Supplementary material for Mondal et al. entitled "Sterols are mainly in the cytoplasmic leaflet of the plasma membrane and the endocytic recycling compartment of CHO cells"

Supplementary Methods and Results:

Replacement of ~50% cellular cholesterol with DHE in M19 cells:

M19 cells are a partial sterol auxotroph CHO cell line (Hasan *et al.*, 1994) which we used to obtain a high degree of sterol replacement (Xu *et al.*, 2005). For long term growth assays in presence of DHE, M19 cells were plated in medium A (1:1 mixture of Ham's F12 and Dulbecco Modified Eagle's Medium (DMEM) containing 100 units/ml penicillin and 100 μg/ml streptomycin sulfate supplemented with 5% fetal bovine serum /1 mM sodium mevalonate/20 μM sodium oleate/5 μg/ml cholesterol in ethanol) at 500,000 cells per 10 cm plate. The following day the cells were washed with Hanks Balanced Salt Solution (HBSS) three times and switched to medium B (1:1 mixture of Ham's F12 and DMEM containing 100 units/ ml penicillin and 100 mg/ ml streptomycin sulfate supplemented with 10% Lipoprotein Depleted Serum (LPDS)/1 mM sodium mevalonate/20 μM sodium oleate and 10 μg/ml DHE in ethanol). Before each use, Medium B was centrifuged at 43,000 x g for 40 minutes at 4° C to remove DHE aggregates. Cells were given fresh media on alternate days, and confluent cells were passaged onto new plates every 4-5 days.

Measurement of cellular sterol content:

For measurement of cellular sterol content by gas chromatography (Shiratori *et al.*, 1994), cellular lipids were extracted twice with hexane:isopropyl alcohol (3:2, v/v). Extracted lipids were dried under argon, resuspended in hexane, and separated on a Hewlett-Packard gas chromatograph (HP 5890 series II; Palo Alto, CA) equipped with a flame-ionization detector. A 15 m x 0.53 mm HP-5 capillary column coated with 1.5 µm film thickness of 5% phenyl methyl siloxane was used to separate free cholesterol and DHE. The injection temperature was maintained at 255°C, and oven temperature was held isothermally at 260°C using helium as a mobile phase at a flow rate of 30 ml/min. The free sterol content of each well was quantified using ß-sitosterol as the internal standard. After lipid extraction, cells were lysed with 0.1 M sodium hydroxide solution, and the protein content of each well was measured using a Bio-Rad DC protein assay kit (Bio-Rad Laboratories, Hercules, CA).

Quenching of PM-associated DHE fluorescence by long chain lipid quencher:

To be certain that our results did not reflect the unique chemistry of a specific quencher, we tested several membrane-associated lipid quenchers. The quencher, 12-SLPC , transferred from albumin into the membrane within a few seconds (Marx *et al.*, 1997), and the transbilayer movement of this lipid is very slow compared to our experimental time scale (Seigneuret and Devaux, 1984). Thus, this long chain lipid quencher would quench fluorescence exclusively in the outer leaflet of the PM. As a control for DHE quenching, we tested the ability of 12-SLPC to quench C_{12}-NBD-SM. NBD-labeled SM is incorporated into the outer leaflet of the PM, and flipping is slow because of the polar headgroup (Koval and Pagano, 1989; Mayor *et al.*, 1993).

Cells were labeled with DHE to steady state and imaged (Supp. Figure 4A). The spin-labeled lipid quencher, 12-SLPC (0.6 mM) was added to the cells, and the same field was imaged again within a minute (Supp. Figure 4B). 12-SLPC did not quench DHE fluorescence efficiently in the intact cells. In contrast, this quencher quenched the fluorescence of PM-associated C_{12}-NBD-SM efficiently (>50%) (Sup. Figure 4C,D). Quantification of the results is shown next to the images. 12-SLPC quenches DHE fluorescence efficiently when both the fluorophore and the quencher are incorporated into liposomes (data not shown). Quenching with 5-SLPC or with the di-brominated lipids 6,7-Br_2PC or 11,12-Br_2PC was similar to that shown here for 12-SLPC (data not shown).

Supplementary Figure Legends:

Supp. Figure 1. Fluorescence intensity of DHE in artificial vesicles made of lipids those found either in the outer leaflet or inner leaflet of the plasma membrane and effect of cholesterol content on the fluorescence intensity of DHE.

Multilamellar vesicles of different lipid compositions were made by dispersion of dried lipids in phosphate buffered saline (10 mM NaH_2PO_4, 140 mM NaCl, pH 7.4) with vortex mixing at 37°C. Compositions of different MLV s are summarized in the figure. All the MLV s contain equal amount (5 mole%) of DHE. If the same increase in quantum yield in outer leaflet lipids occurred in cells, it would lead to overestimation of the relative amount of DHE in the outer leaflet.

Supp. Figure 2. Regions used to measure the fluorescence intensity in the plasma membrane after quencher addition.

TRVb1 cells were labeled with DHE-MβCD (A-F) for 1 min at 37°C, washed and chased for 30 min. Before TNBS addition, DHE was distributed at the PM and the ERC (A,D). 10 mM TNBS was added on the microscope stage, and images of the same fields were taken within 1 min (B,E). Images C & F are the same as B & E respectively, but their intensity values were corrected for photobleaching by multiplying intensity values by an average correction factor (7% and 9% for images C & F respectively) that was determined in control photobleaching experiments. All images were background corrected using off-cell fluorescence intensity. Scale bar: 10 μm. Boxes (0.75 x 0.75 μm) in the ring like plasma membrane regions (A-C) or boxes (1.5 x 1.5 μm) in peripheral regions of the flat cells (D-F) were drawn and average of integrated intensity of those boxes before and after TNBS addition are shown next to the respective images.

Supp. Figure 3. Concentration dependence of quenching plasma membrane associated DHE in permeabilized cells by extracellular TNBS.

TRVb1 cells were labeled with DHE-MβCD and permeabilized with Streptolysin-O (SLO) as for Figure 3. Separate dishes were used for quenching with different concentrations of TNBS. Amount of plasma membrane associated DHE quenching in permeabilized cells has been plotted against TNBS concentration.

Supp. Figure 4. Spin labeled lipid quencher, 12-SLPC, restricted to the outer leaflet of PM, quenches C_{12}-NBD-SM but not DHE fluorescence.

TRVb1 cells were labeled with DHE-MβCD as described in the legend to Figure 2. DHE was distributed at the PM and the ERC (A). The lipid fluorescence quencher, 12-SLPC was added at a concentration of 0.6 mM, from a solution containing Medium 1, 2 g/l (w/v) glucose and 20 mg/ml (w/v) fatty acid free BSA. The same field is shown after the addition of 12-SLPC (B). TRVb1 cells were labeled with C_{12}-NBD-SM at 4°C for 10 min, washed and imaged immediately (C). At this time, C_{12}-NBD-SM only labels the PM. The same field is shown after addition of 12-SLPC (D). All images shown here have been background corrected using off-cell fluorescence intensity as background. Scale: 10 μm. The quenching of fluorescence in 30-50 cells was quantified and has been shown next to the images. Scale bar: 10 μm. Error bars are standard deviations of quenching values.

Supp. Figure 5. 4-SLPC quenches half the DHE fluorescence in large unilamellar liposomes with a symmetrical inter-leaflet DHE distribution.

Large unilamellar vesicles of different lipid compositions that mimic those in either outer leaflet or inner leaflet of plasma membranes, were prepared by extrusion method (Mayer *et al.*, 1986). Composition of vesicles mimicking outer leaflet lipids were POPC: Egg SM: Cholesterol 2.3: 1.65: 1, plus 2.5 mole% DHE and 1 mole% Biotinylated PE (Schroeder *et al.*, 1998; Dietrich *et al.*, 2001). Composition of vesicles mimicking inner leaflet lipids were POPC: POPE: POPS: Cholesterol 1.35: 1.35: 1.35: 1, plus 2.5 mole% DHE and 1 mole% Biotinylated PE(Wang and Silvius, 2001). The liposomes were then immobilized on streptavidin-coated coverslip dishes. The concentration dependence of quenching LUV associated DHE fluorescence by 4-SLPC is shown by solid lines for

outer leaflet lipid mixtures (o) and by dashed lines for inner leaflet lipid mixtures (●). Separate dishes were used for quenching with different concentrations of 4-SLPC.

Supp. Figure 6. Microinjection of 4-SLPC into the cytosol quenches PM and ERC associated DHE fluorescence significantly, but not PM and ERC associated C_6-NBD-SM fluorescence.

TRVb1 cells were co-labeled with DHE-MβCD and Tf-Alexa 633. (A,C) images of DHE and Tf respectively; (B,D) the same fields after injection with 25 mM 4-SLPC (yielding about 1 mM 4-SLPC in the cytosol). The ERC morphology (arrowhead) is not altered after microinjection as judged by the intact Tf distribution and intensity in these cells. TRVb1 cells were labeled with C_6-NBD-SM (E) for 1min at 37 $^{\circ}$C, washed and chased for 15 min, further incubated with Tf-Alexa 633 (G) for 10 min, in order to reach the steady state distribution of both probes at the PM and ERC. (F,H) The same fields after injection with 25 mM 4-SLPC. Note that part of the PM outer leaflet associated C_6-NBD-SM fluorescence was quenched due most probably to continuous flow of SLPC from the needle during injection. Images were background corrected using off-cell fluorescence intensity. Displayed images were corrected for photobleaching. Scale bar: 10 μm.

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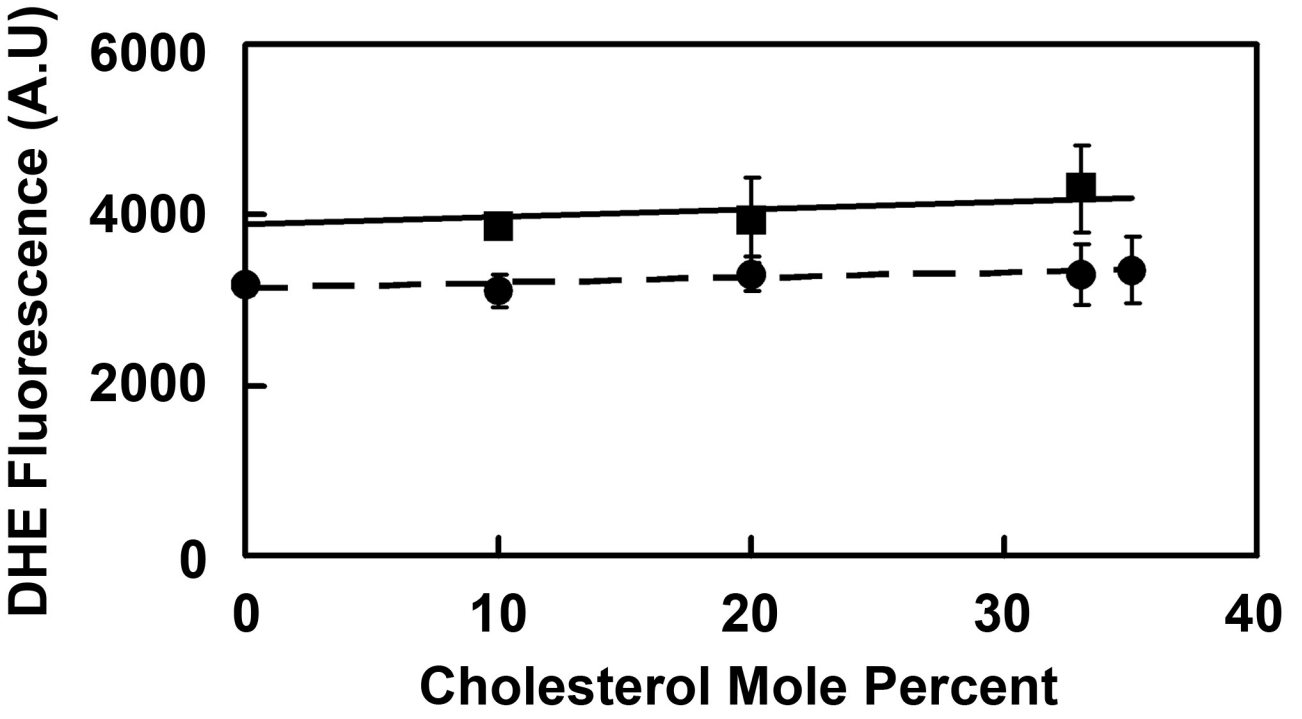


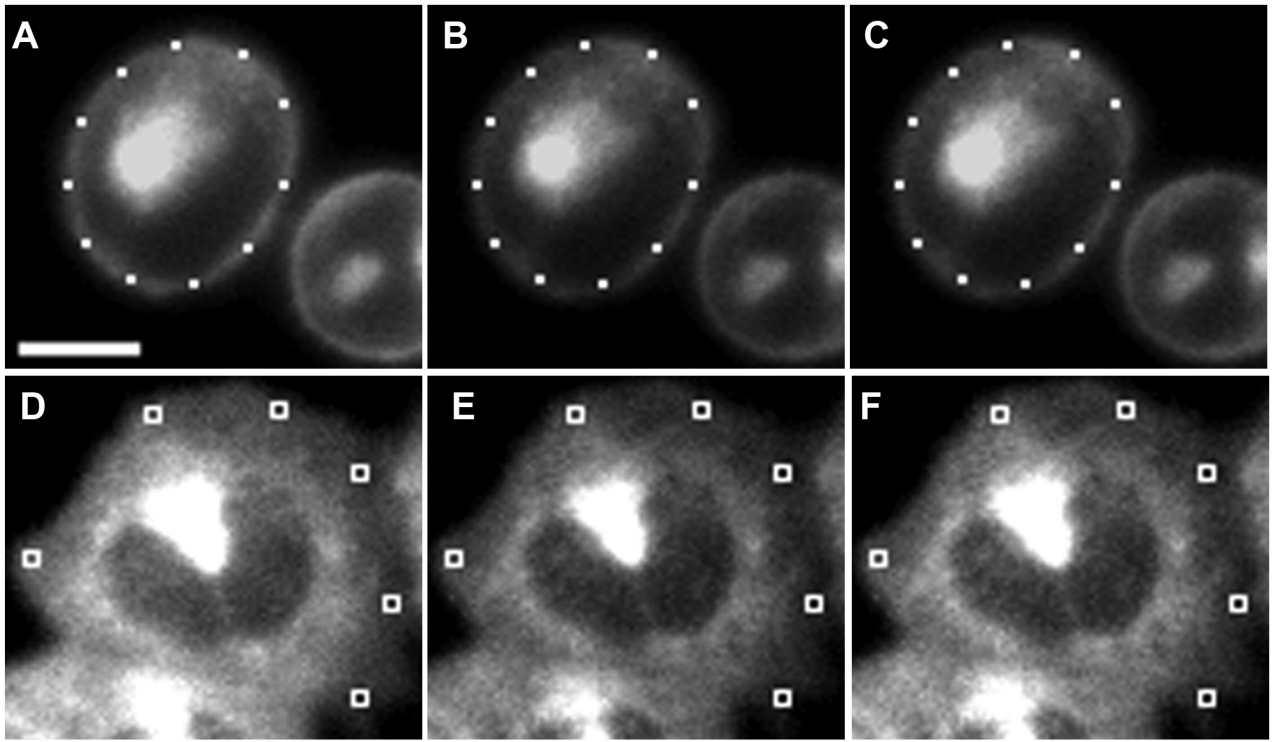
Outer Leaflet-mix liposomes ■

CHL	10	20	33	mol%
POPC	56	46	33	
SM	33	33	33	

Inner Leaflet-mix liposomes ●

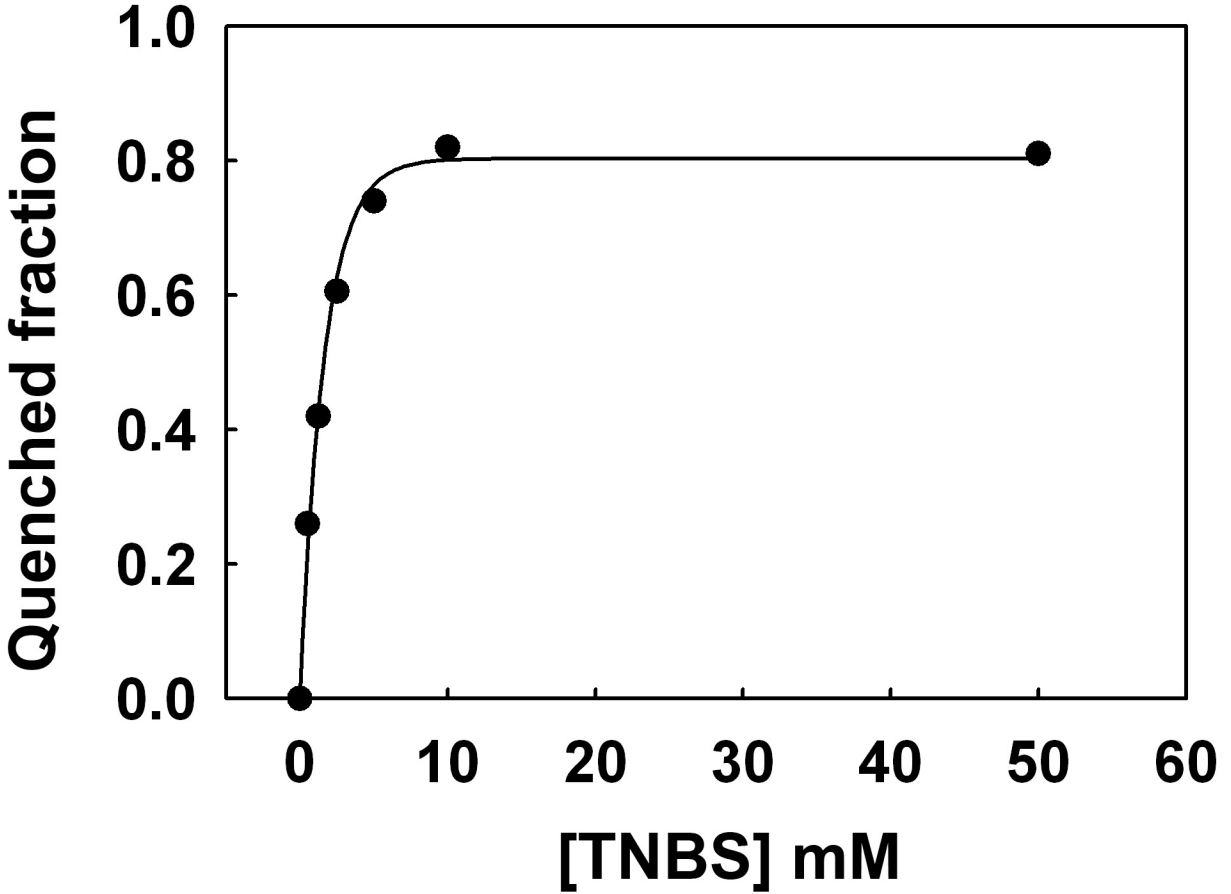
CHL	0	10	20	33	35	mol%
POPC	33	30	27	30	21.6	
POPE	33	30	27	30	21.6	
POPS	33	30	27	7	21.6	

Supp. Figure 1

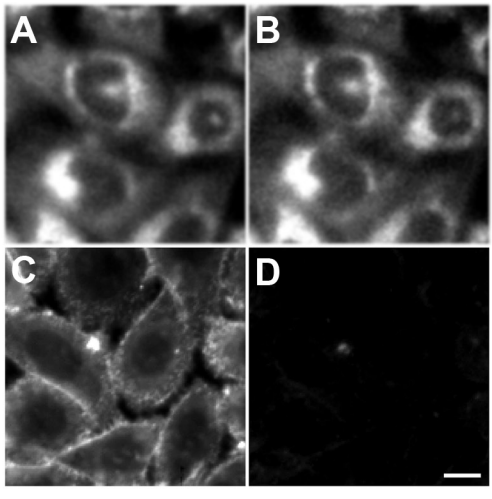


	- TNBS	+ TNBS		Quenching
		Uncorrected	After photobleaching correction	
	1363	993	1066	22 %
	2750	2175	2390	13%

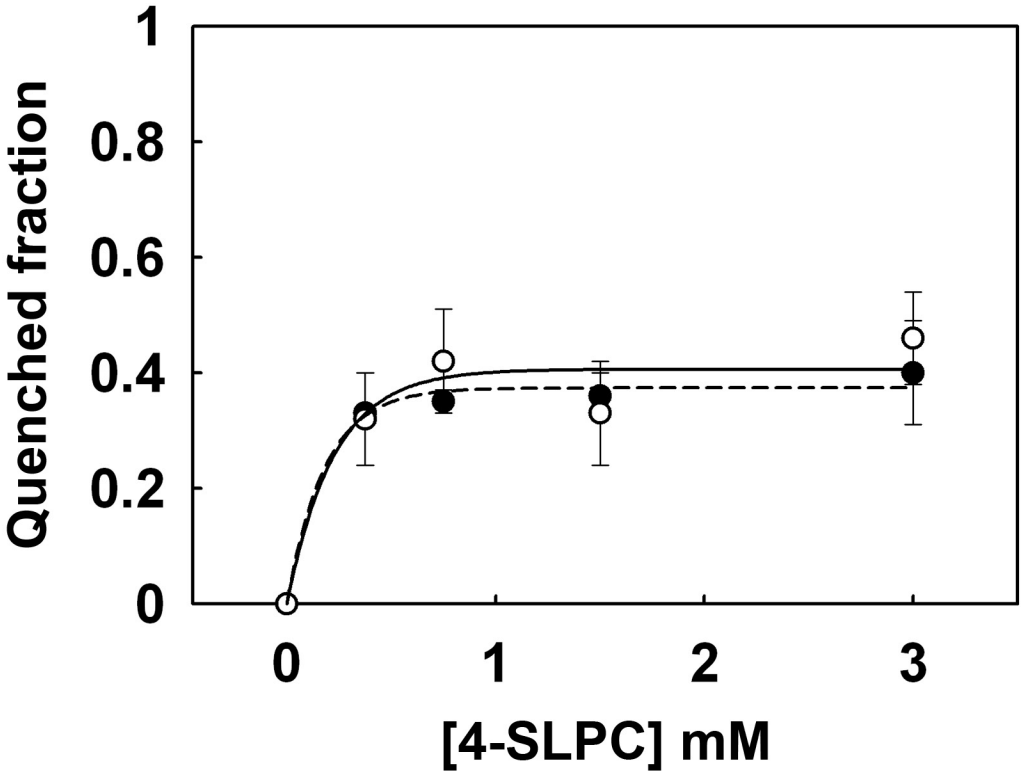
Supp. Figure 2



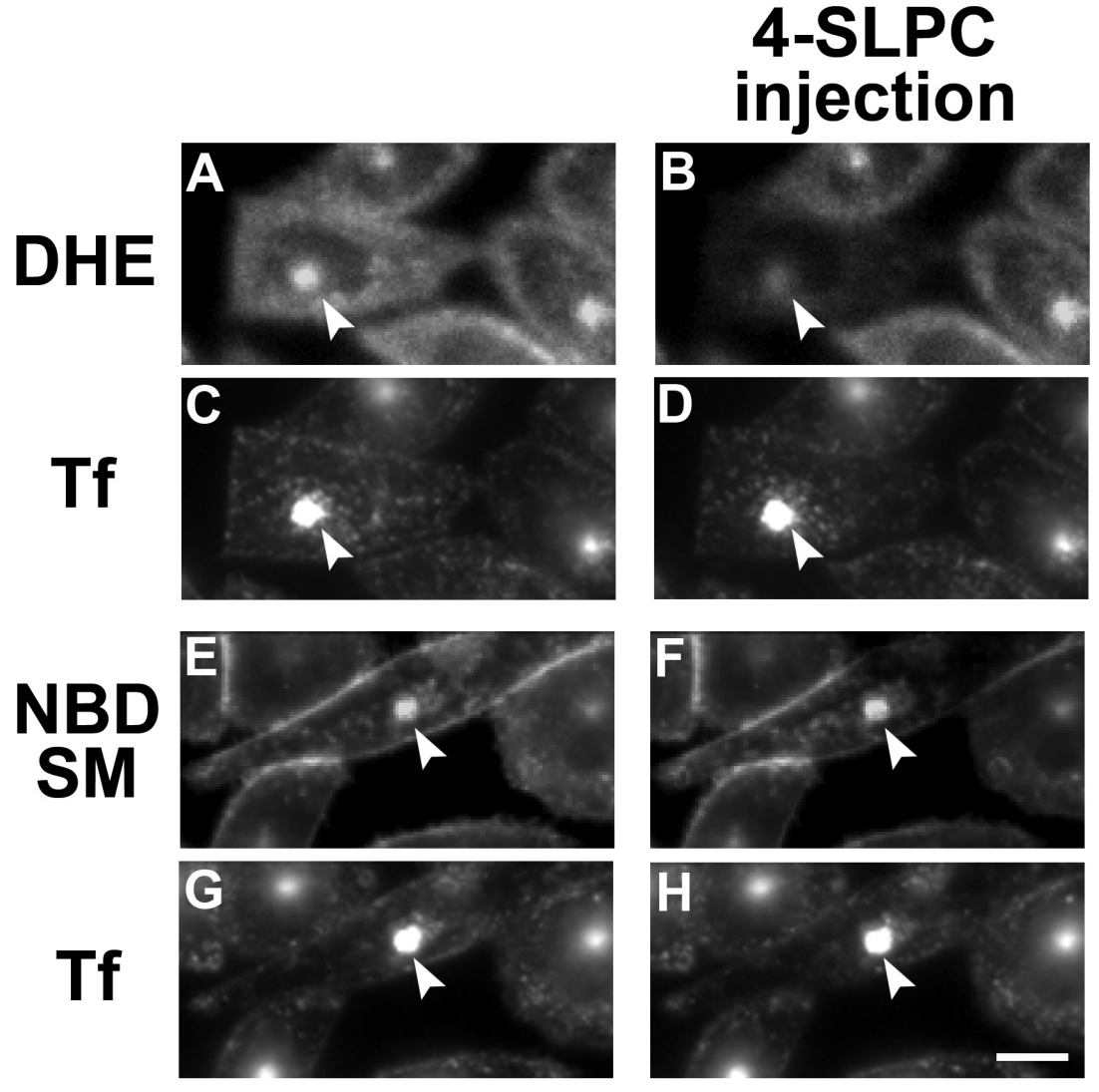
Supp. Figure 3



Supp. Figure 4



Supp. Figure 5



Supp. Figure 6