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Fluorescent labelling of intracellular bacteria in living host cells

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Abstract

The fluorescent reagent, CellTracker, labels metabolically-active cells and was used here to label *Chlamydia* in vivo during their exponential phase of growth in infected cells. HeLa cells infected with *C. psittaci* were labelled with the CellTracker reagents between 15 and 48 h post-infection. The fluorescent label accumulated in the host-cell membrane compartment (inclusion) within which *Chlamydia* reside and replicate, and was also incorporated by the bacteria. Labelling with the CellTracker affected neither the growth nor the differentiation of the chlamydiae, and labelled chlamydiae isolated from infected cells were infectious. Our results demonstrate that the CellTracker could become a valuable tool for in vivo labelling of obligate intracellular parasites for which no genetic tools exist.

Keywords: In vivo labelling; Intracellular bacteria; *Chlamydia*

1. Introduction

Chlamydia are obligate intracellular eubacteria that are widespread in nature and capable of causing a large diversity of diseases in humans and other animals. In humans, they cause pneumonias (*C. pneumoniae*), trachoma (certain strains of *C. trachomatis*), and sexually transmitted diseases (other strains of *C. trachomatis*) (Schachter and Dawson, 1990; Stephens, 1993; Bavoil et al., 1996).

The pathogens exist in two distinct morphological forms: the replicative, intracellular reticulate bodies (RB, 1 μm in diameter), which are not infectious but have metabolic activity; and the infectious elementary bodies (EB, 0.3 μm in diameter) which are metabolically inert (Moulder, 1991). The interaction of *Chlamydia* with eucaryotic host-cells involves attachment, entry, evasion of lysosomal destruction, intracellular growth and exit.

Following internalization, the chlamydiae remain enclosed within membraneous compartments that are subjected to bacteria-induced modifications both in their luminal environment and in their membrane composition, and transported to a perinuclear location (Hackstadt et al., 1997). These modified *Chlamydia*-containing membrane compartments are termed inclusions. The bacteria appear to survive within the host cells through their ability to inhibit fusion of the inclusions with host-cell lysosomes (Scidmore et al., 1996). Within the inclusions, the EBs differentiate into RBs which proliferate to give rise within about 24 h to large inclusions, often reaching a size larger than that of the host-cell nucleus. Between 24 and 72 h post-infection, the

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RBs redifferentiate into EBs and are released into the extracellular space to start a new round of infection.

Due to the lack of genetic tools for manipulating *Chlamydia*, it is not possible to make GFP-labelled bacteria that would permit in vivo studies of the bacteria life cycle, as has been done thus far for other intracellular microbes (Valdivia et al., 1996; Barker et al., 1998; Jacobi et al., 1998; Bubert et al., 1999). Alternative methods for fluorescent labelling of the *Chlamydia* in vivo therefore need to be developed.

We have addressed the possibility that the intracellular chlamydiae could be labelled with theZ CellTracker reagents (Molecular Probes, 1996), which are fluorescent chloromethyl derivatives that diffuse freely across the membranes of live cells. Once inside the cell, these mildly thiol-reactive probes can react with thiols on proteins or peptides and thus become membrane-impermeant, producing aldehyde-fixable conjugates. This reaction is believed to be mediated by glutatione *S*-transferase or an enzyme with similar metabolic activity (Molecular Probes). Many cell types loaded with the CellTracker are both fluorescent and viable for at least 24 h after loading and often through several cell divisions. Although several reports exist describing the labelling of eukaryotic cells with the CellTracker (Cumberledge and Krasnow, 1993; Shelden and Knecht, 1995; Yoshida et al., 1996), no studies have been performed to determine if intracellular microbes (bacteria or parasites) could be labelled with these reagents.

We demonstrate here that CellTracker labels efficiently chlamydiae during their exponential phase of growth in live infected cells. The fluorescent label accumulated in the *Chlamydia* inclusion and was incorporated by the rapidly-proliferating bacteria. Labelling of the *Chlamydia*-infected cells with CellTracker did not have a measurable effect on the growth or differentiation of the bacteria. Finally, *Chlamydia* EBs labelled in vivo adhered to and invaded epithelial host cells.

This new method for in vivo labelling of *Chlamydia* opens numerous possibilities for studying *Chlamydia* infection, especially for real-time measurements with video fluorescence microscopy. The CellTracker could also become a valuable tool for in vivo labelling of other obligate intracellular parasites for which the genetic tools required for labelling with GFP are not yet available.

2. Materials and methods

2.1. Materials and cells

CellTracker green fluorescent BODIPY (8-chloromethyl-4,4-difluoro-1,3,5,7-tetramethyl-4-bora-3*a*,4*a*-diaza-*s*-incadene, Molecular Probes, C-2102), CellTracker orange fluorescent CMTMR (5-(and -6)-(((4-chloromethyl)benzoyl)amino)-tetramethylrhodamine), C-2927) and BODIPY FL C5-ceramide (*N*-(4,4-difluoro-5,7-dimethyl-4-bora-3*a*,4*a*-diaza-*s*-incadene-3-pentanoyl)sphingosine), Molecular Probes, D-3521) were obtained from Interchim (France). Hoëchst was purchased from Sigma (St. Louis, MO) and Mowiol from Calbiochem (La Jolla, CA). FITC-conjugated anti-*Chlamydia* monoclonal antibody was from Argene BIOSOFT (France) and Texas red-conjugated goat anti-mouse antibody was from Interchim (France).

The guinea pig inclusion conjuctivitis (GPIC) serovar of *C. psittaci* was obtained from Roger Rank (University of Arkansas). Cervical carcinoma epithelial (HeLa) cells were obtained from ATCC.

2.2. Labelling of Chlamydia with the CellTracker green BODIPY or CellTracker orange CMTMR

HeLa cells infected with *C. psittaci* GPIC, as recently described by Boleti et al. (1999) were labelled with the CellTracker green fluorescent BODIPY or CellTracker orange CMTMR by incubating with the reagents in DMEM medium supplemented with 10% FBS (30 min up to 1.5 h at 37°C). The CellTracker was added directly to the culture medium at concentrations of 50 μg/ml (50 mg/ml stock in EtOH or DMSO kept at −20°C) for the CellTracker BODIPY or at 0.125–0.250 μg/ml (5 mg/ml stock in EtOH or DMSO kept at −20°C) for the CellTracker CMTMR. These conditions were found to be optimal for the staining of the chlamydial inclusions, while faintly staining the host-cell cytoplasm. Staining of the chlamydial inclusions was verified by fixing the cells with 4% paraformaldehyde and staining either the DNA with Hoëchst or

the inclusions with an anti-*Chlamydia*-specific antibody (Boleti et al., 1999).

2.3. Purification of Chlamydia labelled with CellTracker

Semiconfluent HeLa cell cultures grown in 10-cm dishes were infected with *C. psittaci* (multiplicity of infection of about 1.0) as described previously by Boleti et al. (1999) and were incubated with CellTracker green BODIPY (50 μg/ml) or CellTracker orange CMTMR (0.5–1.0 μg/ml) from 19 to 20 h post-infection up to 48 h post-infection. We used a higher concentration of the labelling reagent CellTracker orange CMTR to ensure that it would not be depleted during the 28 h labelling period. Bacteria were prepared as recently described (Boleti et al., 1999) and stored at −80°C. The fluorescent labelling of *Chlamydia* was verified by an inspection of a small aliquot of the preparation under a fluorescence microscope. To eliminate large aggregates of bacteria, an aliquot of the bacterial suspension was subjected to a centrifugation step ($700 \times g$, 10–15 min, 4°C) and the bacteria present in the supernatant were used for the infection.

2.4. Double labelling with BODIPY-FL C5-ceramide and CellTracker CMTMR

HeLa cells infected with *C. psittaci* GPIC for 24 h were labelled with the CellTracker CMTMR (0.125–0.250 μg/ml, 30 min, 37°C). The medium containing the CellTracker was removed and the cells were washed twice with warm (37°C) culture medium (DMEM complete supplemented with 10% serum). The cells were then incubated with BODIPY FL C5-ceramide (5 μM, from a 250 μM stock in EtOH kept at −20°C) for 15 min at 37°C. The fluorescent lipid was removed, the cells were washed twice with DMEM medium supplemented with 10% serum and 0.34% (w/v) bovine serum albumin (BSA), and fresh medium containing 0.34% (w/v) BSA was added to the cultures. The cells were further incubated at 37°C for periods of 15–30 min up to 1–3 h. For immunofluorescence analysis the cells were washed three times with PBS (20°C) and mounted directly with Mowiol (12% w/v Mowiol, 30% w/v glycerol, 0.1 M Tris, pH 8.5). The coverslips were sealed with nail polish after storing at 4°C for 30 min.

2.5. Adherence and internalization of Chlamydia labelled with CellTracker BODIPY or CellTracker CMTMR

Adherence of the labelled EBs on HeLa cells was performed at room temperature for 1.5 h. Bacteria that had not adhered were washed away and infected cells were incubated further at 37°C for up to 5 h to allow for internalization of adhered *Chlamydia*. The infected cells were subsequently fixed and processed for immunofluorescence as described previously (Boleti et al., 1999). To quantitate internal versus external bacteria the extracellular *Chlamydia* were labelled with an anti-*Chlamydia* monoclonal antibody in the absence of detergent. This prevented access of the antibody to the intracellular bacteria. In the case of infection with *Chlamydia* labelled with CellTracker CMTMR, an antibody conjugated to FITC was used. In the case of *Chlamydia* labelled with CellTracker BODIPY, the first anti-*Chlamydia* antibody was revealed with a secondary goat anti-mouse antibody conjugated to Texas red.

2.6. Fluorescence microscopy

Immunofluorescence and image analysis was performed as recently described (Boleti et al., 1999). The DNA was revealed by labelling with Hoëchst (5 μg/ml, 5 min, room temperature). The samples were examined under an epifluorescence microscope (Axiophot, Zeiss, Germany) attached to a cooled CCD-camera (Photometrics, Tucson, AZ), using a 63× Apochromat lens. The bacteria labelled with CellTracker BODIPY or CellTracker CMTMR were revealed with a FT 510 nm or a FT 580 nm filter, respectively.

3. Results and discussion

3.1. Labelling of early and late Chlamydia inclusions with the CellTracker

HeLa cells infected with *Chlamydia* were labelled with the CellTracker at different stages of the

infection cycle. Accumulation of the CellTracker reagent in the chlamydial inclusions appeared to correlate with the level of metabolic activity of the bacteria, as a function of the developmental stage of the intracellular chlamydiae. Thus, under the same conditions of labelling, chlamydial inclusions 15 and 17 h post-infection were faintly stained and difficult to distinguish from the equally stained host-cell cytoplasm (Fig. 1A–D). In contrast, when labelling was performed at later stages of infection (between 24 and 48 h post-infection), during which time the metabolic activity of the RBs is at its highest level, the reagent accumulated preferentially in the inclusions (Fig. 1E–H).

3.2. No effect of the CellTracker on the growth and differentiation of Chlamydia

To ensure that the CellTracker is not toxic to the chlamydiae and has no effect on the progression of the infection, HeLa cells infected with *C. psittaci* for 24 h were labelled as described in Materials and methods with CellTracker BODIPY and were then allowed to grow an additional 24 h in the absence of CellTracker. At 48 h post-infection, the CellTracker was localized almost exclusively in the inclusion. However, the intensity of staining was not the same in the different inclusions (Fig. 2). It is possible that this correlates with the developmental stage at which the chlamydiae happened to be during the labelling step. Hence, inclusions which contained a higher proportion of EBs would be less strongly stained than inclusions containing the metabolically-active RBs.

3.3. Double in vivo labelling of chlamydial inclusions with BODIPY-FL C5-ceramide and CellTracker CMTMR

The interaction of labelled chlamydial inclusions with intracellular compartments of the host cell was investigated by labelling 24-h inclusions with CellTracker CMTMR (orange) and then analyzing for their ability to accumulate fluorescent C5-Ceramide (green), as described in Materials and methods. C5-Ceramide is a metabolic precursor of sphingomyelin, which is normally transported from the trans-Golgi network to the plasma membrane (Pagano et al., 1991); but in *Chlamydia*-infected cells, it is specifically transported to the inclusion membrane and incorporated in the bacterial membranes (Hackstadt et al., 1996). Interestingly, the CellTracker CMTMR accumulated in the Golgi in the non-infected cells, while it stained preferentially the *Chlamydia* inclusions in the infected cells (Fig. 3). The intensity of staining of the inclusions varied among different cells in the same field. In comparison, the BODIPY-FL C5-ceramide dye, after a 15-min chase, concentrated mainly in the Golgi, but also gave rise to a reticular and punctate pattern of staining. In infected cells, the intensity of staining in the inclusion increased, while the Golgi staining diminished as the chase was prolonged. In cells whose inclusions were stained faintly, a more intense staining of the Golgi was retained (Fig. 3). We observed a small number of cells with inclusions that were strongly labelled with the CellTracker and weakly with C5-ceramide, and vice-versa. However, the majority of the inclusions were strongly stained with both vital dyes after a 2–3-h chase with C5-ceramide. Thus, the inclusions labelled with the CellTracker were competent to accept fluorescent lipids from the Golgi apparatus, and both the CellTracker and C5-ceramide are good indicators for inclusions with high metabolic activity.

3.4. Infectious activity of chlamydiae purified from CellTracker-labelled infected cells

We finally wished to determine if we could prepare CellTracker-labelled chlamydiae that maintained their infectious activity. We therefore measured the ability of the bacteria to adhere to the host cell surface, be internalized, and give rise to productive infections. *Chlamydia* EBs labelled either with the CellTracker BODIPY or the CellTracker CMTMR were purified as described in Materials and methods and tested for their ability to adhere and enter HeLa cells as recently described (Boleti et al., 1999).

A problem faced initially in measuring *Chlamydia* internalization is that the CellTracker-labelled bacteria formed aggregates more easily than unlabelled bacteria (not shown). This problem was minimized by a centrifugation step ($700 \times g$, 10–15 min, 4°C) before the adherence assay. The labelled bacteria

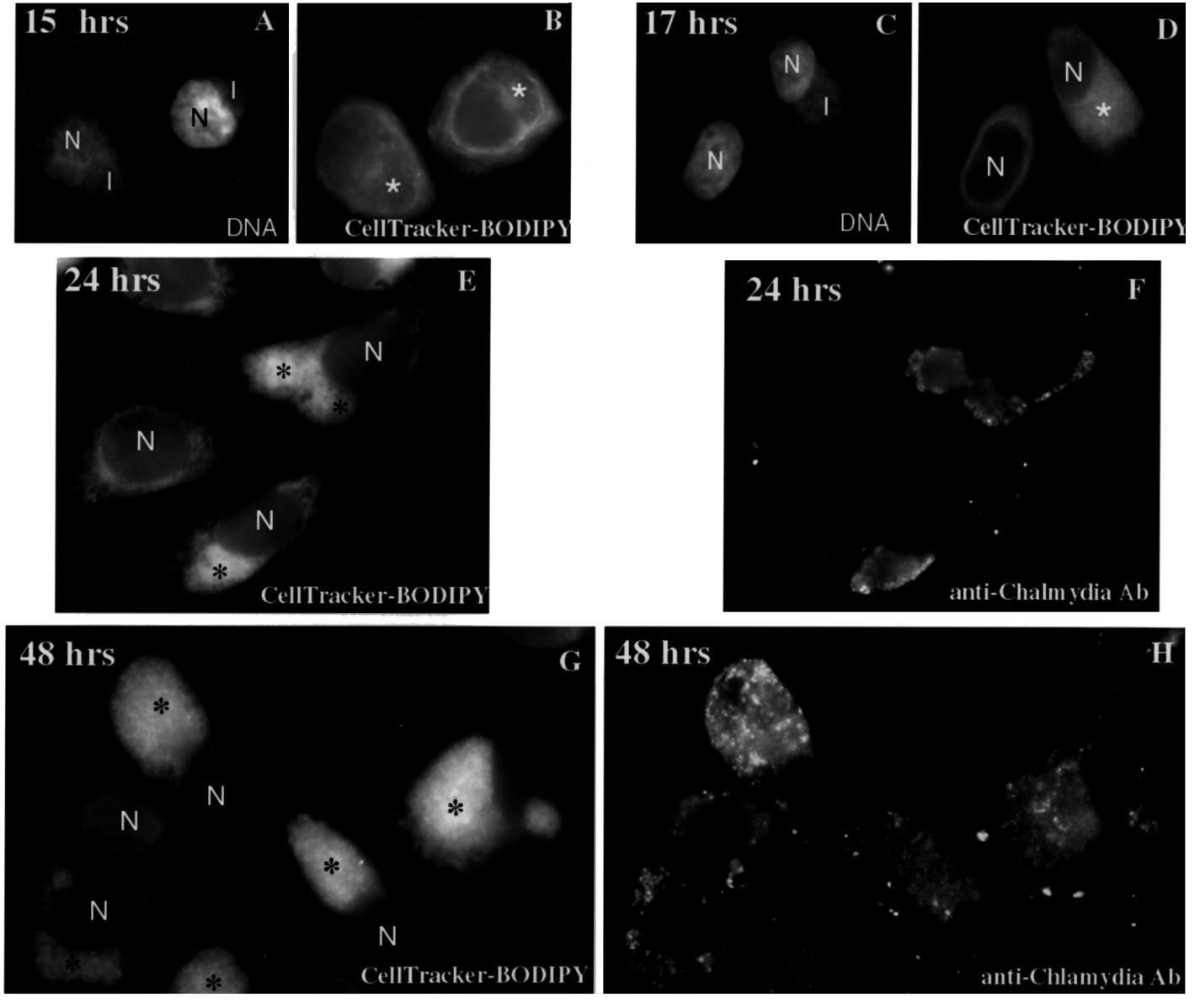

Fig. 1. The CellTracker labels both early and late *Chlamydia* inclusions. HeLa cells were infected with *C. psittaci* and labelled with CellTracker BODIPY (30 min at 37°C) at 15, 17, 24 or 48 h post-infection as described in Materials and methods. Cells were fixed and further stained with the anti-*Chlamydia* antibody revealed with a Texas red-conjugated goat anti-mouse antibody. The DNA was stained with Hoëchst. I or *: inclusion; N: nucleus.

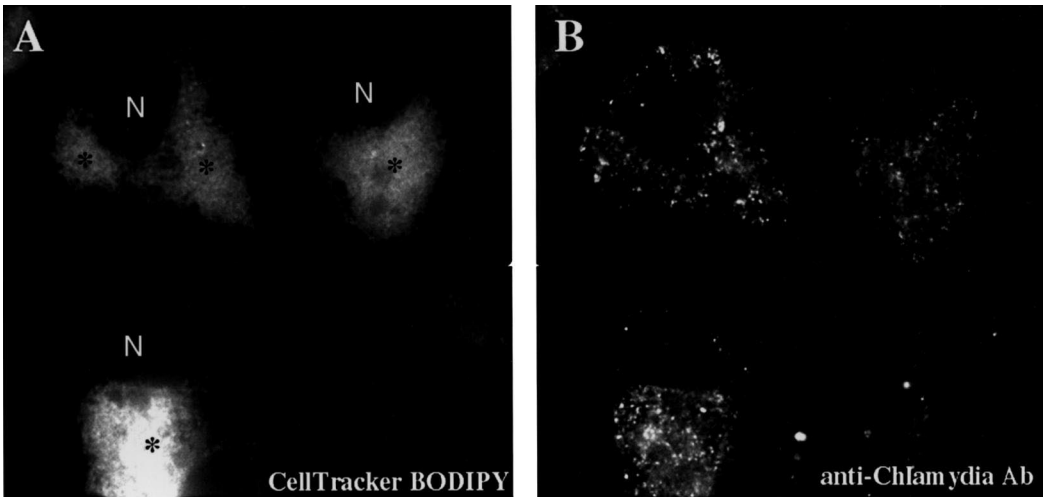

Fig. 2. Labelling with CellTracker does not affect the growth and development of *Chlamydia*. HeLa cells infected with *Chlamydia* for 24 h post-infection were incubated with CellTracker BODIPY (30 min at 37°C), the medium with CellTracker was removed, fresh medium was added, and the infected cells were allowed to grow for an additional 24 h. At the end of the 48-h infection period, the cells were fixed and stained with the anti-*Chlamydia* antibody as described above. A and B show the same field. N: nucleus; *: inclusion.

were internalized (Fig. 4A) with an efficiency that appeared to be twice as high as for the unlabelled EBs (Table 1). This is within the limits of the variability that can be observed between two different unlabelled preparations, or in the same preparation that has been subjected to a single freeze–thaw cycle or simply kept at 37°C for half an hour (unpublished observations). Conversely, a higher number of adhered bacteria/cell were needed to produce the same % of cells with inclusions (24 h post-infection) as compared to the infection with unlabelled *Chlamydia* (Table 1), which is again consistent with the variability that can be observed in different unlabelled preparations (unpublished observations). Nonetheless, in both labelled and unlabelled EB preparations, the % of infected cells increased linearly as a function of the number of bound bacteria (not shown). In addition, when heparin, which inhibits infection with *C. psittaci* GPIC (Ojcius et al., 1998), was used in the adherence step, the adhesion of both the labelled and unlabelled chlamydiae was completely blocked (not shown).

Since some of the inclusions identified by labelling with anti-*Chlamydia* antibodies could have been the result of proliferation of unlabelled bacteria present in the labelled preparation, we also verified that the majority of the bacteria in the preparation were in fact labelled with the CellTracker. Cells infected (5 h) with bacteria labelled with the CellTracker were processed for immunofluorescence and labelled with the anti-*Chlamydia* antibody in the presence of detergent, which allowed the visualization of the adhered and internalized bacteria (Fig. 4E,F). Our results from two experiments showed that the bacteria that were labelled with the anti-*Chlamydia* antibody but not with the CellTracker represented 1.2–3.5% of the total bacteria. This implied that the inclusions produced in the infected cells had arisen mainly from CellTracker-labelled chlamydiae.

On the other hand, only about 25% of the bacteria labelled with CellTracker were also labelled with the anti-*Chlamydia* antibody. Thus, either the antibody labels bacteria with a lower efficiency than the CellTracker and/or has a limited access to epitopes on intracellular bacteria (within the inclusions), or the preparation of *Chlamydia* contained host cell debris labelled with the CellTracker that could be falsely identified as labelled bacteria. This might

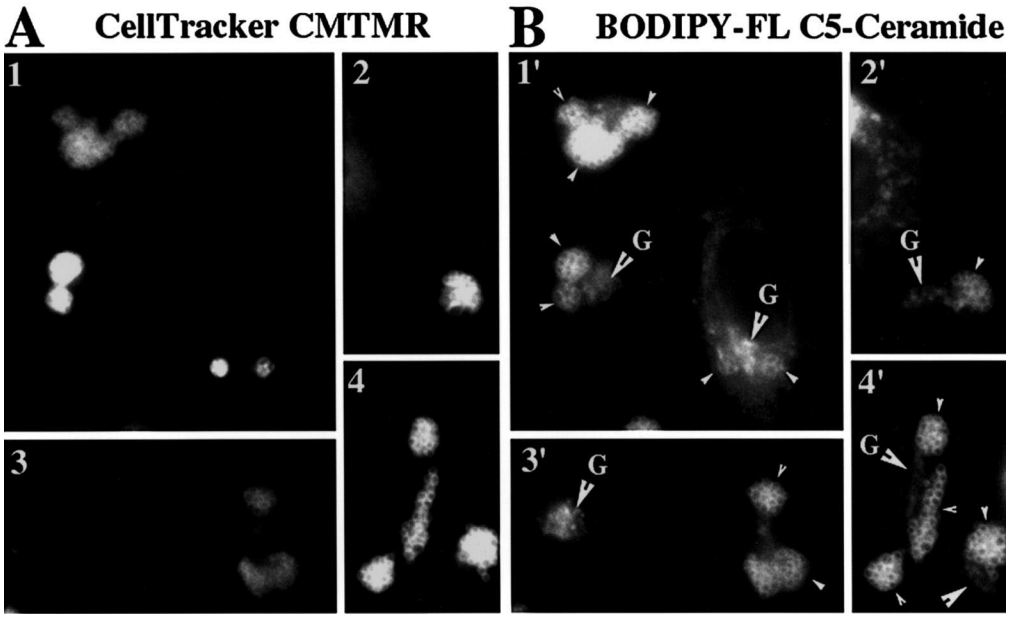

Fig. 3. Chlamydial inclusions can be labelled in vivo simultaneously with BODIPY C5-ceramide and CellTracker CMTMR. HeLa cells infected with *C. psittaci* for 24 h were labelled first with CellTracker CMTMR and subsequently with BODIPY C5-ceramide as described in Materials and methods. The fluorescent lipid was extracted from the plasma membrane with BSA (0.34% w/v) and the intracellular localization of the fluorescent lipid was followed by fluorescence microscopy after 3 h chase. The BODIPY-spingomyelin labels strongly the Golgi in uninfected cells while labelling strongly the inclusions and faintly the Golgi in infected cells. Panels 1, 2, 3, 4 and 1′, 2′, 3′, 4′ show the same fields, respectively. G: Golgi; small arrow heads: inclusions; large arrow heads: Golgi.

partially explain why *Chlamydia* labelled with the CellTracker seem to be internalized with a higher efficiency, even though a higher number of CellTracker-labelled *Chlamydia* than unlabelled bacteria is required to produce the same percentage of cells with inclusions.

3.5. Concluding statements

Incubation of *Chlamydia*-infected epithelial cells with the CellTracker resulted in preferential accumulation of the vital dyes in the *Chlamydia* inclusions. This allows the labelling and identification of chlamydial inclusions in live cells, without the prefixation and permeabilization steps required for labelling with fluorescently-conjugated antibodies, and should permit a characterization of *Chlamydia* development in live host-cells by fluorescence video microscopy. With this purpose in mind, we have demonstrated that the chlamydiae undergo a normal developmental cycle in the presence of the CellTracker, and that the fluorescent bacteria recovered from labelled infected cells retain their ability to adhere to the host-cell surface, to be internalized, and to produce normal inclusions.

While the mechanisms whereby the CellTracker labels cells remain to be fully defined, it is believed that the reactive chloromethyl derivative of the CellTracker is transferred to sulfhydryl groups of cysteine residues via the activity of an endogenous glutathione *S*-transferase-like activity. Labelling of *Chlamydia* proteins with the CellTracker therefore also suggests that chlamydiae possess an enzyme exhibiting the same or equivalent activity. A search in the *Chlamydia* genome (http://chlamydia-www.berkeley.edu/4231/) did not reveal the presence of a sequence homolog of glutathione *S*-transferase, suggesting that a functional homolog may be responsible for chlamydial labelling with the CellTracker.

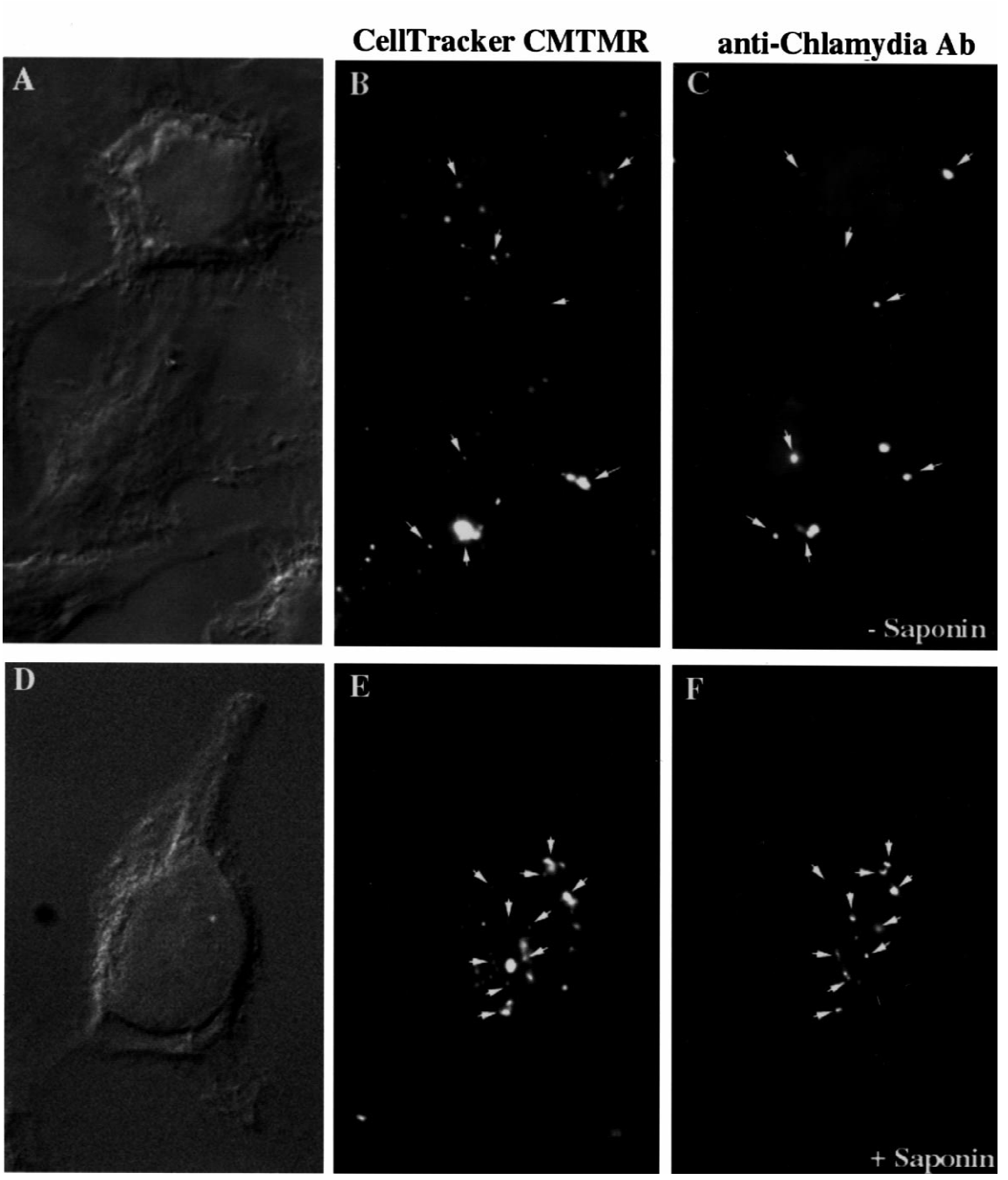

Fig. 4. CellTracker-labelled chlamydiae adhere to host epithelial cells and are internalized. *Chlamydia* EBs labelled with CellTracker BODIPY isolated from infected cells, as described in Materials and methods, were incubated with HeLa cells for 1.5 h at 20°C. The unbound bacteria were washed away and the infected cells were incubated at 37°C for an additional 3.5 h. The cells were fixed and stained either with or without permeabilization with the anti-*Chlamydia* antibody conjugated to FITC. Staining with CellTracker CMTMR represents the total number of bacteria, both those that have simply bound and those that have been internalized. Staining with the anti-*Chlamydia* antibody corresponds to extracellular bacteria when cells were not permeabilized (−saponin), or to both intracellular and extracellular bacteria when cells were permeabilized (+ saponin). A, B and C show the same field. D, E and F show the same field. The arrows indicate bacteria that were labelled with both the CellTracker and the anti-*Chlamydia* antibody. A and D are Nomarski images.

Table 1
Ability of labelled and unlabelled chlamydiae to bind to, enter, and infect host cells

	% Entry (5 h post-infection)	Number of bacteria/cell (5 h post-infection)	% Cells infected (24 h post-infection)
Labelled bacteria	63±1.7 (212)[a]		
Experiment 1		1.9 (67)	9.0 (191)
Experiment 1		4.0 (58)	12.2 (477)
Experiment 1		6.0 (50)	21.3 (403)
Experiment 2		6.1 (50)	33.0 (150)
Experiment 3		7.3 (111)	39.0 (251)
Experiment 2		11.9 (50)	52.0 (214)
Unlabelled bacteria	26.5±4.5 (167)[a]		
Experiment 1		2.6 (102)	53.6 (153)
Experiment 2		4.7 (65)	67.0 (201)

[a] The number of cells analyzed is given within parentheses, and standard deviations are from three independent measurements. Combined results are from three different experiments. A range of multiplicities of infection were tested in the different experiments.

HeLa cells infected (5 or 24 h) with *C. psittaci* GPIC that were unlabelled or labelled with the CellTracker CMTMR were fixed and processed for immunofluorescence at the end of the infection period. The bacteria were stained with the anti-*Chlamydia* antibody conjugated to FITC (Materials and methods). Labelling with the antibody was conducted either in the absence (labelling only of adhered, extracellular bacteria) or presence (labelling of intracellular and extracellular bacteria) of 0.05% saponin. The percentage of infected cells was estimated by counting the cells with inclusions 24 h post-infection. The efficiency of entry was estimated in cells infected for 5 h by counting the extracellular bacteria (green and red) and the total number of bacteria (red) per field taking into account the number of cells in each field. The percentage of internalization for the unlabelled bacteria was done as described recently by Boleti et al. (1999).

Fluorescein isothiocyanate (FITC) labelling of microorganisms has been used extensively as a convenient way to visualize bacteria interacting with mammalian cells (Hed, 1986; Drevets and Campbell, 1991; Weingart et al., 1999). FITC binds covalently to primary amines of amino acids present on the N-terminus of proteins and on lysine residues. It labels only amines in the free base (uncharged state), and a high pH (>8) can therefore be used to increase the efficiency of labelling. However, it has been demonstrated that covalent labelling with FITC can modify the function of biologically important surface proteins, affect the viability of bacteria, or inhibit the activity of virulence factors. Labelling of bacteria through bacterial expression of genes coding for GFP, as already demonstrated for several bacteria, appears to be a less disruptive way of rendering the bacteria fluorescent (Valdivia et al., 1996; Troyer et al., 1999; Weingart et al., 1999). Nonetheless, as appealing as the GFP-labelling strategy may be, there is a number of intracellular microbes, including *Chlamydia* and a number of parasites, for which techniques for genetic manipulation and therefore labelling with GFP are not yet available.

The CellTracker reagent has the advantage over other fluorescent reagents that it can be used for labelling under normal culture conditions, thus avoiding exposure of the microbe to conditions of high pH, as in the case of labelling with FITC. Additionally, subsequent steps of manipulation are limited to the procedure for purifying the microbes. The label is incorporated into the microbe in vivo, while the pathogen is still metabolically active, therefore ensuring high viability and preservation of the microbe, while at the same time minimizing labelling of uninfectious (metabolically inert) microbes. Finally, bacteria can be labelled with two different colors, which could be useful for studying the interaction between microbes and the host cell, the entry of two microbes in the same cell, or other cellular applications.

4. Notation

GFP	green fluorescent protein
GPIC	guinea pig inclusion conjuctivitis
EB	elementary body
RB	reticulate body
Ab	antibody

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