Chapter 2

Using Virtual Filtering Approach to Discriminate Microalgae by Spectral Flow Cytometer

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Abstract

Fluorescence methods are widely used for the study of marine and freshwater phytoplankton communities. However, the identification of different microalgae populations by the analysis of autofluorescence signals remains a challenge. Addressing the issue, we developed a novel approach using the flexibility of spectral flow cytometry analysis (SFC) and generating a matrix of virtual filters (VF) which allowed thorough examination of autofluorescence spectra. Using this matrix, different spectral emission regions of algae species were analyzed, and five major algal taxa were discriminated. These results were further applied for tracing particular microalgae taxa in the complex mixtures of laboratory and environmental algal populations. An integrated analysis of single algal events combined with unique spectral emission fingerprints and light scattering parameters of microalgae can be used to differentiate major microalgal taxa. We propose a protocol for the quantitative assessment of heterogenous phytoplankton communities at the single-cell level and monitoring of phytoplankton bloom detection using a virtual filtering approach on a spectral flow cytometer (SFC-VF).

Key words Spectral flow cytometry, Phytoplankton, ID7000, Virtual filtering, Spectral flow cytometer, Cyanobacteria

1 Introduction

The development of spectral flow cytometry (SFC) expanded our ability to characterize heterogeneous cell populations because of the high spectral resolution achieved by this instrument [1].

The key advantage of spectral flow cytometry (SFC) is that a measurement of a set of emission spectra using different excitation wavelengths is done from individual cells with rates of hundreds and thousands of events per sec [1, 2]. Moreover, SFC analysis makes possible additional differentiation of heterogeneous algal mixtures by size and granularity in a manner similar to conventional flow cytometry (FCM) [1]. The emission spectrum information for every single cell could be combined with light scattering data

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through sequential gating on combinations of standard dot plots and histograms. The populations could now be separated not only by using conventional fluorescent conjugated antibodies but by also using the autofluorescent signal from unstained cells [3].

Since the spectral unmixing algorithm is based on the record of single stained probes [4], it cannot be directly applied to the natural algal probes having bright autofluorescence from different sources, and another approach has to be considered.

In 2019 we developed a novel "virtual filtering" approach (SFC-VF) based on the spectral flow cytometry analysis and use of variable regions of algal autofluorescence spectra in combination with light scattering-related separation of algal populations based on algae cellular size and granularity [5]. We applied SFC-VF to differentiate and characterize microalgae taxa in binary and multi-component mixtures as well as natural environmental microalgae assemblages and were able: (1) to differentiate microalgal cells from different phytoplankton taxa with a similar combination of pigments; and (2) to remove fluorescence signal from contaminating sources using light scatter gating. Moreover, unlike FCM, SFC makes it possible to separate individual algal cells presented in heterogenous algal populations (such as cryptophytes) based on their unique spectral data.

The SFC-VF method relies on identifying of the most variable regions of the spectra of the mixtures of algal strains analyzed pairwise and on creating a matrix of SFC fluorescent channels corresponding to those regions. Spectral differences between single algal strains (morphology—Fig. 1, left column) were captured by both spectral flow cytometer Sony SP6800 (Sony Biotechnology Inc., USA, 405 nm and 488 nm excitation) and spectrofluorimeter (Fig. 1, right column). However, the spectrofluorimeter provided an averaged signal from the population of algal cells, debris, and fluorescent organic matter. The separation of algal mixtures based on the conventional FCM approach and a filter combination used for algal analysis (such as phycoerythrin (PE) bandpass 575/25 nm) versus allophycocyanin (APC) bandpass (660/20 nm) was complicated by the heterogeneity of algal populations.

In the SFC-VF approach, the sensitivity of chlorophyll-associated channels (CH 24–30) captured on the SP6800 was switched to the minimal level. Then, the non-chlorophyll-based spectral differences (from accessory pigments) in the 420–650 nm wavelength range became prominent, enabling better discrimination of algal strains (Fig. 2). Further SFC analysis of algal cultures was continued with the reduced intensity of chlorophyll-associated channels.

Mixtures of algal cultures were analyzed in a pairwise manner generating different algal combinations. Initially, several variants of

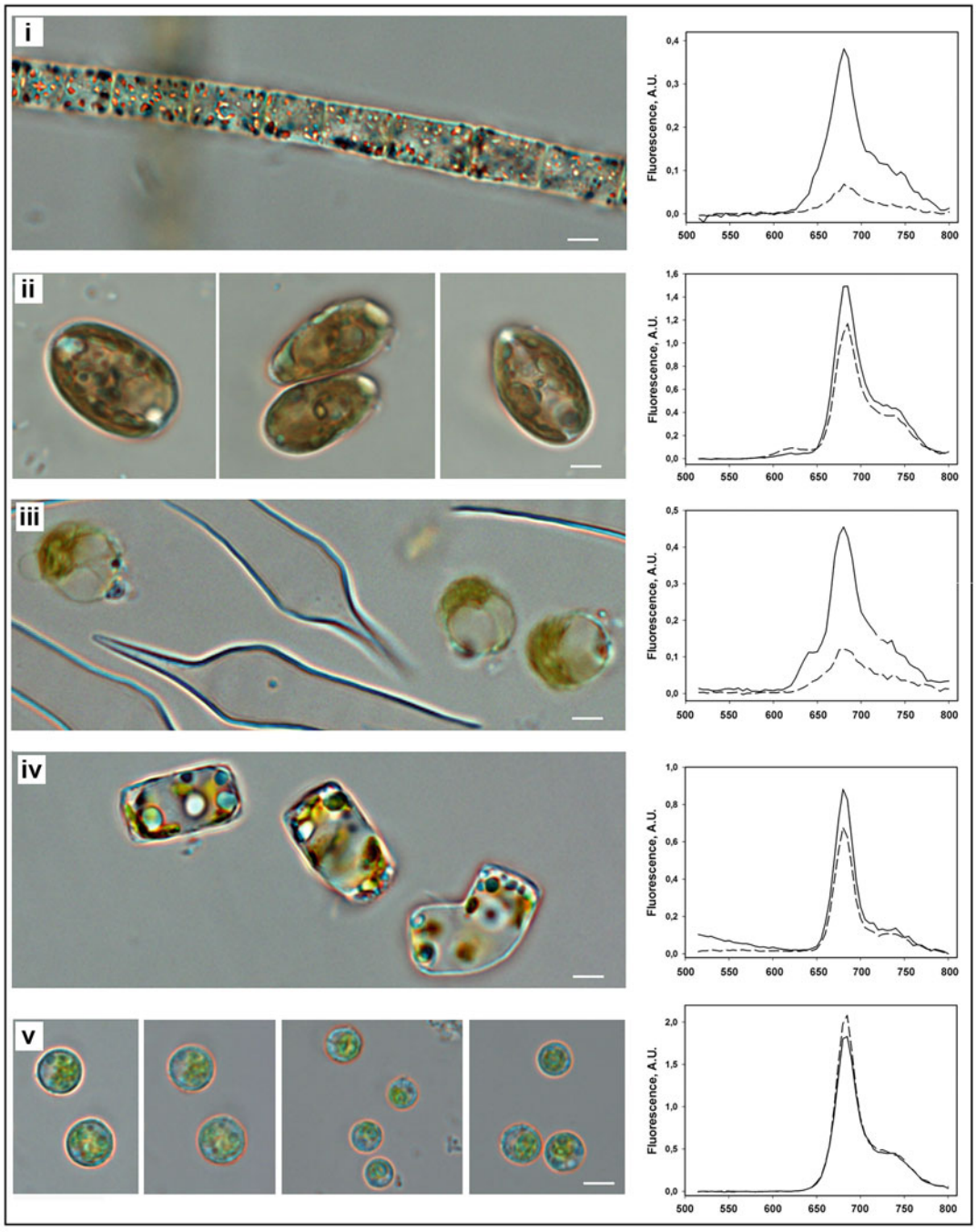

Fig. 1 Light microscopy and spectrofluorometric data of algal cell cultures. (**i**) *Aphanizomenon* sp., (**ii**) *Cryptomonas pyrenoidifera*, (**iii**) *Dinobryon divergens*, (**iv**) *Cyclotella sp*., (**v**) *Chlorella* sp. First column: light microscopy image of algal cultures; second column: spectrofluorometric data of corresponding culture obtained with 407 nm (solid line) and 488 nm (dashed line) excitation. Scale bar 5 μm. Notice significant differences in the relative intensities at the peaks for *Aphanizomenon* sp. and *D. divergens*

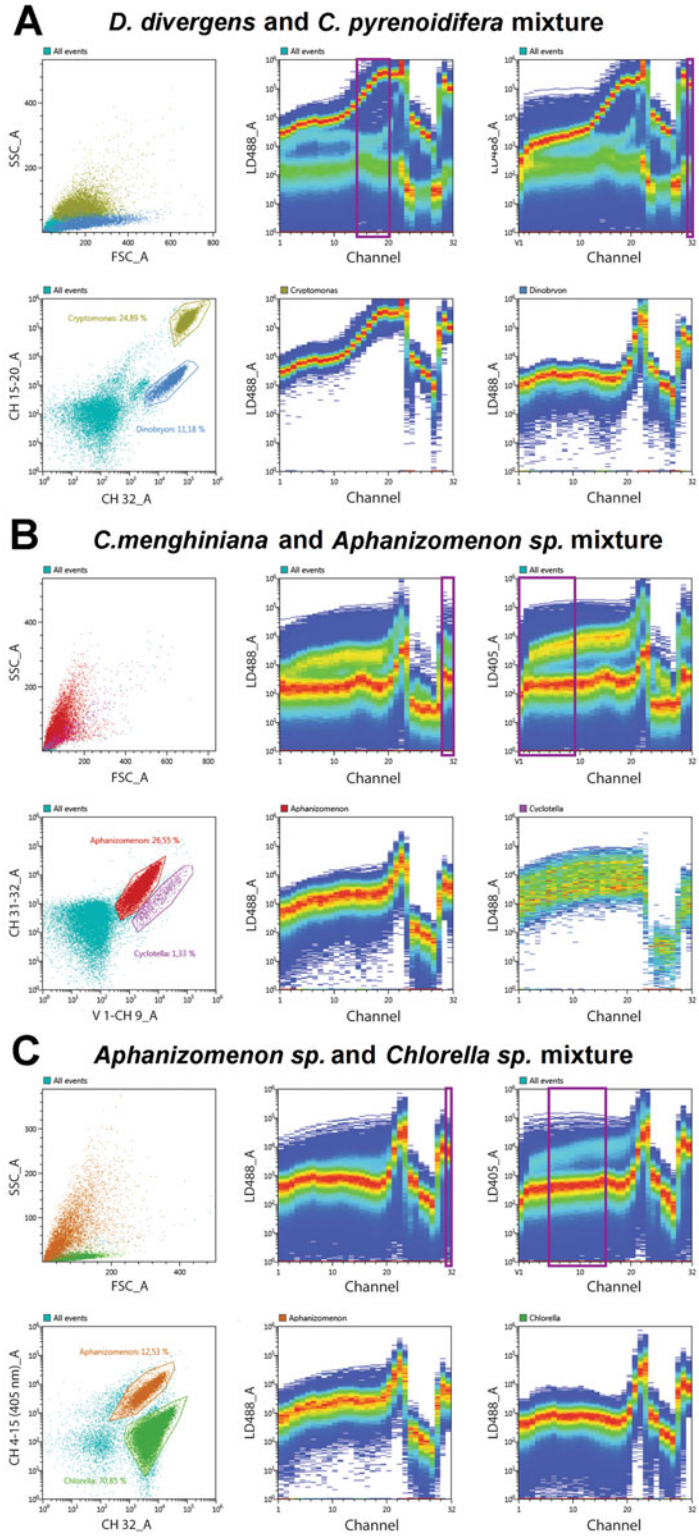

Fig. 2 Spectral analysis of algal culture mixtures *D. divergens* and *C. pyrenoidifera* (**a**), *Cyclotella sp.* and *Aphanizomenon*. (**b**), and *Aphanizomenon* and *Chlorella* sp. (**c**). Spectral data of all cells in the mixture were

a matrix of fluorescent channels corresponding to virtual filters capturing the algal spectra variability regions were created (Fig. 3).

We then selected a combination of fluorescent channels (virtual filter) that provides the best separation of two cell populations by a single dot plot. The spectra of the discriminated populations were further validated with the spectra of single algal culture controls. Furthermore, all five algal strains were mixed together and analyzed using the spectral flow cytometry analyzer. To discriminate all algal taxa, individual plot was not sufficient; instead, we used sequential gating and a combination of fluorescent channels based on virtual filters, previously selected for pairwise culture analysis (Fig. 4).

Using the above mentioned approach, we tested whether a particular microalgae type or species can be traced in the mixture of environmental microalgae populations based on its spectral profile. Different quantities (from 50% to 0.5%) of *Aphanizomenon*

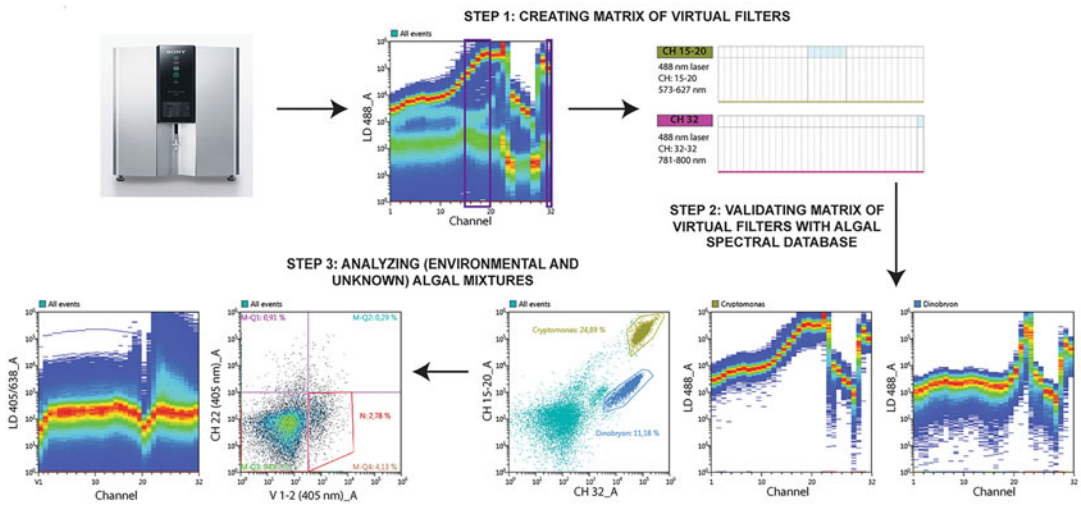

Fig. 3 Virtual filtering analysis algorithm for a mixture of microalgae cells. The mixture of microalgae cultures is analyzed using the spectral analyzer SP6800, and the obtained total spectrum of the mixture is examined for the most variable and elongated regions. A matrix of several virtual filters corresponding to the variable spectral regions is then created, and the combination of the filters providing the best separation of populations was selected (**Step 1**). The spectra of discriminated and gated populations are validated with the spectra in the algal spectral database (control spectra) (**Step 2**). In the environmental sample, virtual filters are applied, and a population different from the major one using an appropriate virtual filter could be analyzed and attributed to the cultured microalgae accordingly (**Step 3**)

Fig. 2 (continued) obtained under 488 nm laser excitation and 405 nm laser excitation spectrum charts. Based on the most variable spectral regions, combination of virtual filters corresponding to spectrum regions in channels 15–20 (488 nm excitation) and channel 32 (488 nm excitation), channels 31–32 (488 nm excitation) and channels V1-CH9 (405 nm excitation), and in channel 32 (488 nm excitation) and channels 4–15 (405 nm excitation) were selected to achieve the best discrimination of the two cell populations. Spectra of gated populations were then plotted to confirm the identity of discriminated populations

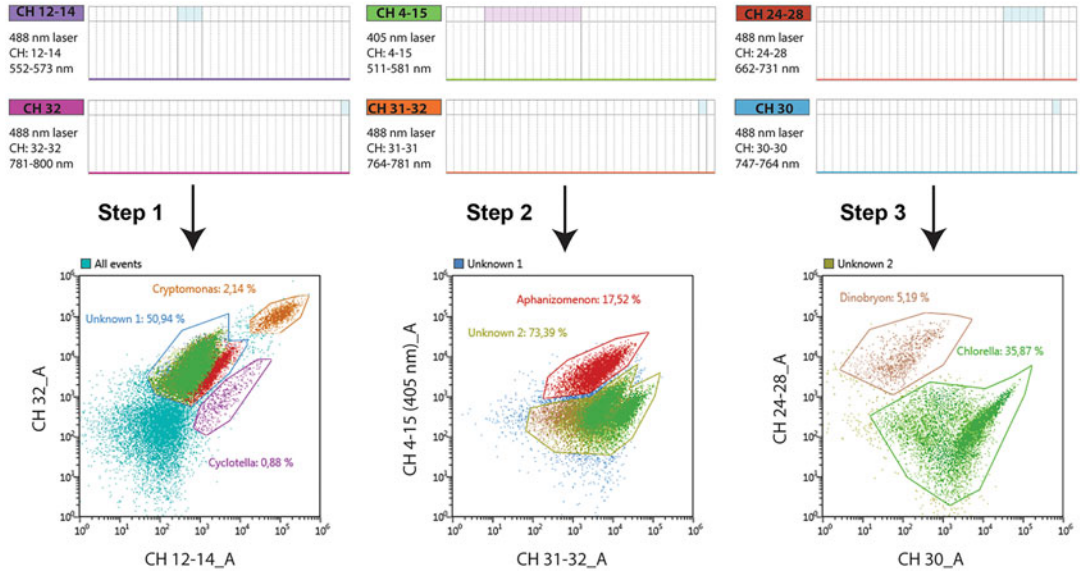

Fig. 4 Spectral analysis of five algal cultures *Aphanizomenon* sp., *C. pyrenoidifera*, *D. divergens*, *Cyclotella sp.* and *Chlorell*a sp. Mixed together. *C. pyrenoidifera* and *Cyclotella sp.* populations were separated within the mixture based on CH 12–14 and CH 32 (488 nm excitation) filters (**Step 1**). Then unseparated part of the mixture (marked Unknown 1) was gated and projected onto CH 4–15 (405 nm excitation) versus CH 32 (488 nm excitation) dot plot to discriminate the cell population of *Aphanizomenon* sp. (**Step 2**). Consequently, the unidentified population (Unknown 2) was gated and visualized on a combination of CH 24–28 and CH 30 (488 nm excitation) filters to detach the last two populations of *D. divergens* and *Chlorella* sp. with very similar spectral profiles (**Step 3**)

sp. culture were mixed with environmental samples and analyzed using SFC-VF. A combination of the virtual filters CH 22 (405 nm excitation) and V1-2 (405 nm excitation) enabled the best separation of *Aphanizomenon* sp. population in the 1:1 mixture of *Aphanizomenon* sp. and environmental sample (50% *Aphanizomenon* cells: 50% pond sample) and was used for the analysis of other volume ratios. Spectra of *Aphanizomenon* sp. cells could be traced in the mixture containing as little as 0.5% proportion relative to the total volume (*see* **Note 1**).

In conventional cytometry, optical bandpass filters are used to separate fluorescent signals during instrument detection. Optimization of fluorescence detection and decreasing the acquisition of signal coming from a region with a high level of autofluorescence (e.g., GFP signal from cellular autofluorescence in a green-range region) require the replacement of a standard optical filter with a modified one [6]. The SFC-VF approach allows the creation of "virtual bandpass filters" with no hardware modification and without spectral unmixing. As a result, it was possible to narrow or widen the spectral signal that is taken into consideration from ~10 to ~300 nm bandwidth (for the SP6800 instrument) and to achieve significant discrimination of algal populations.

Initially, we analyzed representatives of five major groups of microalgae, namely (1) *Cyclotella sp.* from phylum Bacillariophyta (diatoms); (2) *Cryptomonas pyrenoidifera* from phylum Cryptista (cryptophytes; cryptomonades); (3) *Aphanizomenon* sp. from phylum Cyanobacteria ("blue-green algae", cyanoprokaryotes); (4) *Chlorella* sp. from phylum Chlorophyta ("green algae", chlorophytes); (5) *Dinobryon divergens* from phylum Ochrophyta ("golden algae"; chrysophytes) as model microalgal species with a spectral flow cytometer SP6800 (Sony Biotechnology Inc., USA). The data presented show the potential of our approach in the identification and quantitative evaluation of algal mixtures and experimental samples. In our study, we used fresh cultures; however, it is anticipated that different preservation protocols (fixation in paraformaldehyde and freezing in liquid nitrogen) may have a smoothing effect on the shape of emission spectra as it happens for the absorption spectral region related to phycobilins.

A recently introduced ID7000 instrument (Sony Biotechnology Inc., USA) is a significantly improved spectral flow cytometer compared to its predecessor Sony SP6800. It has a larger dynamic range of PMTs and an increased number of lasers (up to 7). These features make the discrimination of the algae species even simpler and more robust.

Since the dynamic range of PMTs in this cytometer is large enough, it was possible to use the standard voltage for chlorophyll channels along with other channels. The absolute amount of chlorophyll in varied species could be significantly different. Thus, to discriminate algae, a chlorophyll signal can be used.

To test the capability of ID7000 in the separation of autofluorescent spectra from different algae, we first recorded individual spectra for all three species used (Fig. 5) and denoted regions of interest there.

Next, after recording the algal mixture, we applied two regions around Chl a maximum channel (Fig. 6) representing each species and compared spectra obtained from these subpopulations with the original ones (Fig. 6d). The spectra obtained from the groups selected by these regions were nearly identical to what was measured in every single sample, proving that such selection allows good discrimination between two species.

2 Materials

2.1 Instrumentation and Accessories

1. Varioscan Flash spectral scanning multimode reader (ThermoScientific, USA).
2. The spectral flow cytometer (spectral FCM) analyzer SP6800 (Sony Biotechnology Inc., USA) was equipped with 40 mW blue 488 nm, 60 mW violet 405 nm, and 60 mW red 638 nm

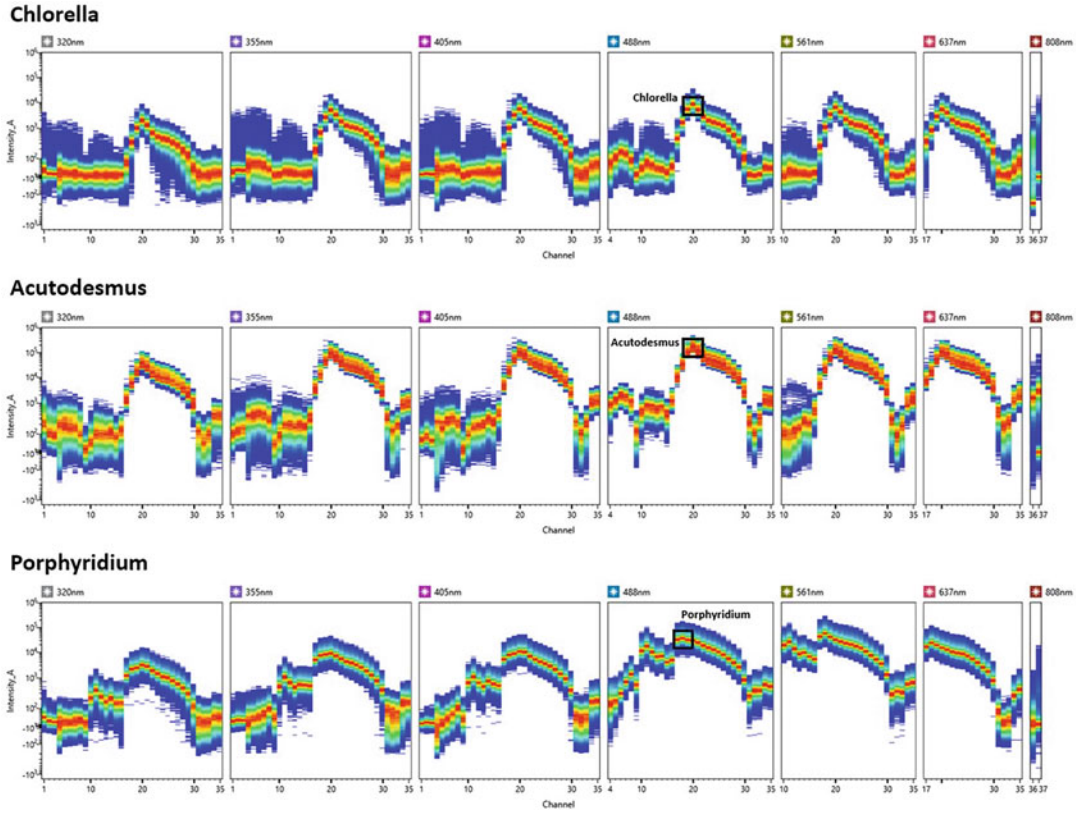

Fig. 5 Spectral analysis of algal cultures *Chlorella* sp., *Acutodesmus obliquus*, *Porphyridium sordidum* with ID7000 spectral flow cytometer (Sony Biotechnology Inc., USA). For further analysis, regions of interest (ROI) were created in the Chl a channels (shown in black in 488 nm spectra)

lasers and a 32-channel linear array photomultiplier (500–800 nm range for 488 nm excitation and 420–800 nm range for 405/638 lasers combination), and acquisition and analysis software

3. The spectral flow cytometer (spectral FCM) analyzer ID7000 (Sony Biotechnology Inc., USA) was equipped with 20 mW deep UV 320 nm, 50 mW UV 355 nm, 100 mW violet 405 nm, 150 mW blue 488, 100 mW yellow-green 561 nm, 140 mW red 637 nm lasers and 150 mW far red 808 nm, 186 detectors: 184 fluorescence channels, one forward scatter, one side scatter, and equipped with ID7000 acquisition and analysis software (Sony Biotechnology Inc., USA).
4. Algae growth and harvesting chamber Percival model AL-30L2 (Percival Scientific Inc., USA) for algal culture incubation (with controlled temperature, light, and humidity conditions).
5. Brightfield microscope Axiovert with a color camera (Carl Zeiss Inc., Germany) (*see* **Note 2**).

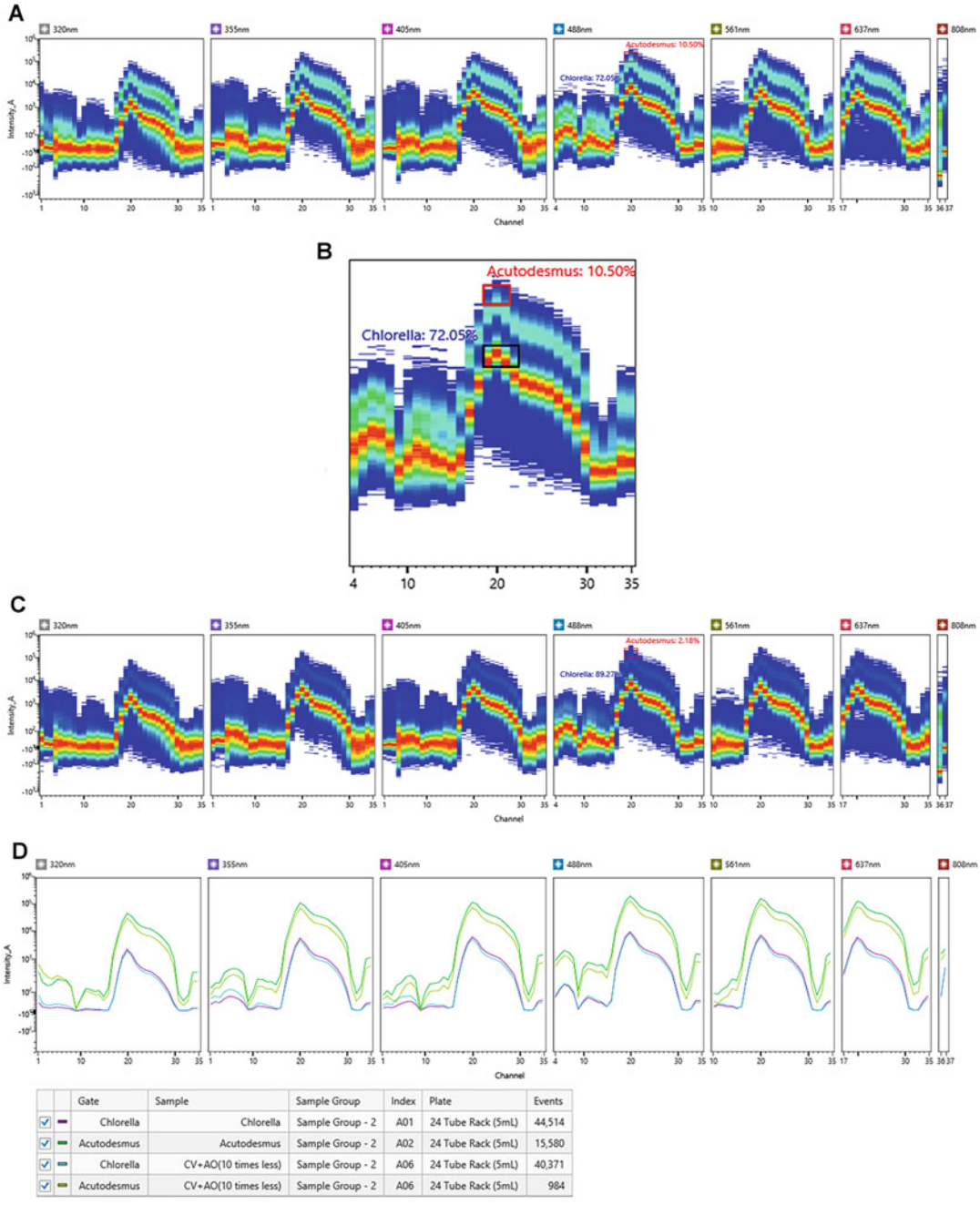

Fig. 6 Spectral analysis of mixed algal cultures *Chlorella* sp. and *Acutodesmus obliquus*, with ID7000 spectral flow cytometer. (**a**) All spectra, mixture in a ratio 1:7. (**b**) Enlargement of the spectra excited from 488 nm laser. ROI used for the selection of each species are shown as black rectangles. (**c**) All spectra, algal mixture in a ratio 1:50. The same ROI were applied for the species selection. (**d**) Comparison of the spectra obtained from pure samples and by selection using ROI

2.2 List of Microalgae Cell Cultures

Microalgae cell cultures from major microalgae taxa, including *Cyclotella sp.* CCMP334, *Chlorella* sp. CCMP251, *Dinobryon divergens* CCMP3055, *Cryptomonas pyrenoidifera* CCMP1177, *Aphanizomenon* sp. CCMP2764, *Acutodesmus obliquus* SAG 276-1, and *Porphyridium sordidum* SAG 114.79 were obtained from the National Center for Marine Algae and Microbiota (NCMA; Bigelow Laboratory for Ocean Sciences, USA) and Göttingen University's collection of algal cultures (Germany).

2.3 Reagents

1. Microalgae cell culture media: (1) DY-V medium; (2) L1 medium; (3) L1 derivative, L1–11 psu medium.
2. Eight peak beads (Sony Biotechnology Inc., USA).
3. Align Check beads (Sony Biotechnology Inc., USA).
4. 12 × 75 mm round-bottom Falcon polystyrene tubes.

3 Methods

3.1 Cultivation of Algae Cultures

Freshwater cultures *D. divergens*, *Aphanizomenon* sp., and *C. pyrenoidifera* were maintained in DY-V medium (modified from Lehman and co-authors [7]) at 14 °C and 20 °C, respectively, under 150 μmoles/m^2/s light irradiance and 12/12 L/D cycle. *Chlorella* sp. and *Cyclotella sp* were maintained in L1 medium and L1 derivative, L1–11 psu medium, respectively, at 14 °C under 150 μmoles/m^2/s light irradiance and 12/12 L/D cycle. 1. Two or more phytoplankton cell cultures (e.g., *Chlorella* sp. CCMP1177, *Acutodesmus obliquus* SAG-276-1 and *Porphyridium sordidum* SAG 114.79) were used for experiments with ID7000 spectral flow cytometer.

3.2 Spectrocytofluorimetric Acquisition of Microalgal Samples

1. Prior to the analysis, spin down each microalgae culture and resuspend it in a small volume. Count algal cells (microscope). For spectral cytometry analysis, 1000 μL volume of each culture should be used to analyze single culture controls.
2. Spectral analysis of algal cell cultures for ID 6800:
3. Prepare mixtures using 500 μL volume of each culture to analyze 10 pairwise culture mixtures and 200 μL volume of each culture to analyze a mixture of all five cultures together (ratio 1:1:1:1:1). Alternatively, mix *Chlorella* sp. and *Acutodesmus obliquus* cultures with relatively equal cell densities in 1:1, 1:10 volume ratio making up to 250 μL sample. The next steps are accommodated for the use of the ID7000 spectral system (Sony Biotechnlogy Inc., USA).
4. Turn on the spectral flow cytometer and run autocalibration using calibration beads. Use Ultra Rainbow calibration beads (Spherotech, USA or Sony Biotechnology Inc., USA) for automatic calibration.

5. Open the Acquisition window in the ID7000 software.
6. Prime the fluidics lines by flushing with sheath fluid.
7. Dissolve two drops of Align Check beads in 450 μL water.
8. Dissolve two drops of eight-peak beads in 450 μL of water.
9. Run the Daily and Performance QC.
10. Spectral analysis of single algal cell cultures. For SP6800 spectral cytometer: Adjust the laser power for 488 nm and for 405 nm lasers; reduce gain for channels 24–32 to the minimum and adjust gain for other channels. Record emission spectra of single cells in the range 420–800 nm using excitation at 405/407 nm and in the range 500–800 nm using excitation at 488 nm for SP6800.
11. Record mixed samples.

3.3 Spectral Analysis of Algal Cell Cultures for ID 7000

1. Choose Template—24 Tube Rack in the Experiment tab.
2. Load adjusted for different microalgae ID7000 settings (FSC to—16, SSC gain to 30, the threshold value to 11%, and fluorescence PMT voltage from 40% to 70%).
3. Set the sample flow rate to 1 under the "Flow Control" tab to keep the intermediate flow velocity.
4. Set the stopping condition to 50,000.
5. Create FSC_A vs. SSC_A dot plot and ribbon plot for all lasers. (*see* **Note 3**).
6. Place the round-bottom tube with the *Chlorella* sp., *Acutodesmus obliquus,* and two mixed cultures at different ratios in 24 Tube rack.
7. Place the rack in the multi-well plate holder and click "Load".
8. Highlight sample positions as a "Target" and move all samples to sample group 1.
9. Choose Set current position in the first sample tube by right-clicking, and then click "Preview."
10. Once the sample is being processed, observe if any parameters from the "Detector & Threshold," e.g., fluorescence PMT voltage and/or FSC/SSC gain, need to be tuned.
11. After tuning, click "Auto Acquire" to record the samples (*see* **Notes 4**–**7**, Fig. 5).
12. Record mixed samples with ID 7000 spectral flow cytometer (Fig. 6).

3.4 Analysis of Microalgal Samples on a Spectral Flow Cytometer (Example with Cultures Acquired with ID7000 Spectral Flow Cytometer)

1. Once the acquisition is completed, go to the Analysis window and open the recorded experiment.
2. Select the tube corresponding to the *Chlorella* sp., and on the FSC/SSC plot, place the gate to exclude debris and by right-clicking on the plot area, choose Sync Scale and Gate.
3. Place the gate on a 488 nm ribbon plot to include the dense events (ap. CH 18–22, 657–712 nm), name the gate "Chlorella", and create a ribbon plot from "Chlorella" gate.
4. Repeat the previous step for *Acutodesmus obliquus* culture.
5. Since all gating is synchronized within one group, go to the *Chlorella* sp. sample and send the "Chlorella" ribbon plot for "Overlay" by right-clicking.
6. Send the "Chlorella" ribbon plot to "Overlay" from the mixed sample.
7. Repeat steps previous steps for *Acutodesmus obliquus* culture.
8. Observe the differences and similarities between spectra in "Overlay" builder.

3.5 Virtual Filtering (Example with Cultures Acquired with ID6800 Spectral Flow Cytometer)

The virtual filtering algorithm is shown in Figs. 3 and 4 and consists of the following steps:

1. Using spectra from individual algal cultures, select two channels (or groups of channels) with a maximal difference in the intensity of the signal between two cultures when using settings determined in the steps above (*see* **Note 8**).
2. Make dot plots using two virtual channels along the X and Y axes for each pair, the mixture, and the environmental sample. The details are shown in Fig. 3.
3. Change virtual filters by adding or removing channels to achieve maximal separation for two populations. The details are shown in Fig. 4. (*see* **Notes 9–10**).

These filters will be further used for PCA (Principal Component Analysis) (Fig. 7) (*see* **Notes 10** and **11**).

3.6 Analysis of Microalgal Environmental Populations

The analysis illustrated an example where *Aphanizomenon* sp. was determined.

1. Record your sample. Analyze it with the virtual filters prepared for mixed strains and try to select the side population (as shown in Fig. 8, upper row).
2. If this side population is similar to one of your strains, then make a mixture of this strain with an environmental sample in 1:1 and 1:10 proportions and run this sample. Your suggestion is correct if this side population in the mixed sample will be enlarged and its position will not be shifted (**Note 11**).

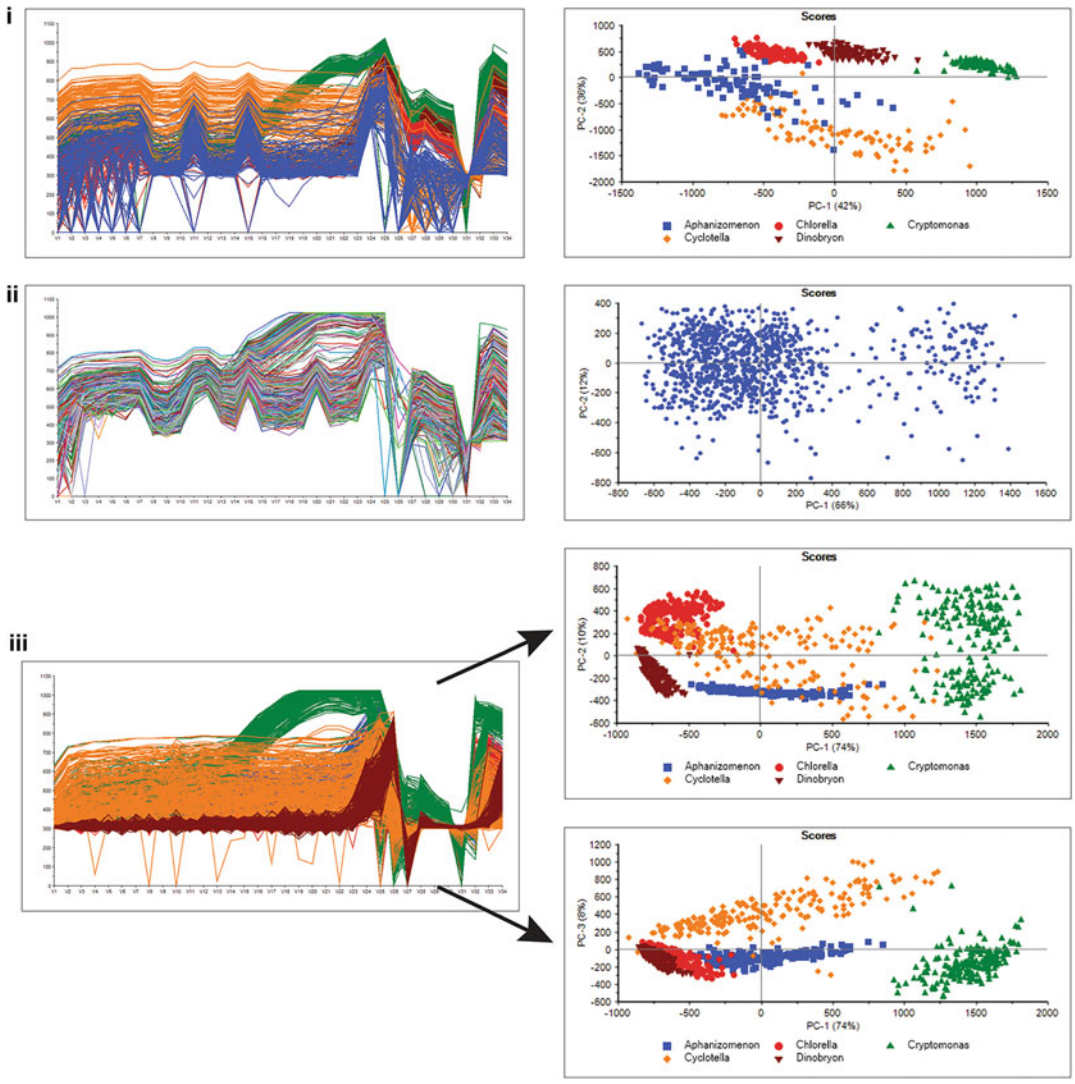

Fig. 7 Principal component analysis (PCA) performed for spectral data of algal cultures *Aphanizomenon* sp., *C. pyrenoidifera*, *D. divergens*, *Cyclotella sp.* and *Chlorell*a sp. (**i**) Projection of spectra of individual cells (left) of artificially mixed algal cultures onto the plane of the first two principal components (PC) (right). (**ii**) Projection of spectra of individual cells (left) of physical mixture of algal cultures onto the plane of the first two PCs (right). (**iii**) Projection of spectra of individual cells (left) of FCM gated populations from the mixture of algal cultures onto the plane of the PC1 and PC2 (top right) and the plane of the PC1 and PC3 (bottom right). Four populations are clearly discriminated on the PC1/PC2 dot plot. The population of *Cyclotella* appears to be heterogeneous. On the PC2/PC3 plot, *Cryptomonas* and *Cyclotella* populations are more compact, but *Chlorella* population cannot be discriminated from *Aphanizomenon* sp. and *Dinobryon divergens*

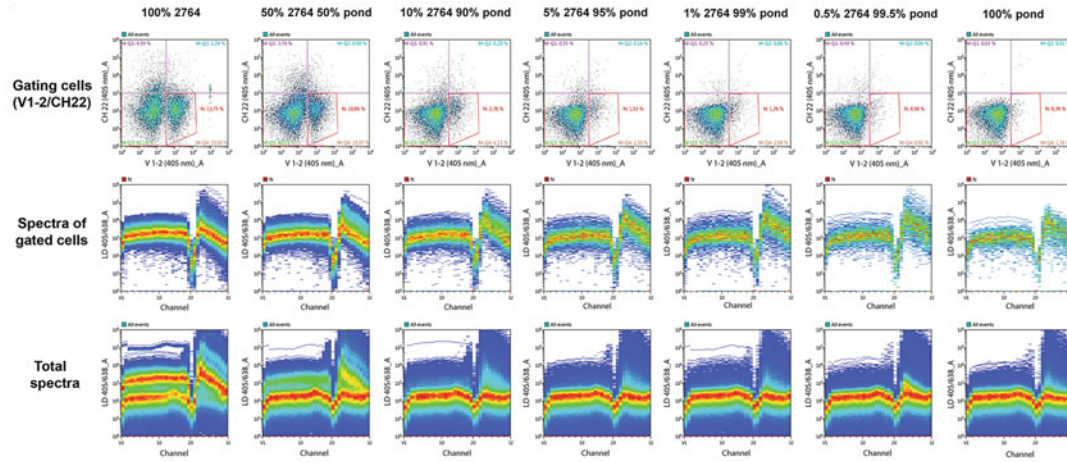

Fig. 8 Tracing different quantities of CCMP2764 *Aphanizomenon* sp. cells in an environmental sample from a pond based on spectral characteristics. From left to right: 100% of 2764 cell culture, 50% volume of 2764 culture and 50% volume of pond sample, 10% volume of 2764 culture and 90% volume of pond sample, 5% volume of 2764 culture and 95% volume of pond sample, 1% volume of 2764 culture and 99% volume of pond sample, 0.5% volume of 2764 culture and 95.5% volume of pond sample, and 100% of pond sample. In the first row: all cells are displayed on channel 22 (405 nm laser excitation) versus channels V1–2 (405 nm laser excitation) density plot, and a region corresponding to 2764 cells region is gated (L). Second row: spectra of gated L regions are displayed on 405 nm/638 nm spectrum plots. Third row: all cells in the sample are displayed on 405 nm/638 nm spectrum plots

3.7 Auto-fluorescence Finder

Algae samples can also be analyzed with an Autofluorescence Finder Tool. The ID7000 software allows to consider autofluorescence as an independent fluorescent parameter, which is particularly important for the taxonomic identification of different algae. Adding the 320 nm laser allows to include in the algal fluorescent signature a unique emission region far from Chl peak, and highly variable for different algae.

The part of ID7000 analytical software—Autofluorescence Finder Tool allows detecting the independent autofluorescent signals (Fig. 6). After finding the best separation between autofluorescent populations, the events are gated, and their emission spectra are checked. Each population should have its unique autofluorescence spectrum (Fig. 9). Autofluorescence Finder Tool in the analysis of algae also helps to separate dead algae from living ones due to the difference in the autofluorescence. The example of *Chlorella* shows three distinct algae populations with unique autofluorescent spectra (AF-A dead, AF-B aggregates, AF-C live *Chlorella*) acquired from seven lasers, including a new 320 nm UV-laser. Dead and aggregated algae can be cut off (Fig. 10), and live populations of algae with a more evident spectrum can be used in further analysis (*see* **Note 12**).

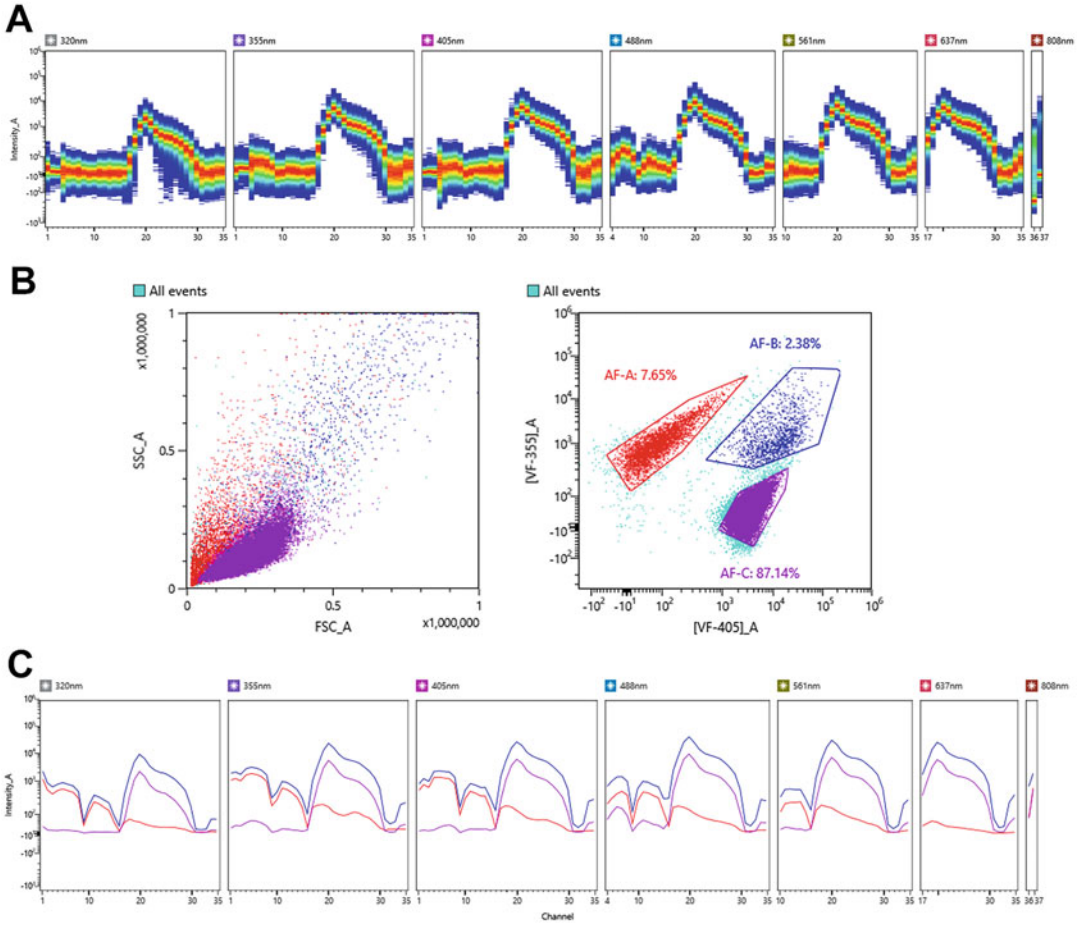

Fig. 9 *Chlorella* sp. analysis using Autofluorescence Finder Tool. (**a**) Ribbon spectral plots for the entire sample; (**b**) Gating by Autofluorescence Finder Tool using 355 nm and 405 nm excitation lasers; (**c**) Three autofluorescence spectra for the selected subpopulations (red: debris and dead cells; blue: large cells with high Chl a level; violet: cells losing Chl a photosynthetic activity)

4 Notes

1. Notably, a small population of cells with a spectral profile similar to *Aphanizomenon* sp. was detected in the gated region of an environmental sample, which can be explained by the presence of similar or same cyanobacteria species in the collected sample.
2. Any type of brightfield microscope equipped with a high NA objective (i.e., 60× or 100× with NA1.3-1.4) and a good enough color camera can be used.

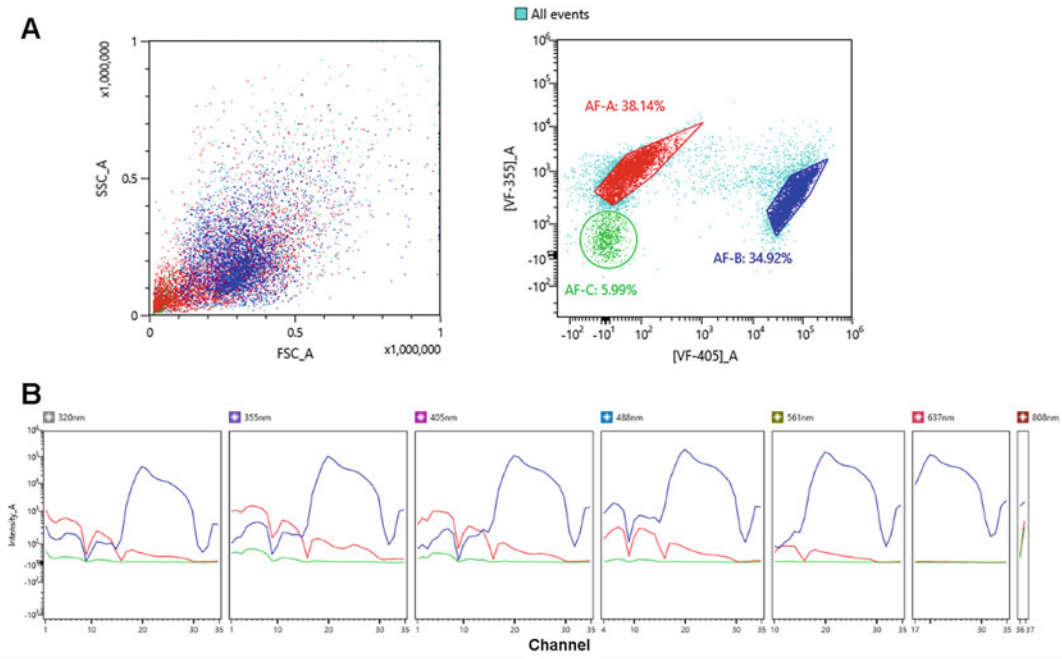

Fig. 10 *Acutodesmus obliquus* analysis using Autofluorescence Finder Tool. (**a)** Gating by Autofluorescence Finder Tool using 355 nm and 405 nm excitation lasers; (**b)** Three autofluorescence *Acutodesmus*. spectra – of debris (green); dying (red) cells losing Chl a autofluorescence, and alive (blue) algal cells

3. Since the maximal difference between algae is determined for the accessory photosynthetic proteins, the maximal gain should be used for the channels with minimal chlorophyll emission (CH 10–24 representing wavelengths 500–630 nm).
4. In the photosynthetic algae or cyanobacteria population, dead cells could be easily removed from the sample using a relatively low Chl a signal.
5. The residual difference between spectra obtained in the single probe and from the region while in a mixture, could be explained by a limited region while the whole distribution used for calculating the mean in the last figure is broader.
6. When recording all samples, you cannot change the threshold and the parameters in a detector.
7. The optimal cell concentration of microalgae cultures is in the 20,000–75,000 cells mL^{-1} range. A higher concentration will result in a high frequency of doublets. Record not less than 50,000 events in each probe using the settings.
8. Virtual filters for the dot plot data could be generated using Sony software v1.6 (Sony Biotechnology Inc., USA) and FlowJo software vs.10.2 (Treestar, USA).

9. We used CH 32 or CH 31 as one virtual filter and different groups of channels for another virtual filter (SP 6800, Sony Biotechnology Inc., USA). The best separation between pairs of algal cultures is achieved using different filter settings.
10. Some of the algal populations (*Chlorella* sp. and *Acutodesmus obliquus*. in our samples) might have a remarkably high number of cells with 0 values in channels 24–27, which may be associated with low chlorophyll signal due to the death of the cells. In order to reduce the cell heterogeneity within the sample, cells with no chlorophyll signal should not be included in the statistical analysis. Only gated chlorophyll-positive populations are shown in Fig. 5 (two upper rows). However, such gating cannot be applied to algae with a low level of chlorophyll.
11. Analysis of the environmental population will be successful only in determining the presence of one of the cultures that have been tested in advance.
12. We used a single step for separation since our algae were different in Chl a fluorescent intensity. However, when median Chl a intensities between compared species are similar, other channels should be taken into account.

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