Supplementary Materials for "Ordered Raft Domains Induced by Outer Leaflet Sphingomyelin in Cholesterol-Rich Asymmetric Vesicles" Qingqing Lin and Erwin London*

Supplementary Figure 1. Analysis of lipid composition and asymmetry of LUV after one round of lipid exchange. (A) HP-TLC analysis of asymmetric egg SM+DOPCo/DOPCi/cholesterol LUVs prepared by a single exchange procedure. After exchange, acceptor LUVs are composed of 37 mol% cholesterol (based on initial lipid composition and assuming a lack of net lipid transfer (15)), an inner leaflet of DOPC and an outer leaflet of a mixture of egg SM and DOPC. (B) and (C) Measurement of lipid flip-flop in asymmetric egg SM+DOPCo/DOPCi/cholesterol LUVs by C_6-NBD-PC protection assay with 1 mol% C_6-NBD-PC relative to total lipids added externally to the vesicles. After 3h or overnight incubation, sodium dithionite was added to reduce outer leaflet NBD, and NBD fluorescence was measured as a function of time. Panel C. shows the percent protection derived from extrapolating percent protection to time zero after dithionite addition. (D) and (E) Measurement of lipid flip-flop in asymmetric egg SM+DOPCo/DOPCi/cholesterol LUVs by C_6-NBD-PC protection assay with C_6-NBD-PC incorporated into both inner and outer leaflets. Both donor and acceptor vesicles contained 1 mol% C_6-NBD-PC before exchange. Lipid exchange was carried out same as for samples without C_6-NBD-PC. After 3h or overnight incubation, sodium dithionite was added to reduce the NBD fluorescence. Panel E. shows the percent protection derived from extrapolating percent protection to time zero after dithionite addition. NBD protected is the fraction of NBD protected, which is the intensity of NBD fluorescence after exposure to dithionite divided by that before exposure to dithionite.

Supplementary Figure 2. Analysis of composition and asymmetry using LUV prepared by two rounds of lipid exchange. (A) and (B) HP-TLC analysis of asymmetric egg SMo/DOPCi/cholesterol LUVs (A) or milk SMo/DOPCi/cholesterol (B) prepared by a double exchange procedure. After two rounds of exchange, ~ 50 mol% of the non-cholesterol lipid in exchanged vesicles were SM. (C) TMADPH anisotropy of (left to right) symmetric egg SM/cholesterol, asymmetric egg SMo/DOPCi/cholesterol, scrambled vesicles, and symmetric DOPC/cholesterol at room temperature. (D) TMADPH anisotropy of (left to right) symmetric milk SM/cholesterol, asymmetric milk SMo/DOPCi/cholesterol, scrambled vesicles, and symmetric DOPC/cholesterol at room temperature. Cholesterol concentration (assuming no net lipid transfer) in all samples was 37 mol%. TMADPH dissolved in ethanol was added to preformed vesicles to a concentration of 0.1 mol% of total lipid. Samples were incubated for 10 min at room temperature before measurement. Average values (mean) and range are shown from duplicates for anisotropy measurements.

Supplementary Figure 3. Some representative vesicles illustrating the occasional (about one half of preparations) heterogeneity of NBD-DPPE partitioning in egg SM+DOPCo/DOPCi/cholesterol vesicles inner leaflet.

Supplementary Figure 4. (A) Confocal microscopic imaging of symmetric 1:1 egg SM/DOPC with 37 mol% cholesterol GUVs having both leaflets labeled with both Rho-DOPE and NBD-DPhPE (upper panel), asymmetric egg SM+DOPCo/DOPCi/~37 mol% cholesterol GUVs having inner leaflets labeled with NBD-DPhPE and outer leaflets labeled with Rho-DOPE (middle

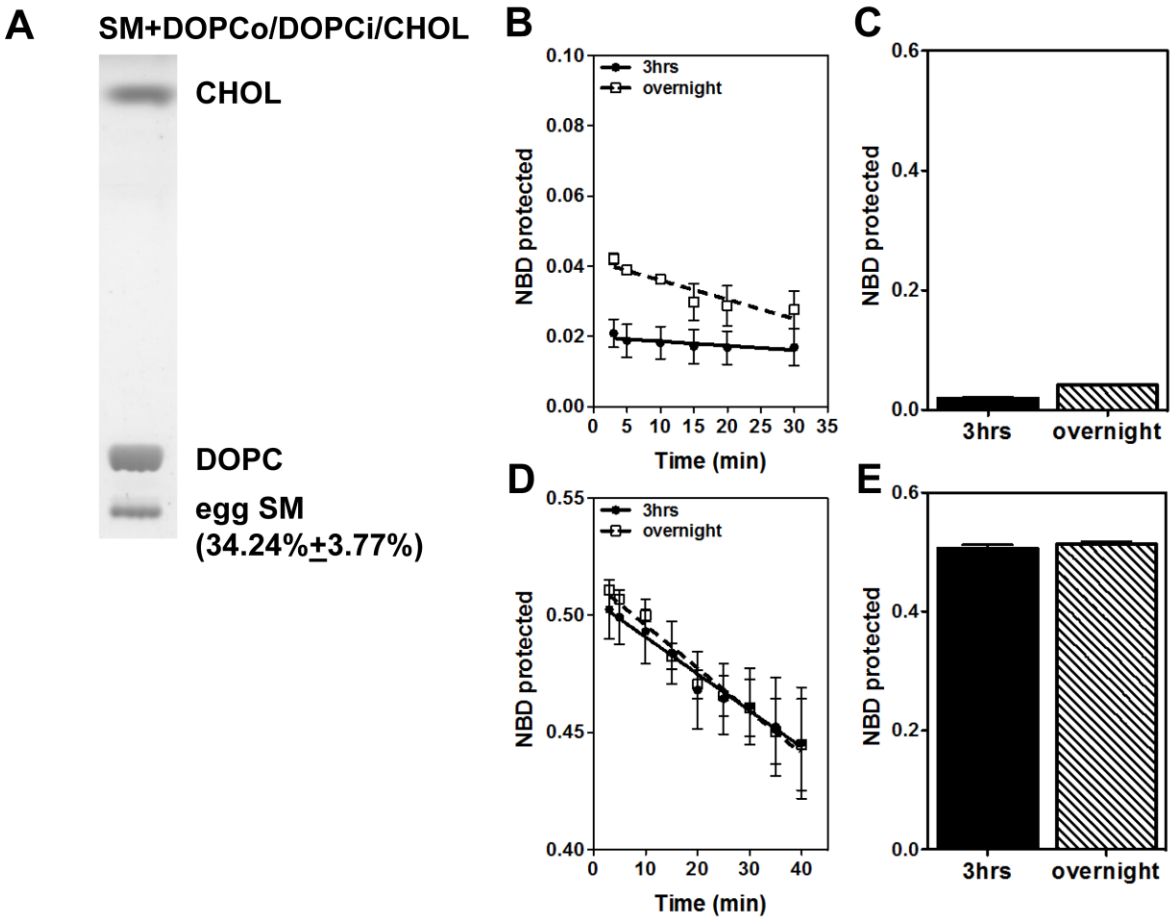
panel), and asymmetric egg SM+DOPCo/DOPCi/~37 mol% cholesterol GUVs having both leaflets labeled with Rho-DOPE and outer leaflets labeled with NBD-DPhPE (bottom panel). (B) and (C) box plot and bar graph representations, respectively, of NBD-DPhPE partition coefficient, Kp (Lo/Ld). N=28 for NBD-DPhPE in symmetric vesicles both leaflets; n=19 for NBD-DPhPE in asymmetric vesicles outer leaflet and n=15 for NBD-DPhPE in asymmetric vesicles inner leaflet. Probe leaflet location: b=both; o=outer; i=inner

Supplementary Figure 5. (A) Confocal microscopic imaging of symmetric 1:1 (mol:mol) egg SM/DOPC with 37 mol% cholesterol GUVs having both leaflets labeled with both Rho-DOPE and NBD-DSPE (upper panel), asymmetric egg SM+DOPCo/DOPCi/~37 mol% cholesterol GUVs having inner leaflets labeled with NBD-DSPE and outer leaflets labeled with Rho-DOPE (middle panel), and asymmetric egg SM+DOPCo/DOPCi/~37 mol% cholesterol GUVs having both leaflets labeled with Rho-DOPE and outer leaflets labeled with NBD-DSPE (bottom panel). (B) and (C) box plot and bar graph representations, respectively, of NBD-DSPE partition coefficient, Kp (Lo/Ld). N=28 for NBD-DSPE in symmetric vesicles both leaflets; n=23 for NBD-DSPE in asymmetric vesicles outer leaflet and n=17 for NBD-DSPE in asymmetric vesicles inner leaflet. Probe leaflet location: b=both; o=outer; i=inner

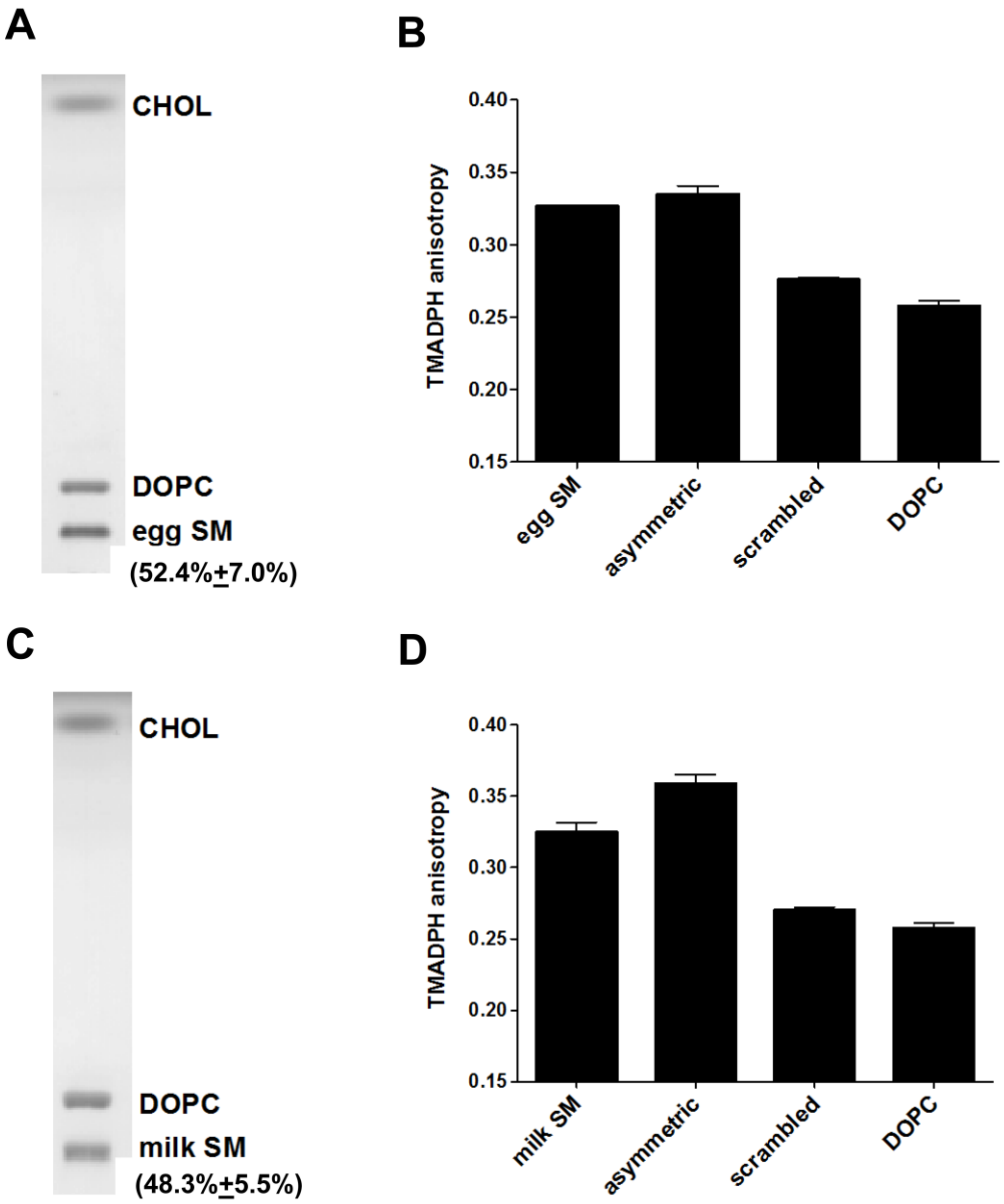
Supplementary Figure 6. Domain formation in symmetric GUVs composed of 1:1 (mol:mol) milk SM/DOPC with different mol% of cholesterol. The vesicles were labeled with Rho-DOPE and NBD-DOPE in both leaflets. Left three panels: Rows from left to right are Rho-DOPE, NBD-DOPE and overlay. Right three panels: 3-D reconstructions. Rows from left to right are Rho-DOPE, NBD-DOPE and overlay.

Supplementary Figure 7. Thermal stability of large domains in milk SM+DOPCo/DOPCi/~37 mol% cholesterol GUVs. Acceptor vesicles contained 63:37 (mol:mol) DOPC/cholesterol labeled with NBD-DOPE. Donor vesicles contained 63:37 (mol:mol) milk SM/cholesterol labeled with Rho-DOPE. NBD in the outer leaflet was reduced with sodium dithionite for 90s to restrict NBD fluorescence to the inner leaflet. Images were taken by Leica LAS AF confocal microscope with the CUBE & BOX temperature control system. Sample temperature was increased in steps, with micrographs recorded 1-2 minutes after the desired temperature was reached.

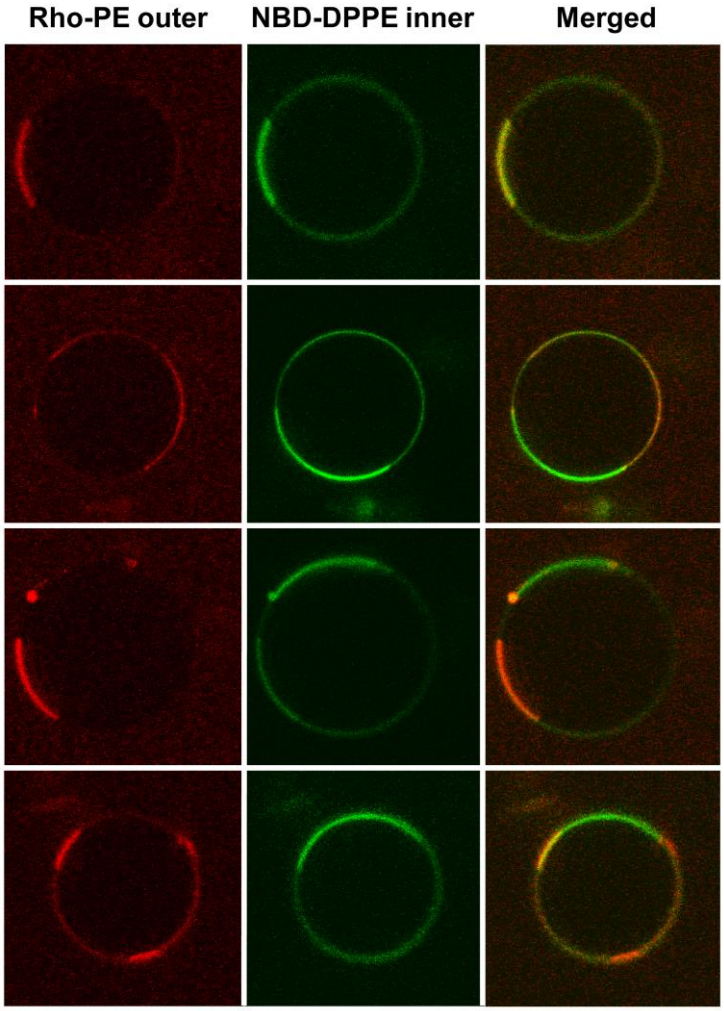
Supplementary Figure 1



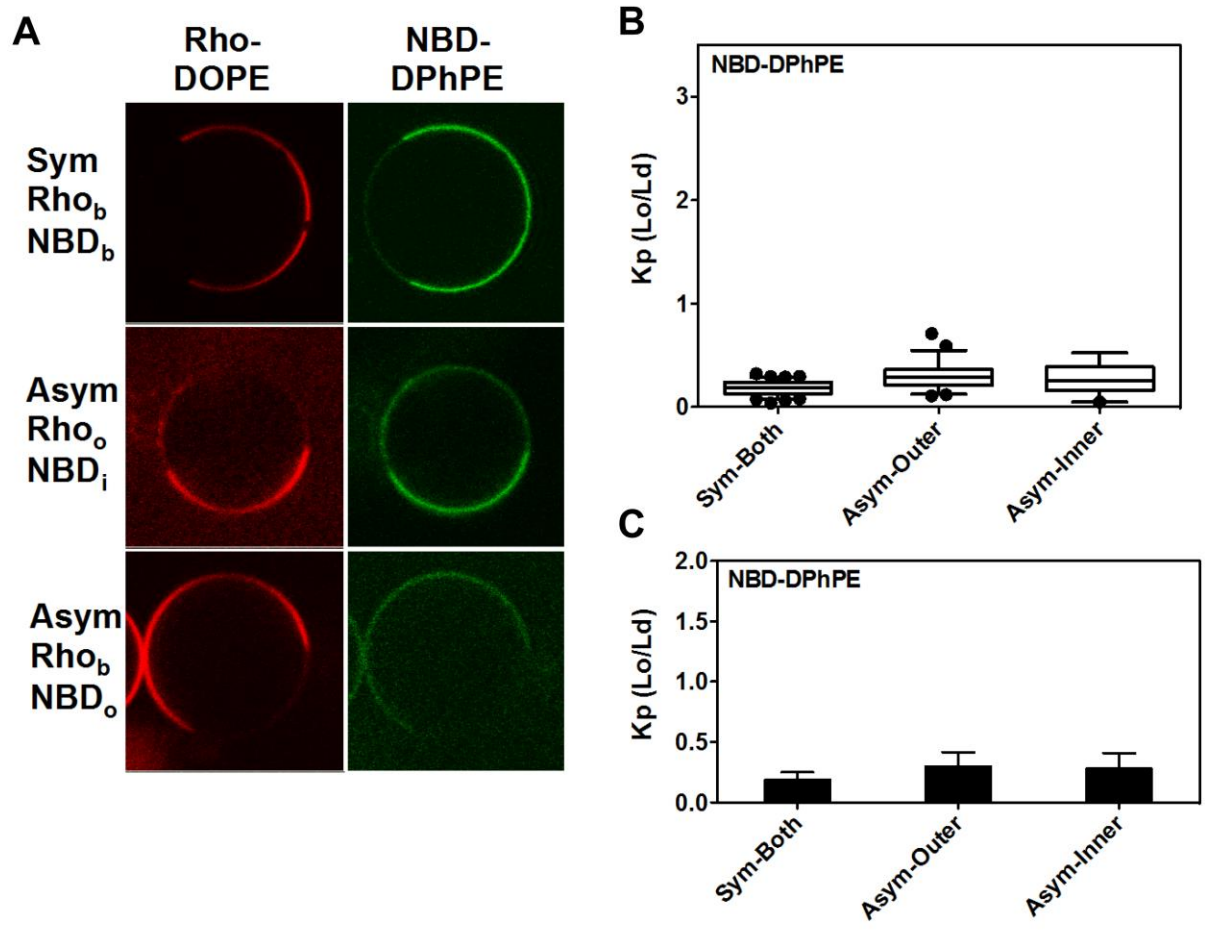
Supplementary Figure 2



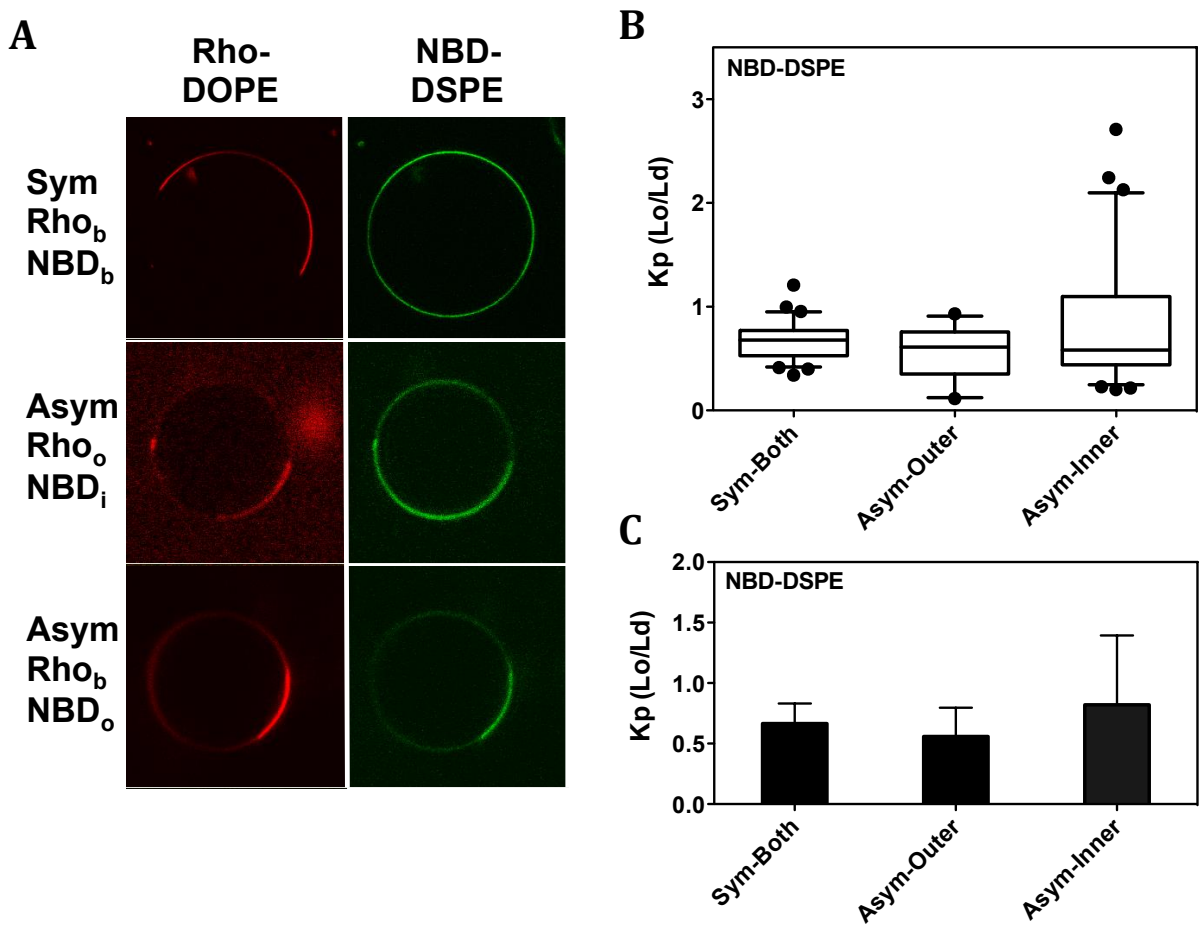
Supplementary Figure 3



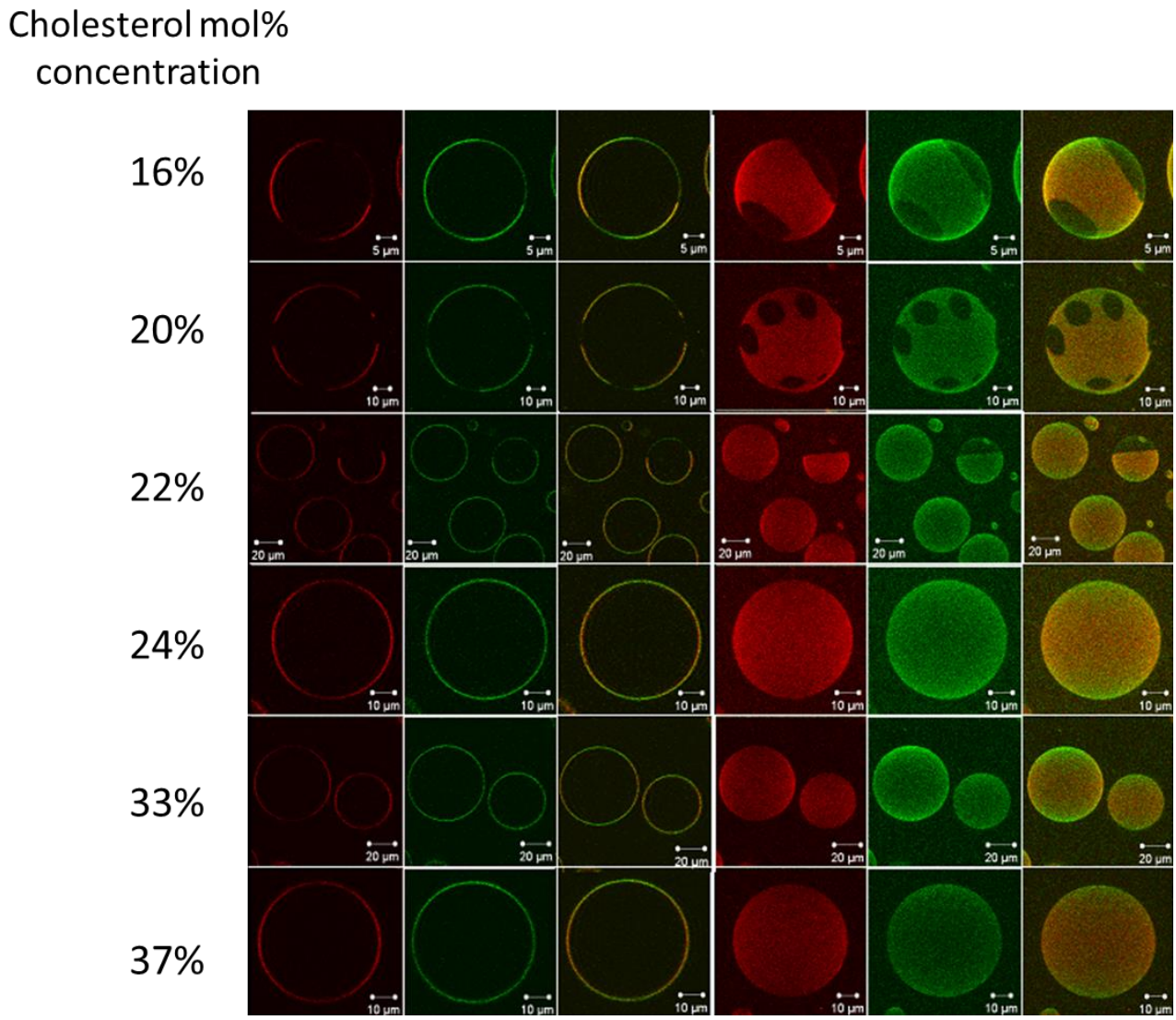
Supplementary Figure 4



Supplementary Figure 5



Supplementary Figure 6



Supplementary Figure 7