

NBD- and BODIPY Dye–Labeled Sphingolipids

Introduction

Fluorescent sphingolipids are versatile probes of lipid trafficking in living cells.^{1,2} In addition, ceramide analogs produce selective staining of the Golgi complex for visualization by fluorescence microscopy. Molecular Probes' fluorescently labeled analogs of ceramides, sphingomyelins and glycosylceramides are prepared from *D-erythro*-sphingosine and therefore have the same stereochemical conformation as natural biologically active sphingolipids. Applications of these fluorescent lipid probes include:

Ceramides

- Fluorescent structural marker for Golgi Complex^{3,4}
- Outlining cellular boundaries to allow observation of morphogenetic movements by confocal microscopy⁵
- Tracing lipid metabolism and trafficking in living cells^{6,8}
- Measuring rates of lipid synthesis by Schwann cells⁹

Sphingomyelins

- Tracing lipid endocytosis pathways by fluorescence microscopy^{7,10,11}
- Analysis of acid sphingomyelinase expression by flow cytometry¹²

Glycosylceramides

- Cellular-level identification of lysosomal lipid storage disorder phenotypes^{13,14}
- Visualization of lipid domains associated with T cell antigen receptor activation¹⁵

The BODIPY FL and NBD fluorophores incorporated in Molecular Probes' fluorescent sphingolipids (Figure 1) have significantly different spectroscopic properties. The BODIPY FL fluorophore produces greater fluorescence output than NBD due to its higher molar absorptivity and fluorescence quantum yield.¹⁶ The BODIPY FL fluorophore is also more photostable than NBD. The photostability of NBD is sensitive to the presence of cholesterol, resulting in weak labeling of the Golgi complex in cholesterol-deficient cells.¹⁷ The most practically important spectroscopic property of the BODIPY FL fluorophore is an aggregation-dependent shift from green- to red-fluorescence emission due to intermolecular excimer formation. Consequently, structures that accumulate large amounts of BODIPY FL–labeled sphingolipids, such as the Golgi complex³ or the lysosomes of sphingolipid storage disease fibroblasts,^{7,13} exhibit red fluorescent staining that is clearly distinct from low-abundance green fluorescent staining. The BODIPY TR fluorophore exhibits red fluorescence (~617 nm) in its normal monomeric state; aggregation-dependent emission shifts corresponding to those of the BODIPY FL fluorophore have not yet been detected.

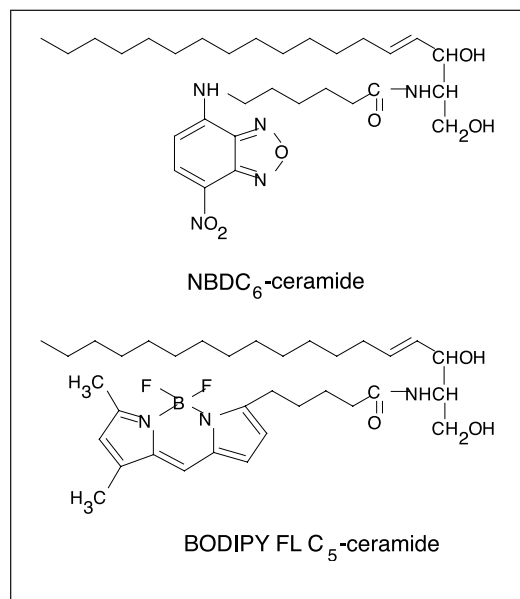
The transport and metabolism of labeled sphingolipids is somewhat dependent on the attached fluorophore.⁶ In some cases,

significant differences have been observed in the metabolic products derived from homologous BODIPY FL– and NBD-labeled sphingolipids.^{3,18} NBD-labeled sphingolipids have higher rates of transfer through aqueous phases than their BODIPY FL–labeled counterparts.¹⁹ Analysis of lipid incorporation and recycling by quantitative removal of labeled lipids from the plasma membrane outer surface (“back-exchange”) is more readily accomplished using NBD-labeled sphingolipids.²

Storage and Handling

With the exception of BODIPY FL C₁₂-sphingomyelin, all fluorescent sphingolipids are supplied in solid form and should be stored at –20°C, desiccated and protected from light. Ceramide analogs can be dissolved in DMSO or chloroform at concentrations of at least 1 mM. Sphingomyelins and glycosylceramides can be dissolved in DMSO or ethanol to the same extent. Delivery in the form of bovine serum albumin (BSA) complexes is recommended for labeling cells (see *Experimental Protocols*). BODIPY FL C₅-ceramide, NBD C₆-ceramide and BODIPY TR C₅ ceramide are also available as ready-made BSA complexes (B-22650, N-22651, B-34400). Additional BSA-complexed sphingolipids include BODIPY FL C₅-ganglioside (B-34401) and BODIPY FL C₅-lactosylceramide (B-34402). These complexes are supplied in lyophilized form and should be stored at –20°C, desiccated and protected from light. Procedures for reconstitution of the lyophilized BSA complexes are described in the *Experimental Protocols*. BODIPY FL C₁₂-sphingomyelin is supplied as a 1 mg/mL solution in DMSO which should be stored at –20°C, desiccated and protected from light.

Figure 1. BODIPY FL– and NBD-labeled ceramide analogs.



Experimental Protocols

Preparation of Sphingolipid–BSA Complexes

For staining of living and fixed cells, it is efficacious to add fluorescent sphingolipids in the form of complexes with BSA.⁴ BSA delivery complexes of fluorescent sphingolipids can be prepared as follows:

1.1 Prepare an approximately 1 mM sphingolipid stock solution in chloroform:ethanol (19:1 v/v). Molecular weights required for conversion of mass to molarity are printed on the product labels.

1.2 Dispense 50 μ L of sphingolipid stock solution into a small glass test tube and dry, first under a stream of nitrogen, and then under vacuum for at least 1 hour. Redissolve in 200 μ L of absolute ethanol.

1.3 Measure 10 mL of serum-free balanced salt solution such as Hanks' buffered salt solution + 10 mM HEPES, pH 7.4 (HBSS/HEPES) into a 50 mL plastic centrifuge tube. Add 3.4 mg (0.34 mg/mL) of defatted BSA.

1.4 Agitate the tube containing the 10 mL of the BSA solution on a vortex mixer. Inject the sphingolipid solution in ethanol (200 μ L) into the vortex. Store the resulting solution (5 μ M sphingolipid + 5 μ M BSA) in a plastic tube at -20°C .

Reconstitution of Ready-Made Ceramide–BSA Complexes (B-22650, N-22651, B-34400)

2.1 Dissolve 5 mg of the ready-made complex in 150 μ L of sterile deionized water. The resulting stock solution contains 0.5 mM sphingolipid and 0.5 mM BSA (1:1 mol:mol). Store unused portions of stock solution at -20°C .

2.2 Prepare a 5 μ M staining solution by diluting the stock solution 100-fold (e.g. add 10 μ L of stock solution to 1 mL of HBSS/HEPES).

Staining the Golgi Complex in Living Cells with Fluorescent Ceramides

3.1 Rinse cells grown on glass coverslips in an appropriate medium (such as HBSS/HEPES).

3.2 Incubate the cells for 30 minutes at 4°C with 5 μ M ceramide–BSA in HBSS/HEPES (prepared in step 1.4 or 2.2).

3.3 Rinse the sample several times with ice-cold medium and incubate in fresh medium at 37°C for a further 30 minutes.

Table 1. Spectral characteristics of NBD-, BODIPY FL- and BODIPY TR-labeled sphingolipids.

Label	Abs *	Em *
BODIPY FL	505	511 †
BODIPY TR	589	617
NBD	466	536

* Absorption and fluorescence emission maxima, in nm, determined in methanol. Values in labeled cells are similar. † BODIPY FL-labeled sphingolipids also emit red fluorescence (~ 620 nm) in cells.

3.4 Wash the sample in fresh medium and examine using a fluorescence microscope. Prominent labeling of the Golgi apparatus and weaker labeling of other intracellular membranes should be seen.

Staining the Golgi Complex in Fixed Cells with NBD C₆-Ceramide

It has been reported that NBD C₆-ceramide is suitable for staining of fixed cells, but that BODIPY FL C₅-ceramide and BODIPY TR ceramide are not.⁴ Conditions for staining appear to be generally noncritical. However, detergents and methanol/acetone fixatives should be avoided.⁴

4.1 Rinse cells grown on glass coverslips in HBSS/HEPES and fix for 5–10 minutes at room temperature in 0.5% glutaraldehyde/10% sucrose/100 mM PIPES, pH 7.0 or 2–4% paraformaldehyde in phosphate-buffered saline (PBS).

4.2 Rinse the sample several times with ice-cold HBSS/HEPES, transfer to an ice-bath and incubate for 30 minutes at 4°C with 5 μ M ceramide–BSA complex (prepared in step 1.4 or 2.2).

4.3 Rinse in HBSS/HEPES and incubate for 30–90 minutes at room temperature with 10% fetal calf serum or 2 mg/ml BSA to enhance golgi staining.

4.4 Wash the sample in fresh HBSS/HEPES, mount and examine by fluorescence microscopy.

Fluorescence Microscopy

Spectral characteristics of NBD-, BODIPY FL- and BODIPY TR-labeled sphingolipids are summarized in Table 1. Note that because BODIPY FL-labeled sphingolipids may emit either green or red fluorescence depending on their intracellular localization, they are generally unsuitable for multicolor imaging applications in which the second probe is a green-fluorescent protein (GFP) chimera or an antibody labeled with FITC (or other spectrally similar dyes). In applications of this type, BODIPY TR ceramide should be used for Golgi complex staining.²⁰

Staining for Electron Microscopy

BODIPY FL Br₂C₅-ceramide (D-7546) is designed for ultrastructural localization by electron microscopy (EM). In this staining procedure, excitation of the fluorophore is coupled to photooxidation of diaminobenzidine (DAB) to produce an electron-dense precipitate.²¹

5.1 Cells for EM staining should be grown in 35 mm-diameter plastic tissue culture dishes, not on glass coverslips.

5.2 Label living or fixed cells following the protocols described above. Living cells should be fixed after completion of the labeling procedure.

5.3 Prepare fresh diaminobenzidine (DAB) staining solution (15 mg of DAB dissolved in 10 mL of 0.1 M Tris, pH 7.6). Keep ice-cold until required for use.

5.4 Wash the cells in 0.1 M Tris, pH 7.6 and add 0.9 mL of DAB staining solution. Cover the culture dish and incubate in the dark for at least 10 minutes.

5.5 Irradiate the sample for 30 minutes on a fluorescence microscope using a low-magnification objective (10X or below).

5.6 Wash the sample at least five times in 0.1 M Tris, pH 7.6. Viewing the specimen under a dissecting microscope, make a mark on the inside of the culture dish using a needle, encircling the location of the brown DAB reaction product.

5.7 After rinsing the sample in 0.1 M sodium cacodylate (C₂H₆AsNaO₂), pH 7.4, treat the specimen with 1% osmium tetroxide (OsO₄) in 0.1 M sodium cacodylate, pH 7.4 for 60 minutes at room temperature.

5.8 Wash the specimen in 0.1 M sodium cacodylate, pH 7.4, dehydrate and embed in epoxy medium.

5.9 Cut out the region of the dish identified by the mark made in step 5.6 and mount for thin-section electron microscopy.

References

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Product List *Current prices may be obtained from our Web site or from our Customer Service Department.*

Cat #	Product Name	Unit Size
B-22650	BODIPY® FL C ₅ -ceramide complexed to BSA	5 mg
B-13950	BODIPY® FL C ₅ -ganglioside G _{M1}	25 µg
B-34401	BODIPY® FL C ₅ -ganglioside G _{M1} complexed to BSA.....	1 mg
B-34402	BODIPY® FL C ₅ -lactosylceramide complexed to BSA	1 mg
B-34400	BODIPY® TR C ₅ -ceramide complexed to BSA	5 mg
N-22651	NBD C ₆ -ceramide complexed to BSA	5 mg
N-1154	6-((N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino) hexanoyl)sphingosine (NBD C ₆ -ceramide)	1 mg
D-3521	N-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-pentanoyl)sphingosine (BODIPY® FL C ₅ -ceramide).....	250 µg
D-7540	N-((4-(4,4-difluoro-5-(2-thienyl)-4-bora-3a,4a-diaza-s-indacene-3-yl)phenoxy)acetyl)sphingosine (BODIPY® TR ceramide)	250 µg
D-7546	N-(2,6-dibromo-4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-pentanoyl)sphingosine (BODIPY® FL Br ₂ C ₅ -ceramide).....	250 µg
D-13951	N-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-pentanoyl)sphingosyl 1-β-D-lactoside (BODIPY® FL C ₅ -lactosylceramide)	25 µg
D-3522	N-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-pentanoyl)sphingosylphosphocholine (BODIPY® FL C ₅ -sphingomyelin).....	250 µg
N-3524	6-((N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino) hexanoyl)sphingosylphosphocholine (NBD C ₆ -sphingomyelin).....	1 mg
D-7519	N-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-dodecanoyl)sphingosyl 1-β-D-galactopyranoside (BODIPY® FL C ₁₂ -galactocerebroside).....	25 µg
D-7547	N-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-dodecanoyl)sphingosyl 1-β-D-glucopyranoside (BODIPY® FL C ₁₂ -glucocerebroside).....	250 µg
D-7548	N-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-pentanoyl)sphingosyl 1-β-D-glucopyranoside (BODIPY® FL C ₅ -glucocerebroside).....	250 µg
D-7711	N-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-dodecanoyl)sphingosylphosphocholine (BODIPY® FL C ₁₂ -sphingomyelin) *1 mg/mL in DMSO*	250 µL

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