Confocal microscopy of FM 4-64 tagged membranes in the living fungus *Trichoderma reesei*

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Visualization of the protein secretory pathway in the high secreting *trichoderma reesei* (*T. reesei*) hyphae using confocal microscopy will lead to better understanding of the cellular mechanisms involved in protein secretion and contribute to the identification of bottlenecks in the secretion of foreign proteins from fungi. An introduction into visualization approaches involved the application of the fluorescent dye FM 4-64 for staining of membrane-based structures in *T. reesei* hyphae. Confocal microscopy studies were carried out with 24-hour old cultures of the *T. reesei* strain Rut-C30. Staining of the fungal hyphae and characteristics of FM 4-64 including spectral properties, time course of the labeling and double labeling with other specific organelle dyes will be discussed.

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Trichoderma reesei (*T. reesei*) is a biotechnologically important organism with an excellent capability of secreting high levels of hydrolytic enzymes, e.g., cellulases into the external medium[1,2], therefore it is well known as an efficient cell factory for protein production. In spite of gram per litre levels of secreted enzymes, products from heterologously-expressed genes have remained much less abundant[2]. Research so far has shown that these proteins are lost somewhere in the secretory pathway, therefore giving an indication of intracellular secretion bottlenecks[2]. Advanced fluorescence microscopy[3,4] techniques now allow novel approaches to address the secretion bottlenecks by the visualization of the secretory pathway and proteins traveling through it. This requires fluorescent tagging of both the organelles and cell structures in the secretory pathway as well as the secreted protein(s) of interest so they can be followed along the secretory pathway using fluorescent microscopy. Since the cell structures and organelles in the secretory pathway (e.g., endoplasmic reticulum (ER), Golgi, and secretory vesicles) are membrane-based, a fluorescent probe is required to visualize the relevant components. After this initial mapping, some of the cell structures such as ER and Golgi can be studied in more detail using organelle-specific fluorophores such as an ER stain $DiOC_6(3)$ and a Golgi stain BODIPY FL C5-Ceramide.

FM 4-64, one of amphiphilic styryl dyes (see http://www.probes.com), is being increasingly used for the identification of firing neurons[5], studying vesicle trafficking and organelle organization, investigating endocytosis and exocytosis, and as fluorescent tracers of cell morphology and fluid flow. It is commonly believed that FM 4-64 enters the cell primarily by endocytosis[4,6]. After internalization, FM 4-64 is distributed to different organelle membranes, probably primarily via the vesicle trafficking network whereby components of the secretory pathway can become labeled[4,7−9]. Importantly, FM 4-64 has been successfully applied in combination, for example, with the molecular fluorophore green fluorescent protein (GFP)[10]. FM 4-64 is membrane-selective and stains many organelle membranes, therefore, it can be used as a vital marker to monitor organelle organization and dynamics in general[11,12]. FM 4-64 has been used extensively in the studies of mammalian[13] and plant cells[10,14−19], and there is a reasonable amount of data on its staining characteristics in a range of fungal species including *Aspergillus nidulans*, *Bortrytis cinerea*, *Magnaporthe grisea*, *Neurospora crassa*, *Phycomyces blakesleeanus*, *Puccinia graminis*, *Rhizoctonia solani*, *Sclerotinia sclerotiorum* and *Trichoderma viride*[10], none of which is an industrially-exploited cell factory. To date, there are no reports on the use of FM 4-64 in living *T. reesei*.

In this work, we focus on the staining characteristics of FM 4-64 in the living *T. reesei* hyphae using confocal laser scanning microscopy. Our aim is to obtain an overview of the membrane network involved in protein secretion in *T. reesei* as a prelude to localization of proteins of interest in the secretory pathway and identification of secretion blocks for heterologous gene products. We will also report on double-labeling using organelle-specific dyes in combination with FM 4-64.

Table 1. Confocal Microscopy Conditions

	Laser Power/Full Intensity (%)	Excitation (nm)	Emission (nm)
FM 4-64			
$xy\lambda$	20	488	510–800
xyt	10	488	560–720
Carbocyanine $DiOC_6(3)$	10	488	500–540
BODIPY FL C5-Ceramide	10	488	500–560
SYTO 40	10	405	410–480

The dyes FM 4-64 (for molecular structure, see Fig. 1), carbocyanine $DiOC_6(3)$, BODIPY FL C5 Ceramide, and SYTO 40 were obtained from Molecular Probes Inc. (U.S.A.). All other chemicals were supplied by Sigma (U.S.A.).

T. reesei Rut-C30 strain[20] was grown in a medium which contained (w/v) 1.5% KH_2PO_4, 0.5% $(NH_4)_2SO_4$, and 1% lactose on an orbital shaker at 250 rpm at 28 °C. The inoculum used was 10^8 conidia/50 ml of growth medium in a 250-ml conical flask. Cultures were harvested by centrifugation after 24 h and the pellet was collected.

The above 24-h old cultures were directly stained with 33 μM FM 4-64 dissolved in 0.9 % NaCl in an incubator at 28 °C for 60 min for the emission spectra scans ($xy\lambda$ experiment). For double-staining, FM 4-64 was combined with $DiOC_6(3)$ or BODIPY FL C5 Ceramide, or SYTO 40. For staining, we used 33 μM FM 4-64 together with 5 μg/ml carbocyanine $DiOC_6(3)$ or 4.2 μM BODIPY FL C5 Ceramide, or 10 μM SYTO 40, incubated for 1 h at 28 °C. All dye solutions were prepared in 0.9% NaCl.

For imaging, the above stained cells were transferred into wells of a 8-well chambered coverslip (Nalge Nunc International Corp., Naperville, IL) and resuspended in a dye-free culture medium containing 1.5% KH_2PO_4, 0.5% $(NH_4)_2SO_4$, 1% lactose, and 0.5% methyl cellulose (MC) which was applied to semi-solidify the medium to stop the cells from moving around during imaging. For the time course scans (xyt experiment), 50 μl of 24-h old cultures in the above semi-solid medium with 33 μM FM 4-64 were transferred into an 8-well chambered coverslip and incubated at 28 °C using a stage with temperature control.

A SPM2 confocal laser scanning microscope from Leica microsystems with full spectral capabilities was employed to study the emission spectra ($xy\lambda$), time course (xyt) of FM 4-64 in living *T. reesei*. To study co-staining of the hyphae with FM 4-64 in combination with other organelle stains, an HCX PL APO CS 100 × oil immersion objective was used. Confocal microscopy conditions for

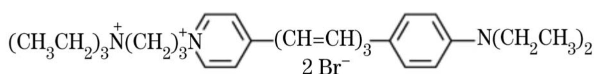

Fig. 1. Molecular structure of FM 4-64.

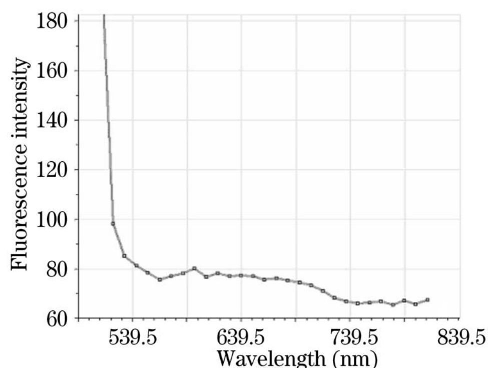

Fig. 2. Emission spectrum of FM 4-64 (330 μM) in 0.9% NaCl without *T. reesei* sample for excitation at 488 nm.

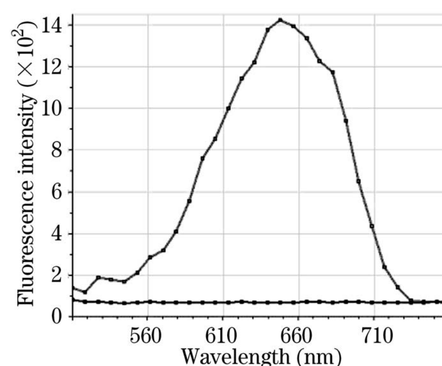

Fig. 3. Emission spectrum of FM 4-64 at 33 μM in living 24-h old culture of *T. reesei* for excitation at 488 nm (green curve represents fluorescent region of interest, purple represents background).

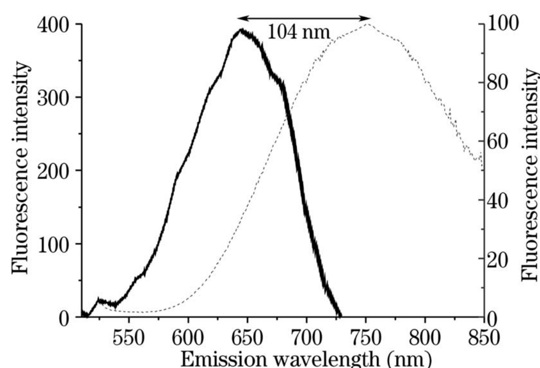

Fig. 4. Comparison of emission spectra of FM 4-64 in methanol (right curve) and *T. reesei* sample (left curve) for excitation at 488 nm. Δ shift = 104 nm.

different dyes and experimental set ups are as shown in Table 1.

The spectral properties of FM 4-64 were investigated by testing the emission spectra for both pure dye solution (330 μM) diluted in 0.9% NaCl and 24-h old *T. reesei* cells stained with 33-μM FM 4-64. No dye emission was obtained at excitation of 488 nm in the FM 4-64 solution without the sample, and the signal intensity was very low (Fig. 2). FM 4-64 in *T. reesei* gave a specific emission curve (Fig. 3) with a similar shape as the pure dye in methanol (see http://www.probes.com). The fluorescence of FM 4-64 in *T. reesei* appeared to have blue-shifted up to 104 nm, with maximum emission at 646 nm (Figs. 3 and 4). The most distinctive emission range was at 560–720 nm for 488-nm excitation (Fig. 4).

Examination of the early dye uptake revealed that plasma membrane staining was immediate (Fig. 5(a)) and staining became more pronounced after 20 min in the dye-containing medium (Fig. 5(b)). Subsequently, light staining of the hyphal cytoplasm could be seen, which increased with time (Figs. 5(c)–(e)). Between 105 and 120 minutes after adding the stain, more fluorescent structures were observed throughout the whole cytoplasm (Figs. 5(f) and (g)). After 135 min, numerous organelle membranes had been stained, and vacuolar membranes were visible as well (Fig. 5(h)). Fluorescence intensity after dye application increased over time

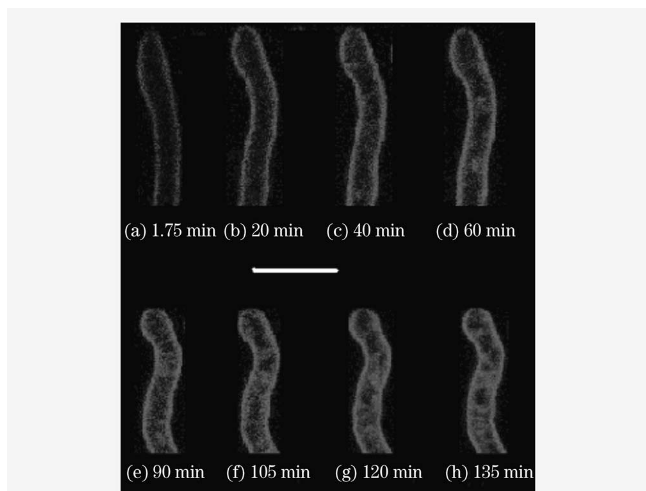

Fig. 5. Time course of FM 4-64 staining in *T. reesei*(continuous loading). Bar = 8.00 μm.

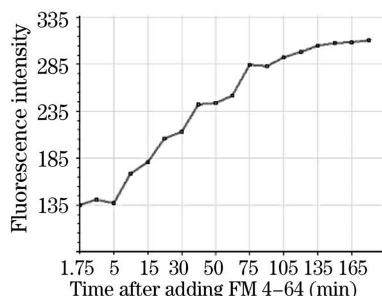

Fig. 6. Fluorescence intensity of FM 4-64 in *T. reesei* over time.

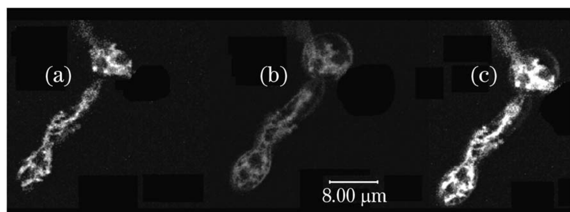

Fig. 7. Double staining with FM 4-64 and DIOC$_6$(3). (a) DiOC$_6$(3); (b) FM 4-64; (c) Superimposed image of (a) and (b).

(Fig. 6). The signal was discernible immediately after 1.75 min and remained constant during the early staining stage. After 5 min, the fluorescence intensity increased dramatically. From 75 min onwards, the intensity increased relatively slowly until a plateau was reached.

Double-labeling experiments with FM 4-64 and some other organelle-specific chemical dyes were applied to identify co-localization of the fluorescent stains. This work was especially directed towards further visualizing individual components, such as ER and Golgi, which are vital organelles involved in secretion. Therefore, the ER-dye DiOC$_6$(3) and Golgi-dye BODIPY FL C5 Ceramide were loaded individually to FM 4-64. It should be noted that discrimination between ER and Golgi through their different morphology using confocal microscopy is beyond the limits of the imaging resolution (maximum: 200 nm)

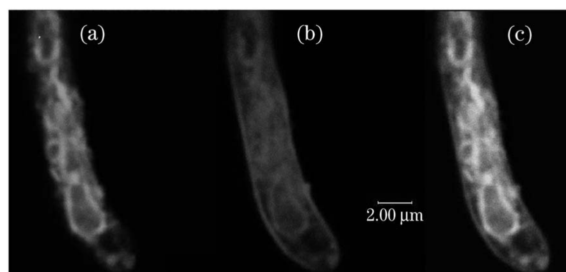

Fig. 8. Double staining with FM 4-64 and BODIPY FL C5. (a) BODIPY FL C5; (b) FM 4-64; (c) superimposed image of (a) and (b).

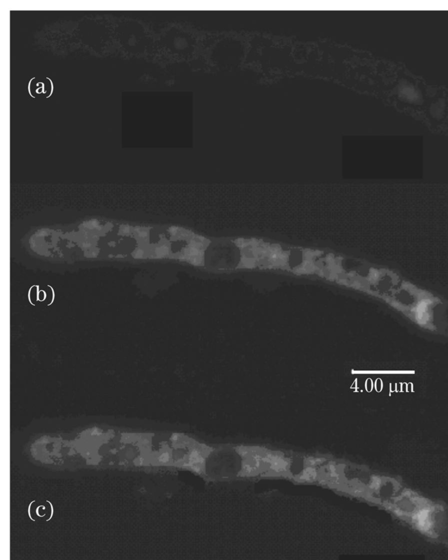

Fig. 9. Double staining with FM 4-64 and SYTO 40. (a) SYTO 40; (b) FM 4-64; (c) superimposed image of (a) and (b).

of the system employed in this study. A DNA-stain SYTO 40 was applied together with FM 4-64.

When stained with DiOC$_6$(3), the *T. reesei* ER appeared as a fluorescent strand-like network within the cytoplasm (Fig. 7(a)). A superimposed image of the dual-labeled hypha (Fig. 7(c)) showed apparent co-localization of the FM 4-64-stained membranes with ER. Plasma membrane was stained only with FM 4-64 (Figs. 7(a)–(c)). The association between Golgi and FM 4-64-labeled membranes gave similar results, indicating co-localization of Golgi and FM 4-64-labled membranes in the cytoplasm but not in plasma membrane (Figs. 8(a)–(c)). There was no overlapping staining using a DNA-specific SYTO 40 and FM 4-64 (Figs. 9(a)–(c)).

The spectral properties of FM 4-64 appeared to change depending on the microenvironment provided by different cell types and growth media[6] because of its aromatic nuclei (Fig. 1). Thus, emission spectra in pure solutions can sometimes be misleading when working with living cells[4,10,18]. FM 4-64 has an excitation peak at 505 nm with an emission peak at 750 nm in methanol. No significant emission curve was observed for FM 4-64 in 0.9% NaCl solution, hence confirming that it emits significant fluorescence only when on a lipid-rich membrane[10].

Therefore, there was no need to wash the dye out of the medium in order to study hyphal staining. This will provide an excellent platform for the next stage of work where a target protein will be traced through the secretory pathway. The emission spectrum of FM 4-64 varies between different cell types. For instance, emission peaks of FM 4-64 at 640 nm and 670 nm have been reported in yeast and plant cells respectively. We found the maximum emission of FM 4-64 in T. reesei to occur at 646 nm indicating its potential to be combined with GFP which is normally fluorescent between 470–560 nm with emission peak at 509 nm[21]. We routinely excite FM 4-64 using 488-nm laser line and image its fluorescence between 560 and 720 nm. GFP provides an excellent candidate for genetic tagging of a secreted protein.

In fungal or other eukaryotic cells, the successive staining of the cell plasma membrane followed by the membranes of different intracellular structures is time-dependent[10] and it is believed that the intake of the dye happens by an active endocytic internalization process rather than unfacilitated diffusion[7,9,10,13,22,23]. A membrane-crossing activity for FM 4-64 is suggested because of the action of a flippase enzyme. Once the dye has been translocated to the inner leaflet of the plasma membrane by flippase activity, lipid transfer proteins may then transport dye molecules to the cytosolic face of the membranes of other organelles[9]. Owing to the water solubility of these dyes and reversible incorporation into many membranes, they could enter the cytosol from the cytoplasmic face of the plasma membrane and then label the external leaflet of the organelle membrane[7,22]. FM 4-64 is assumed to follow this pathway from plasma membrane to targeted organelles such as Golgi and finally to vacuolar membranes[9,10]. Evidence also supports that it recycles back to the plasma membrane[8,16,24]. Our observations of the time-dependent sequence of FM 4-64 staining indicate that the pathway of dye distribution in living *T. reesei* closely resembles the pathway discussed above. This also confirms that FM 4-64 is a powerful experimental tool to label components involved in the secretory pathway.

One of the current debates regarding FM 4-64 staining in live cells is organelle specificity[10]. No staining of nuclear membranes by FM 4-64 has been reported[10]. Our findings with living *T. reesei* support this claim. ER or Golgi membranes have not been previously shown to be stained with FM 4-64[9,10] with two exceptions, a study on *Fucus* cells[16] and on a BY-2 cell line[10]. Our results indicate co-localization of FM 4-64 with ER and Golgi as well, therefore providing another positive example to add to the two cases mentioned above. Further evidence may be obtained by studies involving genetic tagging of ER and Golgi with a fluorescent marker and FM 4-64 staining of the cells.

In conclusion, FM 4-64 can be used as a powerful experimental tool for visualizing membrane-based structures in living *T. reesei*, hence providing a basic organelle map to trace genetically tagged proteins traveling along the secretory pathway. However, the complexity of the staining pattern of intracellular membrane structures has to be taken into account when identifying organelles such as ER and Golgi.

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