

Pagano Lab - Protocol

Endocytosis and Trafficking of BODIPY-LacCer

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A. Preparation of BODIPY-LacCer/BSA complex (see JCB 1994 125:769-781)

1. BODIPY-LacCer is available from [Molecular Probes](http://www.probes.com), catalog no. D-13951 (for structure, see <http://www.probes.com/servlets/structure?item=13951>). Aliquot 250 nmole of **BODIPY-LacCer** into a glass tube, dry under nitrogen, and vacuum for 1 hr (e.g., take 0.5 ml from 0.5 mM stock of **BODIPY-LacCer**).
2. Prepare 300 μ l of HMEM HEPES-buffered minimal essential medium; see below) containing 250 nmole of fatty acid free BSA in a glass tube. [Mix 250 μ l of 1mM (68mg/ml) with 50 μ l of HMEM].
3. Add 200 μ l of ETOH to the dry **BODIPY-LacCer** from step 1 and vortex until **BODIPY-LacCer** is dissolved.
4. Add **BODIPY-LacCer** ethanol solution one drop at a time to the BSA solution from step 2.
5. Dialyse overnight against 1X PBS. Use MW cutoff 12-14,000 dialysis tubing. Change PBS 4-6 times.
6. Spin 2 times in ultra-centrifuge for 20 min at 100,000 x g, 4°C.
7. Determine concentration of **BODIPY-LacCer/BSA** complex by measuring relative fluorescence intensity against known standards.

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B. Fibroblast Cell Culture

1. Normal cells (negative control)-Normal human skin fibroblasts (GM 5659 from Coriell).
2. Positive control for LacCer storage disease assay-Niemann Pick C cells (e.g., GM 3123 from Coriell).
3. Patient sample- fibroblast from skin biopsy.
4. Culture cells in EMEM media containing 10% FBS, penn/strep, L-glutamine, and non-essential amino acids in 35 mm tissue culture dishes containing glass cover slips.
5. Culture fibroblast should be grown to 40-60% confluency for assay.

C. Endocytosis of BODIPY-LacCer- general method

(see JCB 2001 154:535-547, MBC 2003 14:3254-3265, JBC 2003 278:7564-7672)

1. Rinse cell samples 2-3 X with HMEM + glucose (HMEM+G)
2. Incubate 1-5 μ M BODIPY-LacCer with cells at 10°C for 30 min (loads plasma membrane with lipid analog).
3. Rinse cells 2-3 times with HMEM+G at 10°C.
4. Incubate cells at 37° C (various time points; e.g., 1-30 min) (allows endocytosis and subsequent trafficking).
5. Rinse cells with cold HMEM and then back exchange at 11°C, with 5% fatty acid-free BSA in HMEM 6 X over 60 min (removes remaining plasma membrane lipid).
6. View cells under fluorescence microscope. Excitation 450-490nm. Green emission, 520-560 nm (like fluorescein). Red emission, \geq 590 nm. See Protocol on "MICROSCOPE FILTERS FOR OBSERVING BODIPY-FLUORESCENCE" for further details.

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D. BODIPY-LacCer assay for sphingolipid storage diseases (SLSDs) (see PNAS 1998 95:6373-6378; Lancet 1999 354:901-905)

BODIPY-LacCer Pulse:

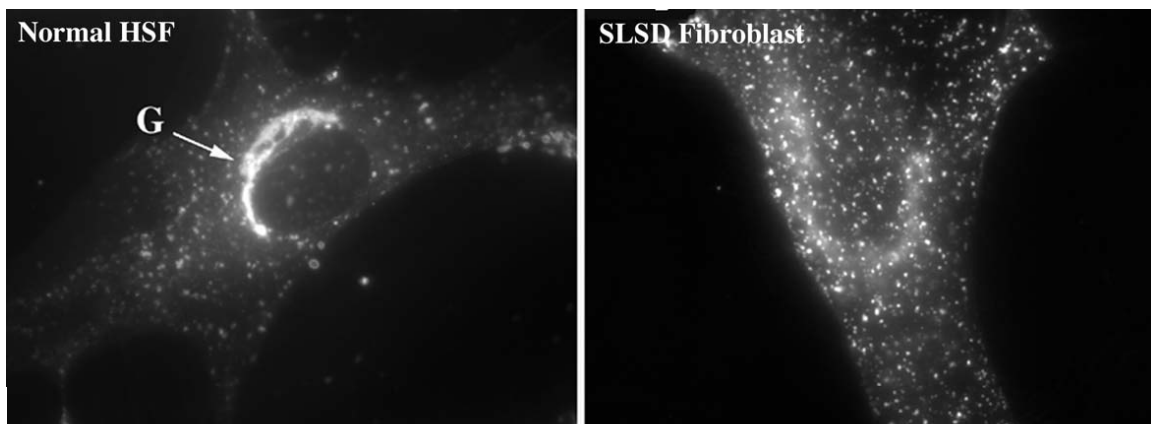
1. Wash cells 2-3 times with 0% FBS/EMEM culture media.
2. Add 5 μ M Bodipy LacCer/BSA in 1 ml of 1% FBS/EMEM to cells.
3. Incubate for 45 min in the 37°C 5% CO₂ incubator.
4. Wash cells with 1% FBS/EMEM 2-3 times.

Chase:

5. Add 1.5 – 2 ml of 1% FBS/EMEM.
6. Incubate 60 minutes in the 37°C 5% CO₂ incubator.
7. Wash cells 1-2 times with HMEM minus glucose.
8. Back-exchange at 10°C, 6 X 10 min with 5% fatty acid-free BSA in HMEM minus glucose.

Observe and Score:

9. Observe under fluorescence scope. Score as normal (predominately bright Golgi fluorescence) or storage disease like (predominately bright punctate endosome/lysosome fluorescence as in NPC cells. Example is shown in Figure below.



BODIPY-LacCer storage disease assay. Normal or SLSD fibroblasts were pulse labeled with BODIPY-LacCer. Note the Golgi staining (“G”) in the normal cell sample and the punctate endosomal staining in the SLSD cell type.

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E. BODIPY-LacCer Assay for NPC Variants

(see Am. J. Hum. Genet. 2001 68:1361-1372)

1. Plate cells as in the Lipid Storage Disease LacCer Assay.
2. Add 150 µg/ml of LDL to normal HSF, NPC and patient cells 24 hours before assay.
3. Proceed with Lipid Storage Disease LacCer Assay (see above).
4. Variant NPC cells will appear normal with mostly Golgi staining without excess cholesterol. Variants loaded with excess cholesterol will have a punctate endosome/lysosome appearance like NPC cells.

F. Preparation of HMEM

Combine in ~500 ml of distilled water

	grams	MW	mM for 10 X	mM for 1 X	
HEPES acid	32.84		238.3	138	13.8
NaCl	80		58.44	1370	137
KCl	4		74.55	54	5.4
Glucose	10		180.2	55	5.5 Exclude for HMEM minus glucose
Glutamine	2.92		146.1	20	2.0
KH ₂ PO ₄	0.6		136.1	4.4	0.4
Na ₂ HPO ₄	0.254		142	1.79	0.18

100 X MEM vitamins **100 ml** from Gibco-BRL cat. no. 11120-052

50 X MEM amino acids **200 ml** from Gibco-BRL cat. no. 11130-051

2. pH with NaOH to 7.4, and then bring up to **1 l final volume**
3. Filter through 0.2 µm filter
4. Store at 4°C (usually make 25-50 ml aliquots)

Separately prepare 100 X CaCl₂ and MgSO₄ stocks

	grams	ml	MW	mM for 100 X	mM for 1 X
CaCl ₂ •2H ₂ O	1.85		100	147	1.25
MgSO ₄ •7H ₂ O	2		100	246.5	8.1

For 1 X HMEM solution to use with cells: Dilute 10 X HMEM, 100 X CaCl₂ and 100 X MgSO₄ stocks in water and filter through 0.2 µm filter (e.g., 10, 1, and 1 ml with 88 ml water).