SINGLE-CELL PIGMENT IDENTIFICATION IN LIVING PHOTOTROPHIC COMMUNITIES BY CONFOCAL IMAGING SPECTROPHOTOMETRY

ROLDÁN M. [a], THOMAS F. [b] CASTEL S. [c] QUESADA A. [d] & HERNÁNDEZ-MARINÉ M.[a]

[a]*Departament de Productes Naturals, Fisiologia Vegetal i Botànica, Universitat de Barcelona, Av. Joan XXIII s/n, 08028 Barcelona, Spain.*
[b] *Institut de Robòtica i Informàtica Industrial (CSIC-UPC), Llorens Artigas 4-6, 2 planta, 08028 Barcelona, Spain.*
[c]*Serveis Cientificotècnics, Universitat de Barcelona, Barcelona Science Park, Josep Samitier 1-5, 08028 Barcelona, Spain.*
[d]*Departamento de Biología, Universidad Autónoma de Madrid, 28049 Madrid, Spain.*

Abstract

In this paper the first data about the utilization of confocal imaging spectrophotometry on algal and cyanobacterial communities are presented. This new technique combines the capabilities of confocal laser scanning microscopy with spectrophotometrical methodologies and is revealed as a reliable and powerful tool which allows in vivo single cell unequivocal taxonomical group identification using its fluorescence signature. The capabilities and the limitations of the technique are explored using pure pigments, cyanobacterial and green algae cultures and natural assemblages from hypogean environments. Our results show that this technique allow the analysis for both global and single fluorescent pixel, providing their three dimensional localization *in vivo*, the direct analysis of fluorescent pigments from a single cell *in situ* into thick samples without isolation and the discrimination of the cells with particular fluorescence signatures within the colony or assemblages. The confocal imaging spectrophotometry is also found as a promising tool with future applications in fields such as aquatic ecology, ecophysiology, oceanography or limnology.

Keywords: spectral imaging, pigment identification, fluorescence, confocal, image analysis, chlorophyll, phycoerythrin, allophycocyanin

Abbreviations: Chls, Chlorophylls; Chl *a*, Chlorophyll *a*; Chl *b*, Chlorophyll *b*; Xant, Xanthophyll; C-PE, C-phycoerythrin; APC-XL, Allophycocyanin Cross-linked; MFI, Mean Intensity Fluorescence; λ_{exc}, excitation wavelength; λ_{em}, emission wavelength; $\lambda_{max,}$ maxima; PSI, photosystem I; PSII, Photosystem II; ROI, region of interest;

Introduction

Phototrophic organisms produce several kinds of photosynthetic pigments, each of them collects photons from a narrow range of the spectrum. A fraction of the energy absorbed by pigments may be emitted immediately at a longer wavelength, a phenomenon known as fluorescence (Keränen et al. 1999). The emitted fluorescence originates mainly from the subantenna of PSII (Govindjee 1995), and results from the inability of the photosystem to use all the absorbed energy (Buschmann et al. 2000). Since photosynthesis and fluorescence are competing processes, changes in the photosynthetic activity are reflected in variations in the fluorescence emission. Therefore, pigment fluorescence can be used as an indicator of photosynthetic processes in plants, algae and cyanobacteria (reviewed by Krause & Weis 1991, Govindjee 1995, Lutz et al. 2001). The fluorescence is a diagnostic tool that allows a description of a complex community in terms of physiological state (Ying et al. 2002), discrimination among phylogenetic groups (Millie et al. 2002), energy transfer and cell evolution (Campbell et al. 1998). However, with most of these techniques the kind of photosynthetic microorganism can only be inferred from its photopigments and simultaneous observation of the samples is not possible.

Traditional epifluorescence imaging reveals the structure and organisation of samples in 2D. This technique is used to study photosynthetic microorganisms and several systems have been described at the microscopic (Gilroy 1997, Yang et al. 1997, Ying et al. 2002) and macroscopic scale (Edner et al. 1995, Lang et al. 1994, Heisel et al. 1996, Lichtenthaler et al. 1996, Saito et al. 2000, Sowinska et al. 1999, Gower 1999, Becker et al. 2002). However, there is sometimes a need to analysemicrostructure in 3D (as in microbial mats, biofilms, etc). To date, confocal imaging spectrophotometry (CIS) has only been used to determine the optimal detection and separation of emission spectra for

either new or known fluorochromes. Here we report a new application of the Confocal Scanner Laser Microscope coupled to a spectrophotometric detector. CIS (confocal imaging spectrophotometry) provides a simultaneous 3D image of photosynthetic microorganisms and their fluorescence signatures, within thick assemblages, due to their multiple excitation wavelengths and free selection of emission wavelength. It allows discrimination *in vivo* among individuals from phylogenetic groups such as chlorophyta, bacillariophyta and phycoerythrin- and non-phycoerythrin-containing cyanobacteria within complex communities by their fluorescence signatures. The potential applications of this tool in complex intact communities are discussed. We also report our results on pure cultures and biofilms from hypogean environments. In intact biofilms a relationship was established between the spectrum and the depth of the cells in the biofilm profile, without needing the isolation of the micro-organisms.

Material and Methods

Pigments

Pure pigments were used for calibrating the CSLM as follows. Liposoluble pigments Chlorophylls *a* and *b* (Chls) obtained from *Spinacia oleracea* and Xantophyll (Xant) from *Medicago sativa* (Sigma, St. Louis, MO, USA) were dissolved in pure ethanol. Hydrosoluble pigments such as R-phycoerythrin (R-PE) from *Porphyra tenera* and C-phycocyanin (C-PE) from *Spirulina* sp. were dissolved in filtered distilled water. Allophycocyanin-XL (APC-XL) stock solution from *Mastigocladus laminosus* was dissolved in ammonium sulphate (60%) and potassium phosphate (pH= 7) (Sigma-Aldrich) with a final concentration 38 mM. Four hundred µl of each pigment solution at a final concentration 1 mg/ml was transferred into one of the 8 wells of a covered-glass bottom chamber (Nunc Lab-Tek™, Nalge Nunc International, Roskilde, Denmark).

Cultures and biofilms preparation

Two monoalgal cultures of *Nostoc humifusum* Carm. (Cyanobacteria) and *Muriellopsis* sp. (Chlorophyta) were cultivated as shown in Table 1. Two cultures of each species, at different stages of growth (exponential - one week - and stationary phase -three weeks-) were used to test the technique. *Anabaena* sp. containing only C-PC was also used as reference to differentiate the species with only C-PC and APC (que yo sepa no hay ninguna ciano que no tenga APC) . In addition, three aerophytic biofilms were used in the CLSM observations to test the method with natural complex assemblages. The biofilms contained different phylogenetic groups (Cyanobacteria, Chlorophyta and Bacillariophyta). The collected biofilms were described, and identified elsewhere (Hernández-Mariné et al. 2003). The first biofilm (BF1) -obtained from St. Callistus Catacomb, (Rome, Italy) - was mainly formed by *Scytonema julianum* (Meneghini ex Frank) Richter and *Leptolyngbya* sp. The second (BF2) -obtained from Domitilla Catacomb (Rome, Italy) - was dominated by the Bacillariophyta *Diadesmis gallica* W. Smith and an unidntified Cyanobacteria of the Chroococcal group. Finally, the third (BF3) -obtained from Zuheros cave (Córdoba, Spain) - was constituted by *Cyanosarcina parthenonensis* Anagnost. (Cyanobacteria) and an unidentified *Chlorella*-like (Chlorophyta). The three biofilms were obtained from artificially illuminated surfaces, although Zuheros biofilm also received natural light, at very low irradiance (<

0.1 µmol·m^{-2}· s^{-1}). Fragments of biofilms were detached from their substrata (plaster, mortar or speleothems) or, rarely, taken together with small pieces of their support.

The biofilms were maintained on a 2 mm layer of 10 % BG11 medium (RIPPKA 1988), agarized (1%, Merck), and processed during the first week. Biofilms and cultures were mounted in cavity microscope slides or placed onto Nunc Lab-Tek™ chambered coverglass. Samples were processed at room temperature in the dark.

Confocal Laser Scanning Microscopy

Confocal Scanning Laser Microscopy (CSLM) was performed with a Leica TCS-SP2 (Leica Microsystems Heidelberg GmbH, Manheim, Germany) using either 63X (NA 1.32, oil) or 100X (NA 1.4, oil) Plan-Apochromatic objectives (range of zoom 1-4). Wavelength scans were performed using the 351- and 364-nm lines of an UV Ar laser; the 458-, 476-, 488- and 514-nm lines of an Ar laser; the 543-nm of a green HeNe laser and the 633-nm of a red HeNe laser. The Leica TCS-SP2 uses spectrophotometric detection that allows the system to perform different scans from 360- to 800-nm of the spectrum using a motorised slit placed in front of the photomultiplier. Although our spectral confocal microscope can acquire spectral images of 5 nm bandwidth between 360-800 nm, each image sequence (wavelength scans or lambda-scan function of the system) was obtained by scanning the same x-y optical section using a bandwidth of 20 nm for emission (λ coordinate of an x-y-λ data sets) to avoid photobleaching. The emission detection was set to 4-9 nm further the excitation wavelength to avoid the laser beam reflectance. Previous experiments showed that samples undergoing more than 400 lambda-scans exhibited photobleaching. As a consequence, although our spectral confocal microscope can acquire spectral images of 5 nm bandwidth between 360-800 nm, we used the system described above.
Scans were performed using the substrate (for UV) or the triple dichroic (488/543/633) filters in the beam splitter. The x, y, λ data set was acquired at the z position in which the fluorescence was maximum. The background fluorescence was measured in areas without sample and, subsequently, used to correct the raw spectra on the thin sections. The laser beam impinged upon the sample perpendicularly and, in order to exclude interfering background radiation (light in the laboratory or light from excitation sources), the images were recorded in total darkness. Gains and offset were the same for

each field at each excitation wavelength and not altered throughout the scanning process.

The variation in intensity of a particular spectral component, encoded using 8 bits, is represented on the screen using a pseudocolor look-up-table. This table is not only used for visualization purposes but also to allow the user to adjust the scanning parameters such as gain and offset. Warm colours such as white and red represent maximum intensities whereas cold colours, like blue, are representative of low intensities (the intensity of each pixel was set to 255 levels of grey). The image size was 512 x 512 pixels.

Three-dimensional projections (extended focus) were made using Imaris software (vers. 2.7.) (Bitplane AG Zürich, Switzerland).

Fluorescence analysis

Mean Fluorescence Intensity (MFI) of the x-y-λ data sets was measured using the Leica Confocal Software, vers. 2.0. The region of interest (ROI) function of the software was used to determine the spectral signature of a selected area from the scanned image. A ROI can also be specified to determine the spectrum of each sample and the software will display the mean intensity of all pixels within the ROI versus the wavelength. For pigment solutions we analysed ROIs of 1000 μm^2 (n= 10 regions). For cell culture and biofilm analysis ROIs of 1 μm^2, taken from the fluorescent thylakoid region inside the cell, were set in each x-y-λ stack of images.

Lambda scans of *N. humifusum* (n= 21 cells), *Muriellopsis* sp. (n= 50 cells) and biofilms (n= 5 cells for each species present in the biofilm) were obtained for each excitation wavelength (λ_{ex}) in at least three independent experiments.

Numerical data were processed with Excel 97 or Excel 2000 (Microsoft). The mean and standard error, for all the regions or cells examined in each λ_{ex}, were calculated. The maxima of the pigments corresponded to their dispersion range, at the different excitation wavelengths ($?_{exc}$).

A parallel experiment to compare the MFI of species in culture, at different λ_{exc}, was carried out. The emission ranges used for the statistical test were: 640-700 nm (351-nm λ_{exc}), 555-605 nm (488-nm λ_{exc}), 580-620 nm (543-nm λ_{exc}). For statistical purposes 25 replicates of each taxa, each of them composed of 20 optical sections at a z step = 0.45

µm, were obtained. These optical sections were processed by Metamorph image analysis software to obtain a composite image, in which the total fluorescence was quantified. The comparisons between the MFI of species in culture at different λ_{exc} were evaluated using unpaired two-tailed Student's *t*-test with statistical significance set at $p<0.05$.

Representation data

Three-dimensional plots -MFI to longer wavelength emission *versus* cell number- were obtained with Matlab 6.0 (Mathworks, Inc.).

Results and Discussion

Fluorescence analysis

Lambda-scans of the pure pigments, cultures or biofilms at different ?$_{exc}$ are shown in Figs. 1-3. Although the fluorescence emission scans of the samples were acquired after the excitation at eight different wavelengths (?$_{exc}$), only the wavelengths with the best signal-to-noise ratio of fluorescence are shown: 351-, 488-, 514- and 543-nm. Other ?$_{exc}$ are not shown because they did not provide much information, 364-, 458- and 476-nm ?$_{exc}$ gave very low signal whereas for the 633-nm ?$_{exc}$ most of the pigments (C-PC, APC and Chls) showed overlapping emission peaks in this region.
In some samples, a deviation of the emission spectrum depending upon the ?$_{exc}$ was observed (Tables 2-4). Both the position and the half-band width of the spectra changed by 5-20 nm with the changes of the ?$_{exc}$ in the range 351-633 nm at room temperature, while spectrum shape was identical (Tables 2-4, Figs. 1-3). These deviations were attributable to the ?$_{exc}$ used as other authors have reported (Ying et al. 2002, Gill & Wittmershaus 1999). The absorption bands of the various photosynthetic pigments overlap strongly but, by changing the excitation wavelengths, the amount of energy absorbed by the various pigments can be changed. These results are translated into modifications of the fluorescence spectra and are related to variation in the distributions of excitation energy between two photosystems (PS1 and PS2) (Goc et al. 2002). However, the fluorescence emission spectrum of the pure pigments in solution should be independent of ?$_{exc}$ (Parker 1968; Talbot & Sauer 1997, MacColl et al. 1996) although our results showed a slight shift in the maximum wavelength emission.

Pure pigments

The extracted pigments show variations in fluorescence spectra when compared to *in vivo* pigments (Sarada et al. 1999). So, due to the methodological character of this paper, we performed a control with pure pigments to compare with the reported studies (Figs 1, 2).

The pure pigment spectra (Table 2) correlated well with published spectra of extracted pigments (Ong & Glazer 1987; Tjioe et al. 2001; Talarico & Maranzana 2000; Canaani et al. 1980). The maximum emission range of Chl *a* (672.4 ± 2.9 nm) and Chl *b* (662.4 ± 2.1 nm) partially overlapped. The maximum fluorescence peak of C-PE was located around 577.2 ± 2.2 nm, with a small shoulder around 660.1 ± 3 nm. This shoulder may correspond to another contaminant phycobiliprotein present in the standard. The maximum fluorescence peak of C-PC, located at 656.1 ± 4.3 nm, appeared shifted with respect to the reported references. However, changes in the maximum peak for C-PC are reported in the literature, depending on the extraction method used. C-PC extracted from fresh biomass showed a major peak at 615 nm while that from dried samples showed an additional peak at 652 nm. C-PC extracted by freezing and thawing showed only one absorption peak while the extraction by homogenisation showed a minor second peak at 678-nm indicating chlorophyll contamination due to disintegration of cells (Sarada et al. 1999). Esta última parte como se relaciona con el shift de longitud de onda de emisión?, realmente nos habla de la contaminación en la extracción no de una variación en los picos.

Both C-PC and Xant presented weak fluorescence. While the weak fluorescence for Xant, was expected because the main de-excitation pathway occurs through the transition to the triplet state, which is non-fluorescent (Schoefs 2002), we could not find the reason for this low fluorescence in the pure C-PC although Meris **(Becker et al 2002)** reported the same problem.

Maximum fluorescence peak for the allophycocyanin-crosslinked (APC-XL) was at 676.2 ± 2.4 nm. The special structure of APC-XL, used to prevent the dissociation of the molecule in dilute solutions, probably affected the maximum emission peak and cannot be related to the field material. On the other hand, this maximum agreed with a specific type of APC reported by Talarico & Maranzana (2000) and Canaani et al. (1980). Other common low-energy forms of APC have fluorescence maximum near 680-nm, which is similar to the emission from intact phycobilisomes (McColl 1998)

whereas APC from cyanobacteria presented an emission maximum at 660 nm (Tjioe et al. 2001).

Cultures

Species belonging to the same phylogenetic group, Cyanobacteria, Chlorophyta or Bacillariophyta, showed similar spectra (Figs. 1-3), due to the presence of characteristic pigments (Millie 2000 and references therein) while all of them had a common Chl *a* fluorescence maximum at 680-690 nm.

Fluorescence properties for x-y-? single sections of *Nostoc humifusum*, corresponding to the emission peak ($?_{max}$), were obtained for each pigment at each $?_{exc}$ (Fig. 1). From these sections the ROIs used to plot 3D and 2D spectral data (Figs. 1B and 1C) were obtained. In 3D plots of *N. humifusum* (Fig. 1B), the $?_{max}$ position of each cell was practically constant, even if the cultures were at a different growth stage-compare image section at 351-nm $?_{exc}$, in which the cultures were 1 week old, with the rest of images, which were 3 weeks old (Fig. 1A)-.
Depending on the choice of excitation wavelengths, the fluorescence of different pigments can be discriminated. Chls fluorescence was mainly determined using UV excitation (351-nm) and was detected at 640-700 nm. For phycobiliproteins orange excitation at 543 nm was used and was detected at 580-620 nm.

The emission maximum of Chl *a* was observed as a weak shoulder, close to the high phycobiliprotein fluorescence maximum, at 663 ± 1.5 nm (Fig. 1C) (Campbell et al. 1998) except for the clear peak obtained at 351-nm $?_{exc}$, because C-PC and APC showed weak fluorescence at this $?_{exc}$.
The 488-, 514- and 543-nm $?_{exc}$, absorbed essentially by the C-PE, resulted in strong orange fluorescence emission *in vivo* at 579 ± 1.6 nm. These results are consistent with the ones reported by Rodríguez et al. (1989) and Wyman (1992).
Muriellopsis sp. (Fig.2) shows a prominent Chl *a* fluorescence peak at 696.5 ± 2.4 nm when excited at any $?_{exc}$ (Fig. 2C). *Muriellopsis* sp. produces other antenna pigments such as Chl *b* and the Xant lutein (Del Campo et al. 2000, 2001). The $?_{max}$ for Chl *a* for all $?_{exc}$ was shifted (~ 10 nm) to a longer wavelength than generally reported. This shift may be a result of the interaction of Xant with Chl *a* (Anderson et al. 1992, Young & Frank 1996). Furthermore, the presence of Chl *b* affected the state of the longer

wavelength-absorbing forms of Chl *a* (Mullet et al. 1980, Bialek-Bylka & Brown 1986). In all $?_{exc}$, a small shoulder was observed at 653-672 nm, which could be assigned to Chl *b*. However, Chl *b* emission in antenna complexes is not usually detected in steady state fluorescence experiments because of rapid and efficient transfer of energy from Chl *b* to Chl *a* (Frank et al. 2001). As in the case of *N. humifusum*, differences were not observed in the $?_{max}$ among the cells (Fig. 2B).

Biofilms

Extended focus images of three stratified biofilms -BF1, BF2 and BF3- showed the differential distribution in depth of the microorganisms in the biofilm (Fig. 3A). Spectra emission for 488-, 514- and 543-nm $?_{exc}$ are shown below each corresponding biofilm (Fig. 3B).

Biofilm BF1. Thin filaments of the red *Leptolyngya* sp. were horizontally oriented on top of the wide *Scytonema julianum* (Fig. 3A). Both cyanobacteria had a wide 658.4 ± 3 nm $?_{max}$, from the overlap of Chl *a* and phycobiliproteins (Fig. 3B). In addition, *Leptolyngybya* sp., but not *S. julianum*, presented an emission peak (579.7 ± 3.8 nm) attributable to the presence of C-PE (Fig. 3B).

Biofilm BF2. CLSM revealed two layers. *Diadesmis gallica* -Bacillariophyta- was mainly concentrated on the top of the biofilm while the unidentified Chroococcal formed a discontinuous bottom layer (Fig. 3A). *D. gallica*, 3 µm in diameter, presented less fluorescence than the cyanobacteria. Their $?_{max}$, at 676.2 ± 5 nm (Fig. 3B), did not coincide with the other groups $?_{max}$ due to the presence of Chl *c*. To avoid photobleaching, caused when these cells were successively excited with different $?_{exc}$, different optical fields were used to obtain the emission spectra at all $?_{exc}$. The unidentified Chroococcal presented the same spectral shape as *Leptolyngybya* sp. and *N. humifusum* (Figs. 1 and 3).

The biofilm BF3 was also stratified with a continuous upper layer of *Chlorella*-like and isolated colonies of *Cyanosarcina parthenonensis* in deeper parts (Fig. 3A). Their $?_{max}$ matched those of the related groups - Chlorophyta and Cyanobacteria, respectively (Figs. 1-3)-.

We could not separate the emission spectra of the Chl *a* and *b*, since the excitation and emission spectra are similar for the different chlorophylls. Gibbs (1979) and Lorenzen & Jeffrey (1980) reported that the interference from Chl *b* is severe (Moberg et al. 2001). As for C-PC and APC, esto está bien?, no te refieres a PE, we identified both

phycobiliproteins in the spectra of *N. humifusum* and field cyanobacteria, by comparing their maxima (Tables 3-4) with the maxima at 649.3 ± 3.3 nm identified in *Anabaena* sp., which does not present PE.(no es cierto, Anabaena también tiene APC)

Differences in mean fluorescence intensity

MFI of emission range 640-700 nm at 351-nm $?_{exc}$, which corresponds mainly to chlorophyll fluorescence, was similar in *Nostoc humifusum* and *Muriellopsis* sp. ($p<0.05$). In contrast, at 488- and 543-nm $?_{exc}$ differences of MFI emission at 555-605 nm and 580-620 nm were statistically significant, ($p< 0.05$), due to accessory pigments present in *N. humifusum* (Table 3).

In the field samples, the cyanobacteria presented higher MFI for the range 640-740 nm at any of the $?_{exc}$, when compared to the Chlorophyta and Bacillariophyta- (Fig. 3B). The cyanobacteria also presented high MFI at 577-580 nm $?_{max}$ (Fig. 3), due to the C-PE. Furthermore, the phycobiliprotein content of cells increases with decreasing irradiance (Albertano 1991, Prezelin et al. 1989), which is the rule in the hypogean habitats where they grow (Bruno et al. 2001).

Each photosynthetic pigment present in the species absorbs the light of a certain wavelength, but on the whole they collect photons from a wide range of wavelengths, when excited at wavelengths from 351-633 nm. Only in the 364-nm (UV) and the 458-nm (blue) $?_{exc}$ a smaller emission response was observed in all the microorganisms. This provides evidence that the minimum efficiency of the cyanobacterial photoreceptors is at 430-460 nm (Tandeau de Marsac & Houmard 1993).

Heterogeneities of the MFI within the same cell and among different cells of the same specie were observed (Figs. 1B and 2B). The fluorescence emission of the phototrophic microorganisms depends on the pigment concentration in a cell although the relationship is not linear. Photochemical and non-photochemical quenching factors and adaptation to environmental factors, such as light, nutrient availability and temperatureare important (Babichenko et al. 2001). In addition, absorption characteristics of the whole organisms (Sosik & Mitchell 1991, Johsen & Sakshaug 1996) and their optical properties determine the penetration of excitation (Buschmann et al. 2000). We did not observe changes in the $?_{max}$ of particular taxa - *Nostoc humifusum*, *Scytonema julianum* or *Cyanosarcina parthenonensis*- when covered by thick sheaths, exopolymeric substances (EPS) or calcareous investments. *N. humifusum*, *Muriellopsis*

sp. and field *C. parthenonensis* presented a permanence of the fluorescence level at 720-730 nm (9-83.8%). This maintenance of the fluorescence emission is attributable to the combined performance of the PSII and PSI (Li et al. 2001). According to Young & Frank (1996), this maintenance is caused by the presence of carotenoids.

Conclusions and Future perspectives

The combination of CLSM with spectrophotometry techniques creates a powerful new tool which enlarges the application fields for both methodologies. This set-up allows: (i) analysis for both global and single fluorescent pixel, providing their three dimensional localisation *in vivo,* (ii) direct analysis of fluorescent pigments from a single cell *in situ* into thick samples without isolation, thus minimizing the artefacts associated with a small sample size and avoiding extraction or blurry substances such as EPS, that cause problems when identifying fluorescence spectra, (iii) establishing a relationship between the fluorescence properties and the position inside the particular microbial assemblage. The result is given as an image cube that contains spectral as well as spatial information, which permits identification of $?_{max}$, (iv) the possible application of eight excitation wavelengths to obtain single cell spectra, providing high-resolution detection and detailed information for the sample, holds great promise for the study of mixed populations or strains with a complex pigment composition, (v) fast access to statistical information on cell numbers and spectral properties of the community, (vi) discrimination of the cells with particular fluorescence signatures within the colony and correlation with the individual cell states.

The fluorescent pigments act as photoactivated fluorescent markers that switch on in response to irradiation by light of a particular wavelength, so that the method can be used to track other fluorescent substances.

Some technical problems have yet to be solved, such as identification of low fluorescence photoprotective pigments, pigments with a low photobleaching threshold or overlapping emission spectra of some pigments. Further, it is important to combine computational algorithms for spectral deconvolution and the development of a database consisting of analysis of spectra pigments to resolve these problems. However, the accuracy of the spectra obtained is sufficient to determine small changes in maxima which are in the practical range for many screening applications, as the unequivocal identification of the composition of different community structures in the smallest size of the phytoplankton.

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The authors's address:
MÓNICA ROLDÁN,
MARIONA HERNÁNDEZ-MARINÉ,

Unitat de Botánica
Facultat de Farmacia
Universitat de Barcelona
Av. Joan XXIII s/n
E-08028, Barcelona, Spain.
e-mail: mroldan@farmacia.far.ub.es

FEDERICO THOMAS
Institut de Robotica i Informàtica Industrial (CSIC-UPC)
Llorens Artigas 4-6, 2 planta
08028 Barcelona, Spain

SUSANA CASTEL
Serveis Cientificotècnics
Universitat de Barcelona, Barcelona Science Park
Josep Samitier 1-5
08028 Barcelona, Spain.

ANTONIO QUESADA
Departamento de Biología
Universidad Autónoma de Madrid
28049 Madrid, Spain.

Figure captions:

Figure 1. CLSM images and Lambda-scans of *Nostoc humifusum in vivo.* Optical sections and spectral profiles derived from $?_{exc}$ of 351-, 488-, 514- and 543-nm. **A**. Pseudocolor confocal x-y-? single sections corresponding to the $?_{max}$ autofluorescence of C-PE (first maximum) and PBS + Chl *a* (second maximum) for each of the four $?_{exc}$.

The pseudocolor scale is shown at the bottom left. Such optical sections correspond with the maxima peaks, when excited with the corresponding ?$_{exc}$, shown in C plots (shady areas). **B**. 3D surface pseudocolor plots of fluorescence spectra: emission wavelength (*x*)-MFI, mean fluorescence intensity (*y*)- number of cells (*z*); the ?$_{max}$ position of each cell practically did not show variability in each of the ?$_{exc}$, even if the cultures were in different state of growth (exponentially grown cultures in 351nm ?$_{exc}$ and stationary state for the others) . **C.** 2D plots representing the MFI spectra for *N. humifusum* and pure pigments. Standard error (n=21cells) and R-PE, C-PC, APC-XL and Chl *a* (n=10 regions) to these ?$_{exc}$ are represented. Ratios of C-PC and APC-XL fluorescence had to be multiplied with factor 2 due to the low signal of fluorescence received by the CLSM. The values shown are averages of MFI from the three independent experiments carried out in the same experimental conditions. Zoom factor: 2. Scale bar = 10 µm.

Figure 2. CLSM images and Lambda-scans of *Muriellopsis* sp *in vivo*. Optical sections and spectral profiles derived from ?$_{exc}$ of 351-, 488-, 514- and 543-nm. **A**. Pseudocolor confocal x-y-? single sections corresponding to the ?$_{max}$ autofluorescence of Chls (*a* and *b*) for each of the four ?$_{exc}$. The pseudocolor scale is shown at the bottom left. Such optical sections correspond with the maxima peaks, when excited with the corresponding ?$_{exc}$, shown in C plots (shady areas). **B.** 3D surface pseudocolor plots of fluorescence spectra: emission wavelength (*x*)-MFI, mean fluorescence intensity (*y*)-number of cells (*z*); the ?$_{max}$ position of each cell practically did not show variability in the ?$_{exc}$, even if the cultures were at different stages of growth (exponentially grown cultures in 351nm and 488 nm ?$_{exc}$ and stationary state for the others). C. 2D plots representing the MFI spectra for *Muriellopsis* sp. and pure pigments. Spectra emission are the mean ± standard error (n= 50 cells) and xanthophyll (Xant) and chlorophylls (Chls *a* and *b*) pigments (n=10 regions) in each ?$_{exc}$ are represented. Xant ratio fluorescence had to be multiplied with factor 2 due to the low signal of fluorescence received by the CLSM. Zoom factor: 2. Scale bar = 10 µm.

Figure 3. CLSM images and Lambda-scans of *in vivo* three aerophytic biofilms from Roman Catacombs (BF1 and BF2) and Zuheros (BF3). Optical sections and spectral profiles derived from ?$_{exc}$ of 351-, 488-, 514- and 543-nm. **A**. Three-dimensional extended focus pseudocolor projections in x-y and orthogonal views in z-direction of

the biofilm. Each image represents the maximum auto-fluorescence emitted in the range of 590-775 nm (shady area) when excited at 543-nm. T= Surface of the sample. Scale bar = 10 µm. **BF1.** 49 x-y optical sections of *Scytonema julianum* and *Leptolyngbya* sp. The volume under observation is 465.03 x 465.03 x 398.73 µm³. Z step: 0.4 µm. Calibration: pixel size (x, y) is 0.46 µm/pixel, z-step is 0.39 µm. Zoom factor: 1. Thickness: 19.54 µm. **BF2.** 66 x-y optical sections of an stratified biofilm, consisting of two strata, the upper epilithic layer composed by colonies of *Diadesmis gallica* and understorey layer formed by Chroococcal colonies. The volume under observation is 75.82 x 75.82 x 98.51 µm³. Z step: 0.1 µm. Zoom factor: 3.86. Thickness: 6.6 µm. **BF3.** 98 x-y optical sections of an stratified biofilm with a continuous upper layer of *Chlorella*-like and a discontinuous bottom layer of *Cyanosarcina parthenonensis* (arrow). The volume under observation is 146.77 x 146.77 x 200 µm³. Z step = 0.2 µm. Zoom factor: 2. Thickness: 19.4 µm.

3B. *In vivo* mean emission spectra of the different species present in the biofilms of Figure A and the standard error (n= 5 cells). The difference emission profiles obtained in biofilms indicate the presence of different groups of algae and cyanobacteria. In the BF2, a strong fluorescence decrease in the 543-nm ? $_{exc}$ is observed. This decrease is attributable to photobleaching after successive Lambda-scans. Zoom factor: 2.

Table 1. List of species in culture with the corresponding characteristics of the origin, medium type, irradiance, temperature, reported pigments and references

Strains	**Sampling sites**	**Medium type**	**Irradiance (µmol·m^{-2}· s^{-1})**	**Temperature (ºC)**	**Reported pigments**	**References**
Nostoc humifusum (=*Anabaena* sp.)	Albufera lagoon [1]	ATCC [3]	49.8[5]	30-35	Chl a PE C-PC APC	Rodriguez et al. 1989
Muriellopsis sp.	Empordà marsh [2]	ATCC[4]	49.8[5]	25-28	Chl a Chl b Lutein (xanthophyll)	Del Campo et al. 2000, 2001

[1] Valencia, Spain

[2] Catalunya, Spain

[3] ATCC medium (ARNON et al. 1974) lacking nitrate and 1% agar (Merck)

[4] ATCC medium (ARNON et al. 1974) 1.2 % agar (Merck)

[5] Both species were subjected to alternating 12-h light and dark cycles.

Table 2. ?$_{max}$ and shoulders for different pure pigments by confocal imaging spectrophotometry in all ?$_{exc}$. Data are mean ± SE (n=10 regions) from the three independent experiments carried out in the same experimental conditions.

Pigments	**? exc (nm)**							
	351	**364**	**458**	**476**	**488**	**514**	**543**	**633**
Chl *a*	674.9	661.0	671.1	676.6	676.4	680.4	660.9	678.1
Chl *b*	658	652.8	664.8	664.6	670.7	660.8	660.9	666.9
Xant	514	528.8	513 569.9*	514.1 568.3*	567.9*	-	-	-
R-PE	573.3 -	578.4 -	582.55 652.1*	580.3 658.6*	573.6 653.6*	569.2 660.8*	583.3 668.6*	- 666.9*
C-PC	649.5	636.2	652.1	658.6	676.4	654.2	660.9	661.2
APC-XL	674.9	677.5	683.8	682.6	682.1	667.3	668.6	672.5

* Shoulder

Table 3. ?$_{max}$ and shoulders of *Nostoc humifusum* (n= 21 cells) and *Muriellopsis* sp. (n=50 cells) species in culture by confocal imaging spectrophotometry in all ?$_{exc}$.

Strains	**? exc (nm)**							
	351	**364**	**458**	**476**	**488**	**514**	**543**	**633**
Nostoc humifusum.	581.7 658	578.4 661	576.2 658.5	586.3 664.6	579.3 665	575.8 667.3	575.5 668.6	- 661.3
Muriellopsis sp.	691.8	702.3	690.1	688.7	693.6	700	699.7	706.2

Table 4. Comparison of the ?$_{max}$ and shoulders of three aerophytic biofilms -BF1, BF2 and BF3- at four principal ?$_{exc}$ (351-, 488-, 514- and 543-nm). BF1: *Scytonema julianum*

and *Leptolyngbya* sp. BF2: *Diadesmis gallica* and Chroococcal unidentified. BF3. *Chlorella*-like and *Cyanosarcina parthenonensis.* Each ?$_{max}$ value were obtained from 5 cells.

Biofilms	**Species**	**?$_{exc}$ (nm)**					
		351	**488**		**514**		**543**
BF1	*Leptolyngbya* sp.	573.6	653.6	582.3	660.8	583.3	660.9
	Scytonema sp.	602.1	653.6	601.9	660.8	606.5	660.9
BF2	*D. gallica*	-	670.7	-	673.8	-	684.1
	Chroococcal	573.6	659.3	582.3	660.8	575.5	653.1
BF3	*Chlorella-like*	-	687.9	-	693.5	-	691.9
	C. parthenonensis	579.3	665	575.8	660.8	583.3	668.6

[1] Corresponding to Csc19c (BF1), Cd15c (BF2) and Z7b (BF3) samples sites, respectively, of hypogean monuments from CATS European project