacterial cells [23]. This fact gives a unique opportunity for operational in-line monitoring (including remote sensing) of the physiological state of photosynthetic microorganisms in variable environmental conditions. Since most photosynthetic pigments possess self-fluorescent properties, thus, the emission spectral composition and fluorescence kinetics can provide important information about photosynthetic activity of living cells and reveal any variations in their physiological state under the influence of external factors.

All fluorescence methods can be divided in to two groups by their degree of organism impact. First group deals with *in vivo* investigations of living cells. Such methods should be noninvasive and very sensitive to study the life as it is, in dynamics of growth and adaptation. The second group deals with dead material. By these methods, chemical, morphological and genetic structure of the cell can be studied in different soluble and nonsoluble chemical components. In this chapter, we will be interested in the first ones. The most naturally suitable and appropriate methods of investigation of photosynthetic organisms are to use the self-fluorescence of photosynthetic apparatus. In this sense, following *in vivo* and *in situ* methods can be suggested: fluorescence spectroscopy (steady-state fluorescence) [24, 25], chlorophyll fluorescence induction analysis (fast and slow kinetics) [26, 27], pulse amplitude-modulated fluorometry (chlorophyll fluorescence quenching) [28, 29], delayed fluorescence [30, 31], flow cytometry [32, 33], confocal microscopic spectroscopy [34, 35], and remote sensing (passive and active, e.g., fluorescence LIDAR technique) [36, 37].

Two main aspects in studying cyanobacteria usually are interested both ecologists and biotechnologists: physiological state of cyanobacteria on population and cell level, and biological diversity of the considered community. Fluorescence methods can successfully solve both problems.

2. Peculiarities of cyanobacterial photosynthetic apparatus

Photosynthetic system of cyanobacteria, in contrast to green algae and higher plants, contains the external membrane light-harvesting complexes. The antenna complex for photosystem II (PSII) and photosystem I (PSI) is extrinsic and is formed as a large multiprotein organelle (phycobilisome), which is located on the stromal side of the thylakoid membrane. The detailed description of the morphology, structure, chemical, and optical properties of light-harvesting complex of cyanobacteria, detached phycobilisomes, and phycobilins can be found in numerous publications [10–15, 38–40]. Here, we only pointed out several main features of its self-fluorescence spectrum that are essential for further discussion.

Phycobilisomes (PBSs) are assembled from 12 to 18 different types of polypeptides, which may be grouped into three classes: (1) phycobiliproteins, (2) linker polypeptides, and (3) PBS-associated proteins. And only first ones bear chromophores and form fluorescence response together with chlorophyll *a* (Chl *a*) of PSII and PSI. PBSs are constructed from two main structural elements: a core substructure and peripheral rods that are arranged in a hemidiscoidal fashion around that core (**Figure 1**). During the energy transfer process through PBS to PSII and PSI, the occasional quenching of the absorbed light by fluorescence

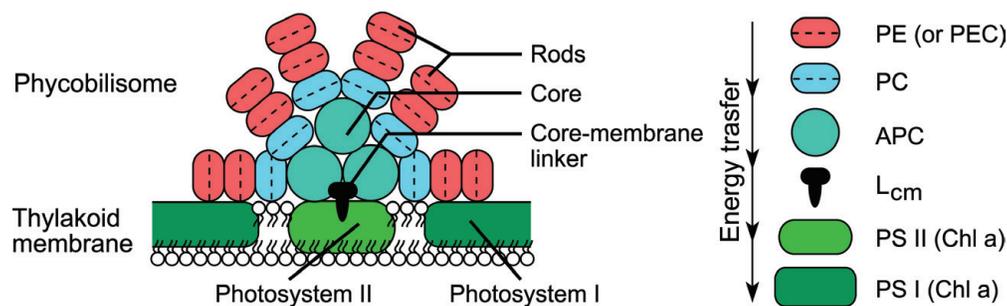


Figure 1.
 Schematic drawing of photosynthetic apparatus and energy transfer in cyanobacteria.

can occur and this is the essential property for fluorescence spectroscopy. Both steady-state and time-resolved fluorescence measurements are used for probing the structure and functioning of photosynthetic system of cyanobacteria via this specific fluorescent response.

The fluorescence of intact living cyanobacterial cells is originated from the efficiency of the energy transfer between all components of the energy transfer chain including the final step, the delivery to PSII or PSI (**Figure 1**). Each transfer step results in the spectrum shape as a peak or shoulder [41, 42]. This is due to the fact that in the course of the energy transfer from the initially photoexcited phycobiliprotein to the reaction center of photosystems PSI and PSII; fluorescence is emitted from almost every type of pigment and can be used as a probe to examine the mechanism of energy transfer within the light-harvesting system and between two photosystems [30, 38–40]. To monitor this energy transfer process, a sample should be irradiated with the light that selectively absorbs by one set of pigments and then the fluorescence, originated from another set of pigments, should be recorded. This type of fluorescence excitation experiment can be used to measure the efficiency of energy transfer from one set of pigments to another, which reflects the species-specific features, if cyanobacteria are in the optimal physiological state. On the other hand, under any external actions this fluorescence “finger prints” will change according to mechanisms of the cyanobacterial response on this action, and the changes in steady-state fluorescence and its kinetics finally can reveal these mechanisms.

As it was mentioned previously, a vast amount of fluorescence techniques were elaborated to study physiological state and biological diversity of photosynthetic organisms, but not all of them can be directly applied to cyanobacteria. Several crucial features of their fluorescence response lead to the necessity of changes in conventional techniques; otherwise, it will lead to insufficient or wrong results. To demonstrate these features of cyanobacteria, we present here several simple but representative examples, which show specific fluorescence response of cyanobacteria compared to green microalgae *Chlorella ellipsoidea* Gerneck CALU 3. It should be mentioned here that the fluorescence response of green microalgae is quite similar to higher plants one, due to the similarity of the structure of their photosynthetic apparatus.

In **Figure 2**, three sets of fluorescence spectra, recorded via standard lambda-scanning procedure of confocal laser scanning microscope (CLSM) Leica TCS-SP5, and microphotographs of corresponding species, obtained simultaneously in fluorescence and transmission detection channels, are presented. The excitation wavelengths, corresponding to the emission lines of three CLSM lasers, are indicated over the curves (405, 458, 476, 488, 496, 514, 543, and 633 nm). It is well seen that depending on the excitation wavelength, the room temperature fluorescence emission spectrum of the intact cyanobacterial cells exhibits various extents of

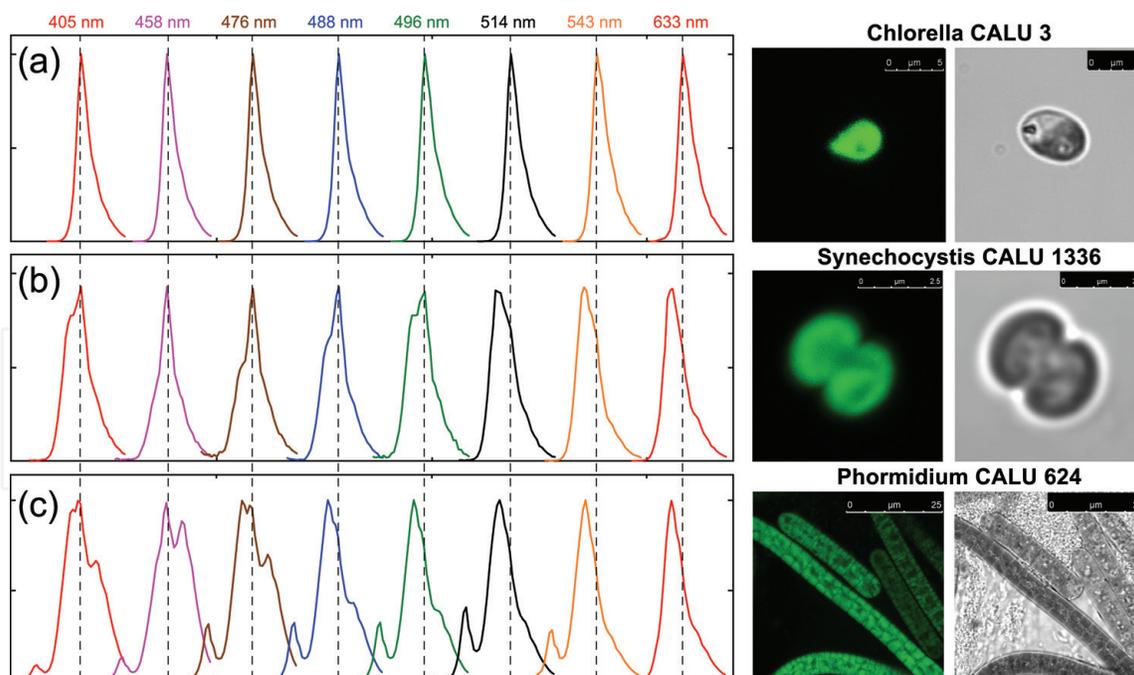


Figure 2.

Three characteristic sets of CLSM single-cell fluorescence spectra and microphotographs of corresponding strains, obtained simultaneously in fluorescence and transmission detection channels. (a) *Chlorella ellipsoidea* Gerneck CALU 3; (b) *Synechocystis aquatilis* CALU 1336; (c) *Phormidium favosum* str. Marvan CALU 624. All spectra are normalized to the maximum intensity and shifted along x-axis for convenience of observation. Corresponding excitation wavelengths are given over the curves. Dashed lines indicate wavelength of Chl a fluorescence maximum at 682 nm. The white bar corresponds to 5, 2.5, and 25 μm , respectively.

contribution of phycobilisome emission to the spectrum, opposite to green algae. If one exclusively excites chlorophyll *a* fluorescence, using a 458-nm excitation wavelength, the emission spectrum of cyanobacterial cells shows no appreciable emission of phycocyanin or allophycocyanin. It is due to the fact that, in cyanobacteria, the 458-nm excitation wavelength is preferentially absorbed by PSI and PSII, bypassing photosynthetic pigments of light harvesting complex (LHC). The excitation by intermediate (blue and green) wavelengths (405, 476, 488, and 496 nm) reveals fluorescent maxima of all photosynthetic pigments, belonging to the corresponding species, since the light in this range is absorbed by all pigment-protein complexes almost in equal portions and fluorescence emits by all steps of energy transfer chain. The direct excitation of cyanobacterial cells in the phycocyanin absorption region at 514 and 543 nm results in emission spectrum with two main peaks at 580 and 656 nm, which are due to phycoerythrin, phycocyanin, and allophycocyanin emission. The spectra for 633-nm excitation wavelength directly give a prominent emission band at 656 nm that originates from phycocyanin, omitting the band at 580 nm, which cannot be excited by 633 nm. Other small emission bands, corresponding to fine pigment structure of antenna complex, are not resolved at the room temperature investigation.

Opposite to cyanobacterial species, green algae demonstrate poor diversity in the emission spectra (**Figure 2(a)**). Comparative analysis of the series of fluorescence spectra for different cyanobacterial species and strains (**Figure 2(b)** and (c)) reveals visible variations in their shape, and thus, species/strains differentiation (so-called ataxonomic analysis) could be carried out [41, 42]. Moreover, this diversity in fluorescence responses on different excitation wavelength in cyanobacterial cells shows that all fluorescence techniques based on the analysis of chlorophyll *a* fluorescence, developed for higher plants (pulse amplitude modulation technique, flow cytometry, remote sensing, etc.), should be strongly modified for studying cyanobacteria.

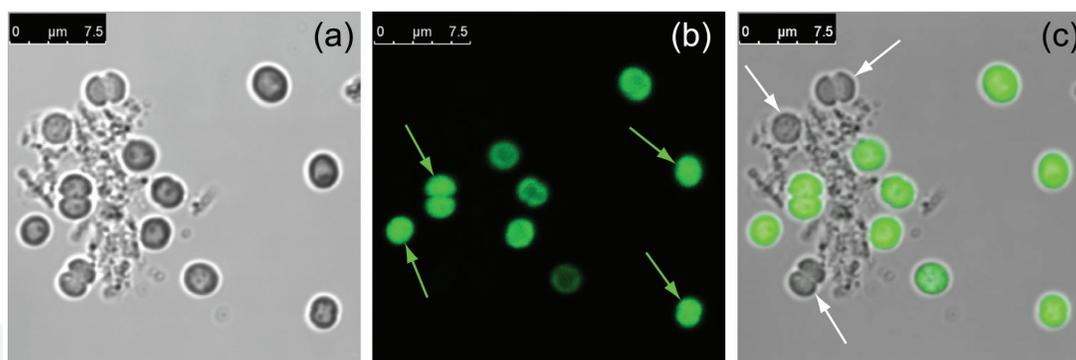


Figure 4. CLSM micrographs of cyanobacterium *Synechocystis aquatilis* CALU 1336, visualized with CLSM. (a) Transmission image, (b) fluorescence false-green channel represents the response from phycobilins and Chl a at 650–680 nm, (c) represents an overlap of fluorescence and transmission channels. Scale bar = 7.5 μm .

Figure 4 illustrates several cells of unicellular cyanobacterial culture *Synechocystis aquatilis* CALU 1336. Diverse physiological states are observed and indicated with arrows. Small fluorescent signal corresponds to healthy cells. Cells in frustrated physiological state (dying cells) have a high fluorescence and are indicated by green arrows. Dead cells have no fluorescence and can be observed only in transmission channel. They are indicated with white arrows.

Here, we have demonstrated only two examples of the differences between cyanobacterial and green-algae fluorescent response. Below some more cases will be considered concerning specific fluorescence techniques.

3. Fluorescence methods to study cyanobacteria

As it was mentioned previously, two main properties of cyanobacterial communities are of great interest during the investigation and control: physiological state (or viability) and biological diversity. And the fluorescence measurements contribute greatly to the study of these issues.

Historically, since the 1960s, researches had noted that changes in physiological state of cyanobacterial cell, occurring when it is grew old or is treated with different external actions, manifest themselves as corresponding changes in the intrinsic self-fluorescence spectrum [43]. Several studies have shown that changes in the pigment fluorescence are associated with the changes in the enzymatic activity of the cell and an increase in the permeability of the cell membrane, which can be used as an indicator of aging for cyanobacterial species [41, 44]. Today, there is no doubt that the *in vivo* analysis of fluorescence characteristics of photosynthetic apparatus is a powerful tool for studying the impact of a wide variety of environmental factors on cyanobacteria [24, 45]. The changes in the steady-state and variable fluorescence reflect the in-time physiological state of cyanobacterial communities, exposed to different external factors [23]. The environmental changes cause the changes in bioenergetic processes occurring in cyanobacterial cells; they significantly affect the kinetics parameters and spectral features of the intrinsic fluorescence spectra. Since early 1980s, chlorophyll fluorescence kinetics has been widely used for investigation of viability of eukaryotic plants [28, 46]; however, the specific features of cyanobacteria only recently began taking into account. Therefore, in some earlier works, it was reported that the results of chlorophyll fluorescence measurements in cyanobacteria did not reflect the condition of photosynthesis at all [29, 47–49].

The fluorescence spectra have been widely used to classify phytoplankton populations since approximately early 1970s [50]. However, because of the generally low

number of available excitation wavelengths in the conventional devices, the rate of species discrimination was relatively low. Researches can separate only algal groups that differ greatly in the structure of the photosynthetic apparatus (e.g., cryptophytes, chlorophytes, and cyanobacteria) but cannot separate groups that are more similarly pigmented (e.g., among cyanobacterial species) [51, 52]. Discrimination between similarly pigmented taxa or even between species within a taxon requires high-resolution and multi-wavelength spectral analysis to reveal small peculiarities in configuration and functioning of photosynthetic system. The rigorous discrimination is possible if the inter-species differences are greater than those within a species. For cyanobacteria, the spectral features of fluorescence, emitted by single cells *in vivo*, depends only on the structure and operational effectiveness of photosynthetic apparatus; thus, they reflect the individual characteristic of cyanobacterial strain and can be used for confident differentiation up to strain level [42]. The possible contribution of environmental adaptation effects to the resulting fluorescence spectra can be minimized by an accurate definition of the corresponding spectral regions of the excitation and emission wavelengths. Recent progress in CLSM methods allows to get spectroscopic data from single cells, instead of the whole population (like in conventional fluorescence spectroscopy); thus, the external interference has a minimal effect on the resultant spectrum.

Several fluorescent methods for *in vitro* and *in situ* investigations will be considered in this part, and the specific features of their use for studying cyanobacteria will be outlined.

3.1 Fluorescence spectroscopy

Steady-state fluorescence spectroscopy can solve many routine problems of limnology and oceanology in a noninvasive manner. For example, the gravimetrically measured dry weight is an invasive method that is unsuitable for monitoring growth over time in the same sample; however, fluorescence methods can successfully replace it by applying calibration curves for *in vivo* chlorophyll *a* fluorescence of different cyanobacteria species [53]. This is the only one simple example of growth rate measurement. In more complicated cases, like monitoring of physiological state or biological diversity of cyanobacterial communities, fluorescence spectroscopy can become the only possible noninvasive method of investigation.

Opposite to the absorption spectra, the *in vivo* fluorescence spectra are much more informative. Moreover, it was shown in [54, 55] that the fluorescence assay would be a preferred alternative to absorbance in quantifying cyanobacterial growth because the fluorescence measures only viable cells and absorbance measures total cell biomass (including dead cells). Fluorescence spectroscopy is a highly sensitive, nearly instantaneous, noninvasive way to study various components and processes *in situ* and *in vivo*. Although the fluorescence spectra contain the information only about photosynthetic apparatus of different algal groups, they include the information about the chemical structure of light harvesting complex (LHC) and accessory pigment-proteins, as well as about the character of links between pigment-protein complexes and the efficiency of energy transfer in the light-harvesting process. When compared with absorption, fluorescence is affected by the excitation wavelength and energy. Thus, the use of different excitation wavelengths can provide more detailed information for the study of single-cell composition (**Figure 5**).

As it was mentioned previously, the specific features of fluorescence measurements of cyanobacterial communities, based on their initial fluorescence, lay mostly in the peculiarities of their photosynthetic apparatus, in particular, of the light-harvesting antennae. The distinct set of photopigments makes cyanobacteria

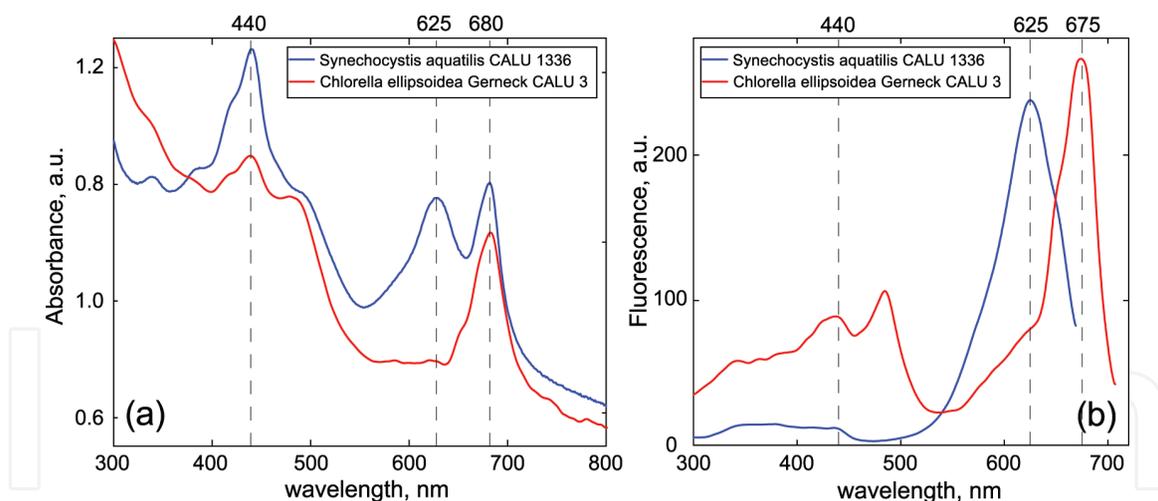


Figure 5.

Absorption (a) and fluorescence excitation (action) spectra (b) for *Synechocystis aquatilis* CALU 1336 (blue line) and *Chlorella ellipsoidea* Gerneck CALU 3 (red line).

an ideal laboratory object for various biological investigations. Fast adaptation and understandable fluorescence response on the environmental changes allow using cyanobacteria as an indicator organism in ecological studies and as a laboratory object in biotechnological applications. However, the structure and functioning of their photosynthetic apparatus differ drastically from eukaryotic plants (**Figure 5**). Thus, the attention should be paid while applying fluorescence methods, elaborated for higher plants, to cyanobacteria and *vice versa*, during the application of the results of cyanobacterial studies to green algae. Let us mention several specific features that should be kept in mind while working with cyanobacteria.

The problem of registration and control of the physiological state of natural communities and the viability of cultures during incubation is a primary task in both ecological and biotechnological fields of application of cyanobacteria. In cyanobacteria, the main pigments responsible for photosynthetic activity are phycobilins; therefore, the methods associated with the analysis of only chlorophyll fraction do not give satisfactory results in the study of cyanobacterial cultures [56–58]. Moreover, the analysis of the fraction of water-soluble phycobilins may give the ambiguous information, due to debris of disrupted cells in the culture medium. The former gives underestimated results, and the latter, overestimated results. Thus, the analysis of the viability of living cyanobacterial cells should be carried out considering a set of fluorescence spectra with different excitation wavelength, to elucidate the impact of each photosynthetic pigment [41, 44, 49]. The examples of one-excitation-wavelength studies are also present in the literature [59, 60]. However, the conclusions made on the basis of such investigations are questionable.

The investigation of phytoplankton communities and the identification of individual algae species, in the presence of different photosynthetic pigments, are completely impossible via one or two fluorescence spectra. Here, only a set of fluorescence spectra with different excitation wavelength can give a sufficient accuracy (see e.g., [42, 61]).

In the last years, so-called excitation-emission fluorescence matrices are often used in investigations of the effect of pollutants on natural algae communities [24], as well as in biological diversity studies [25, 62, 63]. Some researches even develop submersible devices to carry out such fluorescence study *in situ* [64].

3.2 Chlorophyll fluorescence kinetics

A vast amount of works are devoted to the investigation of chlorophyll fluorescence induction (fast and slow kinetics) and chlorophyll fluorescence quenching

analysis via saturation pulse method in green algae and higher plants, but only few of them concerns studies of cyanobacteria [28, 29, 47, 65–67]. Here, we will be interested in the peculiarities of using this technique for studying cyanobacteria. Therefore, we will not discuss here theoretical basics but only pointed out some important features of fluorescence kinetics in cyanobacterial photosynthetic apparatus.

In numerous reviews, the theoretical investigations of chlorophyll *a* (Chl *a*) fluorescence induction in different photosynthetic organisms are presented [26, 27, 65–69]. Basically, three classes of processes modulate Chl *a* fluorescence: photochemical quenching [q(P)], nonphotochemical quenching [q(N)], and fluorescence lowering or fluorescence increase due to state 1 to 2 and state 2 to 1 transitions, respectively. In addition at high actinic light excitation, Chl *a* fluorescence can be subject to various photoinhibitory quenching processes q(I), which are much slower than the previous three.

Differences in the interpretation of cyanobacterial fluorescence induction start from the first step. First of all, the dark adaptation shifts plants and algae toward light state 1 (enlarged PS II antenna, high fluorescence state), whereas in cyanobacteria, dark adaptation shifts toward light state 2 (enlarged PS I antenna, low fluorescence state). This leads to discrepancy in interpretation of the JIP-test results like very small rise of OJIP fluorescence, compared to eukaryotic plants (see **Figure 6**) and very large rise of PSM fluorescence instead of decay for green algae and higher plants [26] Here and hereafter, the notation of main points in polyphasic chlorophyll *a* fluorescence curve (OJIP and PSMT) is taken from the main works of Strasser and Stamatakis [65, 66].

The first recorded signal in fluorescence induction (FI) experiment, F_0 , is considered usually as the maximally oxidized Q_A and as the maximal rate of primary charge separation in the RCII in dark-adapted green algae and higher plants. In cyanobacteria, the O level of fluorescence induction curve is higher (see **Figure 6**) because of C-phycoerythrin and allophycoerythrin fluorescence contributions at 710 nm and also because Q_A is partly reduced in the dark due to equilibration with the PQ pool, which is reduced in darkness by respiratory substrates. This happens because in cyanobacteria, the PQ pool is shared both by photosynthetic and respiratory electron transport (PSET and RSET), contrary to

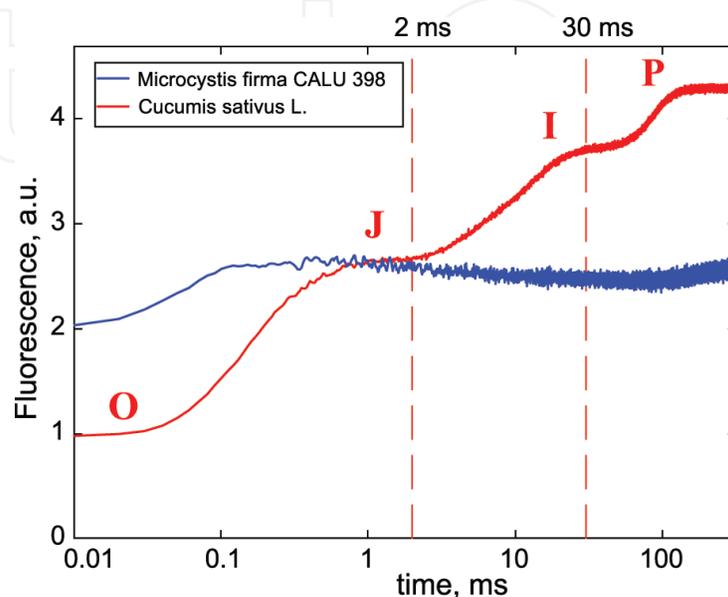


Figure 6. Fast OJIP chlorophyll fluorescence kinetics for *Microcystis firma* CALU 398 (blue line) and *Cucumis sativus* L. (red line). Characteristics were recorded with PAM-2500, Walz GmbH, Effeltrich, Germany.

other photosynthetic organisms, where PSET and RSET functions are segregated in different organelles, plastoquinones are oxidized enzymically in darkness, and their fluorescence quenching effect is enhanced. Also, in cyanobacteria, Chl *a* is more abundant in PSI than in PSII compared to eukaryotic plants. Besides this, a dark-reduced PQ pool shifts cyanobacteria to state 2, in which the excitation which PBS donates to PSI is maximized, increasing the contribution of PSI to F_0 . In other words, in cyanobacteria, F_0 contains a larger fraction of the redox-silent PSI fluorescence than it does in higher plants and algae. Finally, the OP rise is the integrated response of excited Chls *a* to redox signals and electrostatic fields that PSET generates within the thylakoid membrane.

Compared to the OP rise, the PSMT decay (in plants and rise in cyanobacteria) is far more complex (see e.g., **Figure 2** in [47]). In cyanobacteria, lumen acidity-dependent $q(N)$ works in a characteristically different way than in eukaryotic plants. While in the latter, $q(N)$ dissipates primarily the excess Chl *a* excitation in PSII; in cyanobacteria, it dissipates primarily the excess Chl *a* excitation in PSI, which is obtained from PBS when the cyanobacterium is in state 2 (e.g., after dark adaptation). In other words, in cyanobacteria, $q(N)$ quenching occurs as a sequel to the fluorescence lowering during the state 1 to 2 transition [70]. Moreover, in contrast to algae and higher plants, cyanobacteria have the maximum fluorescence signal in point M between O and T, instead of point P for other photosynthetic organisms. This is due to the fact that in cyanobacteria, the PQ pool is reduced by respiratory substrates during dark adaptation, as it is shared by both the PSET and the RSET chains [71, 72]. Thereby, an extensive, and often monotonous, P to T fluorescence decay in higher plants reflects a state 1 to 2 transition. And the opposite situation occurs in cyanobacteria, where the SMT transient is dominant in the total fluorescence induction pattern, with SM rise corresponding to a state 2 to 1 transition. Thus, it is obvious that the F_m parameter for cyanobacteria should be obtained at another point (M), than for higher plants (P). The SM rise in cyanobacteria displays interesting phenomenology, which cannot always be explained adequately. In contrast to green algae, noncyclic and cyclic PSET inhibitors do not block the SM rise in cyanobacteria, although the fluorescence induction kinetic details are changed. When the MT fluorescence decay takes place, a cyanobacterium cell is at state 1 and most likely stays there during the MT decay because it is illuminated continuously. The MT decay in cyanobacteria is observed only under strong actinic illumination, raising the possibility of $q(I)$ (as that part of a light-induced depression of Chl *a* fluorescence that is not reversed by dark adaptation) contributions to it.

Concerning fluorescence quenching analysis based on pulse amplitude modulation technique [73], it should be noted that this method can give detailed information of the main processes in photosynthetic system of cyanobacteria, but inherent simplicity of this method there is a risk of uncritical use which in certain cases may lead to questionable results and conclusions. In several reviews [28, 29, 67, 74, 75], a lot of peculiarities are pointed out, which distinguish cyanobacteria from eukaryotic plants. They are difference in metabolic interactions (i.e., reduced PQ pool in dark-acclimated cyanobacterial cells due to respiratory electron transport), the problems arise from pigment composition (i.e., the impact of phycobilins fluorescence into the overall chlorophyll fluorescence and the dependence of the result on the wavelength of excitation light), the high rate of development of nonphotochemical quenching, the problem with the estimation of electron transport rate (ETR) (due to the difference in distribution of light energy between PSI and PSII in cyanobacteria), discrepancy in the behavior of $q(N)$ (nonphotochemical quenching) and $q(P)$ (the redox state of PQ pool) (because the regulation of state transition in cyanobacteria is due to not only

redox control, but in some way to component in cytochrome b_6/f complex), the role of the blue light intensity in nonphotochemical quenching (nonphotochemical OCP quenching), etc. Some of them were already described previously.

The main problem of chlorophyll fluorescence analysis in cyanobacteria is related with the determination of the actual F_0 and F_m , since cyanobacteria have highly fluorescent phycobilisome and high PSI/PSII ratio; thus, the contribution of “basal” fluorescence should be far higher than in land plants. The method of calculation of the “true” value of F_v/F_m in the absence of the “basal” fluorescence was suggested in [76], and the corrections that are due to phycobilin fluorescence only are presented in [77, 78]. Another problem with right determination of F_m deals with the reduction of PQ pool in the dark-acclimated cyanobacterial cells, since PQ pool and electron transport are shared by photosynthetic and respiration systems. This problem can be solved by using weak blue light illumination, which oxidizes PQ pool, instead of dark. The determination of F_0 also is not so simple. In the dark, cyanobacterial cells are in state 2; thus in order to determine the true F_0 level, they should be brought to state 1. Again appropriate blue light acts as PSI-excitation light and oxidizes PQ pool as well as Q_A in many cyanobacteria [28]. Thus, we should be especially cautious to interpret the differences in chlorophyll fluorescence parameters determined in the dark or under low light in the analysis of cyanobacteria. To demonstrate the difference in chlorophyll fluorescence kinetics between cyanobacteria and higher plants in **Figure 7**, several characteristics for cyanobacteria (*Microcystis firma* CALU 398) and cucumber leaves (*Cucumis sativus* L.) are presented. The characteristics were recorded at blue actinic light for cyanobacteria and at red actinic light for cucumber leaves. Obviously, the fluorescence emission trace for cyanobacterial and cucumber quenching analysis are quite different, as well as ETR, photochemical [Y(II)] and nonphotochemical (Y(NPQ)) quenching dependencies.

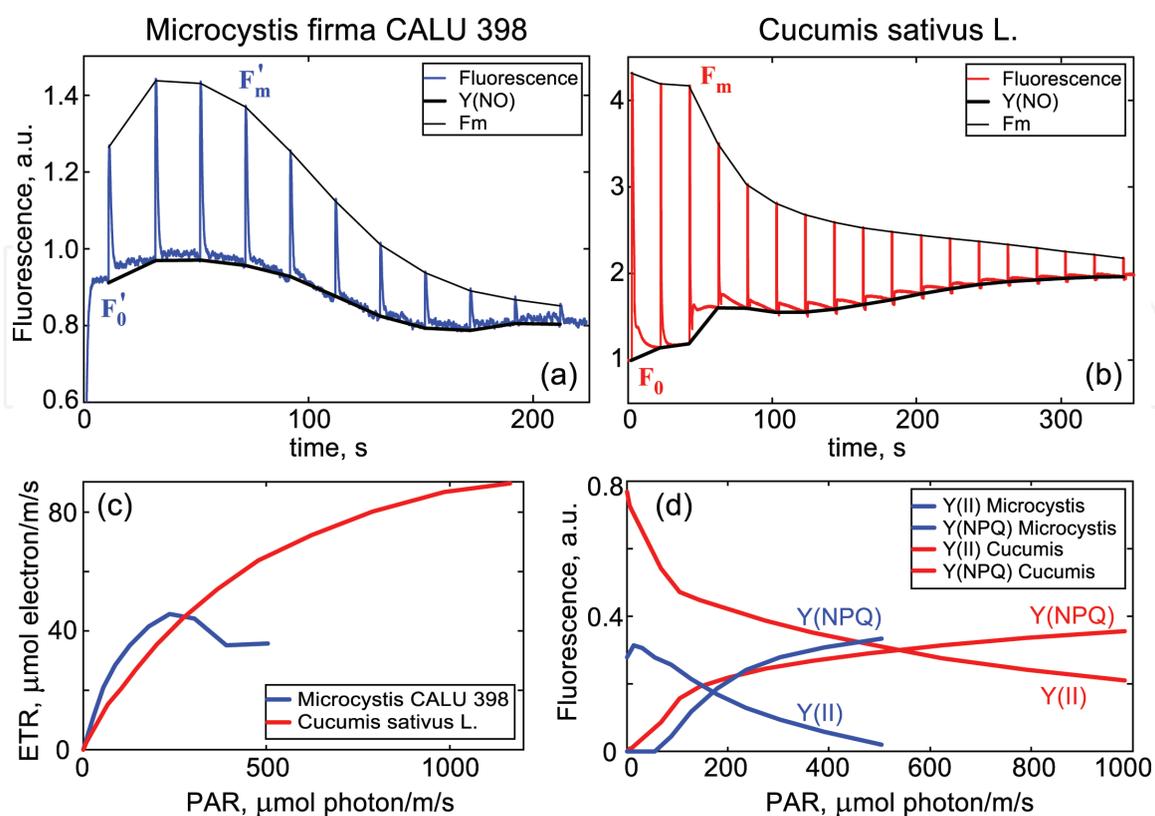


Figure 7. Fluorescence emission trace for quenching analysis (a) *Microcystis firma* CALU 398 (blue lines) and (b) *Cucumis sativus* L. (red lines), (c) ETR, (d) Effective quantum yield Y(II) and nonphotochemical quenching Y(NPQ) dependencies. Characteristics were recorded with PAM-2500, Walz GmbH, Effeltrich, Germany.

The instrumental support of these two fluorescence methods, the fluorimeters, that are currently in use for kinetic fluorescence studies, is based on different approaches to measure the variable Chl *a* fluorescence differing in the manner by which the photochemistry is saturated: shutterless and LED-based instruments for direct fluorometry, as plant efficiency analyzer (HandyPEA, M-PEA, PocketPEA; Hansatech Instruments, Kings Lynn Norfolk, UK) [79], pulse amplitude modulation, PAM fluorometry (Phyto-PAM Phytoplankton Analyzer; PAM-2000, Walz GmbH, Germany) [80], the pump and probe fluorometry [81], the fast repetition rate fluorometry [82], the pump during probe fluorometry [83], and several others that are functionally similar, such as the fluorescence induction and relaxation technique [84], the background irradiance gradient single turnover fluorometry [85], and advanced laser fluorometry [86]. In the last years, a lot of new modifications of these instruments appeared, including multicolor PAM (MULTI-COLOR-PAM and PHYTO-PAM Phytoplankton Analyzer, Waltz GmbH, Effeltrich, Germany) [87–89] and imaging PAM (IMAGING-PAM M-Series, Waltz GmbH, Effeltrich, Germany) [73]. Recently developed multi-color-PAM (Waltz GmbH, Effeltrich, Germany) [87] provides six different colors of measure light and six different colors of actinic light, all of which qualify for highly accurate measurements of fast induction kinetics and assessment of wavelength-dependent F_v/F_m and functional absorption cross-section of PSII.

3.3 Delayed fluorescence analysis

Delayed fluorescence (DF) occurs in photosynthetically active cells and is emitted between 450 and 750 nm with decay times from milliseconds to minutes [30, 31, 52, 90]. DF excitation spectroscopy is a real-time, nondestructive method for rapid estimation of the photosynthetically active pigments—chlorophylls, accessory pigments and phycobiliproteins. It can be used to determine chlorophyll concentration and phytoplankton composition [91–95], as well as to study the changes in electron transport [96], different stress states [97–100], the response to the diurnal light cycle [101], etc. The DF measurement distinguishes between cyanobacteria, cryptophyta, green algae, and chromophyte algae because they have different chlorophylls, accessory pigments, and phycobiliproteins. DF has some specific features in cyanobacteria, due to their specific structural arrangement of the photosynthetic membranes. In particular, in contrast to other algae, some parameters of delayed fluorescence from cyanobacteria show specific changes within the temperature range at which membrane lipids undergo thermophilic phase transitions [102].

The origin of DF is as follows. The accessory pigments, absorbing photons, provide charge separation at the reaction centers of PSII and PSII after the excitonic energy transfer in light-harvesting complex. Then electrons are transported through the electron transport chain to the Calvin cycle. Stopping the illumination initiates processes of the reverse light phase, that is, electrons in the electron transport chain flow back to the oxidized reaction center $P680^+$, leading to an excited state $P680^*$. This excited state $P680^*$ decays to the ground state emitting a delayed fluorescence in 580–720 nm wavelength range. DF can be observed during several minutes until a charge equilibrium between donor side (water splitting complex) and acceptor side (plastoquinone, PSI) is reached by recombination. The resulting decay curve includes the fast decay, produced by recombination of electrons and holes, which are located near $P680^+$, and the long-lasting decay, caused by electron-hole pairs, which are located at greater distances from $P680^+$ (e.g., electrons near PSI together with holes at the Mn^{n+} complex) [96]. Thus, the cellular components, which absorb light, but do not lead to charge separation (like dead cells, or photoprotective pigments—carotenoids), are not detected by DF spectroscopy.

The peculiarities of DF spectroscopy for cyanobacteria are associated with the difference from plants in cellular metabolism, and structure and functioning of their photosynthetic apparatus, which are pointed out in the previous section. The differences in kinetics and spectral characteristics of DF can be easily traced from higher plants taking into account the relation between photosynthetic and respiratory systems in sharing of electron transport chain and the presence of external light-harvesting complex (phycobilisome). The former influences the DF kinetics, and the latter, DF spectral features. For example, the temporal DF dependence (DF decay curve) shows the transient peak with variable, species-specific heights and timing, in contrast to green algae, which have no any peak (see, e.g., **Figure 2** in [100]). The height and timing of this peak is affected differently by different toxicants [100]. Thus, DF can be used as a rapid, reliable, and sensitive phytoplankton toxicity test. In the laboratory experiments, the comparison of four measuring techniques (delayed fluorescence, prompt fluorescence, photometric absorbance, and microscopic cell count) to assess growth inhibition freshwater algae and cyanobacteria shows very high sensitivity of delayed fluorescence excitation spectroscopy [90]. DF as a parameter is used to investigate the phytoplankton community in lakes [95], its seasonal aspect, and vertical distribution [95, 103], as well as continuous biomonitoring of surface waters [94]. Delayed fluorescence excitation spectra, obtained by measuring the intensity of delayed fluorescence at different excitation wavelengths and reflecting the photosynthetic pigment composition, is used to determine chlorophyll concentration and phytoplankton composition [30, 94, 95].

Finally, it should be noted that the delayed fluorescence is the only method that can diverse live and dead cells during on-line monitoring. Its major advantage is that it is emitted only from cells that are photosynthetically active, that is, alive; thus, the additional signals from dead cell debris do not interfere with the measurements. Long-term DF emission also prevents interference problems with fluorescent backgrounds in natural samples [94]. Furthermore, DF can measure nano- and pico-plankton, which may be lost during filtration or may be unaccounted in direct microscopic analysis.

Since 1995, DF excitation spectroscopy has been used in addition to microscopic biovolume estimation to monitor the phytoplankton composition in Germany [104]. Within an EC project “Phytoplankton On-Line,” started in 2000, an on-line version of the DF excitation spectrometer has been developed to monitor continuously the composition of phytoplankton species in Lake Erken, Sweden, Lake Balaton, Hungary and Lake Kinneret, Israel.

3.4 Flow cytometry

Flow cytometry (FCM) is a rapid technique for the quantitative measurement of the properties of individual cells [105, 106]. It is a method for conducting a multi-parameter analysis of cells suspended in liquid and passing through a laser beam. In FCM, the excitation and emission properties of individual cells at one or more wavelength are measured together with the light scatter parameters, such as perpendicular light scatter, forward light scatter, and time of flight. Usually in order to detect any fluorescence, suspended cells require staining with organic dyes. In cyanobacteria, self-fluorescence of photopigments (chlorophylls and phycobilins) not only allows cells to be counted in a rapid manner but also enables a wide range of potential applications in ecological and biochemical studies, such as definition of biomass, distribution, and dynamics of phytoplankton [33, 107–109], discrimination and identification of algae species [110–112], and the investigation of the responses to contaminants [113–116]. It should be noted that the presence of these photopigments limits the possibility to stain cells with synthetic dyes

as their excitation and emission spectra can greatly overlap. Thus, the attention should be paid while choosing appropriate fluorescent dyes with respect to the particular microorganism and the available lasers with which the FCM instrument is equipped [117].

For example, recently, an FCM protocol for quantitative and rapid analysis of *Microcystis* cells and colonies in lake sediment was developed [118]. Because of its many advantages, including rapidity, sensitivity, multiparametric analysis, and cell sorting capacity, FCM has been increasingly applied to environmental studies with aquatic microorganisms.

Various FCM instruments are also coupled with cell sorters [106, 119]. In this case, individual cells can be diverted from the fluid stream based on their properties such as cell morphology, taxonomic position, surface and intracellular protein expression, gene expression, and cellular physiology and collected into homogenous fractions for further analyses [120]. In spite of different cell viability after such a process (especially for fragile species), FCM featuring a sorting device has been successfully applied to isolate different phytoplankton taxa from mixed cultures at a success rate of 80% [121].

Initially, most of the commercially available flow cytometers cannot be directly applied to the study of aquatic biota, due to the large size range and low densities of the field samples. However, in 1989, the so-called Optical Plankton Analyzer has been designed by a group of American researchers [122]. Later, several companies in the United Kingdom and United States began to produce new modifications of flow cytometers for limnological and oceanographical applications [123]. Currently, the major producers in this field include Beckman Coulter, Becton-Dickinson, Thermo Fisher Scientific, and DakoCytomation provide a number of devices equipped with several lasers with different emitted wavelengths that allow studying cells stream flowing at a speed of over 100,000 cells per second. One of the advantages of FCM is the possibility of rapid and automated measurement of very small volume of the tested samples ($1 \text{ cm}^3 \cdot 1 \times 10^6$ cells).

The particularity of studying cyanobacteria via FCM lays in the right choice of excitation and emission wavelengths for recording fluorescent signal, as it was described in previous sections. Measurement of chlorophyll and phycobilin fluorescence via FCM can provide the information on changes in the efficiency of photosynthetic apparatus, and indirectly, it can impart information on the cell's condition and growth state. It can be used to test the impact of different factors on cyanobacteria development, that is pH, toxic metals, herbicides, and other pollutants, under controlled laboratory conditions [114, 115, 124]. In addition to chlorophyll fluorescence, the fluorescence emission of phycocyanin pigment-protein complexes that participate in transferring signals also can be used in environmental stress studies of cyanobacteria, similar to those based on chlorophyll *a* measurement. Thus, cyanobacteria are well suited to flow cytometric analysis.

Considering that FCM equipped with a dual laser is able to measure chlorophyll *a* and phycocyanin, this method could also be of value in ecological studies of cyanobacteria. Once coupled with cell sorting, FCM can be successfully applied to isolate cyanobacteria from mixed phytoplankton samples. The recent emergence of advanced microfluidics, the availability of sophisticated digital image processing techniques, the advent of various optical imaging modalities, and image-guided cell sorting extend the capabilities of FCM from the measurements of one-dimensional signal intensities to multidimensional information-rich images, which can be used to analyze the spatial architecture of single cells in flow in addition to the analysis of the volume samples [125]. However, the presence of autofluorescence in cyanobacterial cells limits the use of staining cells with synthetic fluorochromes due to signal overlap. Moreover, the colonial forms and filamentous cyanobacteria (especially in

freshwater species) are still rather difficult to study using FCM due to their different colony size and trichome lengths. In this case, the light scattering does not allow individual cells to be distinguished.

3.5 Confocal microscopic spectroscopy

Self-fluorescence of the photosynthetic apparatus of cyanobacteria originates from excited states of light-harvesting complex and PSII/PSI that were lost before photochemistry took place. It usually represents a small fraction of the excited state decay in photosynthetic complex of healthy cells. Nevertheless, this small fraction can be easily detected by confocal laser scanning microscopy (CLSM) [126, 127]. With CLSM, a very small excitation and detection areas can be investigated, so that single cells under nondamage conditions can be studied *in vivo*. In addition to above-mentioned techniques that now can be carried out on the single-cell level, CLSM provides some additional facilities. The investigation of self-fluorescence of single living cells reveals the relation between the physiological state and the operational activity of photosynthetic system. The investigation of self-fluorescence gives the information about single-cell processes as well as about the collaboration in cell communities. The changes in spectral characteristics of living photosynthetic cells indicate changes in their physiological state and can be applied for the studies of the results of stress states and external actions. Light-, heat-, ultrasound-, and toxin-induced changes can be distinguished by means of confocal microscopic spectroscopy since all these external actions are stress factors affecting photosynthetic process [44].

Modern CLSMs are unique tools for visualizing cellular structures and analyzing dynamic processes inside single cells. They exceed classical light microscopes in their axial resolution, which enables to acquire optical sections (slices) of a specimen. Thus, the object can be imaged in three dimensions and subsequently visualized as a 3D computer model. CLSM is designed for the quantification and analysis of image-coded information. Among other things, they allow easy determination of fluorescence intensities, distances, areas, and their changes over time. They are capable of quickly detecting and quantitatively unmixing the spectral signatures of fluorescent objects. Many useful parameters as the degree of colocalization of labeled structures or the ion concentration in a specimen can be analyzed [128]. The most interesting feature of CLSM is the capability for single-cell microscopic spectroscopy, which allows obtaining spectroscopic information inside small regions and single cells [35]. Another group of facilities deals with the quantitative analysis of dynamic processes in living cells using techniques such as fluorescence recovery after photobleaching (FRAP) [129, 130], fluorescence resonance energy transfer (FRET), fluorescence lifetime imaging microscopy (FLIM) [131], photo-activation, and photoconversion. Recently, a lot of additional techniques appear in modern CLSMs, which open new perspectives for single-cell investigation, such as white laser, which provides the ability to obtain not only fluorescence emission spectra, but also single-cell excitation and absorption spectra [132]; hyperspectral CLSM that allows more precise fluorescence spectra through the cell thickness and gives more detailed fluorescent pigments location [34, 133]; and STED and multiphotonic techniques, which extend the CLSM abilities to single-molecular studies [134, 135].

However, while working with living cells, the caution must be taken. The studies of the photosynthetic cells require some additional specific skills and techniques to perform measurements and to carry out data processing [35, 41, 42, 44]. The efficiency of photosynthesis and photosynthetic rate are highly dependent on irradiance. This can be seen in the light dependency of various photosynthetic

parameters. Moreover, not only light quantity, but also light “quality” (wavelength) is an important factor [38]. Thus, special spectroscopic methods are required to study the physiology of phototrophic microorganisms [136]. These organisms employ light-dependent photosynthesis as the main energy source for their metabolism, and the detected self-fluorescence finally reflects the diversity in morphological and physiological states of their photosynthetic cells.

The main problem of *in vivo* investigations is to protect the object under consideration from light and heat damage. Since in CLSM the laser light density in the focus point is high, the dwell time and the intervals between the illuminations may influence photodamage and saturation of photosynthesis. It is well known that most chromophores and natural pigments bleach under the high laser excitation energies; thus, a bleach test should be performed [35]. It was shown experimentally that especially phycoerythrin and phycocyanin, as accessory pigments, were very sensitive to photobleaching, while the fluorescence of chlorophyll *a* and allophycocyanin remained stable in the intact cells [137]. Thus, during fluorescence detection of the main accessory pigments of cyanobacteria, the changes in their fluorescence intensity should be controlled during the recording period. The power of individual laser lines should be chosen according to the photodamage they cause. In some experiments, the repeated spectra were obtained under selected excitation power, and in this case, the excitation power would affect the cells (see **Figure 3**). In each case, the optimal zoom and excitation energy parameters should be selected, so the fluorescence spectra did not vary within the experimental error. When excitation energy is increased, both the intensity and shape of fluorescence spectra can change because of photodamage or structure breakdown in photosynthetic systems.

In the experiments, where several laser lines were involved in the investigation (see **Figure 2**), it is useful to record the first spectrum again at the end of each series to control the initial state of the cell. Moreover, the cell fluorescence intensities depend on the sensitivity setting of the photomultiplier, distance from the sample, and the excitation energy (which varies for different laser lines of CLSM); thus, the only qualitative analysis of spectral characteristics is acceptable in multispectra regime.

CLSM gives the opportunity to truly understand the cellular function; thus, we need to extend our imaging capabilities in ways that enable us to follow sequential events in real time, monitor the kinetics of dynamic processes, and record sensitive or transient events. Fluorescence spectra of cyanobacterial photosynthetic pigments are easily recorded by spectral CLSM. The fluorescence shares of individual phycobiliproteins can be reliably determined by spectral unmixing, showing that the spectral resolution of CLSM is well suited for this approach. In addition, CLSM technique allows the investigation not only cultivable but also the noncultivable cyanobacterial species.

3.6 Remote sensing

Methods of fluorescence spectroscopy suit very well for large-scale monitoring and remote sensing. They can be used in ecological studies, water quality control and on-line monitoring of cyanobacterial occurrence, distribution, and spatial and temporal dynamics in the environment [138–140]. Obviously, the presence of cyanobacteria and their photopigments influence reflectance, which is the ratio of radiation leaving the water (reflected) to the total amount of radiation incident on the water, is detected by remote sensing devices. Reflectance can be viewed by remote sensors called spectroradiometers, which can be either multispectral (sampling the electromagnetic spectrum over a relatively wide range of wavelengths in each discrete band) or hyperspectral (collecting and processing information from many

narrow contiguous wavelength intervals across the electromagnetic spectrum). Remote sensing of chlorophyll *a* using its absorbance in the blue and red electromagnetic wavelengths has been successfully applied in the monitoring of phytoplankton blooms, and many single-band or band-ratio-type algorithms have been developed for chlorophyll *a* concentration retrieval from both multispectral and hyperspectral data obtained from ground-based, airborne, or satellite sensors [36]. However, the selective determination of cyanobacteria in the presence of other photoautotrophs requires the use of targeted algorithms (baseline, band-ratio, and semi-analytical nested band-ratio) for retrieval of phycocyanin concentration. Two spectral characteristics should be taken into account: a pronounced absorption peaks near 570 and 630 nm and two fluorescent peaks near 580 and 660 nm. Unfortunately, only in some instances, retrieval of phycocyanin concentrations at high cyanobacterial densities is possible from multispectral data, for example, provided by the MERIS satellite imaging spectrometer [141–143]. More sensitive hyperspectral remote sensing of phycocyanin is applied in cyanobacterial bloom monitoring using shipboard [144, 145] or airborne sensors [146, 147] and is eventually possible with the satellite Hyperion Hyperspectral Imager, which is capable of acquiring hyperspectral data [36, 148]. The methods are being continuously improved in their sensitivity, selectivity, resolution, robustness, and applicability, including new instrument designs or derivation of new algorithms for pigment concentration derivation [146, 149–153].

The most interesting instrument, providing active remote sensing of phytoplankton and dissolved organic matter (DOM) fluorescence, is a laser remote sensing spectrometer (LIDAR) used in shipborne laboratories [154–159]. A LIDAR fluorosensor for marine and freshwater applications is remotely excite and detect laser-induced fluorescence emissions by chromophore groups belonging to different species present in the water body. Four out of 12 available spectral channels, corresponding to water Raman backscattering (402 nm), DOM (450 nm), and phytoplankton pigments (phycocyanin, 650 nm; chlorophyll, 690 nm), were selected in wavelength by placing suitable interference filters. The water Raman peak is used to measure the water turbidity. This allows comparing different seawaters. Some laser transmitters of the LIDAR are also capable of operating in the dual pulse mode, to remotely measure the Chl *a* fluorescence yield according to the pump-and-probe excitation scheme [154].

Thus, the question of the applicability of the remote sensing to monitor the spatial distribution of cyanobacteria can be now answered affirmatively. Recent progress in spatial and spectral resolution of remote sensing allows distinction of cyanobacteria and various groups of algae on the basis of their photosynthetic pigments at a detailed spatial scale. In particular, quantification of cyanobacterial biomass and the recognition of cyanobacterial surface blooms by means of optical detection become true, and these advances allow field studies at the population level [160].

4. Conclusion

In the last years, fluorescence methods became a strong alternative to traditional methods of environmental research of cyanobacteria. They allow selective and sensitive quantification of cyanobacteria to be done quickly and easily in a nondestructive manner *in situ* with real-time data acquisition using fixed, portable, or remote-sensing devices, giving a unique opportunity for automated and continual monitoring of cyanobacterial communities by handheld instruments, shipboard sensors, or airborne and satellite platforms. Data obtained by fluorescence methods can valuably contribute to our understanding of the environmental distribution and

dynamics of cyanobacteria, bloom formation, and cyanotoxin production and to water toxicity risk assessment. Large-scale remote sensing can give the information about the development of cyanobacterial blooms in oceans and freshwater bodies. On the other hand, the laboratory results of the single-cell spectroscopic investigations can give new information to improve remote sensing control. Airborne and satellite data should be also accompanied with shipboard (based phycocyanin fluorescence monitoring) and laboratory studies to provide deep insight to the cyanobacteria nature.

Conflict of interest

The authors declare no conflict of interest.

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