

Pagano Lab – Protocol

Use of Fluorescent Analogs of Ceramide

REFERENCE

Pagano, R.E. and Martin, O.C. Use of fluorescent analogs of ceramide to study the Golgi apparatus of animal cells. *In Cell Biology: A Laboratory Handbook*, Julio E. Celis, Editor. Academic Press, 1994, pp. 387-393.

PROCEDURES

A. Preparation of NBD- or BODIPY-Cer/BSA complexes.

These complexes are used for subsequent labeling of living or fixed preparations of cells (see below). We routinely prepare them as either dilute (5 nmol/ml) or concentrated (0.5 nmol/ μ l) stock solutions. This procedure is also useful for preparing BSA complexes of other NBD- or BODIPY-lipids

Dilute (5 nmol/ml) stock solutions:

Solutions:

1. Approximately 1 mM C₆-NBD-Cer or C₅-DMB-Cer stock solution in chloroform/methanol (19:1, v/v). C₆-NBD-Cer (Cat. No. N-1154) and C₅-DMB-Cer (Cat. No. D-3521) are from Molecular Probes, Inc.
2. 10 ml serum-free balanced salt solution containing 0.34 mg defatted BSA/ml in a 50 ml plastic centrifuge tube. For the balanced salt solution, we routinely use HMEM (10 mM 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid-buffered minimal essential medium, pH 7.4, without indicator).
3. 500 ml serum-free balanced salt solution for dialysis.

Steps

1. Dispense 50 nmol C₆-NBD-Cer or C₅-DMB-Cer in chloroform/methanol into a small glass test tube and dry, first under a stream of nitrogen, and then *in vacuo* for at least one hour;
2. Dissolve dried Cer in 200 μ l absolute ethanol;
3. Inject Cer into the 10 ml BSA solution (while vortex mixing);
4. Rinse Cer/ethanol tube with a little of the Cer/BSA solution and combine with the Cer/BSA complex;
5. Dialyze overnight at 4°C against 500 ml serum-free balanced salt solution;
6. Recover in 10 ml balanced salt solution and store in a plastic tube at -20°C.

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Concentrated (0.5 nmol/ μ l) stock solutions:

Solutions:

1. Approximately 1 mM C₆-NBD-Cer or C₅-DMB-Cer stock solution in chloroform/methanol (19:1, v/v);
2. 450 μ l serum-free balanced salt solution containing 250 nmol DF-BSA in a small glass test tube. For a balanced salt solution we use HCMF (10 mM 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid-buffered Puck's saline without calcium and magnesium).

Steps:

1. Dispense 250 nmol C₆-NBD-Cer or C₅-DMB-Cer from the chloroform/methanol stock solution into a small glass test tube and dry down, first under a stream of nitrogen, and then *in vacuo* for at least one hour;
2. Add 50 μ l ethanol to the dried Cer. Vortex mix to completely dissolve the sample;
3. Using a micropipette, add the ethanol solution of the fluorescent Cer to the DF-BSA solution while vortex mixing;
4. Rinse the tube that contained the ethanol solution with an aliquot of the fluorescent Cer/DF-BSA complex;
5. Transfer the Cer/DF-BSA complex to a plastic conical centrifuge tube and store at -20°C.

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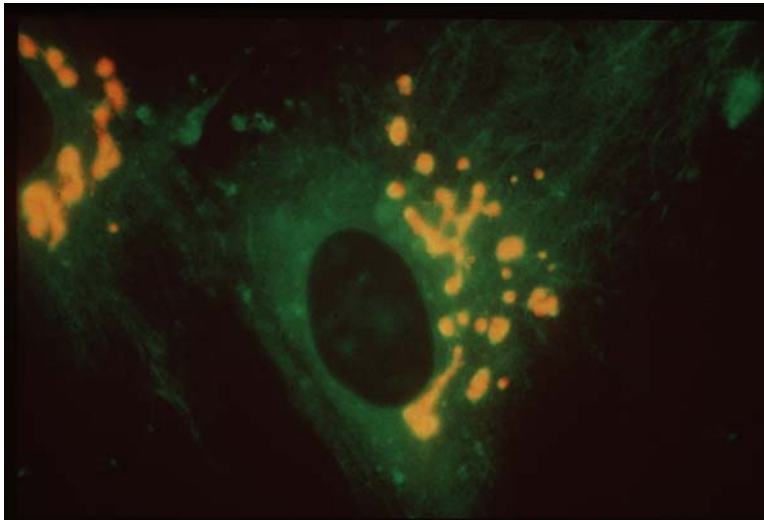
B. Staining the Golgi apparatus of living cells with fluorescent Cer analogs.

Solutions:

1. 5 nmol/ml or 0.5 nmol/ μ l C₆-NBD- or C₅-DMB-Cer/BSA complex;
2. HMEM for cell incubations.

Steps:

1. Cells grown on glass cover slips or on plastic tissue culture dishes are rinsed in HMEM and transferred to an ice-water bath at 2°C;
2. The cells are then incubated for 30 min at 2°C with 5 nmol/ml C₆-NBD-Cer/BSA or C₅-DMB-Cer/BSA in HMEM;
3. The samples are rinsed several times with ice cold HMEM, transferred to 37°C, and further incubated for 30 min;
4. The samples are then washed in HMEM and observed under the fluorescence microscope. Prominent labeling of the Golgi apparatus and weaker labeling of other intracellular membranes by the fluorescent Cers will be seen.



Photomicrograph of a human skin fibroblast treated with BODIPY-Cer and photographed in the living state. Sample was excited with blue light and images were acquired at green + red wavelengths. The Golgi apparatus appears red/orange in color due to excimer formation at high concentrations in the membranes of this organelle.

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C. Staining the Golgi apparatus of fixed cells with fluorescent Cer analogs.

Solutions:

1. HMEM for rinsing cells;
2. 0.5% glutaraldehyde/10% sucrose/100 mM PIPES, pH 7.0;
3. HCMF;
4. *Optional:* freshly prepared NaBH₄ in HCMF (0.5 mg/ml);
5. 5 nmol/ml or 0.5 nmol/μl C₆-NBD-Cer/BSA complex (does not work well using C₅-DMB-Cer/BSA complex);
6. 3.4 mg/ml DF-BSA in HCMF.

Steps:

1. Cells grown on glass cover slips or on plastic tissue culture dishes are rinsed in HMEM and fixed for 5-10 min at room temperature in 0.5% glutaraldehyde/10% sucrose/100 mM PIPES, pH 7.0.
2. The cells are washed in HCMF. [Optional: The samples can be transferred to an ice-water bath and incubated (3 x 5 min) with NaBH₄ in ice-cold HCMF to reduce glutaraldehyde-induced autofluorescence. For most cell types and fixation conditions, the staining of the Golgi apparatus is so prominent and autofluorescence is so low, that this step is not necessary.]
3. The samples are rinsed several times with ice-cold HCMF, transferred to an ice-water bath, and incubated for 30 min at 2°C with 5 nmol/ml C₆-NBD-Cer/BSA complex;
4. The samples are rinsed several times with HCMF and incubated at room temperature (4 x 30 min) with 3.4 mg/ml defatted BSA in HCMF. [This incubation serves to remove (“back-exchange”) excess C₆-NBD-Cer from the fixed cells (Pagano *et al.*, 1989)];
5. The samples are then washed in HCMF and observed under the fluorescence microscope. Prominent labeling of the Golgi apparatus by C₆-NBD-Cer is seen.