Supporting Information

Lipid Vesicles Preparation. The hydrated POPC:rho-DPPE lipid mixture with a lipid concentration of 500 μM (see Materials and Methods) was subjected to 5 freeze-thaw cycles and extruded through a 100 nm pore-size polycarbonate membrane filters 40 times using a mini-extruder syringe device (Avanti Polar Lipids, Inc, Alabaster, AL). The solution containing LUVs was mixed with glycerol in the ratio 6:4. The cover slide was coated with 1 mg/ml bovine serum albumin (Product No. A8549, Sigma-Aldrich, Singapore) to prevent absorption of the lipid vesicles to the glass surface.

Characterizing Excitation Intensity Distribution. For multiplexing in TIR-FCM the excitation intensity distribution should be uniform over the area in which measurements are performed. To measure the excitation intensity profile in the sample plane in TIRF configuration, 100 nM Atto565 dye in DI water was dispensed on a cover slide and excited with 532 nm laser light at 0.8 mW power. The intensity profiles in the horizontal and vertical directions measured with the field aperture partly closed are shown in figure S1a. The percentage deviation in intensity over 40 pixel distance both in horizontal and vertical directions in TIRF configuration is shown in figure S1b. The maximum percentage deviation in the measured intensity is 4.3 %.

TIR-FCM Calibration. To calibrate the TIR-FCM system ACF curves were measured on POPC LUVs diffusing in 40% glycerol. The ACF curves measured in TIR and WF-type configurations are shown in figure S2a using a pixel in the EMCCD camera. The ACF curve measured in TIRF configuration shows a typical correlation function whereas the ACF curve measured in WF-type configuration has no correlation. In TIRF

configuration the detection volume is defined by ω_{xy} and h so that the molecules which diffuse in or out of this volume give rise to fluctuations which are correlated. In the WF-type configuration, the illumination in the z-direction is uniform and extends to a larger distance. This results in a large observation volume and thus weak fluctuations therefore yielding no measureable correlation. The ACF measured in TIRF configuration was fitted to eq 1 and the fit is shown as a dashed line. The number of particles N within the detection volume defined by this pixel is 3.3, the diffusion time τ_z is 10.5 ms, τ_{xy} is 123 ms and the structure factor ω is 3.4. The average diffusion coefficient along the plane from a number of measurements is $D_{xy}=7.4(\pm4.7)\times10^{-10}$ cm^2s^{-1}. The average value of τ_z from a number of measurements on the LUVs in 40% glycerol and the value of D ($9.4(\pm5.7) \times 10^{-9}$ cm^2s^{-1}) determined from an independent experiment in a confocal FCS system employing avalanche photodiode were substituted in eq 2a and h was calculated as 180(±88) nm. Using the value of τ_z measured in TIR-FCS and the value of h, the D_z of any fluorophore can be determined from eq 2a. Taking into account the viscosity of 40% glycerol solution, the value of D is comparable to the reported[i] value measured on POPC vesicles in an aqueous medium. The value of D along the plane (D_{xy}) measured in TIR-FCS is smaller than the D obtained from the confocal FCS experiment possibly due to the influence of the cover slide surface on the mobility of the vesicles in TIR-FCS.

Characterizing the Lipid Bilayer. Next, an exercise was carried out to know if the bilayer used in the present work constitute (a) a lipid layer adherent to the cover slide surface with a discontinuity in the z-direction or (b) it has a lipid density gradient extending to a larger extent in the z-direction. For this purpose, the ACF curves of a bilayer were measured both in the WF-type as well as in TIRF configurations and the

curves are shown in figure S2b. They show a temporal decay of correlations. If the hypothesis (a) is true then the bilayer is a few nanometers thick. The lipid molecules diffuse along the bilayer whereas in the medium above the bilayer there are no fluorescent molecules. This introduces an inherent confinement in the z-direction for fluorescence detection. Due to this reason both the measurements in WF-type as well as in TIRF configurations will show a temporal decay of correlation. On the other hand, if the hypothesis (b) is true then there is no inherent z-confinement and the sample is similar to a liquid droplet in which the fluorescent lipid molecules are undergoing translational motion. In this case only the curve measured in TIRF configuration will show a temporal decay while the curve measured in WF-type configuration will not show any decay. Figure S2b shows the ACF curves measured in WF-type as well as TIRF configurations. Both the curves show measurable correlations although the temporal decay of the two curves is different. This suggests that there indeed is a lipid layer adherent to the cover slide. As the z-resolution of TIR-FCS is a few hundred nanometers, the possibility of multiple lipid layers with the thickness extending up to h can not be excluded. The ACF curve measured in TIRF configuration was fitted to eq 3 (dashed line in fig S2b) and the fit residuals are shown in the lower panel. For this pixel N is 4.1 and τ_{xy} is 14.4 ms. The ACF curve measured in WF-type configuration was fitted to a two-component model based on eq 3. The fit and fit residuals are shown in figure S2b. Out of 7.9 particles in the detection volume, 33% of the particles diffuse slowly (1.7 s) possibly due to the presence of lipid aggregates and the rest diffuse faster with a τ_{xy} (20.6 ms) comparable to the TIRF data.

Supporting Reference

(i) Rusu, L.; Gambhir, A.; McLaughlin, S.; Rädler, J. Biophys. J. **2004**, 87, 1044-1053.

Figure S1

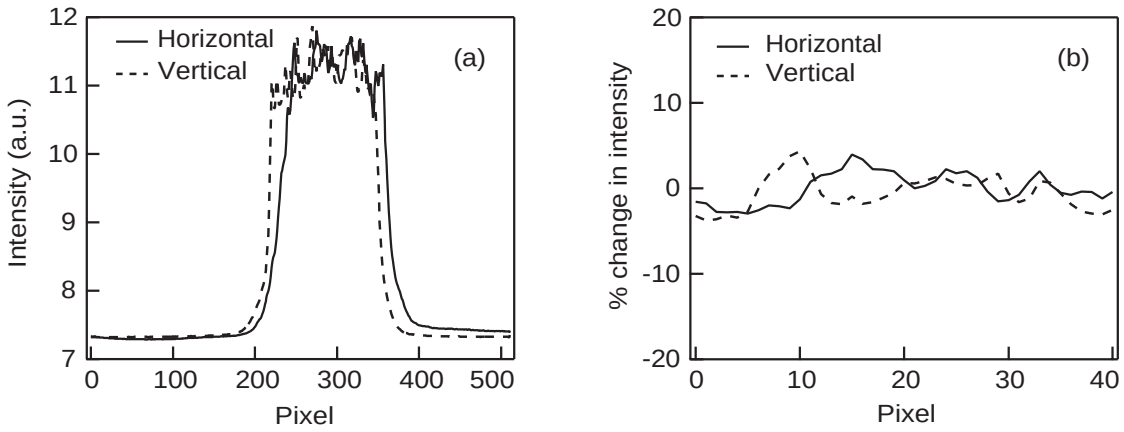
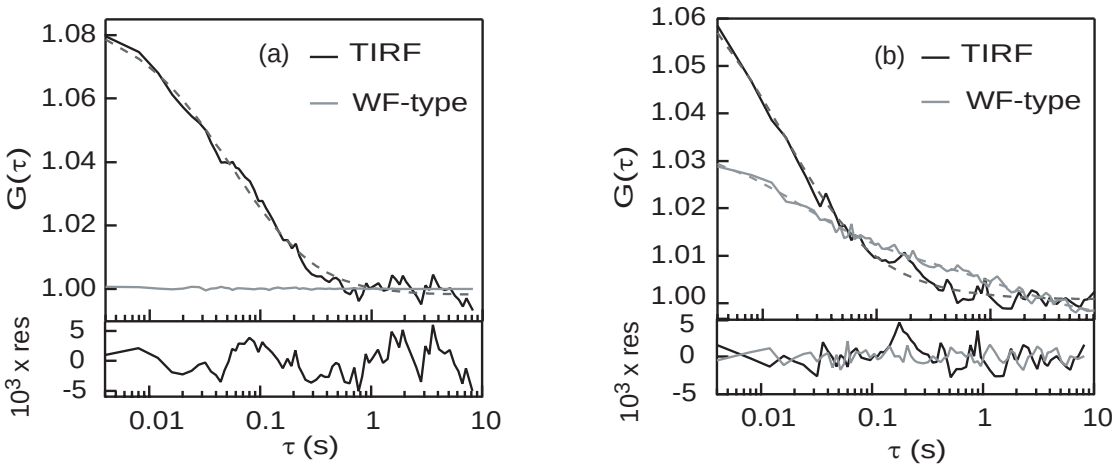


Figure S2



Supporting Figure Captions

Figure S1. (a) Intensity profiles of the excitation field along the horizontal and vertical directions of the CCD chip measured in TIRF configuration with the field aperture partly closed. (b) Percentage variation of intensity over the measurement area (40 pixel distance in horizontal and vertical directions of the CCD chip) in TIRF configuration. Atto565 dye solution at a concentration of 100 nM was excited with 532 nm laser using 0.8 mW power.

Figure S2. (a) Autocorrelation function $G(\tau)$, measured in TIRF and WF-type configurations on lipid vesicles diffusing in 6:4 mixture of water and glycerol. Fit to eq 1 for the TIRF data is shown in dashed line. The lower panel shows the fit residuals. The laser power was 15 mW. The time resolution is 4 ms. (b) Autocorrelation function $G(\tau)$, measured in TIRF and WF-type configurations on a lipid bilayer. Fit to eq 3 for the TIRF data and two-component model based on eq 3 for the WF-type data are shown in dashed line. The lower panel shows the fit residuals. The laser power was 12 mW. The time resolution is 4 ms.