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In Situ Confocal Laser Scanning Microscopy of Protozoans in Cultures and Complex Biofilm Communities

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Summary. A series of novel fluorescent compounds were used to stain protozoans in pure culture and in complex environmental biofilm communities. Confocal laser scanning microscopy was used to image fluor stained, $NiSO_4$ immobilized, living protozoans in situ. Most of the stains, specific for eucaryotic cell structures, such as Paclitaxel-BodipyFL, CellTracker, $DiOC_6(3)$, LysoTracker Red, MitoFluor Green and Rhodamine 6G showed potential for in situ staining of protozoa. However, some of the stains also stained bacterial or polymeric biofilm constituents. Nevertheless after staining, the usually transparent protozoan cells became clearly visible within the complex architecture of environmental biofilms. With respect to staining protozoan cells only and differentiation from other biofilm constituents, CellTracker showed the highest specificity. By using this approach, protozoa can be identified and distinguished within a complex habitat by virtue of (1) specific stains targeted for eucaryotic cell features, especially CellTracker, and (2) staining in combination with information on their size and morphology. With respect to fluorochrome concentration the approach has to be fine-tuned according to the specific characteristics of the sample as well as the identity and physiological status of the protozoa.

Key words: biofilms, confocal laser scanning microscopy (CLSM), fluorochromes, protozoans.

Abbreviations: CLSM - confocal laser scanning microscopy.

INTRODUCTION

Environmental biofilms are very diverse and heterogeneous communities (Lock 1993). Their major cellular components may be bacteria, algae and the heterotrophic eukaryotes e.g. fungi, protozoa and micrometazoa. Furthermore, extracellular polymeric substances (EPS) represent a major component of biofilm systems (Neu and Lawrence 1999). Although standard light and epifluorescence microscopy are effective tools in biofilm and protistan studies, they are limited to the examination of materials that are relatively thin in nature. In contrast, CLSM allows three-dimensional imaging of fully hydrated, living, thick, procaryotic and eukaryotic communities in their natural habitat. The large potential of this technique has been demonstrated for biofilms in a variety of studies (Lawrence *et al*. 1998a, b; Lawrence *et al*. 1991, 1994, 1998a, b; Bott *et al*. 1997; Møller *et al*. 1997, 1998; Neu and Lawrence 1997;

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Manz *et al*. 1999; Neu 2000). To date most studies investigating biofilms by means of confocal laser scanning microscopy (CLSM) have focused on the prokaryotic part of biofilms. Nevertheless, heterotrophic protozoans represent an important yet understudied component of biofilms. Their movement and grazing behaviour results in changes in the biofilm biocoenosis (Jackson and Jones 1991, McCormick 1991, Møller *et al*. 1997, Lawrence and Snyder 1998). However, in situ investigations of motile eukaryotic organisms in biofilms are hampered by methodological limitations.

A range of approaches have been used to study protozoa, direct microscopic examination with bright field or phase contrast optics or general staining and epifluorescence microscopy (Pedersen 1982). The application of specific antibodies to stain the cytoskeleton of protists has also been carried out (Fleury 1991, Jeanmaire-Wolf *et al*. 1993). However, these studies were morphological or developmental in nature and did not focus on the natural environment. In situ hybridisation has also been used to examine and identify protists in situ and in pure cultures (Lim *et al*. 1993, 1996). Most of these methods (i.e., antibodies, hybridisation) have the draw back of requiring fixation resulting in distortion, loss of protists and loss of context or growth habit. As eukaryotes, members of the protista have a variety of structural and chemical characteristics that may be exploited to separate them from bacterial, exopolymeric, algal and other components of biofilm communities. These include an extensive cytoplasm, endoplasmic reticulum (ER), Golgi apparatus, vacuoles, tubulin containing cytoskeleton, numerous mitochondria, ciliary bands etc.

The fluorescent stains often used for investigating microbial communities with epifluorescence microscopy are general nucleic acid or protein stains such as Acridine Orange, DAPI, SYTO or Proflavin (Strugger 1948, Porter and Feig 1980, Sherr *et al*. 1993, Neu and Lawrence 1997, Lawrence *et al*. 1998a). The application of these stains leads to a non-specific binding to all cells present in the biofilm. In the case of algae, the autofluorescence signal of chlorophyll, can be used to distinguished them from bacteria and other biofilm components (Lawrence *et al*. 1998a). Nevertheless, in complex biofilms under most staining conditions it is difficult to distinguish the bacterial signal from the protozoan signal. However, the protozoa, as members of the eukaryotes, offer the possibility to use stains specific for cell organelles and other cellular structures. A variety of cellular stains have been developed in order to visualise and identify subcellular compartments, storage products and other metabolites of eukaryotes. In addition to their specificity for protozoans, these stains also allow the analysis of fully hydrated living biofilms without prior fixation. A further challenge for observation of protozoa in situ using a point scanning CLSM is the motility of these organisms. During the scanning process it is necessary that the cells are stationary, in order to perform optical sectioning and averaging for final three-dimensional reconstruction.

The aim of this study was to find a suitable fluorescent staining protocol for protozoa in pure culture as well as in complex biofilm communities. For this purpose we evaluated a range of potential fluorescent stains specific for eukaryotic cell features. In addition, the staining protocol was tested in combination with an immobilisation technique in order to apply CLSM to motile protozoan cells.

MATERIALS AND METHODS

Cultures. The ciliates (*Paramecium caudatum*) and heterotrophic flagellates were co-cultivated in Petri dishes with Eau de Volvic (French table water) enriched with some rice grains. For examination an aliquot of culture medium (500 or 1000 µl) containing cells was used to fill four well coverslip chambers (Nunc, Roskilde, Denmark). Immobilisation tests and staining were carried out in these chambers.

Biofilms. The complex biofilms were grown in a rotating annular reactor (RAR) on polycarbonate slides (Sinis, Dettingen, Germany). The reactors were fed with raw water from the South Saskatchewan River, Saskatchewan, Canada. For further details of the operating conditions see Neu and Lawrence (1997).

Microscopy. A MRC-1000 CLSM (BioRad, Hemel Hempstead, UK) equipped with an argon-krypton laser and mounted on a Microphot SA microscope (Nikon, Tokyo, Japan) was used to obtain images of biofilms and stained protozoa. Observations of RAR slides were made with water immersible lenses including a 63x, 0.9 numerical aperture (NA) Zeiss lens and 10x 0.3 NA, 20x 0.4 NA, and 40x 0.55 NA Nikon lenses. The system was controlled by a Pentium host computer with the operating software Comos 6.01. Pieces of the polycarbonate slides (*ca* 1.0 cm^2) with biofilm (grown for 38 to 48 days) were glued in Petri dishes and covered with filtered river water for subsequent staining. Images were obtained using green, excitation (ex) 488/32, emission (em) 522/32), red (ex = 540, em = 580/32 nm) and far red (ex = 647, em 680/32). In addition, phase contrast transmission laser images were obtained. Images were also obtained with a TCS-4D CLSM system (Leica, Heidelberg, Germany) equipped with an argon-krypton laser and mounted on a DM-IRBE inverted microscope (Leica). For observation, the following lenses were available: 20x 0.6 NA, 40x 0.75 NA, 63x 1.2 W NA and 100x 1.4 NA. The CLSM system was controlled by Scanware Ver. 5.1A (Leica).

Preparation of stains. A variety of potential stains were obtained from Molecular Probes Inc. (Eugene, Oregon, USA) and are listed in Table 1. The stains were stored according to the instructions

of the supplier (at 4 °C or -20 °C), warmed to room temperature, and if provided as a solution, briefly centrifuged. Stock solutions were prepared by adding distilled water, phosphate buffered saline (PBS), DMSO or ethanol to the stain (see Table 1). The stock solution was further diluted with filter-sterilised distilled water, river water, Eau de Volvic or PBS-Buffer to create a working solution. The working solutions were directly applied to the culture medium or the biofilm. Incubation time was 5-10 min., although for MitoFluor Green we used an incubation time of 30 min.

Immobilisation. A solution of nickel sulfate (stock solution 1% w/v for $NiSO_4$) was used for the immobilisation of protozoa (Lee *et al*. 1985). The solution was applied at a final concentration of 0.002% (w/v) to culture medium containing ciliates or flagellates. To the biofilm samples 50-100 µl of a 0.01 % working solution was added.

RESULTS AND DISCUSSION

For staining eucaroytic cells a large number of fluorescent stains are now available for use in combination with epifluorescence microscopy or confocal laser scanning microscopy (Haugland 1999). These stains target many of the organelles and other structures present in eukaryotic cells but have not been extensively tested using protists either in pure culture or in environmental microbial communities. In the present study we assessed whether some of these stains are suitable for staining and detecting protists in natural biofilm communities and in pure culture. Staining protocols were optimized for the specific biofilm system and cultures used. The dilution factors and concentration of fluorochromes listed in Table 1 are intended as guidelines. We recommend testing various concentrations of stains and incubation times in order to achieve the best results. In the examples given, the protozoan cell was visualized by concurrent transmission/fluorescence imaging.

Paclitaxel-BodipyFL. Paclitaxel (approved generic name for the anti-cancer pharmaceutical Taxol) is known to interfere with tubulin polymerisation. It also blocks cells in the G_2 and M phases of the cell cycle. It has been reported that live cells incubated with Paclitaxel-Bodipy FL showed a staining pattern that may correspond to labelled tubulin filaments (Haugland 1999).

We observed that the cytoplasm was labelled to a certain degree with Paclitaxel-BodipyFL (Figs 1A, 2B). Cilia were also brightly stained, as were the fibrillary structures located near or connected to the peristomal regions such as the oral region of hypostome ciliates (Fig. 1A). To our knowledge, PaclitaxelBodipyFL has not previously been used as a vital stain for protozoan cells. Although we did not observe the characteristic ciliation pattern observed after silver staining, the bright staining of cilia and fibrillary peristomal structures indicates that microtubules/tubulin may be a major target of this stain in protists. Consequently this compound may be a useful stain for various applications.

Rhodamine 123. Rhodamine123 has been applied to a variety of cell types, e.g. live bacteria, plants and human cells. This cationic fluorescent dye accumulates in active mitochondria (e.g. Johnson *et al*. 1980) without inducing cytotoxic effects (Haugland 1999). Other studies have used Rhodamine123 to label the mitochondria of the protozoan malarial parasite *Plasmodium* (Divo *et al*. 1985) as well as the mitochondria in phytoplankton cells (Klut *et al*. 1989).

Rhodamine123 applied to ciliate cultures and biofilms did not work as a specific stain. We found that application of Rhodamine123 at high concentration to cultured ciliates resulted in a negative staining similar to that described by Caldwell *et al*. (1992) when staining biofilms and cells with fluorescein. In the case of pure cultures there was a rather bright background staining with protozoan cells appearing dark. The application to complex biofilm communities resulted in staining of the whole biofilm, particularly bacterial cells, with no evident staining of ciliates or other protozoa (data not shown).

Rhodamine 6G chloride. This stain is a rhodamine derivative which has been shown to be selectively accumulated in mitochondria (Johnson *et al*. 1981). Haugland (1999) suggested that at low concentrations lipophilic rhodamine dyes selectively stain mitochondria in live cells, whereas at high concentrations they stain the ER of animal cells. Thus this dye may have some potential for applications in studies of protozoa.

In our studies the stain was effective when applied to pure cultures of both ciliates (Fig. 1B) and flagellates. In contrast, when staining protozoa in biofilms the signal was not very intense and non-specific binding to microbial polymers and bacterial cells was observed. This indicated that the stain has some potential for pure culture studies but was not really suitable for environmental applications.

$DiOC_6(3)$, Dicarbocyanine. This stain is a carbocyanine with short (C1-C6) alkyl-chains. Terasaki and Reese (1992) confirmed that $DiOC_6(3)$ labelled the ER and other membrane compartments in epithelial cells. They suggested that $DiOC_6(3)$ stains all intracellular membranes. $DiOC_6(3)$ has also been reported to stain mitochondria in live yeast and other eukaryotic cells, as well as sarcoplasmic reticulum in beating heart cells (Haugland 1999).

In our applications this fluorochrome stained ciliated protozoans both in pure culture and in complex microbial biofilms. Observations in environmental biofilm samples indicated that staining also occurred in bacterial and algal cells. This makes it difficult to separate the protozoan signal from other signals if digital image analysis is applied to quantify certain features (Fig. 2A).

LysoTracker Red. LysoTracker probes consist of a fluorophore or biotin moiety linked to a weak base that is only partially protonated at neutral pH. According to Haugland (1999) this compound is thought to be freely permeable through cell membranes and typically concentrates in spherical organelles.

CLSM observations indicated that LysoTracker Red stained specific regions of the cytoplasm which may correspond to the lysomal vesicles. This can be clearly seen in the image of a *Paramecium* (Fig. 1D). LysoTracker Red was particularly suited for staining of heliozoans in biofilms were the cytoplasm was brightly stained and even the axiopods were visible (Figs 2 C, D).

MitoFluor Green. Mitofluor Green has a chemical structure similar to MitoTrackerGreen. The MitoTracker probes are cell permeant mitochondrion-selective dyes. They are non-fluorescent in aqueous solutions and become fluorescent once they accumulate in the lipid environment of mitochondria. Mitochondria are visualised in live and fixed cells and exhibit bright green, fluorescein-like fluorescence (Haugland 1999). MitoTracker probes were previously used to stain mitochondria in protist cells (Vassella *et al*. 1997) as well as arthropod and mammalian cells (Hoth *et al*. 1997, Pereira *et al*. 1997).

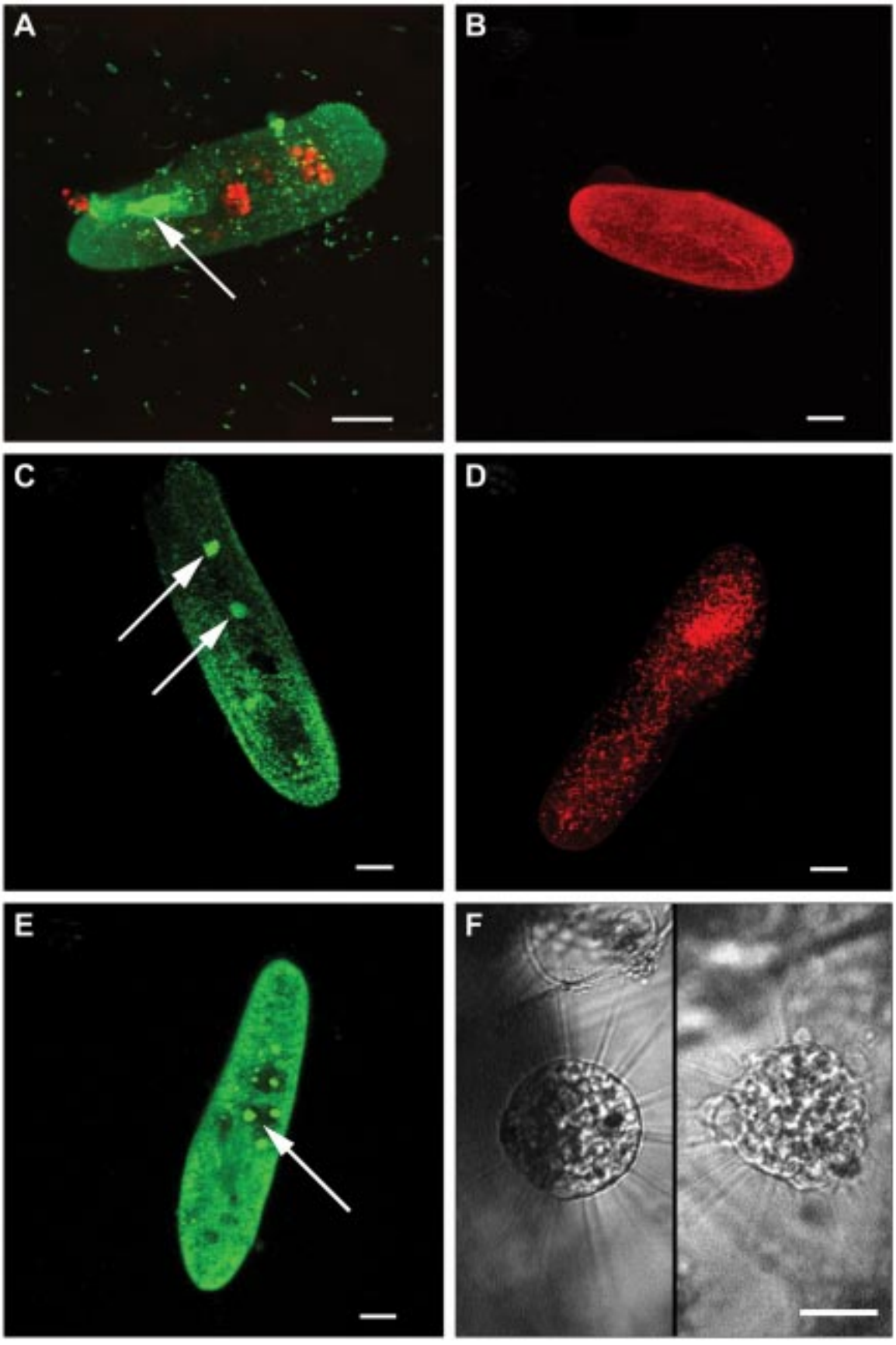
In our experiments MitoFluor Green labelled *Paramecium* cells in cultures (Fig. 1C) as well as peritrich ciliates and the cell body of heliozoans in complex biofilms. The staining was bright, but faded quickly. Some peritrich ciliate cells stained brightly but labelling was concentrated at the outer rim of the cells. MitoFluor Green was not suited for all ciliate groups, due to the fact that specimens of the Scuticociliatida or Hypostomatida were not stained at all.

CellTracker, CMFDA. Chloromethylfluorescein diacetate (CMFDA) is a fluorescent chloromethyl derivative that freely diffuses through the membranes of live cells. Once inside the cell, these mildly thiol-reactive probes undergo what is believed to be a glutathione S-transferase-mediated reaction to produce membrane-impermeant glutathione fluorescent dye adducts. Haugland (1999) indicated a variety of applications for these dyes including cell tracing in mixed cultures (Yoshida *et al*. 1996), long-term viability assays (Poole *et al*. 1996) or measuring cellular glutathione content using flow cytometry (Hedley and Chow 1994). Furthermore, CMFDA was used to label live protozoan cells during grazing experiments (Li *et al*. 1996).

Both in cultures and in the biofilm samples, CellTracker stained the whole cell body of various protozoan groups (Figs 1E, 2E). In some cases structures like nuclei, oral organelles or basal bodies (flagellates) were more intensively stained. Staining of protozoans in the biofilm was very specific, and was limited to protozoans only. No other signals were visible using epifluorescence microscopy with different filter settings or the various excitation/emission options of the CLSM. Cell bodies of heliozoans were well stained and the vacuolized structure of the cytoplasm became visible. The axiopods were usually weakly stained but in some cases not at all. CellTracker may be also combined with LysoTracker which may reveal additional structural details (Fig. 2F).

Imaging of Protists. The application of CLSM to observe protozoans in situ presents special challenges due to their motile and responsive nature. Bacterial cells have proven relatively insensitive to scanning during CLSM (Caldwell *et al*. 1992). In contrast, we frequently observed that during the scanning process protozoan cells tried to escape from the beam or scanned region. We observed that sessile ciliates, frequently contracted their stalks, *Paramecium* exhibited fast swimming movements, and even semi-sessile heliozoa moved away. Immobilization could be achieved by adding a nickel sulfate solution to the samples. The reaction of cells differed from treatment to treatment, possibly depending

Fig. 1 A-F. Staining of cultured *Paramecium* sp. with different fluorescent stains. **A** - Dual channel image with Paclitaxel-Bodipy signal (= green) showing brightly stained fibrillary structures in the peristomal region (arrow) and autofluorescence inclusions (= red). The small green rods surrounding the protozoan cell are stained bacteria. **B** - Single channel image after staining with Rhodamine 6G chloride, staining may indicate mitochondria. **C** - Single channel image after staining with MitoFluor Green, small bodies may be mitochondria, large bright fluorescent bodies may indicate food vacuoles (arrows). **D** - Single channel image after staining with LysoTracker Red, staining may indicate lysosomal vesicles. **E** - Single channel image after staining with CellTracker (CMFDA), large brightly fluorescent bodies may indicate food vacuoles (arrow), other fluorescent regions are presumably thiol-rich areas. **F** - Effect of constant radiation with the laser beam on the integrity of heliozoan cells. Image shows transmission micrograph before (left) and after (right) exposure to laser light. Scale bars - 20 μm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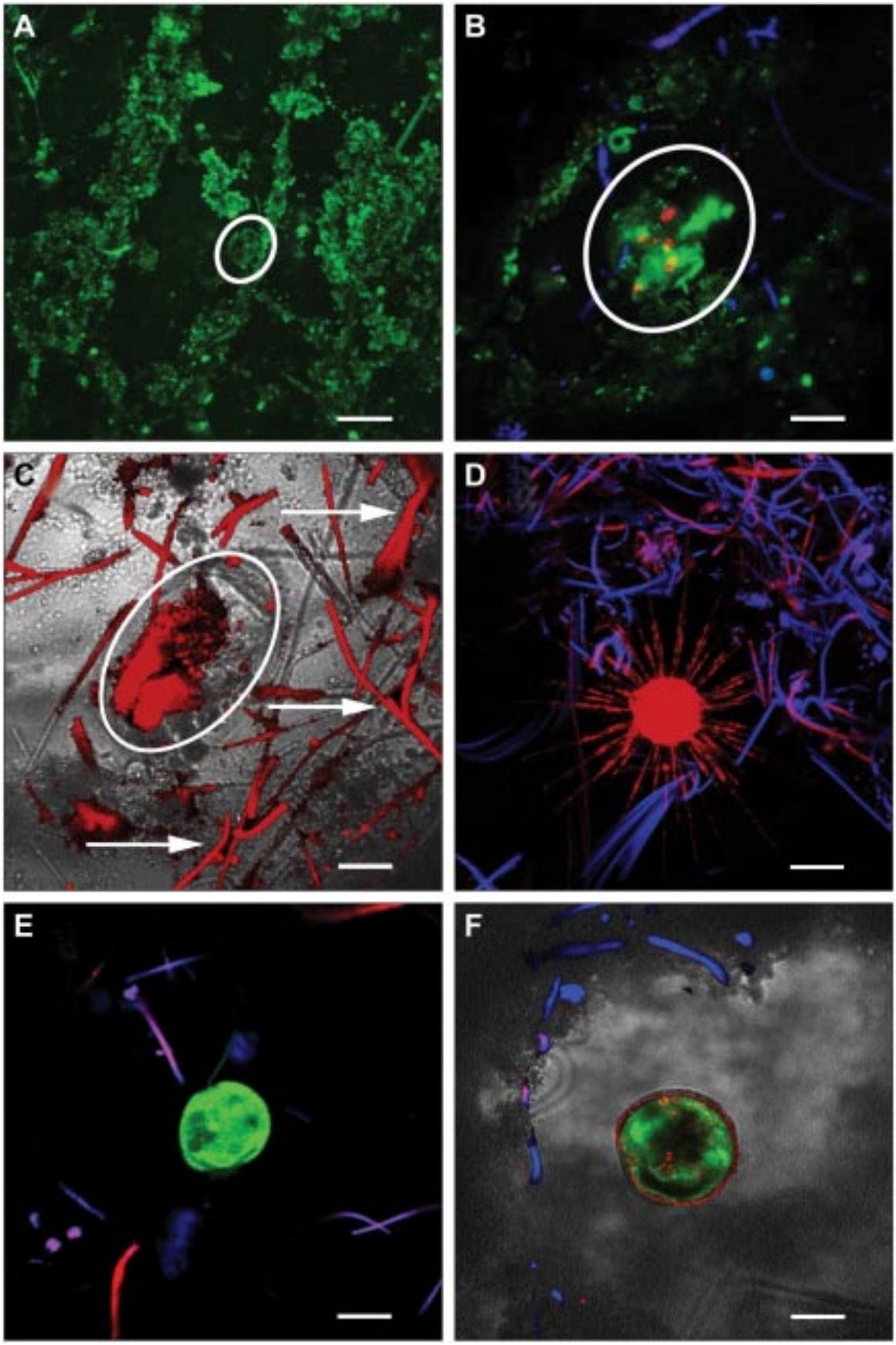
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Table 1. List of stains used with indication of proposed target within the cells, wavelength of absorption and emission maximum, concentration of stock solution and dilution

Fluorochrome	Target (proposed)	Absorption [nm]	Emission [nm]	Stock-solution/ Dissolving reagent	Working solution	Amount of working solution	
						Cultures [µl/ml medium]	Biofilm [µl/sample]
Paclitaxel-BodipyFL	Tubulin	504	511	10 µM Ethanol	-	40	10-50
Rhodamine 123	Cytoplasm, Mitochondria	507	529	2,6 mM Water	1:100	100	100
Rhodamine 6G chloride	Mitochondria, Endoplasmatic Reticulum	528	551	1,7 mM Water	1:10	100	10
$DiOC_6(3)$	Intracellular Membranes, Endoplasmatic Reticulum, Mitochondria	484	501	2,6 mM Ethanol	1:100	100	10
LysoTracker Red	Acid Organells and Vesicels, Lysosoms	577	590	1 M DMSO/ Water	1:10, 1:20[a]	10	5 (stock) 100 (work.)
MitoFluor Green	Mitochondria	489	517	1,65 mM DMSO	1:200	40	100-400
CellTracker, CMFDA	Proteins and Peptides, (Thiols, e.g. Glutathione)	490	520	2,15 mM DMSO	1:10[a] to 1:40	10	100-200

[a]Dilutions used for biofilm samples only

on the physiological condition of the cells. In some instances cells reacted with lysis after a rather short incubation time, whereas others were not affected over a longer period of time. For example, with some cell types such as heliozoans, lysis occurred within a short interval (< 1 min) after the beginning of scanning indicating the limitation of this imaging technique with this specific group (Fig. 1 F). Nevertheless, by stepwise lowering the concentration of nickel sulfate it was possible to immobilise the protozoa without lysis. In most cases staining and immobilisation alone had no negative impact on the protozoan cells and if observed by epi-

Fig. 2 A-F. Staining of protozoa in complex lotic biofilms grown with river water. Differentiation of biofilm and protozoa was done by concurrent imaging in the fluorescence and transmission mode. **A** - Single channel image after staining with $DiOC_6(3)$ showing equal staining of biofilm material and protozoa (circled). **B** - Triple channel image after staining with Paclitaxel-Bodipy demonstrating equal staining of biofilm bacteria and protozoa (circled), autofluorescence signals (red and blue) can be separated. **C** - Transmission image and single channel fluorescent image after staining with LysoTracker Red. The autofluorescence of algae and cyanobacteria (arrows) and the protozoan signal (circled) appear in the same fluorescence channel. **D** - Dual channel image after staining with LysoTracker Red, the 2-channel mode separated the autofluorescence of algae (blue) and cyanobacteria (pink) from the signal of the protozoan cell (red). **E** - Triple channel image after staining with CellTracker. The 3-channel mode clearly separated the autofluorescence of phototrophic biofilm cells from the signal of the protozoan cell (green). **F** - Transmission image and triple channel fluorescent image after staining with LysoTracker (red) and CellTracker (green). In the 3-channel mode the autofluorescence of phototrophic biofilm cells can be clearly separated from the signal of the double stained protozoan cell. Scale bars - 20 µm

fluorescence, phase contrast, or bright field microscopy no lysis was observed. Furthermore, it was usually possible to perform CLSM on most samples.

CLSM allowed detailed observation of protozoa in pure culture and in most cases in complex biofilm communities. Not all of the stains selectively stained protists in complex microbial communities. Although stains such as Paclitaxel-BodipyFL, $DiOC_6(3)$, Lysotracker, MitoFluor Green, and Rhodamine 6G demonstrated some specificity for protists they lacked the broad range necessary for general staining of all protozoa. In particular staining of small ciliates remained difficult. The most suitable fluorochrome for generally staining protozoa in complex environmental biofilms was CellTracker. This stain allowed specific staining of eukaryotic cells without non-specific binding to other biofilm constituents. The conditions for staining listed in Table 1 may be used as guidelines and require assessment for each specific application. For example, the various protozoa react differently to the stains, most ciliates and heliozoans are well stained, whereas some of the flagellates and amoebae are often only weakly or not stained at all. When staining protozoa with stains specific for mitochondria it becomes difficult to distinguish between ingested bacteria and mitochondria. In this case the bacteria fed to the protozoa may be pre-stained with nucleic acid specific fluorochromes having an emission signal in a different channel. Finally, the autofluorescence signal of chlorophyll allowed observation of grazed algae, cyanobacteria or endosymbionts within the protozoans.

In conclusion, this study reports the application of an in situ approach using CLSM for imaging protists in complex interfacial communities. The staining procedure offers the possibility of imaging protozoa alive without fixation and/or embedding. In some cases the fluorochromes also stained bacterial or polymeric biofilm constituents. Despite this fact, the usually transparent protozoan cells became visible within the complex architecture of environmental biofilms. By using this approach, protozoa can be identified and distinguished within the complex interfacial habitat by specific stains targeted for eucaryotic cell features (CellTracker) and by staining in combination with the information on protozoan size and morphology. The approach as suggested may have to be adjusted according to the fluorescence characteristics of the sample and the growth status of the protozoa. Finally, there is the potential to stain bacterial and polymeric biofilm constituents with other fluorochromes and fluor conjugated probes (Lawrence *et al*. 1998a, Neu *et al*. 2001) which then may be clearly separated from the protozoan signal by co-localisation.

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