Chapter 2

Fluorescence Microscopic Spectroscopy for Investigation and Monitoring of Biological Diversity and Physiological State of Cyanobacterial Cultures

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Additional information is available at the end of the chapter

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Abstract

In this chapter, a novel technique for investigation of natural and laboratory cyanobacterial cultures is presented. The technique is based on a strict relation between the intrinsic single-cell fluorescence emission spectra of cyanobacteria and the physiological state of the whole culture. It will be shown else that the single-cell fluorescence spectra for different species are steady enough to conduct a taxonomic analysis of cyanobacterial cultures based on a common statistical data evaluation among the parameters extracted from a set of such spectra. Several examples are given to illustrate the power and simplicity of a new technique, which can become a promising tool for automation of production in the cyanobacterial biotechnology, as well as give a valuable contribution to the development of innovative approaches in environmental monitoring of harmful algal blooms.

Keywords: cyanobacteria, confocal laser scanning microscopy, single-cell fluorescence spectrum, biological diversity, physiological state, environmental monitoring, biotechnology, harmful algal blooms

1. Introduction

Cyanobacteria have gained huge attention in recent years because of their potential application in biotechnology [1–5]. For example, cyanobacteria are considered as a rich source of biologically active compounds with antiviral, antibacterial, antifungal and anticancer activities. Several strains of cyanobacteria were found to accumulate polyhydroxyalkanoates, which can be used as a substitute for nonbiodegradable petrochemical-based plastics. Recent studies showed that oil-polluted sites are rich in cyanobacterial consortia capable of degrading

oil components. Cyanobacterial hydrogen has been considered as a very promising source of alternative energy and has now been made commercially available. Cyanobacteria are also used in aquaculture, wastewater treatment, food, fertilizers, agriculture, production of secondary metabolites including exopolysaccharides, vitamins, toxins, enzymes and pharmaceuticals. In addition, the ecological aspect of the harmful bloom monitoring and control makes an important contribution in this rising interest to cyanobacterial problem.

In the last few years, the investigation of taxonomy, physiology, morphology and genetics of cyanobacteria attracts a considerable attention. A vast amount of different techniques were elaborated to achieve, nowadays, an insight into the physiological processes that rules cyanobacterial life and their genetic background. Large-scale industrial production of the cyanobacterial products requires optimization and more detailed control of incubation conditions in order to increase productivity. Future research will be focus on isolating and study of new cyanobacterial strains and the improvement of different treatments that will support or inhibit their growth.

In this chapter, the novel and most powerful part of the optical spectroscopy, which could make a considerable contribution to the future investigations—a confocal microscopic spectroscopy—will be presented and illustrated by several examples of application. It should be noted, that due to space limitations, only few citations could be incorporated in this chapter. They represent a limited selection from a large amount of works and should be used as a source for further references.

As it was pointed out earlier, there are two opposite aspects in the cyanobacterial problem: first, to prevent ecological hazards, that is, toxic cyanobacterial blooms, and, second, to improve industrial incubation of cyanobacteria, involved in such important applications, as food and fuel production. The former deals with the study and treatment of natural samples and includes the investigation of biological diversity, monitoring of physiological state of natural communities, record and analyze the results of external actions and changings in environmental conditions. In many situations, taxonomic phytoplankton composition is of crucial importance when toxic or other harmful substances might be produced by cyanobacteria. The latter aspect concerns the registration and control of the optimal physiological state and viability of the laboratory or industrial culture in specified conditions. Thus, there are two main problems that are involved in all areas of application mentioned above and meet some obstacles while using conventional methods of investigation. They are: a correct classification and discrimination of present and new cyanobacterial strains, and monitoring of physiological state of cyanobacterial cells in natural communities and in laboratory cultures during industrial incubation.

All these different tasks deal with a big data processing and possibility of process automation are quite desirable. Confocal microscopic spectroscopy gives a unique opportunity for direct automation of all these processes or, otherwise, indirect application of the results of the detailed single-cell spectroscopic investigations for implementation in innovative devices or technique.

The taxonomic composition of cyanobacterial communities is of interest in water-quality field and ecology, where the effect of nutrient pollution on coastal and freshwater resources should be controlled [6, 7], as well as in industrial biomass production, where the additional undesirable strains may appear during the long cultivation process. In nature, the composition of phytoplankton communities can be highly variable in space and time [8, 9]. Characterization of the

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community composition, therefore, requires frequent, high-resolution sampling. Historically, community characterization has been done by chemical preservation of samples and analysis by bright-field or epifluorescence microscopy. Although optical microscopy allows direct measurements of cell size and identification to species level it is laborious and time-consuming, limiting the number of samples that can be analyzed in a day. Minor variations in the composition of phytoplankton are consequently not revealed when using optical microscopy technique. More recently, *in situ* flow cytometric instruments capable of automated characterization of phytoplankton communities have been developed [10, 11]. These instruments have excellent resolution over a wide range of cell sizes but have a great disadvantage of high requirements for sample preparation and no possibility of cell-viability control. Alternative methods that are based on differences in accessory pigments among phytoplankton taxonomic groups [12] such as chemotaxonomic and spectrofluorometric methods have been proposed and included. First requires the high pressure liquid chromatography (HPLC) analysis of pigment contents [13–15]. The introduction of pigment analyses by HPLC facilitated easy and accurate separation, identification, and quantification of phytoplankton pigments. The large number of samples that can be processed by HPLC allows a more thorough examination. However, it does not allow for high-resolution data acquisition and again give no information about physiological state of single cells. And the last, spectrofluorometric method, although enables low-cost, rapid measurements, but till now deals with fluorescence-based chlorophyll *a* (*Chla*) quantification methods, that were proposed by several authors in the early 1960s and were applied either *in vitro* or *in vivo* to continuous measurements of algae and higher plants [16–22]. Unfortunately, these methods cannot be directly applied to cyanobacteria and usually give incorrect results.

Recently, attempts to conduct the discrimination among microalga on the base of absorption or fluorescence spectra were reported [19, 21, 23–26]. Most of them use only absorption spectra. Absorption spectrum includes the information only about the chemical structure of photosynthetic cells, so it results in a rough discrimination of big classes of phytoplankton: diatoms, dinoflagellates, prymnesiophytes, euglenophytes, prasinophytes, raphidophytes, cryptophytes, chlorophytes, chrysophytes, and cyanobacteria. However, among the species of one class, for example, cyanobacteria, the chemical characteristics are quite similar, except several cases when phycoerythrin occurs in phycobilisome in addition to phycocyanin as an accessory pigment. In the last case, the only differentiation can be made among two big groups: the species containing phycoerythrin and those who lack it. All other differences are so small that cannot be used for further differentiation of cyanobacterial species and strains, so more precise classification, that is, among cyanobacterial species and strains, is impossible using only the absorption spectra.

Opposite to the absorption spectra, the *in-vivo* fluorescence spectra are much more informative. Fluorescence detection is undoubtedly a powerful tool owing to the existence of natural fluorescence from phycobilins and chlorophylls. It 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.

Fluorescence spectra have been used to classify phytoplankton populations since the approximately early 1970s [20]. However, because of the generally low number of available excitation wavelengths in the conventional devices the rate of species discrimination was relatively low. Researches again can separate only algal groups that differ greatly in pigmentation and, therefore, in fluorescence spectra (e.g., cryptophytes, chlorophytes and cyanobacteria), but cannot separate groups that are more similarly pigmented (e.g., among cyanobacterial species) [22, 27]. Discrimination between similarly pigmented taxa or even between species within a taxon requires high-resolution spectra and the use of a set of excitation wavelengths to reveal small peculiarities in configuration and functioning of light harvesting system. The rigorous discrimination is possible if the inter-species differences are greater than those within a species. These requirements can be fulfilled only when for each species a set of single-cell fluorescence spectra, excited by a number of wavelengths, are obtained and analyzed. The possible contribution of environmental adaptation effects to the resulting fluorescence spectra can be minimized by an accurate definition of the corresponding spectral regions in the spectra under consideration.

The problem of registration and control of the physiological state of single cells in natural communities and the viability of cultures during incubation is a primary task in both ecological and biotechnological fields of application of cyanobacteria. This problem is more complex than the species discrimination because it deals with very weak variations in chemical and optical characteristics of single cells and a culture as a whole. Despite the importance of this problem in vast amount of tasks, the methods for studying and monitoring of physiological state of cyanobacteria are still based mainly on traditional optical methods of registration, as well as on the analysis of fixed or dissociated samples [28–31]. These approaches do not allow one to register small changes in the physiological state of cyanobacterial cells, which are extremely important during weak external treatments or environmental changes. Usually, this study is reduced to a manual counting of the total number of cells in the experimental samples and determining the total volume of chlorophyll [32–34].

The last method is very effective for algae and higher plants, where chlorophyll is a main pigment, determining the viability. It quickly disintegrates in dead cells and therefore can really serve as an indicator of the viability of single plant. In cyanobacteria, the main pigments responsible for photosynthetic activity are phycobilins, and chlorophyll does not disintegrate for a long time in dead cells, even after their disruption. Therefore, the methods associated with the analysis of the chlorophyll fraction do not give satisfactory results in the study of cyanobacterial cultures. The analysis of the light fraction of water-soluble phycobilins may give some additional information, but it is also ambiguous, since the pigment-protein complexes in the disrupted cells degrade rapidly and carry no information about the initial viability of living cells. With such approach, weak changes in the physiological state of cyanobacterial cells cannot be detected, since the results of the experimental treatment are more influenced by the used methods of investigation than the directional external action, which is studied.

The only method that seems to be appropriate for physiological state investigation and can diverse live and dead cells is the delayed fluorescence (DF) technique. Delayed fluorescence is the long-term emission of light from cells triggered by illumination [27, 35, 36]. It has the same emission spectrum as chlorophyll *a* fluorescence, but occurs with a time delay (from milliseconds to minutes) [37]. The major advantage of DF is that it is emitted only from cells

that are photosynthetically active, that is, alive. Thus, 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 [38]. Furthermore, DF can measure nano- and pico-plankton, which may be lost during filtration or may be unaccounted in direct microscopic analysis. However, by means of DF, only the ensemble spectra of the whole culture can be measured and the physiological state of individual cells is unavailable as before. Thus, a new precise, nondestructive and sensitive method for registration of weak reversible and irreversible changes in the physiological state of cyanobacterial cells should be elaborated.

Historically, since the 1960s, investigators have noted that changes in the physiological state of cyanobacterial cells occurring when it is damaged are reflected in the corresponding changes in the intrinsic fluorescence spectrum [39]. Several studies have shown that a decrease in the pigment fluorescence is associated with a decrease 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 [31]. However, this fact has not yet been widely used to assess the viability of individual cells of cyanobacteria and cultures.

To date, there is no doubt that the *in vivo* analysis of fluorescence parameters of light-harvesting complexes is a powerful tool for studying the effect of a wide variety of environmental factors on photosynthetic organisms. The intensity of fluorescence emitted by single photosynthetic cells *in vivo* depends only on the structure and operational effectiveness of photosynthetic apparatus, reflecting the individual characteristic of cyanobacterial strain and in-time physiological state of the cells under consideration. 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 of photosynthetic apparatus. Thus, the intrinsic fluorescence spectra of a particular type of cyanobacteria, the so-called "fluorescent fingerprints," can be used to identify photosynthetic pigments and to determine the viability of individual cells, as well as for preliminary taxonomic analysis of full-scale samples [40, 41]. These "fluorescent fingerprints" can be easily obtained by the routine lambda-scanning at most of confocal laser scanning microscopes.

We present a novel technique based on a strict relation of the physiological state of cyanobacterial cells and their genera affiliation with the intensity and shape of the intrinsic single-cell fluorescence spectra, obtained by means of confocal microscopic spectroscopy. The nondestructive spectral analysis conducting *in vivo* at a cellular level allows to obtain more complete information about special features of individual cyanobacterial cells and supports the registration of very weak variations in their physiological state. The application of this technique for automation of control processes gives an additional opportunity to rise an effectiveness of production in biotechnology and brings in a valuable contribution to the development of innovative approaches in environmental monitoring.

In this chapter, two main application problems are investigated by means of fluorescent microscopic spectroscopy. Firstly, how the composition of photosynthetic pigments affects the shape of *in-vivo* single-cell fluorescence spectra and, secondly, how the differences in the fluorescence response of cyanobacterial cells may be used for investigation of their physiological state and biological diversity.

2. Fluorescence microscopic spectroscopy

Modern fluorescence microscopic spectroscopy (FMS) or confocal laser scanning microscopic spectroscopy provides a unique opportunity to obtain high-resolution images and intrinsic fluorescence emission spectra from single cyanobacterial cells [42–45]. Moreover, using spectral unmixing, the fluorescence of individual spectral components can be resolved, and their relative intensities can be calculated [46–48]. Unfortunately, most of the researches use confocal laser scanning microscopy only for imaging [49–54]. In this part, the attention is paid mostly to the spectroscopic studies by means of CLSM, and we will give some guidelines on methods of investigation and sample preparation.

It is well known that measurement and analysis of fluorescence is one of the most powerful ways to probe photosynthetic systems because it reports on the energy transfer and trapping. This fluorescence originates from excited states that were lost before photochemistry took place. It usually represents a small fraction of the excited state decay in a functional photosynthetic complex. Nevertheless, this small fraction can be easily detected by CLSM. With the confocal fluorescence microscopy, a very small excitation and detection areas can be investigated, so that single cells under non-damage conditions can be studied *in vivo*. Although, the pigment structure of different cyanobacterial strains has been intensively investigated, the variations of *in-vivo* operation of photosynthetic apparatus for different cyanobacterial species have not been analyzed yet. We suppose that the best way to investigate the operation of photosynthetic system *in vivo* is a single-cell fluorescence spectroscopy. Single-cell detection can provide the information on small peculiarities that is regularly buried in normal ensemble average experiments. This is thus a good way to study the time evolution process and spectroscopic properties of individual cells. Both steady-state and time-resolved fluorescence measurements can be used for probing the organization and functioning of photosynthetic systems by means of CLSM.

2.1. CLSM parameters

In the presented investigation, Leica TCS-SP5 was used for spectral CLSM of living cyanobacterial cells. Fluorescence emission spectra of the intact cells were measured at eight excitation wavelengths corresponding to all available laser lines. The excitation wavelengths are: 458, 476, 488, 496, and 514 nm—the lines of Ar laser, 405 nm is the line of diode UV laser and 543 and 633 nm are the lines of HeNe laser. In the experiments, presented below in this chapter, laser power settings were as follows: 29% of Ar laser power was reflected onto sample with acousto-optical tunable filter (AOTF) and further power percentage for its laser lines was: 30% of 458 nm laser-line and 10% for all other lines. 405 nm line of diode UV laser was reflected onto sample with 3%, HeNe laser lines 543 and 633 nm were reflected with 10 and 2%, respectively. An acousto-optical beam splitter (AOBS) was used to transmit sample fluorescence to detector. Emission spectra between 520 and 785 nm were recorded using the lambda scan function of the "Leica Confocal Software" by sequentially acquiring a series ("stack") of 38–45 images, each with a 6-nm fluorescence detection bandwidth and with 6 nm wavelength step. For obtaining fluorescence-intensity information images of 512 × 512 pixels were collected with a 63× Glycerol immersion lens (glycerol 80% H_2O) with a numeric aperture of 1.3 (objective HCX PL APO 63.0 × 1.30 GLYC 37°C UV) and with additional digital zoom factor

5–9. 1 pixel corresponds to 53.5 × 53.5 nm. The photomultiplier (PMT) voltages were used in range from 900 to 1100 V. The fluorescence emission images were accompanied with the transmission images (in the parallel channel), collected by a transmission detector with the photomultiplier voltages ranged from 300 to 500 V. For better signal yield, lambda scans were performed with "low speed" setting (400 Hz) in bidirectional scan mode and with a pinhole setting of 1 Airy unit (the inner light circle of the diffraction pattern of a point light source, corresponds to a diameter of 102.9 μm with the lens used (see [46]). Regions of interest (ROIs) representing single cells or subcellular regions were used to calculate fluorescence spectra.

2.2. Prevent photobleaching

In CLSM applications, the laser light density in the focus point is high. But, generally, it is difficult to compare the excitation energies used in CLSM with those from methods developed to measure photosynthetic parameters. In CLSM, light is deposited in short "dwell times" during the laser scanning process. Dwell time and the intervals between the illuminations may influence photo-damage and saturation of photosynthesis. Thus, since most chromophores bleach under the high laser excitation energies, a bleach-test should be performed [43]. It was shown experimentally that especially phycoerythrin (PE) and phycocyanin (PC), as an accessory pigments, were very sensitive to photo-bleaching, while the fluorescence of Chlorophyll *a* (Chl *a*) and allophycocyanin (APC) remained stable in the intact cells [43]. During the detection, the fluorescence of the main accessory pigments for each cyanobacterial strain should be controlled and the changes in their fluorescence should not exceed 10–20%. The power of individual laser lines should be chosen according to the photodamage they cause. In our experiments, the repeated spectra were obtained under selected excitation power at a fixed point in a cell to check whether the excitation would affect the cells. It was shown that at the above chosen excitation energies, the fluorescence spectra did not vary within the experimental error during 10–15 records. When excitation energy was increased, both the height and the center of the bands varied enormously with time because of photodamage or structure-breakdown in photosynthetic systems. In the experiments, where several laser lines were involved for the investigation, the first spectrum was recorded again at the end of each series to control the initial state of the cell. To compare different cells in one physiological state, the fluorescence spectra were taken from the cells of one strain cultured at different days and it was established that the variations in spectrum shape and intensity are not considerable. To visualize differences between strains with higher spectral and spatial resolution, lambda scans were performed with 6 nm bandwidth and with 6 nm steps. As far as the fluorescence intensities depend on the excitation energy (which varies for different laser lines), sensitivity setting of the photomultiplier, and the distance from the sample, all spectra were usually normalized to their maximum and only qualitative analysis was performed.

2.3. A set of excitation wavelengths should be used during the investigation

It is well known that phycobilisome contains several kinds of biliproteins, and its absorption and fluorescence spectra reflects the contribution of each. On the other hand, as a result of energy transfer among the tightly coupled biliproteins in the phycobilisome, fluorescence of the intact living cyanobacterial cells is originated from the efficiency of the energy transfer between these components and each transfer step appears in the spectrum shape as peak or

shoulder (**Figure 1**). Moreover, depending on the excitation wavelength, the room temperature fluorescence emission spectrum of intact cyanobacterial cells exhibits various extents of contribution of phycobilisome emission to the spectrum. If one exclusively excites Chl *a*, using a 458 nm line of an Ar laser, the emission spectrum by cyanobacterial cells shows no appreciable emission of PC or APC. In cyanobacteria, the 458 nm excitation is preferentially absorbed by photosystem I (PSI) that contains more Chl *a* than by photosystem II (PSII) and is stoichiometrically more abundant than PSII. However, because reaction center of PSI turns over faster than the PSII, it has lower fluorescence intensity than the PSII antenna. This is indicated by PSI emission band at 715 nm which is much weaker than the PSII emission band at 682 nm. The excitation by intermediate (blue and green) wavelengths (405, 488, and 496 nm) reveals fluorescent maxima of all photosynthetic pigments, as 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 (**Figure 1**). The direct excitation of cells in the PC absorption region at 514 and 543 nm, results in emission spectrum with two main peaks at 580 and 656 nm, which are due to PE, PC and APC emission and for species that lack PE the emission accumulates

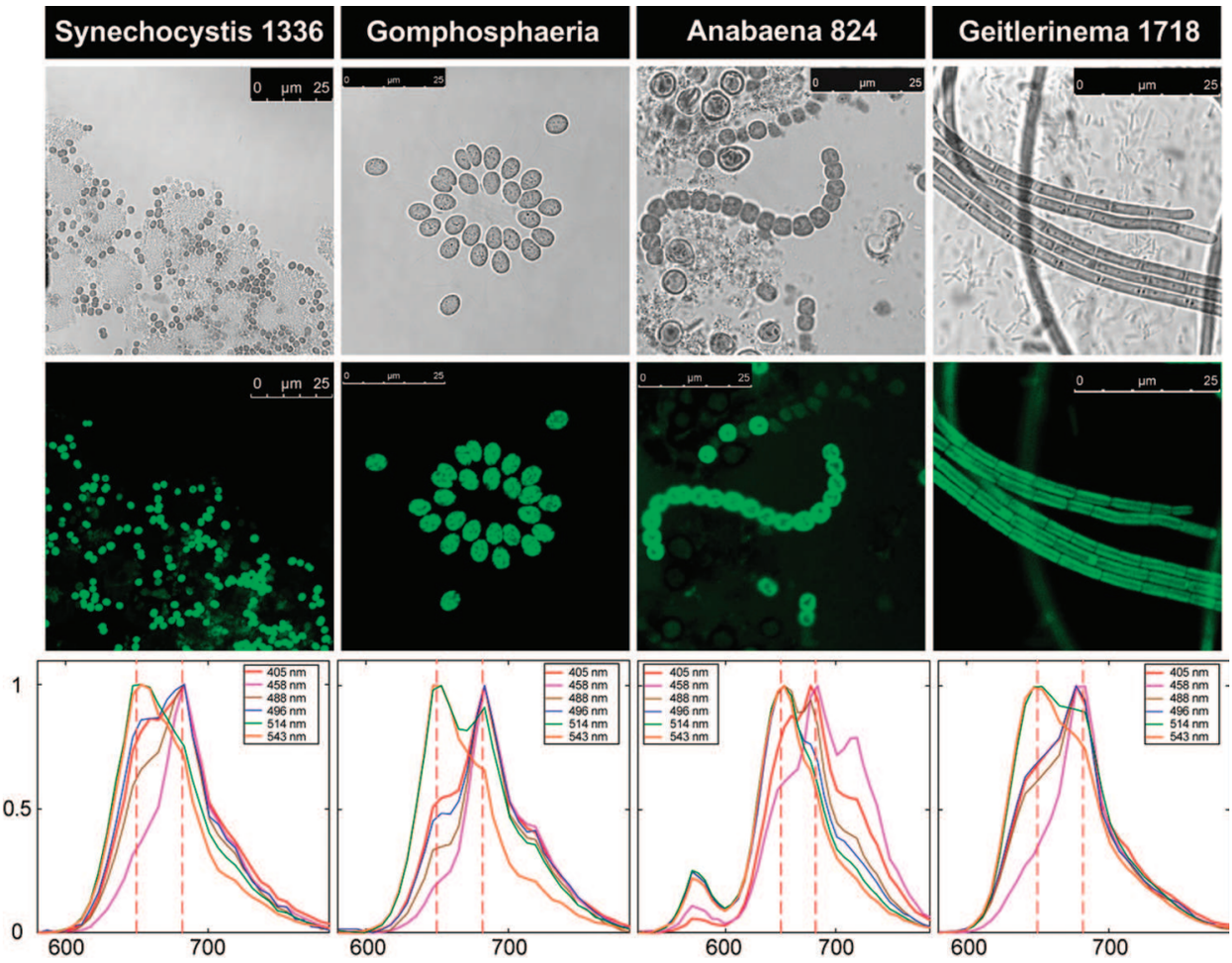

Figure 1. The examples of CLSM images and normalized single-cell fluorescence spectra for four cyanobacterial strains. The white bar corresponds to 25 µm. Corresponding excitation laser lines are indicated in plot legend. Dashed lines indicate fluorescence wavelengths of PC and Chl *a* fluorescence at 656 and 682 nm, respectively.

mostly near 656 nm. The spectra of the 633 nm excitation (not shown here) directly give a prominent emission band at 656 nm, that originates from PC, omitting band at 580 nm, which cannot be excited by 633 nm, even for species that have PE. Other small emission bands, corresponding to fine pigment structure of antenna complex, are not resolved in the room temperature investigation. Comparative analysis of the series of fluorescence spectra for different cyanobacterial species and strains reveals visible variations in their shape (**Figure 1**). If the fluorescence spectra were taken from alive cells in normal physiological state, which are cultured in the same growth environmental conditions, then the interspecies variations in pigment/chl *a* ratios are more pronounced than variations within the individual species. Species/strains differentiation could be carried out on the base of fluorescence analysis.

2.4. Investigation of physiological state of single cell

As it was pointed out, the single-cell fluorescence spectra depend not only on the selected strain, but also on the physiological state of the chosen cell. CLSM is the only method that can accurately diverse alive and semi-dead cells (**Figure 2**), and compared to other direct fluorescence measurements, records the fluorescence emission spectra from only active pigments in alive cells and does not acquire information from dissolved pigments, organic substances, and debris around. Considerable differences in shape and intensity of the fluorescence emission spectrum of cyanobacterial cells within one strain in normal and depressed physiological states allow to estimate the viability of the whole culture relying not only on the visual methods, but also on the accurate spectral analysis. In **Figure 2** (right panel), three typical fluorescence spectra for three different physiological states of one microcystis cell are presented. Spectrum I corresponds to the alive cell, spectrum II is the spectrum of cell in the depressed physiological state, and spectrum III is a spectrum of the dead cell. All spectra were excited by 488 nm laser line. Here the normalization to maximum intensity was not made, so that to illustrate the considerable difference in intensities between normal and depressed cells. On the left panel, corresponding transmission and fluorescent images of the cell under

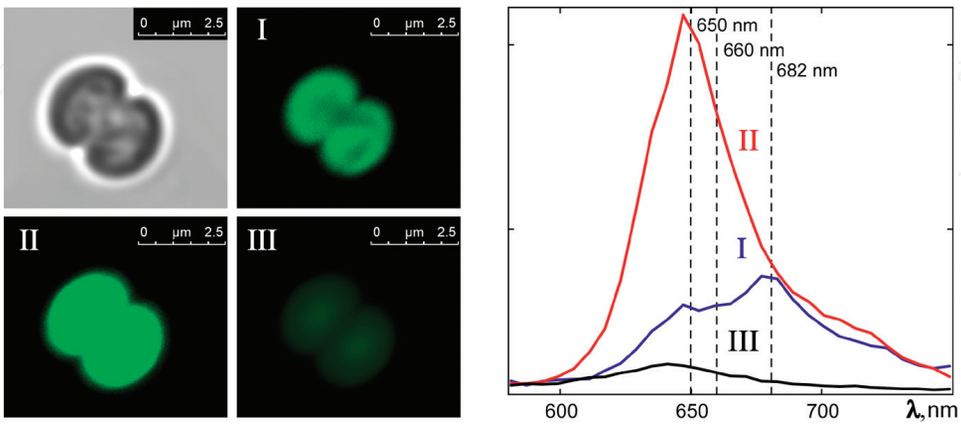

Figure 2. CLSM images and single-cell fluorescence spectra (in relative units) for cyanobacterial strain *Microcystis CALU 398*, obtained at three different physiological state of the same cell: I—spectrum of the alive cell, II—spectrum of the cell in depressed physiological state, and III—spectrum of the dead cell. The white bar corresponds to 2.5 µm. Dashed lines indicates fluorescence wavelengths of APC, PC and Chl *a* at 650, 660 and 682 nm, respectively.

investigation are shown. Analyzing the shape of the ensemble average fluorescence spectrum and counting the relative number of alive and semi-dead cells the conclusion about the viability of the whole culture at given developmental stage could be made.

2.5. Sample preparation

Special attention should be paid to the sample preparation, as well as we work with alive objects. Coverslip should be pressed very carefully to prevent any glass slide, which can cause cell damage. On the other hand, one should keep in mind that cyanobacteria can move and glide; so to fix the object, the coverslip should be pressed hard enough to prevent any motility of the investigated object, which sometimes have a diameter near 1 μm (e.g., microcystis and synechocystis cells).

2.6. Spectral unmixing

There is another very powerful tool implemented in CLSM—spectral unmixing. Unfortunately, in living cyanobacterial cells, it meets some difficulties. The authors of [43] pointed out that a lot of problems arise during the spectral unmixing procedure, which is based on the spectra of isolated phycobiliproteins. These problems are caused by the fact that the light absorption and emission properties of isolated phycobiliproteins are rather different from those of the intact phycobilisomes in the living cyanobacterial cells. In living cells, the spectral properties of pigments from certain organisms may differ crucially from the properties of the dissolved ones, for example, spectra of the components can vary in peak widths and may be shifted in wavelength due to different pigment-protein and linker connections. Thus, the analysis based on the initial fluorescence spectrum without any decomposition is preferable for living cells.

3. Examples of fluorescent microscopic spectroscopy application

The detailed description of the morphology, structure, chemical and optical properties of light-harvesting complex of cyanobacteria, phycobilisomes and phycobilins can be found in numerous publications [55–72]. On the other hand, the fluorescence properties of the intact living cyanobacterial cells differ drastically from the properties of the detached phycobilisomes and its components and originate from the efficiency of the energy transfer between all components of the energy transfer chain included the final step, the delivery to PSII or PSI. Each transfer step result in the spectrum shape as a peak or shoulder. Moreover, fluorescence of photosynthetic pigments in the intact cells is affected by physicochemical and physiological processes that occur within and across the thylakoid membranes. Here we demonstrate on several examples how these peculiarities can be used for investigation of physiological state and biological diversity of cyanobacteria.

The correct identification of cyanobacterial cultures and estimation of their physiological state are quite important in the environmental monitoring and industrial applications. The ability to detect small variations in the physiological state of cyanobacterial culture under weak external treatments is quite desirable in both field and laboratory experiments. The fluorescent CLSM technique is a very powerful tool that can support any on-line field, laboratory

and technological study. In addition, the results of single-cell spectroscopic analysis are much more suitable for further statistical and analytical calculations then the conventional optical methods of investigations. In this chapter, we present several examples of practical application of the described CLSM technique.

3.1. Monitoring of physiological state of single cyanobacterial cell and a whole culture

Since the first broad-scale spectroscopic investigations, the authors of many articles note the dependence of the intrinsic fluorescence spectra of cyanobacteria on the developmental stage of the culture and physiological state of single cells. It is well-known that the light-harvesting and energy-transfer capacities of phycobilisomes can react to the environmental changes, as well as to the changes in physiological state of the living cells induced by stress conditions [39, 64, 73–75]. However, this effect has not yet been widely used to assess the viability of the culture. Several authors pointed out that, although a single-cell fluorescence spectra for the diverse physiological states differ significantly, the physiological state of the given cell cannot be estimated correctly because of the absence of a full set of reference spectra [40, 76, 77]. Moreover, the authors of [40, 76, 77] pointed out that while comparing spectra of individual cells and the results of the ensemble average experiments at a culture as a whole (so-called integral spectra), a significant difference was observed. Obviously, this difference is owing to a wide diversity of single-cell physiological states in the bulk growing culture, which in sum gives different integral fluorescence spectra for a specified strain at different developmental stages because of the variations in cell's proportions. This, of course, should be taken into account. On the other hand, the whole culture in addition to a set of single living cyanobacterial cells consists of metabolites, dissolved pigments, other organic substances and cellular debris. All these substances form undesirable and unpredictable fluorescent background in volume samples.

Actually, the intensity of fluorescence emitted by single photosynthetic cell *in vivo* depends only on the structure and operational effectiveness of photosynthetic apparatus, tracing in-time physiological state of the cyanobacterial cell. Thus, the fluorescence emission can be used effectively to monitor various physiological processes. **Figure 3** illustrates the temporal changes of in-vivo fluorescence spectrum taking place in one living cell of cyanobacteria strain *Synechocystis CALU 1336* under light and heat stress. On the other hand, this timeline set of fluorescence emission spectra illustrates all stages of cyanobacterial cell degradation, that is, all possible physiological states. It is obvious that during the evolution of the culture and aging of each cell all this stages will be presented in the natural samples simultaneously.

Several newer publications [76–80] clearly demonstrate that the variations in the fluorescence shape and intensity of living cells, presented in **Figure 3**, indicate the consequent degradation in the light harvesting chain (antenna complex—reaction center) and following dissociation of the detached antenna complex. It can be seen that in the alive cell, the chlorophyll *a* fluorescence prevails over the fluorescence of the pigment-protein complexes of phycobilisome. While the single-cell physiological state changes for the worse, the photosynthetic apparatus shows instability in operation, that is, the most part of the absorbed energy emits as fluorescence at the early stages of light harvesting. At the last stages, the changes in fluorescent spectrum, shown in **Figure 3**, are the same as it was demonstrated in the works [80–82], where

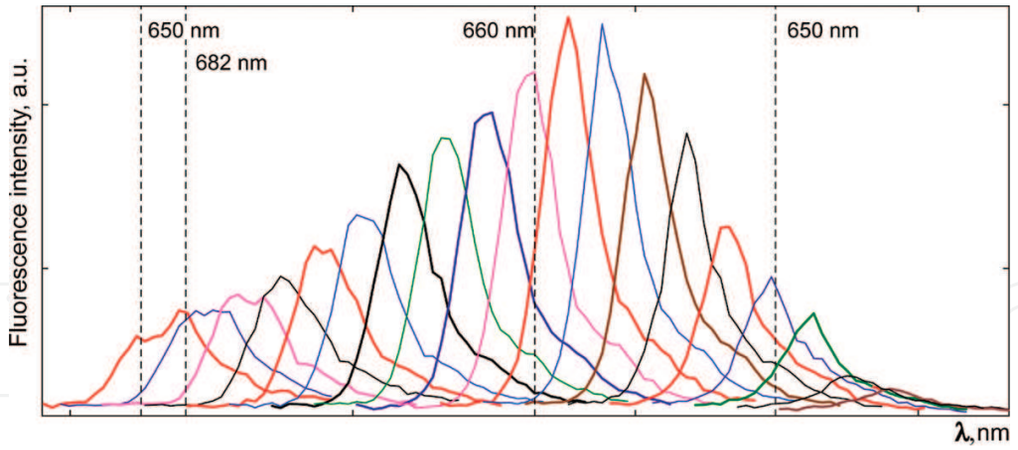

Figure 3. Time degradation of living cell of cyanobacterial strain *Synechocystis CALU 1336* under light and heat stress. Spectra were recorded at the excitation wavelength 488 nm and with the time step 2 min. Spectra are shifted along *x*-axis relative to each other for convenience of observation.

the dissociation of phycobilisomes was investigated. Thus, the estimation of the viability of single living cell and the whole culture is possible via investigation of the changes in fluorescence emission spectra.

Here we present a novel technique, recently elaborated by authors of this chapter, which is based on a strict relation between the shape and intensity of a single-cell fluorescence spectra of cyanobacteria and the physiological state of this cell. This technique is a direct extension of the previously elaborated visual methods of the estimation of the physiological state of cyanobacterial cells by the color of their fluorescence conducted by means of conventional fluorescent microscopy [39]. In contrast to the latter, which is very subjective, a new technique provides a detailed spectroscopic information and variations in "color" of the fluorescence can be measured in nanometers of light wavelength. A general character of the presented technique makes it possible to use it for investigation of any species of cyanobacteria, regardless of their habitat or cultivation conditions. Also the influence of scattering particles and preillumination effects, which are very important in the ordinary fluorescent methods, are absent in single-cell microscopic spectroscopy technique. Moreover, the use of the novel methods of microscopic spectroscopy allows to estimate the viability of colonies of noncultivated cyanobacterial species in natural samples according to the physiological state of individual cells. This fact can considerably facilitate the work with small concentrations of objects under consideration in environmental probes.

According to the proposed technique, the research process can be divided into three steps:

Step 1. Obtaining of the complete set of single-cell fluorescence spectra for given cyanobacterial strain in different physiological states by recording a series of spectra during single-cell degradation by means of CLSM at a certain excitation frequencies (e.g., at 458, 488 and 514 nm), as it was previously shown for 488 nm laser line in **Figure 3**. These sets will serve as reference spectra while determining the rate of cell degradation. At the same time, the most informative spectra for further investigation should be selected, which reflects the physiological state of the cells of a given strain.

Step 2. Recording of several sets of fluorescence spectra for single cells from the tested sample, which belongs to different physiological states, according to obtained reference spectra for

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this given strain. For accuracy of further estimation of the viability of the whole culture, these spectra should be recorded at several excitation wavelengths for each cell (in our investigations it was set of eight laser lines) and then each one should be averaged through a number of cells. The fluorescence spectra of a single cell excited by one laser line is not enough to have a complete information about deviations in photosynthetic process and pigment composition.

Step 3. Direct study of the tested sample in order to determine the viability of investigated culture or colony. A representative random sampling is used for the cells from a given strain and single-cell fluorescence spectra excited by chosen excitation wavelengths are recorded. At this stage, two variants of investigation are possible according to the final purpose.

If the aim is to estimate the viability of a specified culture or colony, one can directly count the rate of normal and depressed cells by their fluorescence emission characteristics and make a decision about the viability of the whole culture. In different periods of culture development, the percentage of living and depressed cells changes significantly. If the percentage of alive cell spectra prevails in the sample, then the culture is supposed enough viable. If the sample has more spectra of sick and dead cells, then the culture is weak and incapable of active reproduction. Alternatively, one can make a linear combination from several spectra, belonging to different physiological states, and compare this result with the integral spectra of the whole culture, obtained via conventional fluorimeter. The fitting coefficients in this linear combination will show the rate of the viability of the considered culture. The second way is less accurate, due to the undesirable fluorescent background in the whole culture mentioned above, and can be applied only for the fresh culture at the early growth phase.

If the main purpose of the investigation is to reveal the influence of weak external actions or environmental changes on the physiological state of single cells from the tested sample, the comparative analysis of single-cell fluorescence spectra from the control and treated culture should be carried out. In this case the investigation of the cells in normal (good) physiological state is enough. If any changes in the shape or intensity of single-cell fluorescence spectra are registered compared to the reference sampling, thus the influence takes place. Independently to the form and the sign of this changes, one can fix the result of the external action; however, it is impossible to consider the origin of this result. For the latter, the additional precise investigation by means of other physical or chemical methods has to be done.

Let us illustrate the effectiveness of this technique on a concrete example of the estimation of the viability of the culture of cyanobacterial strain *Synechocystis CALU* 1336 (from CALU collection), provided by of the Core facility Center for Culture Collection of Microorganisms of Saint-Petersburg State University.

The experiment was carried out in the following way. After passing through steps 1 and 2, for the considered culture the spectra of the individual cells in the normal and depressed physiological states were recorded by means of CLSM at eight excitation wavelengths and averaged for each state (**Figure 4a–c**). Usually, for the culture in exponential growing phase, the range of physiological states includes almost all developmental stages presented in **Figure 3**, but only two main single-cell physiological states—"healthy cells" and "cells in strongly depressed state"—can be selected for further calculations, as it was previously shown in **Figure 2** (states I and II, respectively). These two basic sets of spectra for the main physiological states are shown in **Figure 4a–c**. Blue lines show healthy cell in good physiological state, red lines correspond to the strongly depressed cells. For the clarity of further narration, only three of the

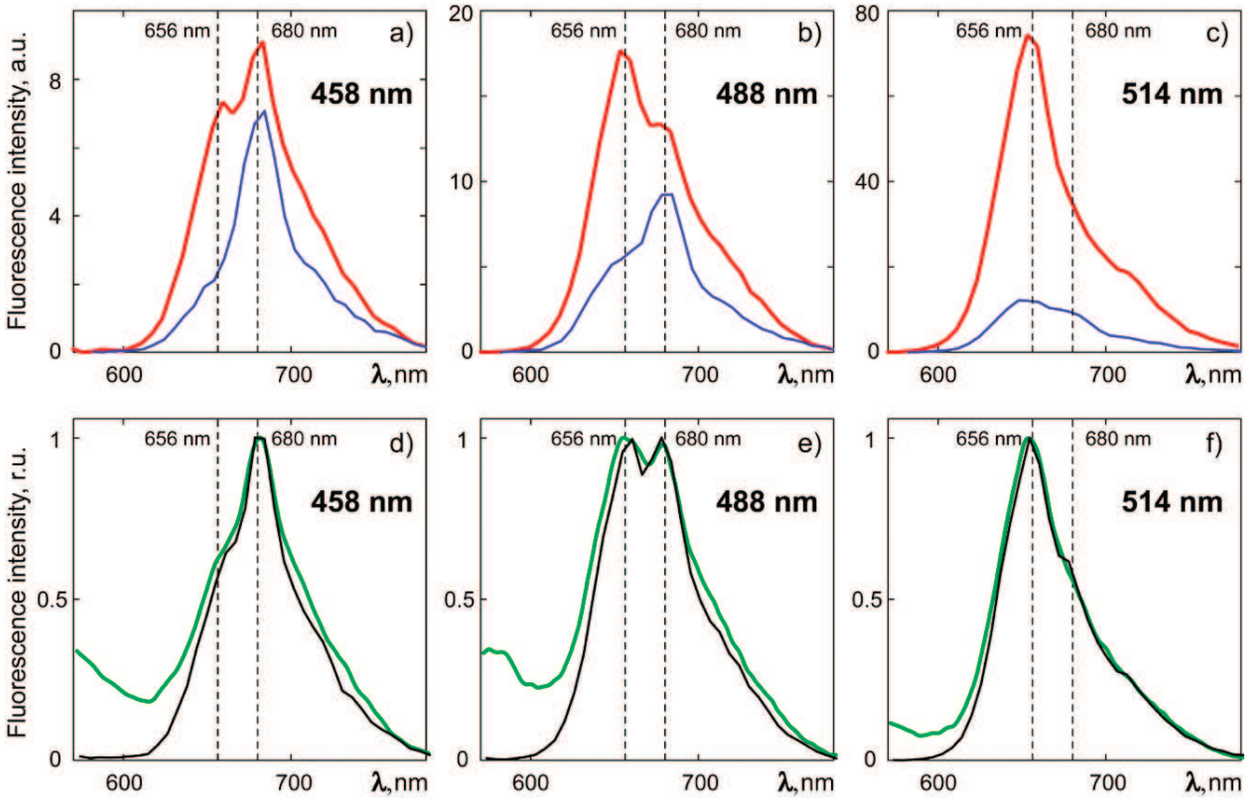

Figure 4. The illustration to the process of the culture viability estimation. (a–c) Averaged SFS for cyanobacterial strain Synechocystis CALU 1336, excited by three laser lines 458, 488, and 514 nm. *Blue* lines show spectra of alive cells, *red* lines are for the cells in the depressed physiological state. (d–f) Fitted and integral normalized spectra for the whole culture under consideration. *Black* lines show a linear combination of CLSM spectra of alive and depressed cells summed in ratio 2:1. *Green* lines show the integral spectra of the whole culture obtained using Cary Eclipse fluorimeter (Varian Cary) at corresponding excitation wavelengths. At all plots dashed lines indicate the fluorescence maximum of chlorophyll a (680 nm) and phycocyanin (656 nm), respectively. For SFS averaging was carried out over 10–15 cells.

eight obtained spectra are presented in **Figure 4**. Then, the integral spectra of the whole culture were obtained at corresponding excitation wavelengths using Cary Eclipse fluorimeter (Varian Cary) (**Figure 4d–f**; green lines). Two sets of eight fluorescence spectra for cells in normal and depressed physiological state were taken to obtain a linear combination for fitting procedure a set of integral spectra. Each spectrum in two sets was averaged over 10–15 cells. It should be noted that to raise accuracy of calculations, the curve fitting process was done over the whole set of the eight spectra simultaneously, so that to exclude any ambiguity. The fitting coefficients for alive and depressed cells were about 2 and 1, correspondingly. Thus, the whole culture can be considered as a healthy and being in the exponential or logarithmic growth phase.

As it is following from the plots of **Figure 4a–c**, the degree of cell damage is determined mostly by the relative fluorescence intensity of phycobilins at 656 nm and chlorophyll-binding proteins at 680 nm. It is especially clear from the excitation wavelength 488 nm (**Figure 4b**), where the fluorescence spectra for healthy and depressed cells have the mirror shape. For the 458 nm excitation, the shape and the intensity of fluorescence spectra for this two states differ not so much because this wavelength excites better the peak of chlorophyll-binding proteins in PS II (680 nm), which is not affected. On the other hand, the 514 nm wavelength strongly excites the fluorescence of the antenna pigments and the fluorescence spectra intensity differs significantly for the healthy and depressed cells.

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Despite the near qualitative character of the presented analysis, the accuracy and stability of this method are ensured by the simultaneous calculations over a series of eight spectra. If several variants are possible while fitting one spectrum, then simultaneous fitting of eight spectra will provide a sufficient accuracy. This small example demonstrates that the shape of single-cell fluorescence spectra reflect the physiological state of cyanobacteria and the sum of single-cell contributions represents viability of the whole culture because these phenomena are strictly related with the correct or incorrect functioning of the photosynthetic apparatus.

Finally, it should be noted that the presented technique can be modified for obtaining any developmental stage of the selected cyanobacterial culture, by considering the reference set of fluorescence spectra to be fitted. For instance, in the technological process of the industrial incubation of cyanobacteria, involved in food production, it is quite desirable to estimate the optimal stage of the culture development when the accumulation of biologically active compounds attains its maximum. This process can be controlled via online recording of several fluorescence spectra and comparing them with reference ones. Moreover, in the environmental monitoring, the online estimation of the viability of the cyanobacterial colonies in the field samples via fast and effective fluorescence technique can assist the prediction and prevention of the hazardous cyanobacterial blooms.

More detailed description of the presented technique and its application can be found in [83].

3.2. Ultrasonic treatments

In recent years, several environmentally friendly methods for preventing of toxic cyanobacterial "blooms" of water bodies have appeared. One of them is a weak ultrasound treatment. Unfortunately, due to the low intensity of the applied sonication and its constant but weak influence on the biophysical parameters of the cyanobacteria most of the results of the previous investigations are quite ambiguous [32–34, 84–89].

Despite the significance of the problem of cyanobacterial blooms and a variety of applied methods to control them, the sufficient principles of investigation and monitoring the results of various external actions on cyanobacteria are not developed. Currently, the most of all studies are based mainly on traditional visual methods of obtaining results or on the analysis of fixed or dissociated environmental samples. However, standard methods can only record the presence of the bloom in reservoir, but cannot determine at what evolution stage it is situated, or, all the more, to predict the possibility of further cyanobacterial bloom. This owes to the use of rather crude methods of monitoring of the physiological state of the culture during the experiment. All previously elaborated monitoring methods either destroy cells or change significantly their physiological state just before the measurements, so it is impossible to determine the initial physiological state of the treated culture.

For example, in the paper [87], the ultrasonic inhibition of *Microcystis aeruginosa* cell growth and extracellular microcystins release was examined. The authors reported the decrease of antenna complexes like cyanobacterial chlorophyll a and phycocyanins (PC), and the oxygen evolution rate. The conclusion about slowing down of the photoactivity and damaging of the antenna complexes was made according to the measuring of the growth rate of the whole culture. This is not the case because there is no any confirmation that the single-cell physiological state really

changes. May be the whole culture died due to other reasons or simply several cells were destroyed during sonication. Moreover, the pointed sonication power settings (about 0.32 W/mL) cannot be considered as a weak and environmentally friendly treatment. It cannot be applied to the natural reservoirs, so that to carry out a real control on cyanobacterial blooms in open water. Obviously, this high intensity of sonication was used to obtain any possible results because used methods are not precise enough. Thus, this example shows that the standard methods do not give the correct results, and new precise, nondestructive *in vivo* methods for monitoring of the physiological state of cyanobacterial cultures are required for such investigations.

In the previous section, we present a novel noninvasive technique for estimation of the physiological state of single living cyanobacterial cells. Let us demonstrate the results of the application of this technique to the ultrasonic treatment experiments with cyanobacterial cultures.

In this investigation, the photosynthetic activity of living cyanobacterial cells treated by ultrasonic radiation was studied. A strain *Synechocystis CALU 1336 aquatilis* from CALU collection of core facility Center for Culture Collection of Microorganisms of St. Petersburg State University was used in the experiment. After 8 days incubation, the tested culture was divided into two parts and placed in the same light, temperature and nutrient conditions. One part was a control sample and another part was sonicated via original, specially designed, ultrasonic laboratory device shown in **Figure 5**.

The sonication was performed in a 35-mm plastic Petri dish placed on the ultrasound emitter. Ultrasound emitter consists of a ceramic resonator connected with handheld pocket frequency generator HPG1 (Velleman Instruments Inc.), it has an emitting surface area about 13 cm^2. The ultrasonic field inside the dish was measured out with a standard calibration ultrasound-needle-hydrophone connected to a TDS 3000 oscillograph (Velleman Instruments Inc.). For each experiment, 10 mL cyanobacteria solution was filled in a dish and kept at 25 ± 2°C. The ultrasound frequency and power density were about 60 kHz and 5.85 mW/mL, correspondingly, and the sonication time was near 24–30 h. Such sonication conditions were

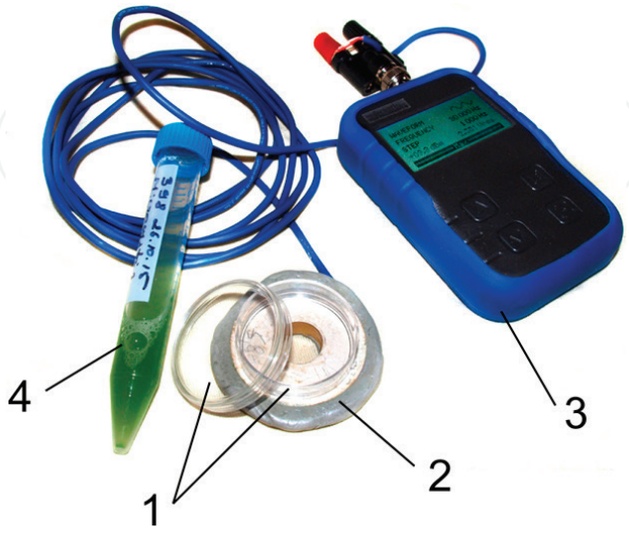

Figure 5. The developed device for laboratory ultrasonic treatment of cyanobacterial culture. (1) Plastic petri dish, (2) ultrasonic emitter, (3) generator of ultrasonic frequencies and (4) sample culture.

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chosen following the literature reports [32, 86] and based on our preliminary tests. During sonication, the cyanobacteria solution was carefully shuffled and taken for analysis just in the process of sonication. Control sample without any treatment was kept under exactly the same conditions during the whole period of the experiment.

In **Figure 6**, single-cell fluorescence spectra for two emission wavelengths (488 and 543 nm), obtained by standard lambda-scanning using Leica TCS-SP5 CLSM, are shown for control and treated samples. Blue lines demonstrate the spectra of the control suspension, whereas red lines show the spectra of the experimental one. It is clear that the fluorescence intensity in the control group differ from the experimental one. According to the preliminary studies, enhancement of the fluorescence at 660 nm in the experimental culture indicates that this culture is in a depressed physiological state in comparison with the control group. Each spectrum in **Figure 6** was obtained by averaging over 15 cells. Spectral analysis on the cell level using CLSM makes it possible to obtain more comprehensive information on the small variations in physiological state of both single cells and the entire culture exposed to ultrasonic treatment. During ultrasonic treatment, single cyanobacterial cells were studied at the first day to obtain the reference spectra of the initial cells in a good physiological state. Then, after 24–30 h, both control and treated specimens were studied and their fluorescence spectra were compared (**Figure 6**).

Let us mention here again that the treated and control probe initially were taken from one cultural sample and were placed at the same environmental conditions. Else, all the obtained results were double controlled via conventional fluorometric methods such as pulse amplitude modulation (PAM) and absorption and fluorescent characteristics of the whole culture [90]. These measurements were conducted using the light curves method on the PAM 2500 pulse spectrofluorimeter (WALZ, Germany) and a standard spectroscopic technique using a Cary Eclipse fluorimeter (Varian Cary), correspondingly. The dependences of the electron transport rate (ETR) and the quantum yield of photosystem PS II (Y(II)) on photosynthetically active radiation (PAR) under blue actinic light, obtained via WALZ PAM, show that ETR and

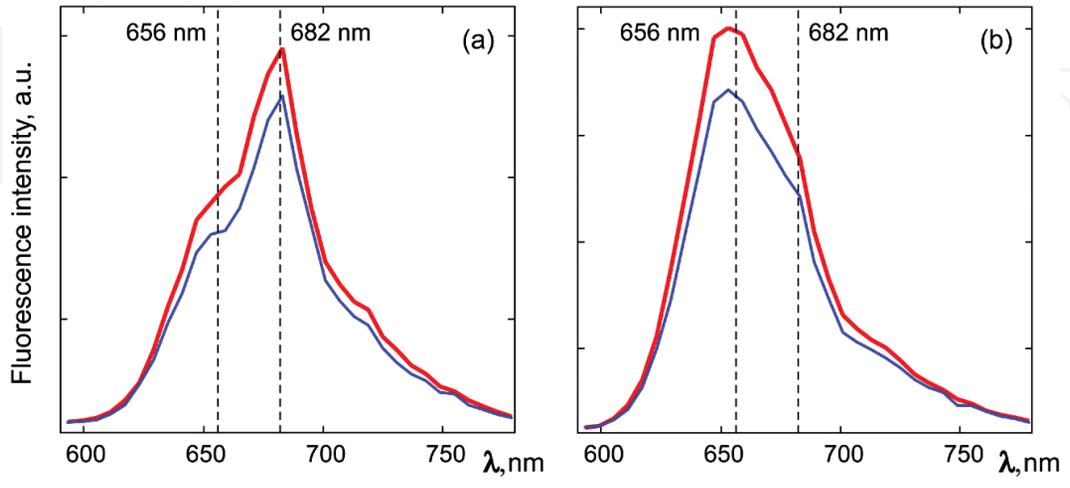

Figure 6. Single-sell fluorescence spectra for cyanobacterial strain Synechocystis CALU 1336 obtained at two excitation wavelengths (a) 488 nm, (b) 543 nm. Red lines—the culture exposed to ultrasound; blue lines—the control culture. Each spectrum was obtained by averaging over 15 cells.

Y(II) decrease in the sonicated culture, which indicates that the physiological state of the culture under sonication is depressed. At the same time, the nonphotochemical quenching of the absorbed light by the fluorescence rises considerably for the treated culture. Comparison of the results of the CLSM spectroscopic measurements with those obtained using conventional fluorimeter and pulse-amplitude modulation approaches confirmed the inhibitory effect of low ultrasonic frequencies (~60 kHz) on the physiological state of cyanobacterial cells and whole cyanobacterial cultures.

It can be concluded with confidence that the results obtained via the CLSM technique are correct, and the reduction of the photosynthetic activity and dumping of single-cell physiological state occur as a reply on the external ultrasonic action. The results presented here demonstrate the experiments conducted with the strain Synechocystis CALU 1336 aquatilis; however, similar results were already obtained for another unicellular cyanobacterial species (*Microcystis CALU 398*). Thus, the treatment presented here may refer to a rather diverse group of unicellular cyanobacteria.

It should be noted that ultrasonic treatment is widely used not only for inhibition of cyanobacteria growth diring harmful blooms, but also for enhancing of protein content and the whole biomass in the industrially cultured strains [91, 92], depending on power-frequency characteristics. Thus, it is very important to obtain on-line correct information about the influence of the ultrasound with given power and frequency on the specified cyanobacterial strain. The noninvasive fluorescent technique presented here gives the opportunity to detect any weak variations in the physiological state of single cyanobacterial cells in real time during sonication.

3.3. Differentiation of cyanobacterial cultures on the base of single-cell fluorescence spectra

The automatic on-line differentiation of cyanobacterial species is a key problem in both industrial biomass production and environmental monitoring. In this section, we present a novel technique for taxonomic discrimination of cyanobacteria based on the numerical analysis of *in-vivo* single-cell fluorescence spectra. An optimal set of the parameters is considered, which is sufficient for determination of the taxonomic position of cyanobacteria by means of mathematical statistics. On the base of the linear discriminant analysis obtained spectroscopic data for 21 cyanobacterial strains from *CALU* collection were analyzed. It was shown that the presented technique allows an accurate differentiation of cyanobacteria up to the species/strain level and enables to distinguish automatically potentially harmful strains.

Since the early 1950s, three different methods are commonly used to characterize phytoplankton and cyanobacterial samples taxonomically: high performance liquid chromatography (HPLC) [13–15, 23, 93]; flow cytometry [10, 11, 94, 95]; and optical microscopy. Various methods have been developed with the aims of increasing accuracy and yielding qualitative information. However, all of them have different limitations. Till now, the best taxonomic differentiation is still obtained using classical inverted microscopy. Unfortunately, this method is time-consuming, human-based and requires appropriate technical skills, and this eliminates the possibility of its application for continuous on-line monitoring. Nearly single-cell flow cytometric analysis is based on light scattering by the cells and fluorescence of the chlorophylls and the phycobilins. It can be easily automated, but it is appropriate only

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for unicellular species and is useless for numerous industrially cultured filamentous strains. HPLC is the only method, of the three, that is based on the chemical constituents in the sample. The problem is that during the chemical sample preparation, most of the information about the peculiarities of individual species is lost and the residual part of the information is not enough for species/strain classification inside cyanobacterial genera, and is suitable only for the rude differentiation of big classes of phytoplankton. As it was mentioned earlier, several factors contribute to the spectroscopic properties of the phycobilins: the number and chemical nature of the bilins attached to the polypeptide chains; the effects of protein conformation or aggregation state; and interaction between the bilins. Any of this feature can be unpredictably changed during the extraction and purification procedure [96]. Thus, only spectroscopic properties of the intact living cells can give pure unspoiled information about distinctive features of light harvesting complex in specified cyanobacterial strain.

Analysis of the *in-vivo* absorption and fluorescence spectra is an alternative way of obtaining qualitative information about the phytoplankton abundance and composition, which is continuously demonstrated by various publications [21, 23–27, 36, 97–100]. The relative phytoplankton abundance can be calculated once initial assumptions about the phytoplankton classes present and their pigment compositions have been made [10, 24, 25, 36, 100]. However, the correct classification of cyanobacterial species on the base of their fluorescence signature was hampered by alterations in pigment composition within one strain, which depends on the environmental conditions [93]. On the other hand, several researchers show that the nutrient and light limitations do not significantly change the initial fluorescence spectra and cannot impede the species discrimination [98, 101].

May be the first attempt to use phycoerythrins as chemotaxonomic markers was done by Glazer et al. [96] for red algae in 1982, but until now fluorescence spectra of phycobilins do not appear to be useful at familial, ordinal and class levels in taxonomic studies. Although the investigation in [96] concerns only purified high molecular weight phycoerythrin from red algae this work clearly demonstrates the possibility of the correct taxonomic analysis on the base of phycobiliproteins structural differences, which can serve as intrinsical fingerprints for taxons and genera in phytoplankton diversity. Later the correlation between the distribution of the biliproteins and the genera of *Cryptophyceae* was discussed in [102]. In 1985, Yentsch and Phinney [19] proposed an ataxonomic technique that utilized the spectral fluorescence signatures of major ocean phytoplankton. Seppälä and Olli [97] used spectral fluorescence signals to detect changes in the phytoplankton community. In 2002, Beutler et al. reported a reduced model of the fluorescence from the cyanobacterial photosynthetic apparatus designed for the in-situ detection of cyanobacteria and presented a commercially available diveable instrument for on-line monitoring of phytoplankton structure [21].

We elaborate a strict procedure for recording and processing single-cell fluorescence emission spectra, which eliminates the most of mentioned above difficulties and has a quite high classification accuracy. As well as according to our technique the fluorescence spectroscopic information is obtained via CLSM, the initial data has less variations and can be accurately sorted. Any objectionable and unpredictable impact can be eliminated at the first step of obtaining fluorescence spectra. Since noninvasive and nondestructive method is used the information about vital cell operation (e.g., light harvesting) can be additionally taken into account. All this allows one to obtain the desirable result directly following the procedure.

The classification procedure consists of three steps: (1) obtaining single-cell fluorescence spectra and creation of reference database; (2) data processing and extraction of classification parameters; (3) statistical analysis and evaluation of classification procedures.

Step 1. To illustrate the usual form of spectra in Ref. database, in **Figure 7** several characteristic sets of single-cell fluorescence spectra are presented. Here four sets related to four cyanobacterial strains are shown: *Microcystis CALU 398*, *Merismopedia CALU 666*, *Leptolyngbya CALU 1715* and *Phormidium CALU 624*. Cyanobacterial strains are labeled according to *CALU* collection of the Core Facility Center for Culture Collection of Microorganisms of Saint-Petersburg State University. Each spectrum in the set was obtained by means of CLSM Leica TCS-SP5, using corresponding laser-line for excitation (405, 458, 476, 488, 496, 514, 543, and 633 nm). Corresponding excitation wavelengths are given over each spectrum. All spectra are normalized to the maximum intensity and shifted along x-axis for the clarity of observation. Four characteristic wavelengths, corresponding to the fluorescence maximum of different pigments can be easily distinguished at each spectrum: (1) peak near 580 nm corresponds to the fluorescence of phycoerythrin (is absent for *Microcystis* and *Leptolyngbya*), (2) peak near 656–560 nm corresponds to the fluorescence of phycocyanin and allophycocyanin in common (they are undistinguishable at room temperature), (3) peak near 682 nm corresponds to the fluorescence of chlorophyll *a* of PSII, and (4) peak or shoulder near 715 nm represents the fluorescence from PSI.

To obtain the representative fluorescent signature for given cyanobacterial strain by means of CLSM several points should be kept in mind. Each set of fluorescent spectra for single cell includes 4–8 spectra, obtained at different excitation wavelengths. One or two spectra in series is not enough for further differentiation. Thus, the low power settings should be used at all laser lines in order to eliminate cell damage during the record. Moreover, the excitation

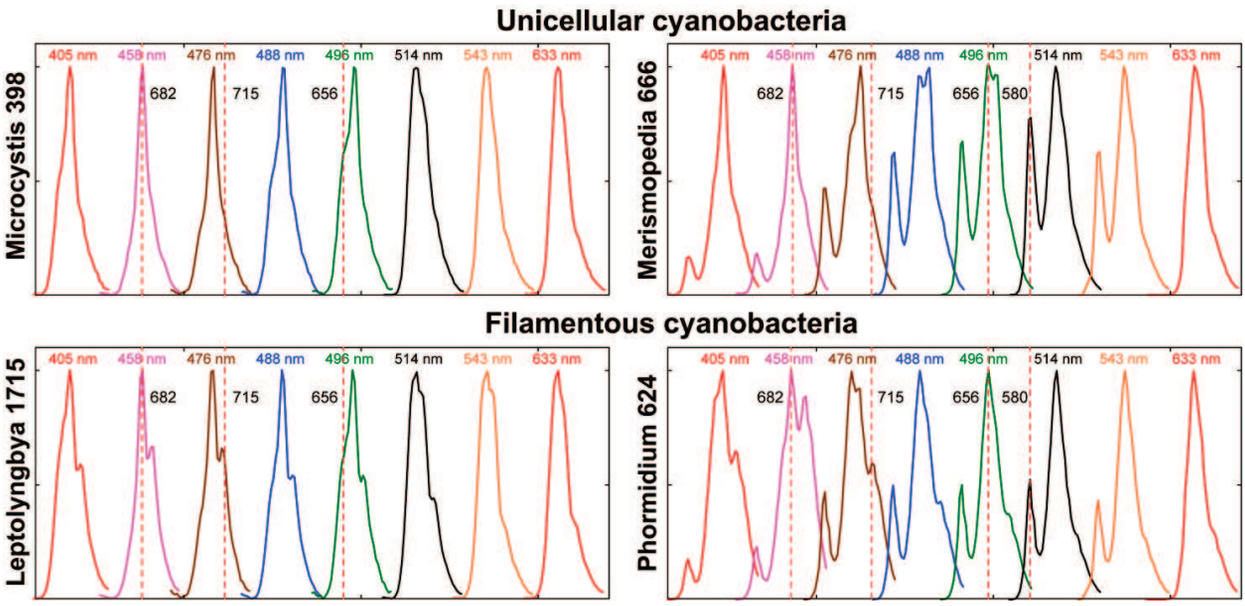

Figure 7. Four characteristic sets of single-cell fluorescence spectra, corresponding to unicellular and filamentous cyanobacterial strains. The excitation wavelengths (405, 458, 476, 488, 496, 514, 543, and 633 nm) are given over the curves. All spectra are normalized to the maximum intensity and shifted along x-axis for convenience of observation.

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wavelengths, that excite mostly one pigment (514, 543, and 633 nm), give less information than other laser lines and can cause more damage due to over-excitation. So, such excitation wavelengths should be used at the end of the record. To create a reference database of fluorescence spectra only the cells in normal physiological state should be used (if the study of the depressed physiological state is not a case of current investigation).

If the database is formed from the cultured species, it is desirable to obtain reference spectra several times at different days and for various developmental stages of the culture to exclude any discrepancy and to take in account all possible variations in spectrum shape. The experimental sampling for each strain should include the sets of fluorescence spectra for more than 30–50 cells, to evaluate the statistical analysis. For the cultured species or for the strains from culture collection specified nutrient, temperature and light conditions should be applied, identical for all samples involved in classification. This is strongly required to exclude any adaptation effects.

If the investigation is conducted over natural samples (for environmental monitoring), the reference database should be recorded for each tested reservoir because the difference in nutrient and light conditions could change the initial fluorescence spectra considerably. Moreover, this database should be extended by new experimental data constantly. While the reference spectra are available, the routine environmental monitoring is acceptable.

The whole procedure of obtaining intrinsic single-cell fluorescence spectra used in this study was designed to minimize preparatory manipulation, so as to conduct a noninvasive investigation of small amounts of experimental material and to prevent any damage of living cells.

Step 2. While as the initial database is completed, the extraction and selection of classification parameters is carried out. To extract from the initial single-call fluorescence spectra, a set of classification parameters recently a computer program in the MATLAB software application [103] was elaborated. By means of this program, interpolation and smoothing of the raw spectra were carried out to eliminate the random noise and metering fluctuations. All spectra were reduced to the same scale and size of data array by the procedure of normalization and extrapolation. The first derivative was taken over smoothed and interpolated spectra and the fast Fourier transform was performed to exclude random noise, owing to the low intensity of the exciting and emitting energy. Else, some specific values characterizing the shape of the curves and the spectral composition of their derivatives were calculated, such as asymmetry and excess.

In **Figure 8**, several plots are given to illustrate the process of extracting parameters. At the left panel, normalized characteristic *in-vivo* single-cell fluorescence emission spectra at excitation wavelength 488 nm for two cyanobacterial species *Leptolyngbia CALU 1713* (blue lines) and *Nostoc CALU 1817* (red lines) are presented. Shaded regions mark the ranges of averaging for corresponding fluorescence intensities (575–586; 654–658; 679–685; and 714–723 nm). While getting the mean values at the corresponding area, one can calculate the impact of each region into the whole fluorescence intensity in percents. This will be the first set of classification parameters named fluorescence emission percentage contribution for individual pigments. For eight laser lines and for four zones as a result, we have a set of 32 parameters. The usual parameters for characterizing the shape of the curves asymmetry and excess (AE)—give another 16 parameters. Finally, the last set—the FFT-contributions in three specified regions of wavevector domain—is formed analyzing corresponding Fourier-transforms for each first derivation

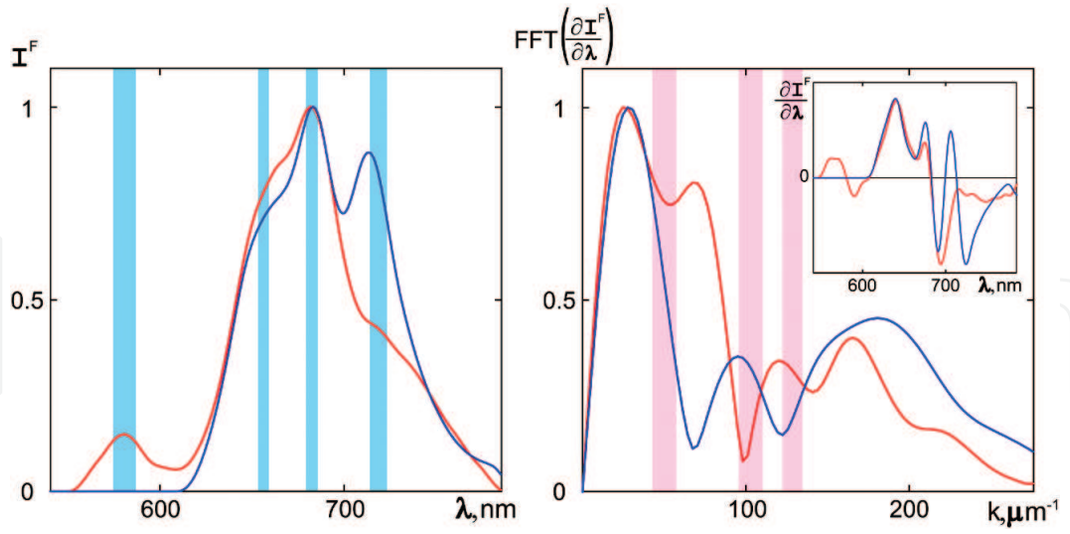

Figure 8. The illustration for the classification parameter's calculation. Left panel: normalized in-vivo single-cell fluorescence emission spectra of two representative cyanobacterial species: blue lines—*Leptolyngbia CALU 1713*, red lines—*Nostoc CALU 1817*. Right panel: Fourier transformants normalized at maximum for the corresponding first derivations of the initial fluorescence spectra. In the inset the first derivation curves are plotted. Excitation wavelength 488 nm. Shaded regions show the bands of averaging.

plot (see **Figure 8**, left panel). The inset in **Figure 8** shows the first derivation curves for corresponding spectra. Three regions (43–58 μm^{-1}; 95–110 μm^{-1}; and 123–135 μm^{-1}) were chosen and the mean value inside each was calculated. Thus, the last set of the parameters includes 24 values for each observation. Finally, for each spectra set, we extract 72 parameters, which are quite enough for evaluating classification by means of the linear discriminant analysis.

It should be noted that this procedure of the parameter's extraction varies according to the obtained data. The set of extracted parameters presented here is only an example of successful solution of formulated classification problem. Moreover, after statistical data analysis by means of hierarchical cluster analysis and stepwise linear discriminant analysis, the extracted set was reduced to 57 items to exclude a strict correlations between the parameters.

Step 3. The last step includes the evaluation of classification procedure. Here we apply linear discriminant analysis (LDA), which is well known, and often applied to various biological objects. The procedure involved creating linear combinations of parameters with normal errors that best discriminate between site groups of cyanobacteria defined *a priori*. LDA was performed with the computer program designed in the MATLAB software [103], in which combinations of initial parameters were selected to maximize the ratio of group means discriminant scores to within-group variance [99, 104].

For this investigation, 314 sets of 8 spectra corresponding to 21 strains and 15 genera of cyanobacteria from the *CALU* collection were used. The results of Fisher discriminant analysis evaluated over 57 parameters is presented in **Figure 9**. The upper panel show 3D-plots in the space of discriminating functions. It is clear that the discrimination between species is sufficiently good. Moreover, the closely related species (e.g., *Spirulina* and *Oscillatoria*, *Synechococcus* and *Chlorogloea*, *Microcystis* and *Myxosarcina*) appear close to each other. Such species as *Leptolyngbia*, *Geitleninema* and *Oscillatoria*, which includes several strains, form a big groups. However, inside these groups, single strains also can be discriminated. In the lower

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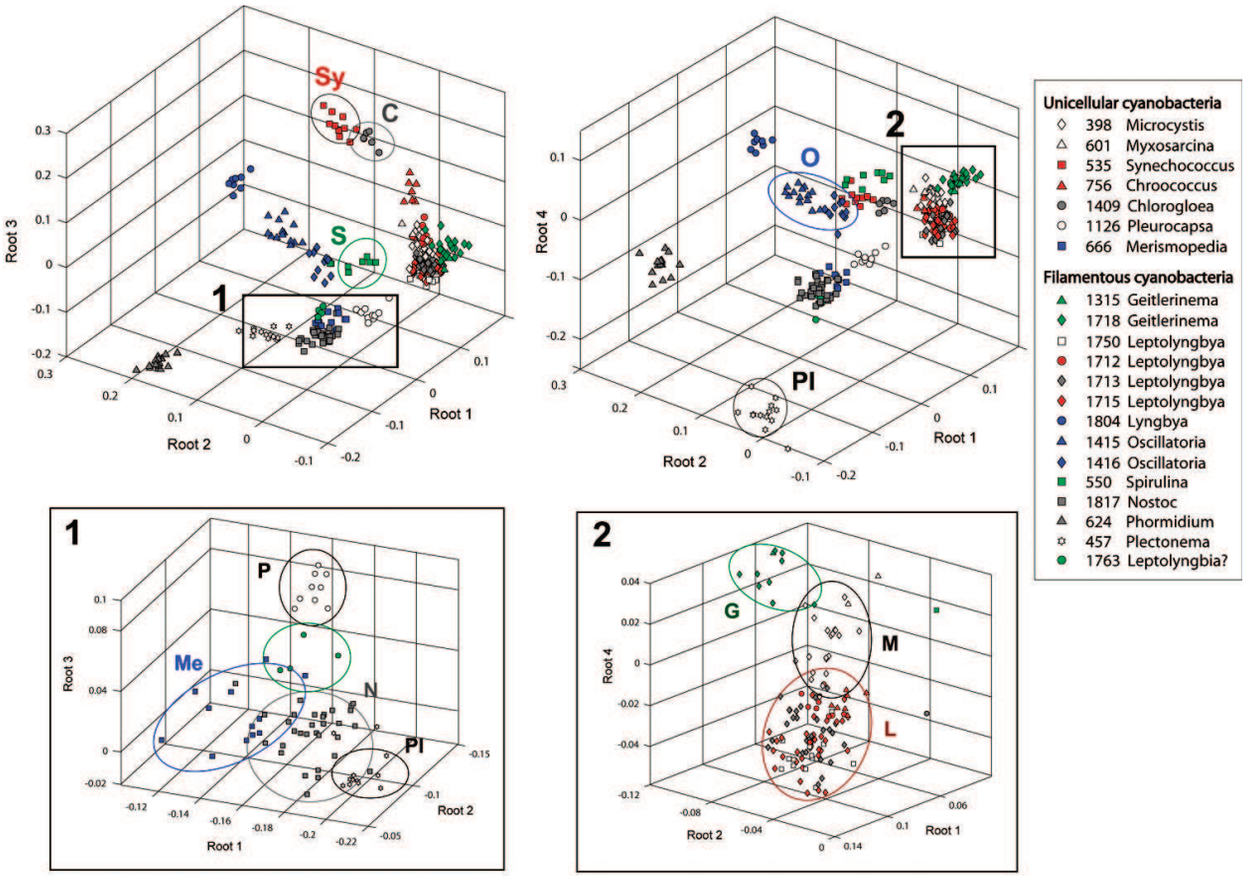

Figure 9. The results of linear discriminant analysis. Solid curves bounded the regions, occupied by specified species: G—*Geitlerinema*, L—*Leptolyngbya*, O—*Oscillatoria*, M—*Microcystis*, Me—*Merismopedia*, P—*Pleurocapsa*, Pl—*Plectonema*, N—*Nostoc*, S—*Spirulina*, C—*Chlorogloea*, Sy—*Synechococcus*. The lower panel shows the scaled view of the corresponding regions.

panel, the corresponding scaled regions are presented. In the legend, all used cyanobacterial strains are named and enumerated according to *CALU* collection. Solid curves bounded the regions, occupied by specified species: G—*Geitlerinema*, L—*Leptolyngbya*, O—*Oscillatoria*, M—*Microcystis*, Me—*Merismopedia*, P—*Pleurocapsa*, Pl—*Plectonema*, N—*Nostoc*, S—*Spirulina*, C—*Chlorogloea*, Sy—*Synechococcus*.

The classification accuracy in the presented example is near 98.3%. However, for the limited set of laser lines (4 instead of 7) and classification parameters (24 instead of 57), the classification accuracy does not reduce considerably—93.7%. The high classification accuracy is due to the fact that LDA works with distribution functions for classification parameters and their statistical characteristics, which allows to build a good classification model.

It should be noted here that the limited set of laser lines and classification parameters was considered subjecting to further possible application of the presented technique in the microelectronic device for on-line data collection in environmental monitoring. During this investigation, the possibility of the implementation of data collection and data processing in one software-hardware application device on the base of on-a-chip technology was examined. Obviously, for the production of such device, it is quite desirable to have less radiation emission sources and limited number of detection spectral ranges.

The universality of the considered technique makes it possible to use it for investigation of any phytoplankton species irrespective of their habitat or cultivation. Utilizing data from several fluorescence spectra, instead of one, results in more fingerprint information which leads to the taxonomic differentiation on a finer scale. Classification procedure, presented here, was carried out by means of statistical analysis on the base of mathematical characteristics of intrinsic fluorescence spectra of living single cells; therefore, it is free from usual subjectivity, which can occur while using methods of direct optical microscopy. Moreover, formalization of data processing gives a wide opportunity for automating of the classification procedure of cyanobacterial strains in field samples, while on-line monitoring of water bodies is conducted.

Undoubtedly, the data set should be expanded to include more species and phytoplankton classes/divisions, grown under different nutrient and light conditions. However, this work already demonstrates the potential of the discrimination of phytoplankton classes by fluorescence microscopic spectroscopy. This work lays the foundation for determining cyanobacterial abundance by direct fluorescence measurement of sea- and freshwater. Combining the knowledge of phytoplankton structure along with taxon-specific measurements of photosynthetic activity and biochemical cell composition, can lead to new models which increase the reliability of on-line monitoring.

4. Conclusion and outlook

In this chapter, the most powerful CLSM method for investigation of cyanobacteria—the fluorescence microscopic spectroscopy—was presented. This method allows to study living cyanobacterial cells via noninvasive and nondestructive technique and obtained in-vivo information about weak variations in single-cell functioning. It should be noted that CLSM provides some other interesting techniques, which can give deep insight into physiological processes that rules cyanobacterial life, such as FRAP [68–69, 105, 106] and hyperspectral microscopy [44]. FRAP is used to measure the mobility of phycobilisomes in intact cyanobacterial cells and hyperspectral microscopy helps to determine pigment localization and distribution in living cyanobacterial cells. Moreover, several time-dependent techniques for investigation of the dynamic properties of photosynthetic apparatus of cyanobacteria, such as PAM, can be implemented at a single-cell level by means of CLSM.

A limited number of examples, presented here, of possible fluorescence microscopic spectroscopy implementation (e.g., the investigation of biological diversity and monitoring of physiological state of cyanobacterial cultures) can significantly rise an effectiveness of the routine procedures in environmental monitoring and industrial culture production. Confocal microscopic spectroscopy gives a unique opportunity to introduce automation into these processes.

On the other hand, the indirect application of the presented results of the single-cell spectroscopic investigations can give a new information to improve remote sensing control. Spectral information recorded by satellite-carried sensors is already used for mapping of algae distribution, and due to the high frequency of data collection provides a database for estimation of phytoplankton dynamics over large areas [107]. Presented investigation gives an opportunity to control also cyanobacterial communities. The elaborated technique can be supported with algorithm that includes a new mathematical fitting strategy which automatically can

cope with the environmentally caused variations of the cyanobacterial fluorescence spectra. Moreover, an additional fluorescence information on the physiological state of cyanobacterial cultures provides a new information for predictive modeling and aquatic management, alternatively to the delayed fluorescence described in [108].

The formalization of the genera identification and cultural physiological state analysis give an opportunity to develop a compact on-a-chip nanoelectronic device for preliminary on-line investigation of the field samples in situ and in vivo and for controlling of the laboratory cultures during industrial incubation.

Obviously, the proposed methods require further development, including evaluation of more species representing more phytoplankton classes, and including non-taxonomic features, such as photoadaptation. Although the quantitative measurements were not performed in this study, they could be possible while all stages will be standardized. However, this work already demonstrates a high potential of fluorescence microscopic spectroscopy. We suggest that CLSM methods have potential application for several of the approaches noted earlier and also other studies regarding photosynthetic apparatus of cyanobacteria. We hope that the unique cell-biology of cyanobacteria will encourage further investigations because of their growing importance in rural biotechnology and commercial production.

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