Evidence for Ciliary Pigment Localization in Colored Ciliates and Implications for Their Photosensory Transduction Chain: A Confocal Microscopy Study

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ABSTRACT In this study we report for the first time the localization of a photoreceptor pigment in the cilia of the colored heterotrich ciliates *Blepharisma japonicum* red and blue form, *Fabrea salina*, and *Stentor coeruleus*, as result of a confocal microscopy investigation. Optical sectioning confocal microscopy has been used for studying the spatial distribution of the pigment in the cell body, surprisingly showing that, besides its expected presence in the cortical region immediately below the cell membrane, it is located in the cilia too. In order to ascertain possible differences in the pigment fluorescence properties along the cell body, we have measured emission spectra from different parts of it (anterior, posterior, and cilia). Our results clearly indicate that in all cases the spectra are the same, within experimental errors. Finally, we have evaluated the pigment relative fluorescence efficiency of these ciliates. In an ordered scale from lower to greater efficiency, we have *S. coeruleus*, *B. japonicum* blue, *B. japonicum* red, and *F. salina*. The possible implications of our findings for the process of photosensory transduction are discussed. *Microsc. Res. Tech. 70:1028–1033, 2007.*

INTRODUCTION

Many unicellular organisms are able to detect spatial and temporal variations in external illumination and to react to the light stimuli by modifying their movement in a typical photosensory response. Besides bacteria and algae (Häder and Lebert, 2001), some colored ciliates belonging to *Heterotrichida* family also show the capability of reacting to light stimulation. Both dark adapted (red) and dimly irradiated (blue) forms of *Blepharisma japonicum* show a clear step-up photophobic response and positive photokinesis; *Stentor coeruleus* can orient itself with respect to light direction, directly by means of negative phototaxis and indirectly by means of step-up photophobic responses; the marine ciliate *Fabrea salina* moves by means of positive phototaxis toward lighted areas, where it more easily feed on green algae (Lenci et al., 2001). All these ciliates are deeply colored, and the pigments responsible have been termed according to the name of the species; so we refer to fabrein in *F. salina*, stentorin in *S. coeruleus*, red blepharismin in dark adapted cells of *B. japonicum* and blue blepharismin (also called oxyblepharismin) in light adapted *B. japonicum* (Lenci et al., 2001). The general molecular structure of these pigments is similar to that of hypericin, while the lateral substituents change in the different cases. The complete molecular structure has been determined in the case of blepharismin (for both red and blue form) (Checcucci et al., 1997; Maeda et al., 1997) and stentorin (Spitzner et al., 1998), while it is still unknown in the case of fabrein (Marangoni et al., 1996). In all cases the photoreceptor pigment was described as confined in pigment granules located below the plasma membrane (Marangoni et al., 1996; Matsuoka et al., 1994; Song, 1981) and it was thought that light stimulation caused a proton release in the cytoplasm, which, in an unknown way, could finally alter the membrane potential and cause modifications of the ciliary beating. Moreover, it was suggested that at least in *B. japonicum* there could have been a different distribution of the pigments along the cell body (Matsuoka et al., 1997). The rationale behind the present work was to investigate the pigment distribution and its fluorescent properties along the three-dimensional cell body and to make a comparative study of the fluorescence efficiency in these ciliates. This was done optically sectioning the cells by means of confocal laser scanning microscopy (Brakenhoff et al., 1985), which allowed us to measure the cell fluorescence in the different regions of the cell body and to give an estimate of the pigment fluorescence efficiency. We also performed some preliminary

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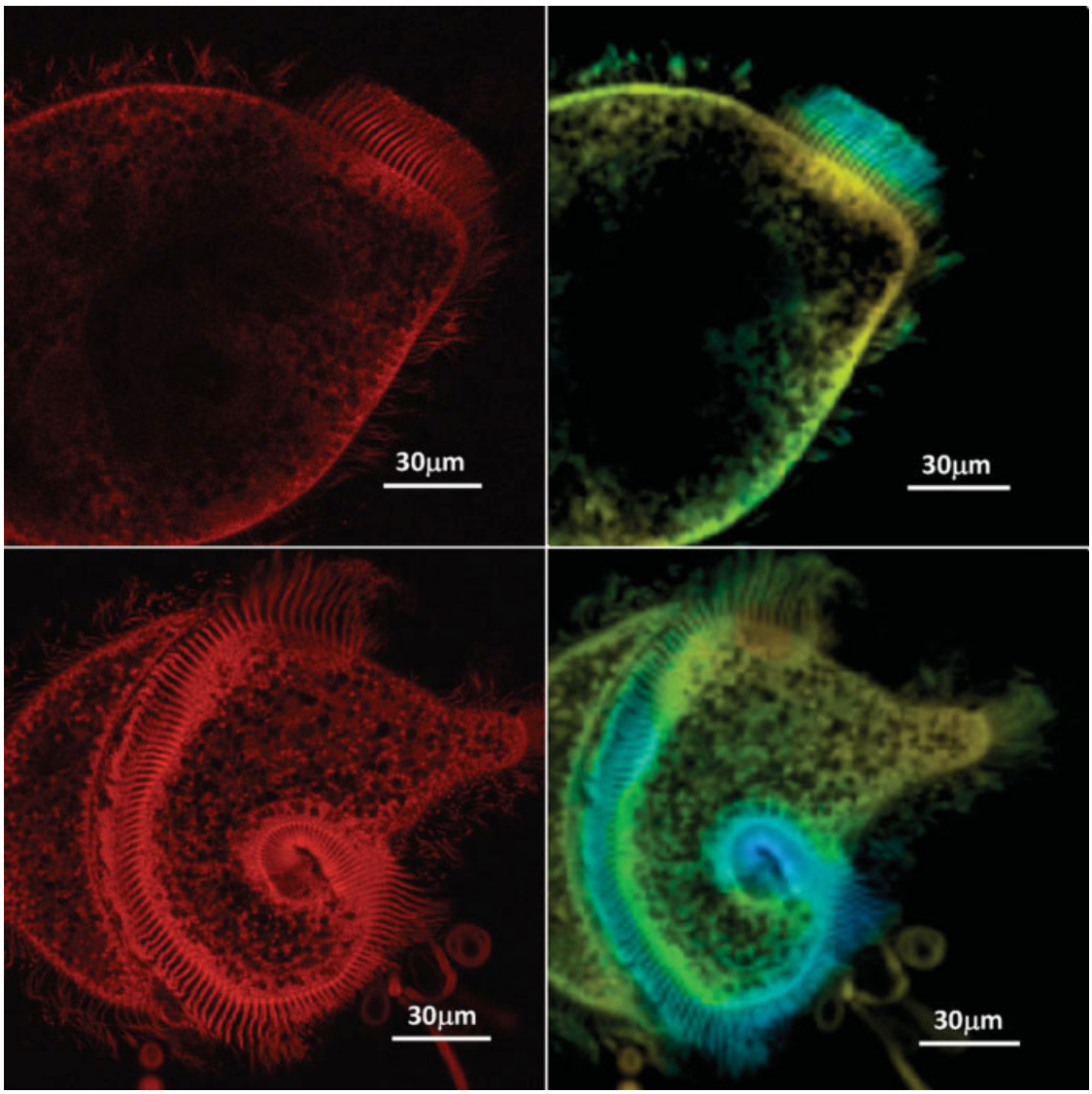

Fig. 1. Fluorescence confocal images of *F. salina* (left column) and preliminary false color lifetime map distributions (right column).

fluorescence lifetime measurements that are still in progress.

MATERIALS AND METHODS

Confocal measurements were performed by means of a Leica TCS SP2/AOBS confocal laser scanning microscope equipped with 457–476–488–514–543 nm laser lines and an HeNe (543 nm). Laser scanning transmission images were obtained using the 488 nm laser line at moderate power. Cells were fixed with 4% paraformaldehyde in saline phosphate buffer and normally examined with an HCX PL APO 100×/1.4 oil immersion objective (Leica Microsystems, Mannheim, Germany). Photobleaching experiments were carried out by illumination with high intensity laser light for 15 min, i.e., about 1.5 mW average power at the focal plane, as compared with 0.4 mW used in imaging mode. The excitation light was set at 488 nm. Emission spectra of fixed cells were determined with a spectral resolution of 10 nm, analyzing the data by means of the LCS software (Leica Microsystems, Mannheim, Germany). In imaging experiments, we collected all cell fluorescence in the spectral range 560–680 nm. Occasionally, lifetime maps were collected under two-photon excitation (2PE) by means of a time-correlated single-photon counting (TCSPC) approach using a SPC-730 module (Becker & Hickl, Berlin, Germany). 2PE was performed using the infrared port of the Leica SP2 AOBS confocal microscope directly coupled, by means of a home-made optical pathway, to a fs-pulsed (140 fs) Ti:sapphire laser Chameleon-XR (Coherent, Santa Clara, CA, USA) operating at 820 nm and at 90 MHz of repetition rate. For each cell type, we have repeated the measurement at least 5–8 times. The data

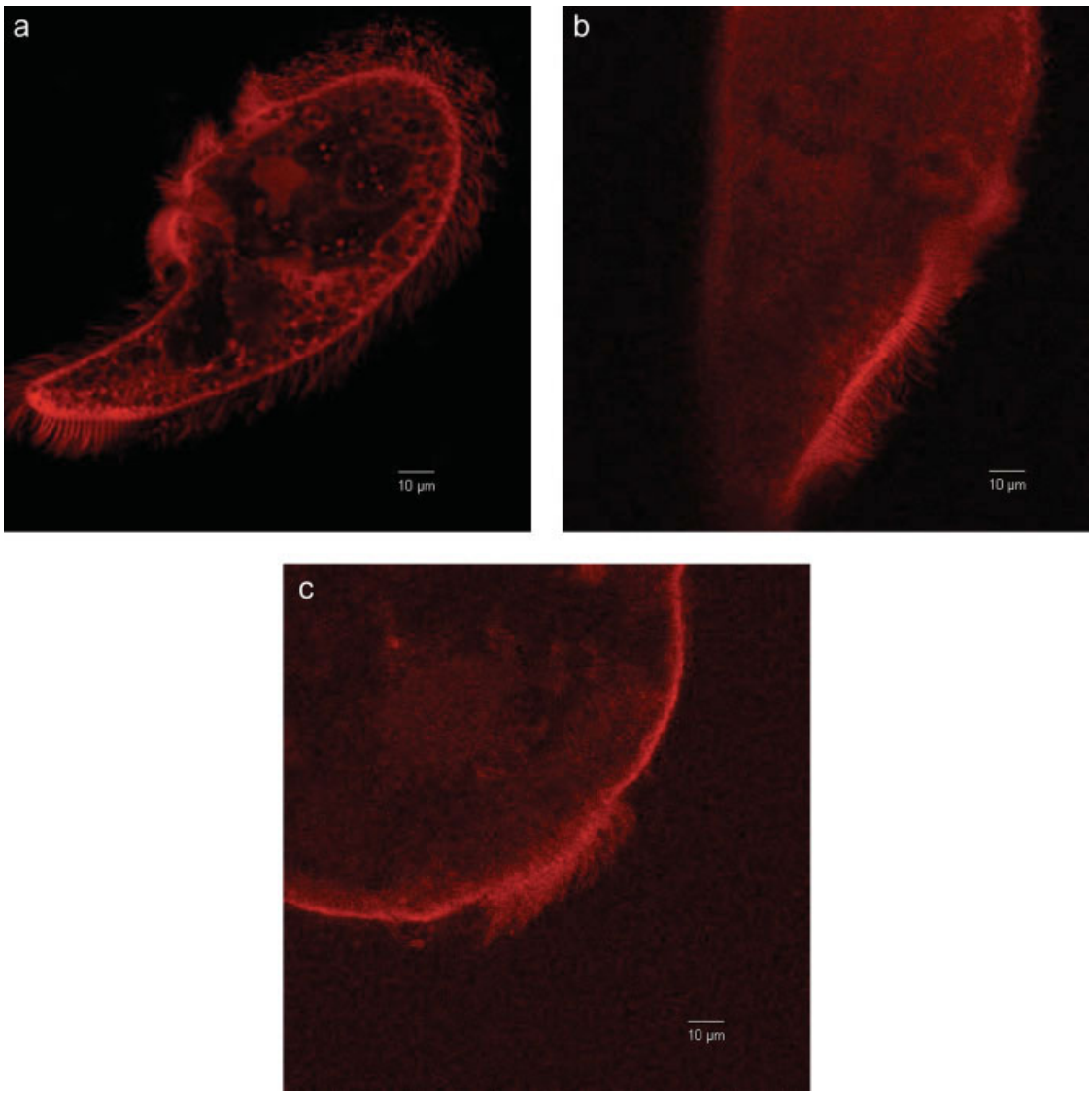

Fig. 2. The fluorescence of the cilia: (**a**) Fs – *F. salina*; (**b**) Bj – *B. japonicum*; (**c**) Sc – *S. coeruleus*.

were analyzed by means of the freeware software Imagej (rsb.info.nih.gov/ij), originally developed by NIH (Bethesda, MD, USA). *F. salina*, *B. japonicum*, and *S. coeruleus* were grown as described elsewhere (Marangoni et al., 1996; Gioffré et al., 1993; Walker et al., 1979). Blue *B. japonicum* was obtained by exposing cells to white light of about 10 W/m^2 for about 24 h.

RESULTS AND DISCUSSION

The very preliminary fluorescence lifetime images simply record different lifetimes depending on the different environmental conditions of the pigments. Ciliary fluorescence could, in principle, be ascribed to an artifact due to cell preparation. However, a possible effect of cell fixation can be excluded since also in cells immobilized by increasing medium viscosity with hyaluronic acid or by water evaporation the cilia are fluorescent (data not shown). Moreover, some related experiments show that cilia do fluoresce also in freely swimming cells (data not shown). Figure 1 shows confocal microscope images and false colored lifetime maps of different *F. salina*. It can be observed that besides the cell body also cilia do fluoresce, clearly indicating the presence of the photoreceptor pigment in the motor organelles. As well, it is apparent that the pigment contained in the cilia has different lifetimes (corresponding right column in Fig. 1) from the rest of the cell body, in particular a longer fluorescence (from red to blue, lifetime ranges from 200 to 3000 ps) is characteristic of the adoral membranes. In order to get more information on the pigments present in these ciliates, we have determined their relative fluorescence emission, since the kind of measurements performed does

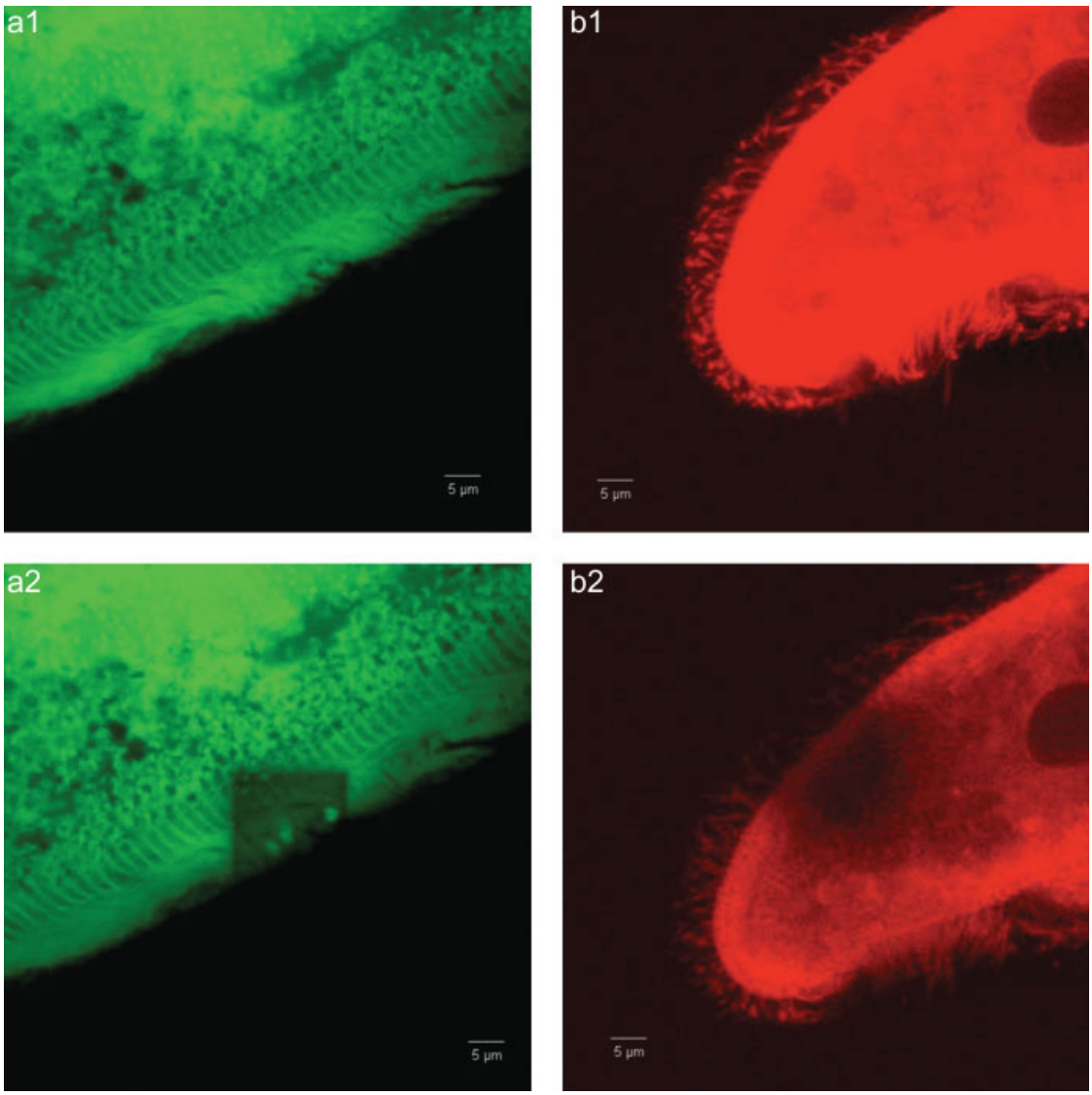

Fig. 3. Photobleaching induced in the ciliar regions: (**a1**, **b1**) before photobleaching; (**a2**, **b2**) after photobleaching.

not allow us to determine real fluorescence quantum yields. Shortly, we have used the confocal microscope in two different configurations: in scanning transmission mode to estimate the cell transmittance, and in confocal scanning fluorescence mode to get the fluorescence emission within the three-dimensional sample. In order to be able to compare the different measures, we have always kept constant the experimental settings of the microscope (such as pinhole, laser power, objective a.s.o.). We have then calculated the ratios of the absorbed to the emitted light. Imaging of cells of *F. salina*, red and blue *B. japonicum*, and *S. coeruleus* was obtained at several different focal planes clearly showing that in all cases the pigment is present below the cell membrane, embedded in granules with an apparent average radius of about 1 µm. To our great surprise, we found that the cilia were fluorescent too (see Fig. 2). To the best of our knowledge, this has never been described before in the literature, though, reexamining the images taken in a previous work of ours (Marangoni et al., 1996) at the light of the present evidence, we can find a very faint evidence of this. In order to completely exclude possible artifacts when considering the fluorescence signal, we have performed photobleaching experiments within the ciliary region. In all cases our results show that photobleaching takes place and is, in fact, limited to the region of irradiation (see Fig. 3). As further confirming detail, there was no fluorescence recovery after bleaching. This was not surprising at all, since the cells were fixed with paraformaldehyde; in these conditions pigments cannot freely diffuse, probably because they are tightly bound to subcellular structures immobilized by the fixation procedure. Optical sectioning of the ciliates showed

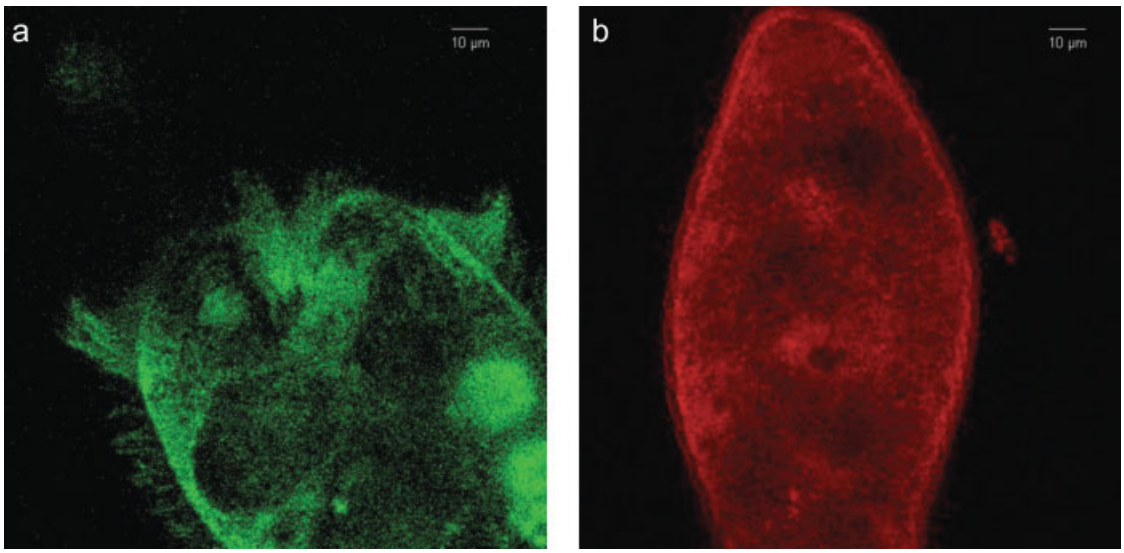

Fig. 4. Optical sectioning of the ciliates: (**a**) Fs and (**b**) Bj. The figure shows that the pigment is localized, besides the cilia, in the cortical region, that is the cell region immediately below the cell membrane.

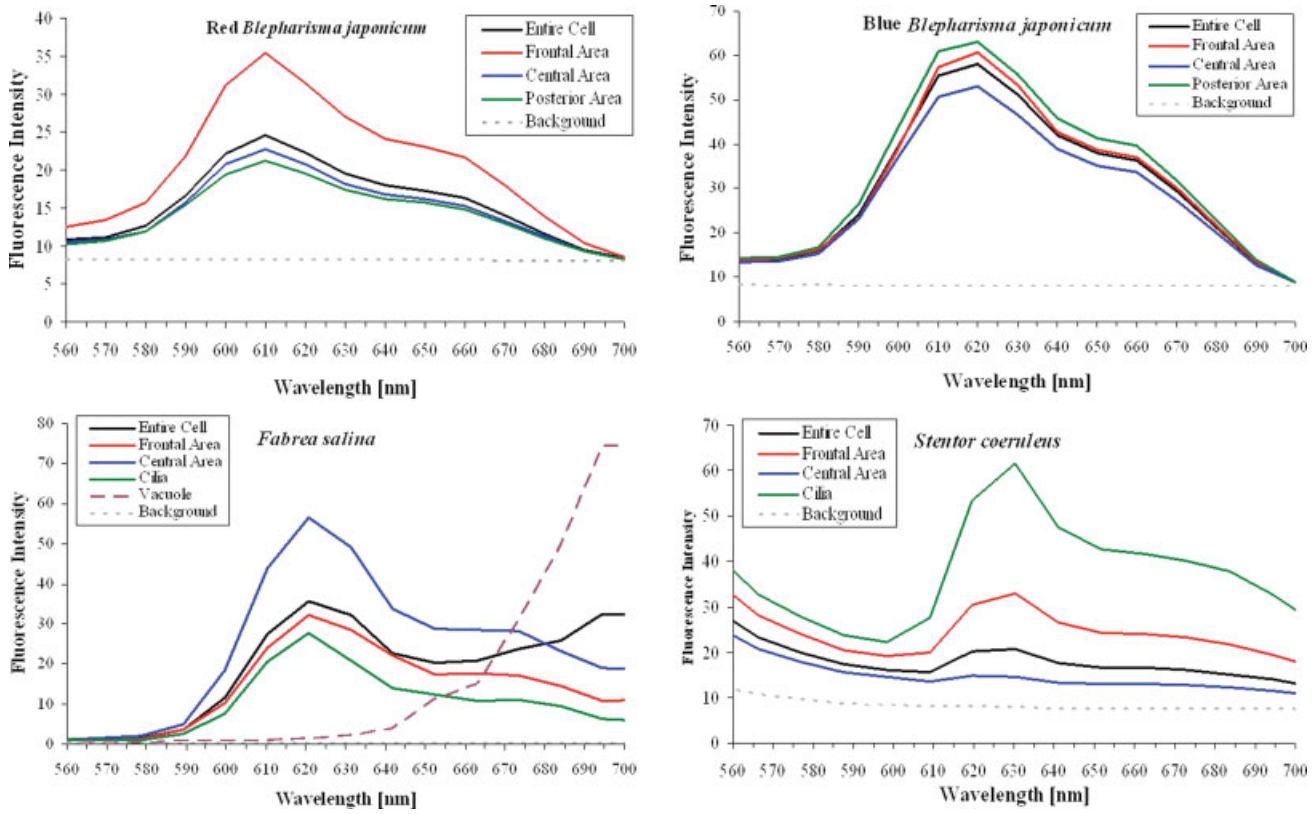

Fig. 5. Fluorescence emission spectra from different cellular compartments.

that the pigment is localized, besides the cilia, in the cortical region, that is the cell region immediately below the cell membrane (see Fig. 4). This is in good agreement with the evidence given by electron microscopy studies, which, on the other side, did not give any indication of the presence of pigments in the cilia. Fluorescence emission spectra were determined for all ciliates. The results are shown in Figure 5. All the spectra

TABLE 1. Relative fluorescence emission from the entire body of the ciliates

	Relative emission	SD
S. cereuleus	1.0	0.4
Blue *B. japonicum*	2.1	0.1
Red *B. japonicum*	3.3	0.9
F. salina	3.4	0.3

have common features, such as a major peak at about 615–620 nm and a shoulder around 660 nm. This is in good agreement with the hypericin-like nature of the fluorophores and with the results obtained by Cubeddu et al. (1993) and Marangoni et al. (1996). The emission spectra of different portions of the cell body (anterior, posterior, and cilia, see Fig. 5) of all the ciliates are substantially identical within experimental errors.

CONCLUSIONS

To sum up, as far as the fluorescence spectral emission characteristics are concerned, there is no significant difference in the pigment distribution along the cell body. Fluorescence emission efficiencies in a relative scale range from 1.0 for *S. coeruleus* to 3.5 for *B. japonicum* and *F. salina* (see Table 1). There are no data in the literature to compare these results to. It looks as though *F. salina* and red *B. japonicum* have similar fluorescence emission efficiency. Taken together with fluorescence emission spectra, this could indicate a substantial similarity between the pigment of these two ciliates. The significantly lower emission of the pigments of blue *B. japonicum* and of *S. coeruleus* could indicate a substantial diversity of these forms of hypericins. It has been suggested (Sgarbossa et al., 2002, and references therein) that the mechanism of photosensory transduction at least in *B. japonicum* and *S. coeruleus* involves the activation of a complex chain of events in the cell cytoplasm, starting from pigment excitation by light. It has been hypothesized that an electron release from the excited hypericin-like pigment might cause a pH alteration in the cytoplasm and the subsequent activation of a series of molecular events leading to the final signal, which somehow should migrate to the motor organelles, the cilia, altering their beating pattern. In fact, no direct proof has ever been given of how this might happen and it is also difficult to imagine how a light-induced alteration of cytoplasmic pH should not be immediately buffered. Our findings can be used for a better understanding of the photomotile reaction. When the photoreceptor pigment is the hypericin-like molecule, and since it has been shown to be located in the motor organelle, all the processes can take place there, without the need to invoke unnecessary complications. It could also be thought that the pigment is capable of a direct interaction with the calcium channel of the cilia and that its activation by light could trigger a modulation of the activity of these channels.

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