Cryptococcus neoformans is an encapsulated yeast that causes systemic mycosis in immunosuppressed individuals. Recent studies have determined that this fungus produces vesicles that are released to the extracellular environment both in vivo and in vitro. These vesicles contain assorted cargo that includes several molecules associated with virulence and implicated in host-pathogen interactions, such as capsular polysaccharides, capsule, laccase, urease, and other proteins. To date, visualization of extracellular vesicles has relied on transmission electron microscopy, a time-consuming technique. In this work we report the use of fluorescent membrane tracers to stain lipophilic structures in cryptococcal culture supernatants and capsules. Two duallylcarbocyanine probes with different spectral characteristics were used to visualize purified vesicles by fluorescence microscopy and flow cytometry. Dual staining of vesicles with duallylcarbocyanine and RNA-selective nucleic acid dyes suggested that a fraction of the vesicle population carried RNA. Use of these dyes to stain whole cells, however, was hampered by their possible direct binding to capsular polysaccharide. A fluorescent phospholipid was used as additional membrane tracer to stain whole cells, revealing punctate structures on the edge of the capsule which are consistent with vesicular trafficking. Lipophilic dyes provide new tools for the study of fungal extracellular vesicles and their content. The finding of hydrophobic regions in the capsule of C. neoformans adds to the growing evidence for a structurally complex structure composed of polysaccharide and nonpolysaccharide components.
and flow cytometry, might be able to overcome this limitation and allow faster and more versatile observation of fungal extracellular vesicles and their cargo. In this work we report the use of fluorescent probes to visualize the extracellular vesicles produced by *C. neoformans* and provide insights about their cellular location and content.

**MATERIALS AND METHODS**

**Fungal strains and media.** *C. neoformans* isolates H99 (serotype A), 24067 (serotype D), B3501 (serotype D), and CAP67 (a B3501-derived acapsular mutant) were used in this study. The cells were grown in either Sabouraud broth or minimal medium (15 mM glucose, 29 mM KH2PO4, 10 mM MgSO4, 13 mM mann) were used in this study. The cells were grown in either Sabouraud broth or minimal medium (15 mM glucose, 29 mM KH2PO4, 10 mM MgSO4, 13 mM mann) and 3 mM thiamine [pH 5.5]). Capsule growth was induced by incubating *C. neoformans* cells in 1:10 Sabouraud broth-MOPS (morpholinepropanesulfonic acid), pH 7.3, for 2 days at 37°C (27).

**Probes.** DiI (1,1'-dioctadecyl-3,3',3'-tetramethylindocarbocyanine perchlorate; Vybrant DiI cell labeling solution), DiD-DS (1,1'-dioctadecyl-3,3',3'-tetramethylindocarbocyanine-5',5'-disulfonic acid), BODIPY-PC [2-(4,4'-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-pentanol]-1-hexadecanoyl-sn-glycero-3-phosphocholine]. DiIC1(5) (1,1',3,3',3'-hexamethyldioctadecylindocarbocyanine iodide), and SYTO RNASelect were purchased from Invitrogen. Uvitex 2B, a probe that detects chitin in the fungal cell wall, was purchased from Polysciences Inc., and AF488-18B7 was prepared by labeling the anti-GXM immunoglobulin G1 18B7 with Alexa Fluor 488, according to the manufacturer's (Invitrogen) instructions. Structures of the membrane probes used in this study are shown in Fig. 1.

**Vesicle purification and staining.** Extracellular vesicles were purified by the method described before (19) with slight modifications. In brief, *C. neoformans* cells were removed from cultures in minimal medium (3 to 6 days, 30°C with shaking at 150 rpm) by centrifugation and filtration with a 0.4-

**Staining of purified vesicles.** To assess the usefulness of dialkylcarbocyanine dyes as probes for extracellular vesicles, we stained vesicular preparations with either DiI or DiD-DS.
and studied them by fluorescence microscopy. Purified vesicles in buffer suspension stained brightly enough with the probes to be readily observed by confocal microscopy as bright dots on a dark background (Fig. 2A). Both probes are also useful for staining the vesicles for flow cytometry (Fig. 2B). Although both DiI and DiD-DS stained the vesicles, DiI staining was brighter than DiD-DS staining, which resulted in better separation between unstained and stained vesicles by flow cytometry. As controls, we also incubated the dialkylcarbocyanine dyes in buffer alone and stained purified vesicles with DiIC1(5), a dye similar to DiD-DS but without the two alkyl tails. As expected, no dye precipitation was observed in both cases, confirming that the probes are indeed staining purified vesicles based on their affinity for lipidic bilayers.
Having determined that these probes were useful for flow cytometry, we devised an experiment to explore the utility of the technique. As mammalian exosomes have been shown to contain RNA (25), we analyzed vesicles that were costained with DiI and the membrane-permeable RNA probe SYTO RNASelect. The results revealed a subset of events that stained positive with both dyes (Fig. 3A). To discard the possibility of nonspecific binding, we used the same pair of dyes to stain commercial liposomes, which stained with DiI only. Unstained vesicles from the same preparation were negative in both channels. Formation of micelles, vesicles, or aggregates by the probes themselves in solution was ruled out with a control consisting of DiI and SYTO RNASelect dissolved in PBS only (data not shown).

**Staining of C. neoformans cells.** Having determined that the dialkylcarbocyanine dyes DiI and DiD-DS stained vesicles, we attempted to visualize and localize them in *C. neoformans* cells using fluorescence microscopy. Staining of intact or capsule-induced cells with DiI or DiD-DS revealed punctate structures located mostly on the outer edge of the capsule in encapsulated strains and on the surface of the cell wall in acapsular mutants (Fig. 4A and B). Some of these structures stained very brightly, while others were much dimmer. Another staining pattern, consisting of a uniform layer on top of the cell wall and stalklike attachments to the cell body (Fig. 4C), was observed much less frequently. No staining for the cytoplasmic membrane or any intracellular membrane structure was observed. Intracellular membranes in *C. neoformans* spheroplasts were labeled (Fig. 4D), indicating that the probes were unable to penetrate the cell wall.

To test the stability of these hydrophobic regions, we extracted the cells with methanol and chloroform and repeated the staining. Surprisingly, organic solvent extraction resulted in cells that still stained brightly with DiI (Fig. 5A). Flow cytometry was used to quantify the amount of probe bound to each cell, revealing that prior extraction with solvents increased binding of both dialkylcarbocyanine probes to the cells (Fig. 5B). Staining of intact cells with DiIC1(5) also revealed similar dots, confirming that in this case the binding was not dependent on the dialkylcarbocyanines’ affinity for lipid bilayers. In contrast to DiI and DiD-DS, DiIC1(5) readily penetrated the cell and stained intracellular structures much more brightly than the extracellular dots (Fig. 5D). To test whether the probe was binding directly to capsular polysaccharide, we incubated different preparations of purified GXM with DiI, which resulted in formation of a stained precipitate (Fig. 5C). Fluorescence microscopic examination of the DiI-GXM precipitate revealed large brightly fluorescent aggregates (data not shown).
Unlike the experience with purified vesicles, the dialkylcarbocyanines appeared not to be good probes to study extracellular vesicles in intact cells because of their propensity to stain capsule structures in delipidated cells. With this caveat in mind, we tested a second membrane tracer, the fluorescent phospholipid BODIPY-PC.

**FIG. 5.** Staining of delipidated cells and polysaccharide. *C. neoformans* cells were extracted with methanol and chloroform to remove lipids and then stained with both DiI and DiD-DS. (A) Extracted and nonextracted cells were stained with DiD-DS and imaged under the same conditions by laser scanning confocal microscopy. Extraction did not abrogate the binding of the dye. Staining with DiI produced the same result (not shown). Bar, 5 μm. (B) Native and extracted cells stained with DiI and DiD-DS and then analyzed by flow cytometry to measure the amount of dye incorporated per cell. Bars represent the median fluorescence intensity of approximately 30,000 cells in each condition. (C) Purified capsular polysaccharides were stained with DiI and then centrifuged. From left to right, the photograph shows that the dye does not precipitate by itself but forms aggregates with GXM purified from culture supernatants (CTAB-GXM) and from the capsule (DMSO-GXM), even after delipidation of polysaccharide samples with chloroform. (D) *C. neoformans* cells were stained with DiIC1(5), which resulted in bright staining of intracellular structures and dim staining of extracellular dots. The panel on the right corresponds to the image on the left after linear brightness and contrast adjustment to highlight the dimmer extracellular dots (arrow).
BODIPY-PC

Merged

Brightfield

DiI

Merged

Brightfield

FIG. 6. Double staining of intact cells with DiI and fluorescent PC. (A) C. neoformans cells that were stained with a green fluorescent phospholipid probe, which resulted in a punctate staining on the edge of the capsule. (B) C. neoformans cells (B3501 isolate, capsule induced) were stained simultaneously with BODIPY-PC and DiI. The double staining reveals limited colocalization of PC and DiI. Images in both panels were linearly adjusted for brightness and contrast.

also revealed punctate structures on the outer edge of the capsule, albeit with much lower brightness and with a lower frequency (Fig. 6A). Double staining with DiI (Fig. 6B) revealed that some DiI-positive structures also stained with BODIPY-PC but that the majority of the spots did not colocalize. In contrast to DiI, BODIPY-PC readily penetrated the cell and stained intracellular membranes.

DISCUSSION

Dialkylcarbocyanines are amphiphilic molecules composed by a polar fluorescent head linked to two alkyl tails. The hydrophobic alkyl chains insert into the lipophilic core of phospholipid bilayers, whereas the positively charged fluorophore rests close to the polar head groups, parallel to the membrane surface (2, 13). This binding mechanism makes them excellent probes for lipidic membranes, a property that made dialkylcarbocyanines attractive as fluorescent probes to detect extracellular vesicles. Our results established that purified vesicles were efficiently labeled with two different probes from this family: DiI, which has excitation (Ex) and emission (Em) maxima similar to those of rhodamine (Ex/Em, 549/565 nm) and the sulfonated DiD-DS (Ex/Em, 644/665 nm). Epifluorescence and confocal microscopy was used to image the stained vesicles as bright dots. The fact that solid dots were observed instead of the ring that would be expected from a vesicular structure can be explained by the fact that the diameter of the vesicles is close to or even lower than the maximum theoretical resolution achieved by optical microscopes.

The dyes are also suitable for staining vesicles for flow cytometry, a technique that permits fast quantitative analysis of a large number of particles with different fluorophores. To test the validity of this method, we costained purified extracellular vesicles with DiI and an RNA-selective nucleic acid probe, revealing a subpopulation of vesicles that may be associated with RNA. This finding could be explained by the presence of rRNA, as ribosomal proteins have been detected in vesicle proteomics studies (18); alternatively, it could be an indication that these vesicles might carry mRNAs and microRNAs, resembling recently described exosomal shuttle RNAs, which are involved in mammalian cell-to-cell transmission of mRNAs and regulatory microRNAs (25).

In addition to staining purified vesicles, we attempted to probe for lipophilic structures in the capsule of C. neoformans using dialkylcarbocyanines. Although the capsule was previously considered to be composed solely of polysaccharide, recent evidence indicates that it contains chitinlike material (17) and osmiophilic protuberances containing 3-OH oxylipins (21, 22). The latter structures appear as distinct globules and protuberances on the capsule of C. neoformans when stained with antibodies to 3-OH oxylipins. DiI staining revealed a variable number of discrete punctate structures, located mostly at the very edge of the capsule on encapsulated strains or right on top of the cell wall in acapsular strains. A control experiment, however, revealed that both DiI and DiD-DS bound to the capsule of cells that had been sequentially extracted with organic solvents, which theoretically should be enough to strip off lipid membranes. We considered various explanations for the resistance of these lipophilic structures to organic solvents. One explanation is direct binding of the probes to the capsule polysaccharide, forming aggregates via interaction between the positively charged nitrogen atom at the head of the dialkylcarbocyanines and negatively charged glucuronic acid residues in capsular polysaccharide. Consistent with this possibility is the finding that staining intact cells with DiI at(5) results in similar dots on the edge of the capsule, as well as the aggregates formed by purified GXM and DiI, even after the polysaccharide was delipidated by extraction with ethyl acetate. Given that these dyes are amphipathic, they may function like CTAB in precipitating soluble GXM. Alternatively, the dye may be staining the same type of osmiophilic protuberances in the capsule, described as containing 3-OH oxylipins, which may be resistant to the organic solvents used. Consistent with this interpretation was the observation that some lipophilic structures had stalklike attachments reminiscent of the protuberances described earlier (21, 22).

Because the binding of DiI to the capsule hindered its usefulness for observing vesicles in intact cells, we used a second probe for lipid bilayers. This probe, a PC molecule labeled in one of its alkyl tails with a green fluorescent hydrophobic fluorophore, labeled similar punctate structures in the edge of the capsule. Double staining with DiI revealed some DiI-pos-
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