USE OF DANSYL-CHOLESTANOL AS A PROBE OF CHOLESTEROL BEHAVIOR IN MEMBRANES OF LIVING CELLS[†]

SUPPLEMENTAL MATERIALS

Supplemental Method 1.

DChol (6-dansyl-cholestanol) Synthesis.

DChol was synthesized and purified based on a previously published method (1) with modifications. Briefly, 6-ketocholestanol was stirred with 5 x molar excess of dansyl hydrazine and catalytic amounts of concentrated HCl in methanol at 65^{o}C overnight. The progress of the reaction was monitored by TLC (Hexane:Ethanol = 8:1). Methanol was evaporated, and DChol was separated from the majority of the starting material and other impurities by flash column chromatography using 60Å, 230-400 mesh silica gel (VWR) eluded with Hexane : Ethanol = 8 : 1 (v/v). DChol was further purified by repeating flash column chromatography as described above and recrystallization from methanol. The purity of the final product was checked by TLC (not shown) and HPLC to be >95%. The molecular mass of the purified DChol was verified by positive atmospheric pressure chemical ionization (APCI+) mass spectrometry (Mass spectrometry service center, Texas A&M University). The M+ ion of DChol exhibited a mass of 649 kDa, consistent with that expected from the structure. Fluorescence excitation and emission maxima of the purified DChol in ethanol were 336 nm and 522 nm, respectively, as expected for the dansyl group.

Supplemental Method 2.

Determination of Critical Micelle Concentration (CMC) of Cholesterol and DChol.

The CMC of cholesterol and DChol were determined by measuring light scatter as a function of increasing cholesterol or DChol concentrations in water. The sterols were added from an ethanolic stock solution (0-2 μl, less than 0.1% v/v) with a Hamilton syringe into 2 ml of water under vigorous vortexing. Light scatter was measured using with Cary Eclipse Fluorescence Spectrometer (Varian Inc., Palo Alto, CA) with excitation and emission wavelengths both set at 380 nm. The following control experiments were carried out, (i) light scatter was measured at increasing DChol concentration in dioxane, under which condition there was no micelle formation; (ii) light scatter was measured when increasing amount of ethanol (0-2 μl, without DChol) was added to water. In both cases no significant light scatter increase was observed (data not shown). Light scatter, after correction for light scatter from water, was plotted versus lipid concentration. The CMC was determined as the x intercept of linear regression line where rapid light scatter increase was observed.

Supplemental Method 3.

Determination of Apparent Binding Constants of Cholesterol and DChol to Methyl-β-cyclodextrin (MβCD).

The dissociation constant (K_d) of cholesterol and DChol binding to MβCD was determined based on an established method (2). Briefly, cholesterol (or DChol) stock solutions (in $CHCl_3$:MeOH = 1:1) were measured into acid washed brown glass vials, dried under N_2, resuspended in water, and bath sonicated for 15min. The suspensions were divided into aliquots, to which different amounts of MβCD were added to yield samples with cholesterol (or DChol) final concentration of 0.15 mM and MβCD final concentrations of 0-40 mM. The samples were kept in the 37^{o}C shaker for 16 hours, cooled to room temperature, and absorbance at 450 nm

(due to turbidity) was measured. The binding curves were constructed by plotting the absorbance at 450 nm (due to turbidity) versus the concentration of MβCD. The K_d was calculated with GraphPad Prism software using the following equation:

$$A = \frac{A_{max}}{2\,[C]} \left\{ [C] - (K_d + [M\beta CD]) + \sqrt{(K_d + [M\beta CD] + [C])^2 - 4[M\beta CD][C]} \right\}$$

where A is the absorbance (450nm) at different MβCD concentrations [MβCD], A_{max} is the maximum absorbance, and [C] is the cholesterol (or DChol) concentration.

Supplemental Method 4.

Quantitative imaging and colocalization analysis

DChol fluorescence in cholesterol-rich microdomains was calculated as follows: threshold was applied to the cholesterol-rich microdomain marker image and DChol image; a binary image of the cholesterol-rich microdomains was created to make the pixels representing cholesterol-rich microdomains 1 and all other areas 0; "Logical And" was performed with the binary cholesterol-rich microdomain image and DChol image to yield an image contained only the colocalized pixels of DChol and cholesterol-rich microdomains; areas of interest were drawn around the plasma membrane, and the fluorescence intensity was calculated. Similarly, DChol fluorescence in cholesterol-poor microdomains was calculated by creating a binary image of the cholesterol-poor microdomains to make the pixels representing cholesterol-rich microdomains 0 and all other areas 1 and repeating the procedure above. Finally the ratio of DChol average fluorescence intensity in cholesterol-rich / cholesterol-poor microdomains was calculated.

Quantitative Colocalization Analysis of Confocal and Multiphoton Images. Quantitative colocalization analysis was performed on the colocalization images. After background correction, regions of interest (ROIs) were drawn around fluorescently labeled cellular plasma

membranes and the average pixel intensities were determined such that the threshold was set to remove all the pixels that were below the average intensity for each ROI. By doing so, most of the noise and low intensity pixels were removed with retention of only the peak intensity pixels. Three types of quantitative colocalization analyses were performed: (i) Mander's colocalization coefficients M_{red} and M_{green} were determined as described (3). The Mander's colocalization coefficient M_{red} represents the sum of the intensities of red pixels that colocalized with the green pixels divided by the total intensities of all red pixels. M_{green} is the sum of the intensities of green pixels that colocalized with the red pixels divided by the total intensities of all green pixels. Thus, Mander's colocalization coefficients indicate the percentage of red fluorescence intensities colocalized with the green fluorescence, and vice versa. (ii) Costes Randomization test was performed as described (4) using ImageJ (Rasband, W.S., ImageJ, National Institutes of Health, Bethesda, Maryland, USA, http://rsb.info.nih.gov/ij/, 1997-2004.) and the Colocalization Test plugin module written by Tony Collins and Wayne Rasband. The Costes approach determines how colocalization coefficients compare with the value expected based on chance alone. The observed colocalizations (R_{obs}) were considered significant if R_{obs} was greater than R_{rand} more than 95% of the time. (iii) For each pair of fluorescent probes (DChol and one membrane domain probe), the ratio of the observed percentage of colocalized peak intensity pixels to the predicted percentage of colocalized pixels assuming a random distribution was calculated. A ratio close to one would indicate that the observed colocalization was most likely due to random distribution while a ratio greater than one would indicate that the two probes were preferentially colocalizing within the same pixel. The higher the ratio, the higher the tendency would be for the two probes to colocalize. In contrast, a ratio much less than 1 would indicate greater mutual exclusiveness.

Supplemental Method 5.

Mathematical calculation of efficiency of energy transfer for a random distribution of DChol relative to DHE in LUV bilayer.

In order to determine if the experimental results of efficiency of energy transfer between DChol and DHE reflect a random distribution of DChol relative to DHE in LUV bilayer, the efficiency of energy transfer is determined mathematically (5): $E = 1 - \frac{\int_0^\infty F(t)dt}{\int_0^\infty F(0)dt} = 1 - \frac{1}{\tau_0}\int_0^\infty F(t)dt$

where the fluorescence intensity can be described by for a spherical bilayer as is the case in large unilamellar vesicles.

$F(t) = F(0)e^{-\frac{t}{\tau_0}}\left[fe^{-\sigma(S_1(t)+S_2(t))} + (1-f)e^{-\sigma(S_3(t)+S_4(t))}\right]$ where

$$S_1(t) = \int_{\theta_e}^{\pi}\left[1 - e^{-\frac{t}{\tau_0}\left(\frac{R_0}{8r_e^6(1-\cos\theta)^3}\right)}\right]2\pi r_e^2 \sin\theta d\theta$$

$$S_2(t) = \int_{0}^{\pi}\left[1 - e^{-\frac{t}{\tau_0}\left(\frac{R_0}{\left(r_e^2+r_i^2-2r_e r_i\cos\theta\right)^3}\right)}\right]2\pi r_i^2 \sin\theta d\theta$$

$$S_3(t) = \int_{\theta_e}^{\pi}\left[1 - e^{-\frac{t}{\tau_0}\left(\frac{R_0}{8r_i^6(1-\cos\theta)^3}\right)}\right]2\pi r_i^2 \sin\theta d\theta$$

$$S_4(t) = \int_{0}^{\pi}\left[1 - e^{-\frac{t}{\tau_0}\left(\frac{R_0}{\left(r_e^2+r_i^2-2r_e r_i\cos\theta\right)^3}\right)}\right]2\pi r_e^2 \sin\theta d\theta$$

$$\theta_e = \arccos\left(1 - \frac{a^2}{2r_e^2}\right)$$

$$\theta_i = \arccos\left(1 - \frac{a^2}{2r_i^2}\right)$$

$$f = \frac{r_e^2}{\left(r_e^2 + r_i^2\right)}$$

r_i and r_e are the inside and outside radii for donor fluorophore lying within the LUV bilayer, σ is the surface density of the acceptor calculated as a function of the acceptor to total lipid molar ratio (A/TL), a is the distance of closest approach for the acceptor to donor, τ_0 is the lifetime of the donor, and R_0 is the Förster distance for DHE and DChol.

The FRET parameter R_0 was calculated to be 15.5 Å using the dynamic averaging condition for the orientation factor of $\kappa^2 = 2/3$, an index of refraction $n = 1.48$ for phospholipid (6), DHE quantum yield of $Q = 0.04$ (7), and a molar extinction ratio of 4600 $M^{-1}cm^{-1}$ for dansyl cholesterol (8) using a software routine written in this laboratory using LabView 6*i* (National Instruments, Austin, TX). In order to determine the range of κ^2 and hence the error in the calculated R_0, time-resolved measurements were made of the anisotropies of the donor and acceptor probes (See Supplemental Methods 6). The following equations were used to calculate the possible range of κ^2 (dipole-dipole spatial orientation factor) from the measured time-resolved parameters of DHE and DChol (8).

$$d_i^x = \left(\frac{r_i}{r_0}\right)^{\frac{1}{2}}$$

$$\kappa^2_{\min} = \frac{2}{3}\left[1 - \frac{\left(d_D^x + d_A^x\right)}{2}\right]$$

$$\kappa^2_{\max} = \frac{2}{3}\left(1 + d_D^x + d_A^x + 3d_D^x d_A^x\right)$$

$$R_{\min} = \left(\frac{\kappa^2_{\min}}{2/3} \right)^{\frac{1}{6}} R_{2/3}$$

$$R_{\max} = \left(\frac{\kappa^2_{\max}}{2/3} \right)^{\frac{1}{6}} R_{2/3}$$

In calculating *F(t)*, the lifetime of the donor was set to 1 ns. An estimated distance for interaction across a bilayer between fluorophore systems, Δr = 32 Å (as defined by the location of the B ring of the sterols within the bilayer), in combination with external diameter of the LUVs as measured previously (9) was used to determine the external and internal radii of interaction: r_e = 525 Å, and r_i = 493 Å. The different surface densities of the acceptor, σ_i, were calculated by dividing each of the molar concentrations of the acceptor (A/TL) by the averaged lipid surface area using the averaged areas of cholesterol (6.3 Å) and phospholipid (8.4 Å) (10) based upon the phospholipid and cholesterol composition of the LUV. MathCAD 14 (PTC Corporation, Needham, MA) was used for numerical calculations to determine efficiency of energy transfer based upon the calculated $R_0 = R_{2/3}$, R_{min}, and R_{max} and the values were subsequently graphed as a function of DChol% (mol%).

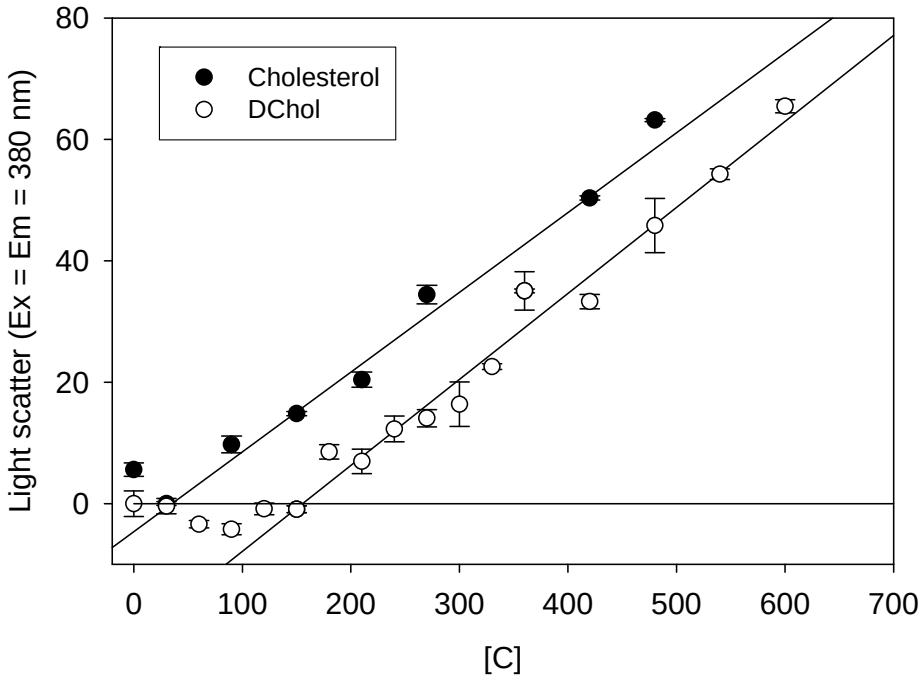
Supplemental Method 6.

Fluorescence Lifetime and Rotational Anisotropy

Lifetime and Rotational Anisotropy Measurements were performed using an ISS Multifrequency cross-correlation phase and modulation fluorometer (ISS, Inc., Champaign, IL) equipped with a LED source that emits at a peak centered at 288 nm further filtered by a U-340 filter (Hoya Optics, Fremont, CA). Emission was collected from the samples in 10mM PBS (pH 7.2) at 15 frequencies from 1-350 MHz through a KV389 emission filter (Schott Glass Technologies Inc.

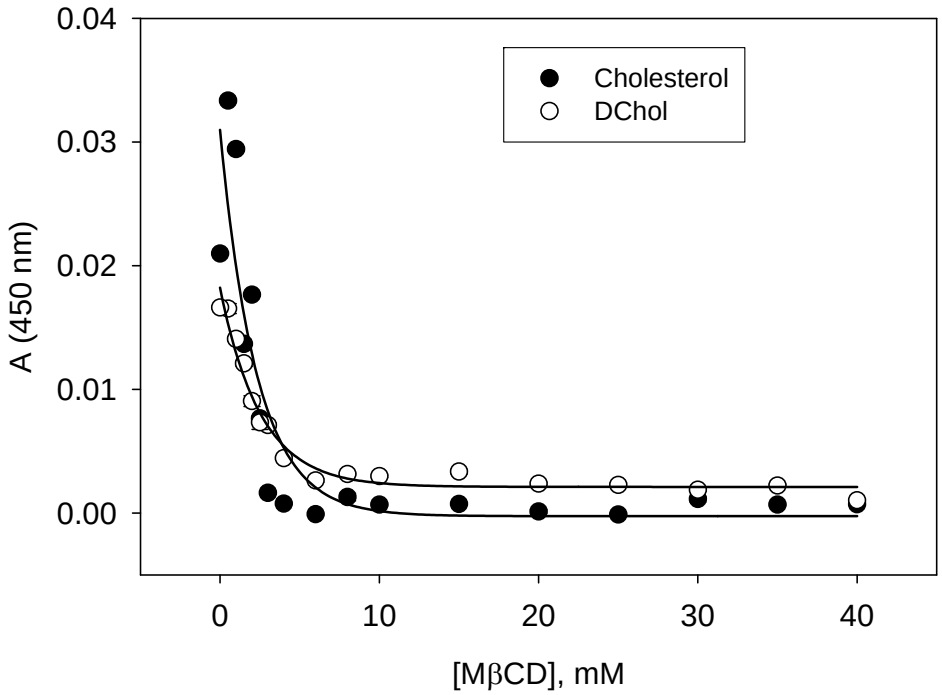
Duryea, PA). Lifetime measurements were collected through vertically oriented excitation polarizer and with emission polarizer oriented at 54.7º. The lifetime reference standard used for these experiments was dimethyl-POPOP with τ = 1.45 ns in absolute ethanol. Rotational anisotropy was examined at similar frequencies by measuring the difference in phase and ratio of modulation of vertical and horizontal components of the emission. Analysis was performed with Vinci 1.6 (ISS, Inc., Champaign, IL) using one or two component systems.

Supplemental Figure 1



Supplemental Figure 1. Critical micelle concentration (CMC) of cholesterol and DChol in water. Micelle formation was monitored by measuring light scatter with a fluorescence fluorimeter at excitation and emission wavelengths of 380nm. Light scatter was plotted versus concentration, and CMC value was calculated as the x intercept of the linear regression line where rapid increase in light scatter was observed.

Supplemental Figure 2.



Supplemental Figure 2. Cholesterol and DChol binding to MβCD. Different amount of MβCD (final concentration 0-40 mM) were added to cholesterol (final concentration 0.15 mM) and DChol (final concentration 0.15 mM) suspensions. The samples were kept in 37^{o}C shaker for 16 hours. The absorbance at 450 nm (due to turbidity) was measured and plotted versus MβCD concentration. The Kd was calculated as described in Methods.

Supplemental References:

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