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Lipophilic Dye Staining of *Cryptococcus neoformans* Extracellular Vesicles and Capsule▽

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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, 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 dialkylcarbocyanine probes with different spectral characteristics were used to visualize purified vesicles by fluorescence microscopy and flow cytometry. Dual staining of vesicles with dialkylcarbocyanine 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.**

Cryptococcus neoformans is an important cause of life-threatening systemic mycosis (5). It is believed that the fungus is acquired by inhalation and causes mild respiratory symptoms before establishing a dormant state. In individuals with immune deficiencies, such as seen with AIDS or cancer chemotherapy, latent infections can reactivate and disseminate (5). This unicellular yeast is distinctive among other eukaryotic pathogens because it is coated with a polysaccharide capsule, composed primarily by glucuronoxylomannan (GXM), with galactoxylomannan and mannoproteins (3) as minor components. The capsule is considered its most important virulence attribute because it confers upon the yeast cell both defensive and offensive attributes in its interaction with mammalian hosts. The capsule provides resistance to phagocytosis and to phagocyte fungicidal reactive oxygen species (3). Capsular polysaccharides are also shed into host tissues, where they mediate a variety of immunomodulatory effects that undermine the capacity of the host to fight infection (10). In addition to the capsule, other major *C. neoformans* virulence attributes include its ability to synthesize melanin, a cell wall pigment that augments resistance to oxidants and to antifungals, and several secreted enzymes, such as urease (9) and phospholipases (6, 8, 23).

GXM is synthesized inside the cell and subsequently exported to the capsule (11, 12, 26). Because GXM fibers can have molecular weights of more than a million (14), their passage through the cell wall, which is required for capsule assembly, could present a formidable transport problem. Rodrigues et al. recently proposed that trans-cell wall polysaccharide export occurs by an extracellular vesicular system (19). These extracellular vesicles are formed in cytoplasmic multivesicular bodies and cross the cell wall into the surrounding environment, where they presumably open to deliver their contents (19). Vesicles purified from in vitro culture supernatants contained GXM that could be recognized by specific antibodies and formed a capsule around acapsular mutants (19). These vesicles vary in size, some being up to 200 nm in diameter, and are heterogeneous in ultrastructural morphology, a hint that there might be different types of vesicles for different types of cargo (18). In fact, further studies detected laccase, urease, and acid phosphatase enzymatic activities in these vesicles, which along with detailed proteomic analyses demonstrated that they carry a large number of proteins involved in virulence and form "virulence factor delivery bags" (18). Biochemical studies of vesicular composition revealed glucosylceramide, ergosterol, and phospholipids such as phosphatidylcholine (PC), phosphatidylserine, and phosphatidylethanolamine (1, 19). Genetic evidence for different vesicular transport systems comes from the observation that *C. neoformans sec6* mutants have defective extracellular laccase transport, despite having intact capsules (16).

The discovery that these vesicles are involved in the transport of several important virulence-associated components has led to a surge in interest in their study. Extracellular vesicles have been detected in the culture supernatants of *Histoplasma capsulatum*, *Candida albicans*, *Candida parapsilosis*, *Sporothrix schenckii*, and *Saccharomyces cerevisiae* (1). Current studies of fungal vesicles are hindered by the difficulties inherent to observation of such small structures, which is possible only by using time-intensive electron microscopy methods. We reasoned that assays based on fluorescence, such as microscopy

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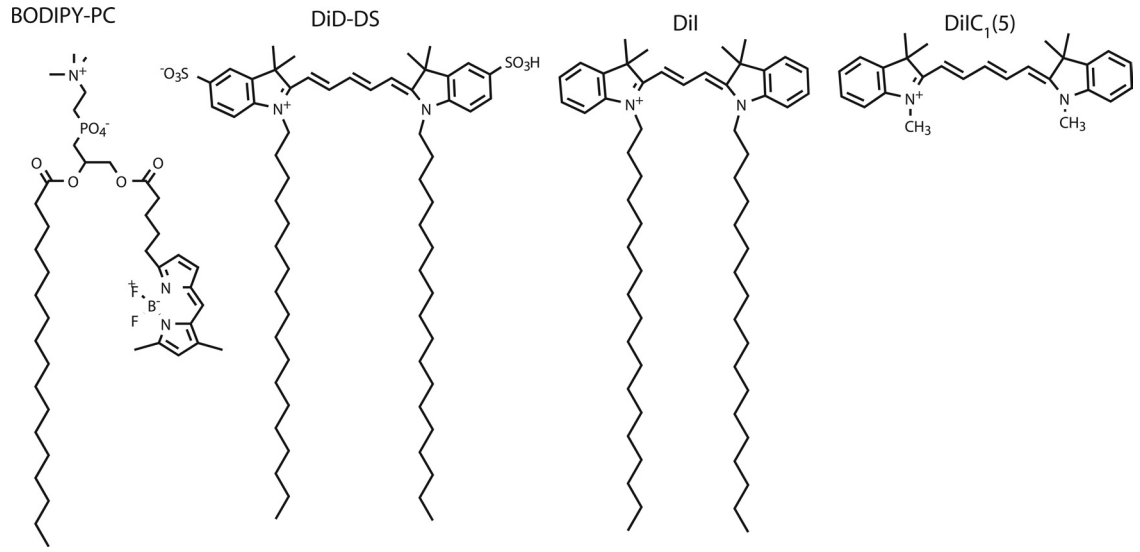


FIG. 1. Structure of the four membrane probes used in this study.

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 KH_2PO_4, 10 mM $MgSO_4$, 13 mM glycine, and 3 μM 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′,3′-tetramethylindocarbocyanine perchlorate; Vybrant DiI cell labeling solution), DiD-DS (1,1′-dioctadecyl-3,3,3′,3′-tetramethylindodicarbocyanine-5,5′-disulfonic acid), BODIPY-PC [2-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-pentanoyl)-1-hexadecanoyl-sn-glycero-3-phosphocholine], $DiIC_1(5)$ (1,1′,3,3,3′,3′-hexamethylindodicarbocyanine 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-μm filter, and the cell-free supernatant was concentrated by ultrafiltration on 100-kDa membranes. Vesicles were separated from this concentrate by ultracentrifugation at 100,000 × *g* for 1 h. The vesicles were then washed twice with phosphate-buffered saline (PBS). The purified vesicles were then stained for 1 h in PBS containing an appropriate dye [5 μM DiI, 5 μM DiD-DS, 5 μM $DiIC_1(5)$, or 1 μM SYTO RNASelect] and washed twice with PBS by centrifugation (1 h at 100,000 × *g*). The dye-stained pellet was then photographed with a Nikon D90 digital camera and suspended in PBS for flow cytometry or in mounting medium (0.1 M propyl gallate in PBS plus 50% glycerol) for fluorescence microscopy. A commercial preparation of liposomes containing PC and ergosterol (Encapsula Nano Sciences) was used as a negative control.

Flow cytometry and microscopy. Dye-stained vesicles were analyzed with a BD FACSAria cell sorter equipped with four lasers emitting at 407, 488, 561, and 635 nm. Data were analyzed offline with FlowJo software (www.treestar.com/flowjo). Stained vesicles in mounting medium were imaged by epifluorescence microscopy on a Zeiss Axioskop 200 inverted microscope equipped with a cooled charge-coupled device using a 63×, 1.4-numerical-aperture (NA) objective. Images were collected and deconvolved using a constrained iterative algorithm with AxioVision (Zeiss) software. Alternatively, a PerkinElmer UltraVIEW RS-3 spinning disk laser confocal microscope equipped with a cooled charge-coupled device camera and a 100×, 1.4-NA objective was used. For experiments with whole cells, *C. neoformans* cultures in Sabouraud broth were collected, washed, resuspended in PBS, and stained with the appropriated probes [5 μM DiI, 5 μM $DiIC_1(5)$, 100 μg ml^{-1} Uvitex 2B, 10 μg ml^{-1} AF488-18B7, 1 μg ml^{-1} BODIPY-PC] for 1 h. Spheroplasts were generated by overnight digestion with 40 mg ml^{-1} *Trichoderma harzianum* lysing enzymes (Sigma) in 1 M sorbitol and 0.1 M sodium citrate, pH 5.5, buffer and stained with 5 μM DiI. After washing and resuspension in mounting medium, the cells were imaged on the Zeiss microscope described above or on a Leica SP2 laser scanning confocal microscope equipped with a 63×, 1.4-NA objective. Deconvolved and confocal Z stacks were analyzed with AxioVision or ImageJ (http://rsbweb.nih.gov/ij/) and tridimensionally reconstructed using VOXX (http://www.nephrology.iupui.edu/imaging/voxx/) software. Postcollection manipulation of the images involved only linear brightness and contrast adjustments, which are indicated in the appropriate figure legends.

Delipidation of whole cells. Lipids were extracted from intact H99 cells grown in Sabouraud broth by sequential incubation in organic solvents. The cells were collected, washed, and resuspended in PBS. Methanol and chloroform were added to a final proportion of 0.8:2:1 water-methanol-chloroform, followed by 2:1 methanol-chloroform, pure chloroform, and pure methanol. Each extraction was carried out with rotation at 37°C from 20 min to 2 h. The cells were then washed twice in PBS and stained and imaged by laser scanning confocal microscopy as described above. Flow cytometric analysis was carried out in a BD LSRII flow cytometer equipped with 488- and 633-nm lasers.

Purification and staining of capsular polysaccharide. Supernatant GXM was purified from H99 culture supernatants by precipitation with cetyltrimethylammonium bromide (CTAB) (7). Capsular polysaccharide was stripped from H99 cells by treatment with dimethyl sulfoxide (DMSO) (4). To remove lipids, 1 mg of the DMSO-purified polysaccharide was diluted in PBS and extracted with ethyl acetate at room temperature for 1 h with vortexing. All polysaccharide samples were diluted in PBS at 1 mg/ml and centrifuged for 30 min at 20,000 × *g* to remove particulate matter. One milliliter of each solution and a control containing only PBS were then stained with 5 μM DiI for 1 h at 37°C and centrifuged again at 20,000 × *g* for 30 min.

RESULTS

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

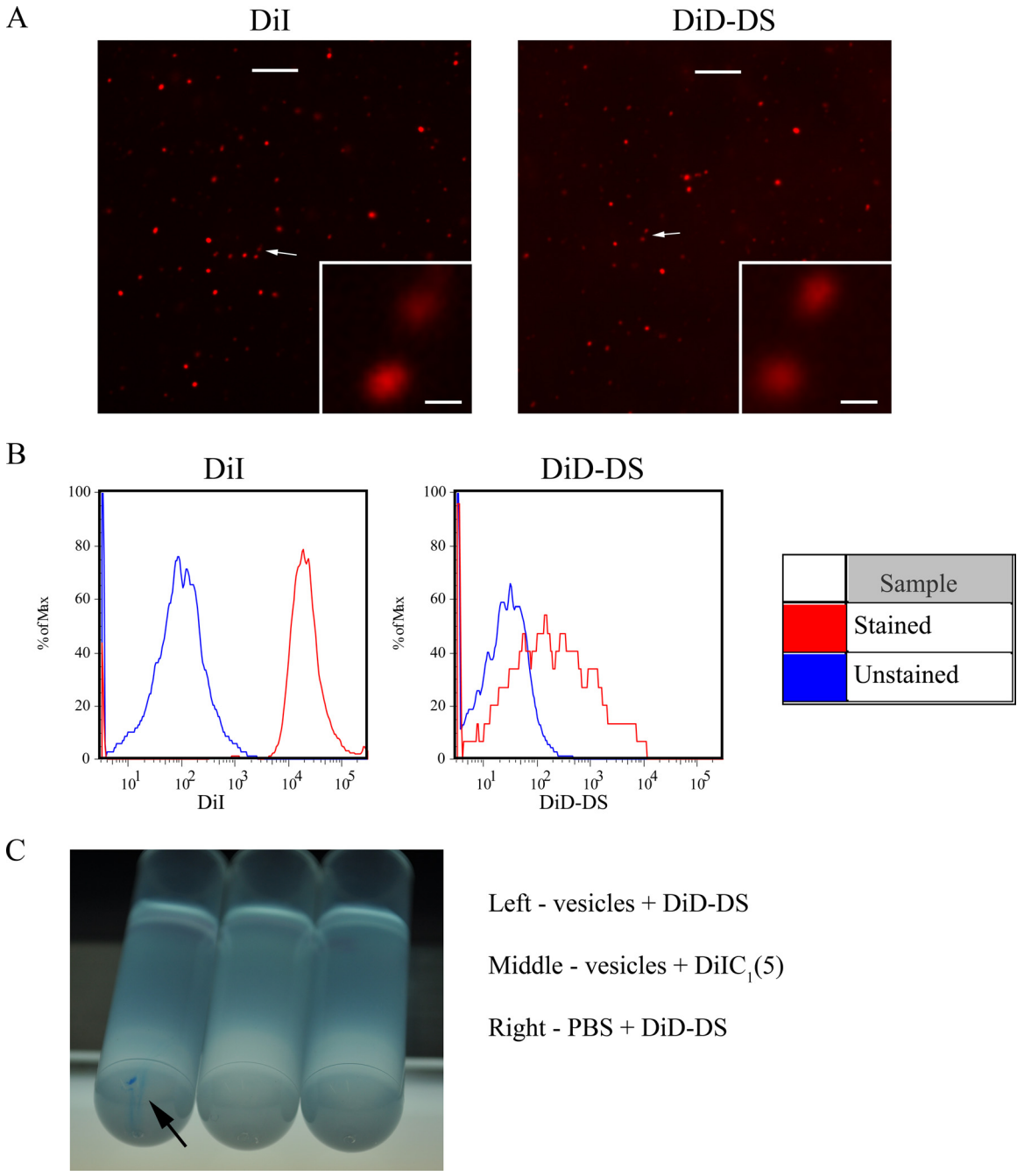
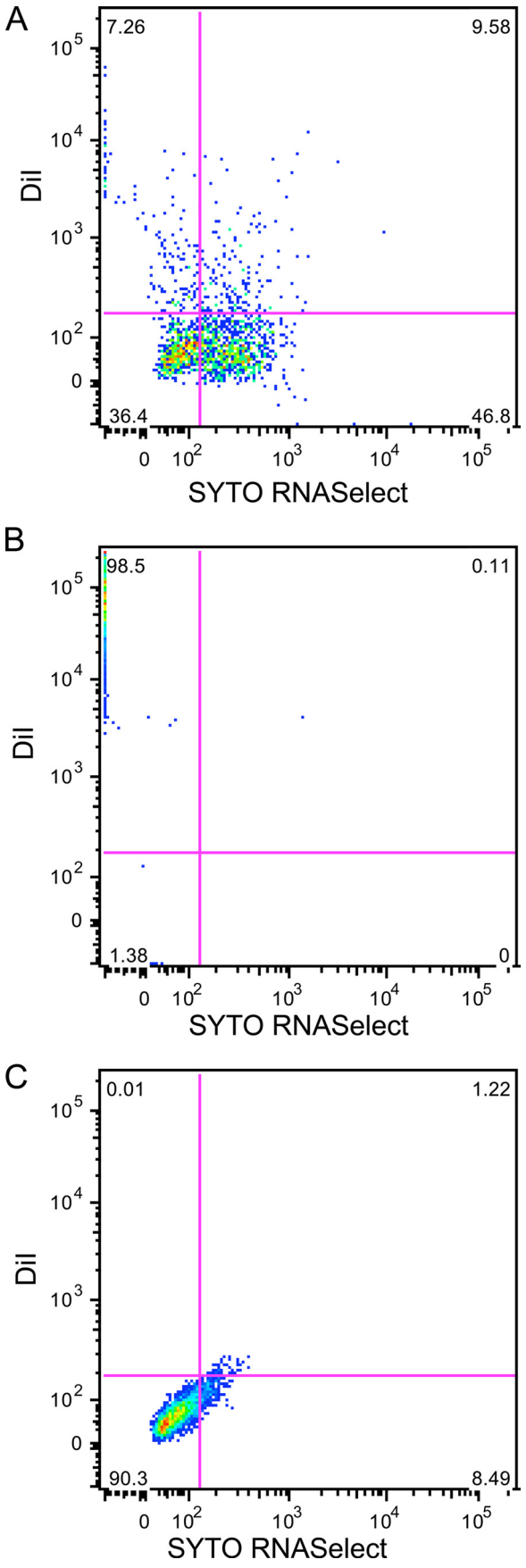


FIG. 2. Staining of purified vesicles with dialkylcarbocyanines. (A) Vesicles purified from *C. neoformans* supernatants after staining with DiI or DiD-DS and observation by confocal microscopy, showing small brightly fluorescent spots. Arrows indicate vesicles that are magnified in the insets. The image was adjusted after collection for brightness and contrast to enhance clarity. Bars, 5 μm (main parts of images) and 400 nm (insets). (B) Similarly stained vesicles were analyzed by flow cytometry, showing that individual vesicles can be observed, quantified, and analyzed. (C) Purified vesicles that were stained with DiD-DS and concentrated by centrifugation, resulting in visible staining of the 100,000 × *g* pellet (arrow). No staining of the pellet was observed with $DiIC_1(5)$, and no pellet was visible when DiD-DS in solution was centrifuged in the absence of vesicles.

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 $DiIC_1(5)$, 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 show 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 $DiIC_1(5)$ 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, $DiIC_1(5)$ 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). Florescence microscopic examination of the DiI-GXM precipitate revealed large brightly fluorescent aggregates (data not shown).

FIG. 3. A population of vesicles stains with a probe for RNA. (A) Purified vesicles were simultaneously stained with DiI and the RNA-selective nucleic acid stain SYTO RNASelect. Double-positive events corresponding to RNA-loaded vesicles can be observed in the upper right quadrant. (B) Commercial liposomes made with PC and ergosterol were stained using the same protocol; as expected, they stained only with DiI. (C) Control with unstained vesicles. All panels are density plots, in which red-shifted colors code for increased density of cells in an area.

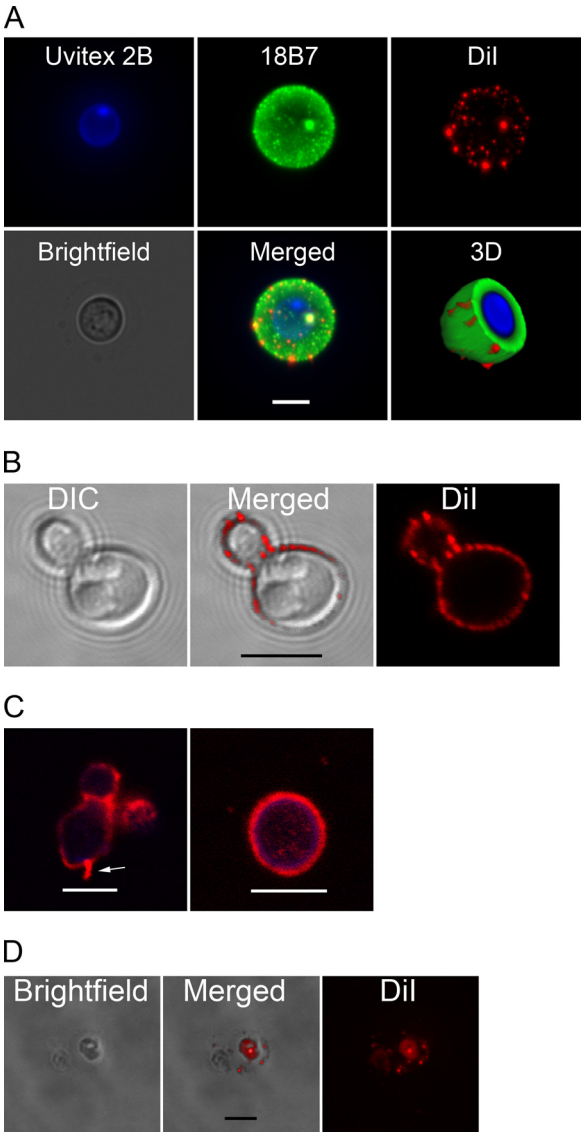


FIG. 4. Staining of whole cells with DiI. (A) Encapsulated *C. neoformans* cells (B3501 isolate, capsule induced) were stained with DiI, Uvitex 2B, and the anticapsular antibody 18B7 conjugated to Alexa Fluor 488. Fluorescence images are Z projections of deconvolved stacks, which were also reconstructed in three dimensions. DiI-positive spots can be observed at the edge of the capsule. The images were adjusted linearly for brightness and contrast. (B) Acapsular CAP67 mutants that were stained with DiI and observed in a laser scanning confocal microscope. The DiI-positive spots are directly on top of the cell wall. (C) Encapsulated isolate H99 was stained with DiD-DS and Uvitex 2B and observed in a laser scanning confocal microscope. A layer on top of the cell wall was uniformly labeled, as well as a projection (arrow). (D) *C. neoformans* spheroplasts stained with DiI and imaged by epifluorescence microscopy followed by deconvolution. The cells, which lost their shape and shrank due to the disruption of the cell wall, are stained. Bars (all panels), 5 μm.

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. The staining with BODIPY-PC

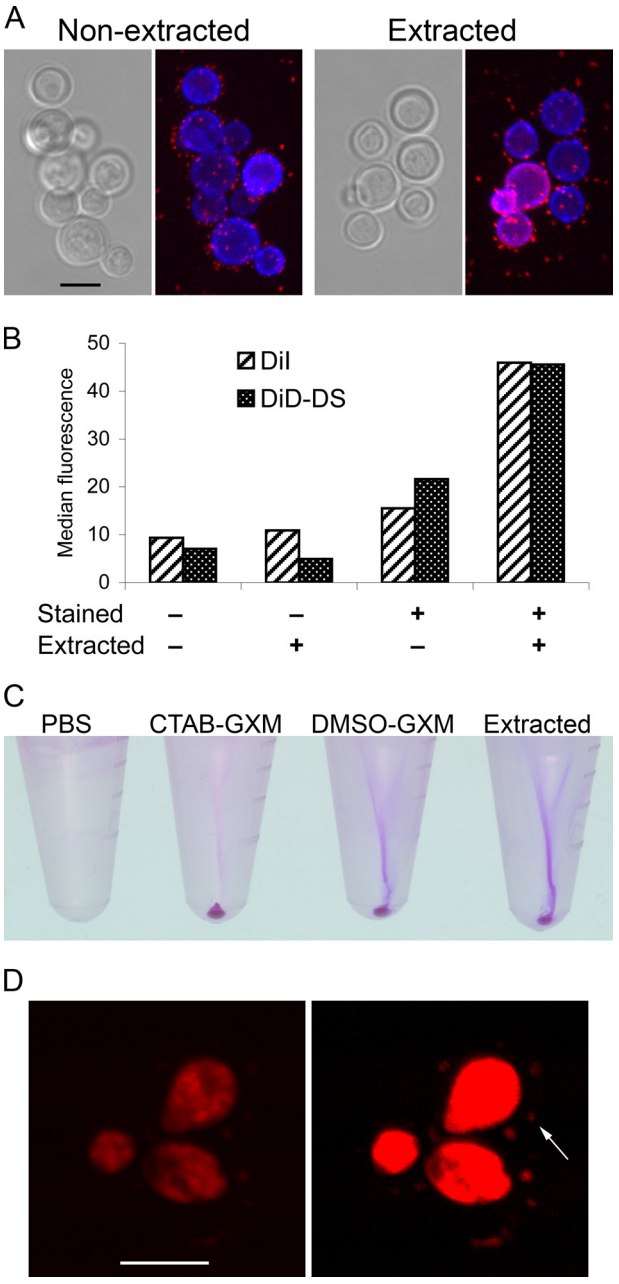


FIG. 5. Staining of delipidated cells and polysaccharide. *C. neoformans* cells were extracted with methanol and chloroform to remove lipids and then stained with both DiI and DiD-DS. (A) Extracted and nonextracted cells were stained with DiD-DS and imaged under the same conditions by laser scanning 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 $DiIC_1(5)$, 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).

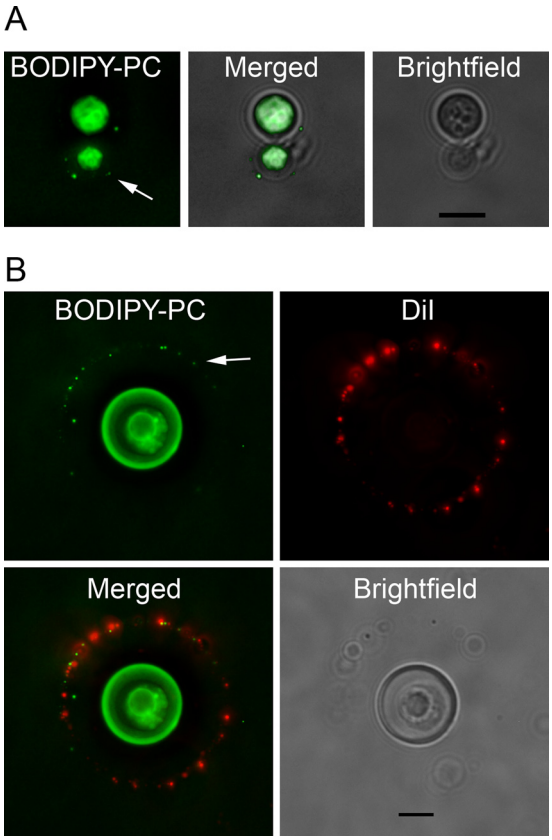


FIG. 6. Double staining of intact cells with DiI and fluorescent PC. (A) *C. neoformans* cells that were stained with a green fluorescent phospholipid probe, which resulted in a punctate staining on the edge of the capsule. (B) *C. neoformans* cells (B3501 isolate, capsule induced) were stained simultaneously 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 capsular 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 $DiIC_1(5)$ 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-

itive spots that were also stained with fluorescent PC, but without complete colocalization. The location of the stained structures matches previous ultrastructural experiments, which have occasionally located vesicles (19) and 3-OH oxylipin globules (21, 22) near the edge of the capsule in encapsulated *C. neoformans* cells. The punctate pattern we observed with BODIPY-PC also resembles the one reported after staining with human antibodies specific for 3-OH oxylipins (21, 22). Although these results do not allow us to conclusively link the punctate BODIPY-PC-stained structures with either vesicles or lipid-containing protuberances (21, 22), the results are consistent with both possibilities and indicate the existence of lipophilic regions in the capsule.

There were significant differences in the staining of *C. neoformans* with DiI, DiD-DS, and BODIPY-PC. Both DiI and DiD-DS were useful for studying purified vesicle preparations. However, when used to study whole cells, they bound to capsular polysaccharides and cell wall structures. In contrast, BODIPY-PC appears to be the most specific for cell-associated lipids, with the caveat that its staining is not as bright or stable as that of the other dyes. Hence, dialkylcarbocyanine dyes are more useful with purified vesicle preparations while BODIPY-PC is more suitable for studying whole cells. Staining of the cell wall with the dialkylcarbocyanines, but not with BODIPY-PC, was observed occasionally. Rodrigues et al. (20) reported the presence of glucosylceramide in the cryptococcal wall, which could provide the stained sites we observed. Lipophilic dye staining of the cell wall is both consistent with and supportive of the existence of lipids in the cell wall. The finding of lipids in the cell wall is relevant to models by which glycosylphosphatidylinositol-containing cell wall proteins are anchored in cell wall structures, with the important caveat that these dyes could also bind to cell wall polysaccharides given the affinity of DiI for isolated GXM.

Extracellular vesicles are increasingly recognized as a new secretion system in fungi that is likely to have a critically important role in fungal pathogenesis (15). In addition to the original discovery in *C. neoformans*, extracellular vesicles have been found in *Histoplasma capsulatum* and several other ascomycetes (1) and also in the parasite *Trypanosoma cruzi* (24), making them attractive for studies on pathogenesis and eventual development of novel therapeutic tools. The finding of hydrophobic regions on the polysaccharide capsule raises the question as to their physiological function and/or role in capsule structure. The lipophilic regions visualized with fluorescent probes on the capsule edge bear a tantalizing resemblance to vesicles and the lipophilic protuberances described by others (21, 22). Although the detailed answer to what these structures are must await future studies, we lean toward the interpretation that the lipophilic regions are related to the previously described 3-OH oxylipid-rich protuberances (21, 22) and/or transcapsular vesicles (18, 19).

In summary, we report the use of two dialkylcarbocyanines and a fluorescent phospholipid to study *C. neoformans* extracellular vesicles and cells. The results establish the feasibility and versatility of fluorescent lipid probes for the study of fungal vesicles. They also suggest that nucleic acids could be added to the list of possible vesicular cargo. The availability of lipophilic dyes that are suitable for light microscopy and flow cytometry significantly expands the range of available methodologies for vesicular and capsular studies.

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