Supporting Information for

Fluorescence anisotropy based single liposome assay to measure molecule-membrane interaction

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ABSTRACT This supporting information file includes 3 figures. Figure S-1 describes the effect of APD saturation on the measured count rate. In Figure S-2 the molecule-membrane interactions of Atto dyes is visualized by means of giant vesicles. Figure S-3 shows the effect of bleaching on fluorescence anisotropy.

Saturation of APDs

Avalanche photo diodes (APDs) are very sensitive photon detectors. At high incoming light level, APDs are not responding linear, due to the dead time (t_d). In homogeneous medium the microscope settings can be easily adjusted to avoid saturation of APDs. Unfortunately, the images of liposome samples contain pixels with a heterogeneous distribution of intensities, therefore the non-linearity behavior of the APDs cannot completely avoided. We compensate for APD saturation by multiplying the count rate (CR) in each image pixel with a correction factor of $1/(1-t_d*CR)$ as described in the literature [1]. Since we had not direct access to the real count rate with the used commercial software from Leica, we performed an experiment, which allowed us to determine an intensity correction factor (k) for a given microscope setting to transform the measured intensity (I) in real count rate: $CR = k*I$. We imaged the fluorescence intensity of solutions with increasing dye concentration. This allowed us to increase the fluorescence signal without changing the measurement settings. The used dye concentration in the nanomolar range prevents undesired intermolecular dye interactions. Such interaction could lead to self-quenching, which occurs typically in millimolar range and would effect the fluorescence signal as well the fluorescence anisotropy. The fluorescence signal was split with a polarization beam splitter in a parallel and perpendicular emission pathway and detected by APDs. Due to the different detection efficiencies of the parallel and perpendicular emission pathway in the microscope, one APD (APD2) detected always a higher signal than the other (APD1). In Figure S-1a the ratio of the intensities in both APDs are plotted versus dye concentration. Because APD2 saturates faster than APD1, the ratio increases with increasing dye concentration. The intensity correction factor was adjusted until a constant APD ratio was obtained for the different dye concentrations. The measured fluorescence intensity and the corrected intensity are shown in Figure S-1b for the different dye concentrations. It is evident that the corrected fluorescent signals follow more precisely a linear dependency. The correction of the saturation of the APDs is crucial for anisotropy measurement, because the anisotropy value would otherwise be strongly biased by the magnitude of the fluorescence and would not reflect the rotational freedom of the dye molecule.

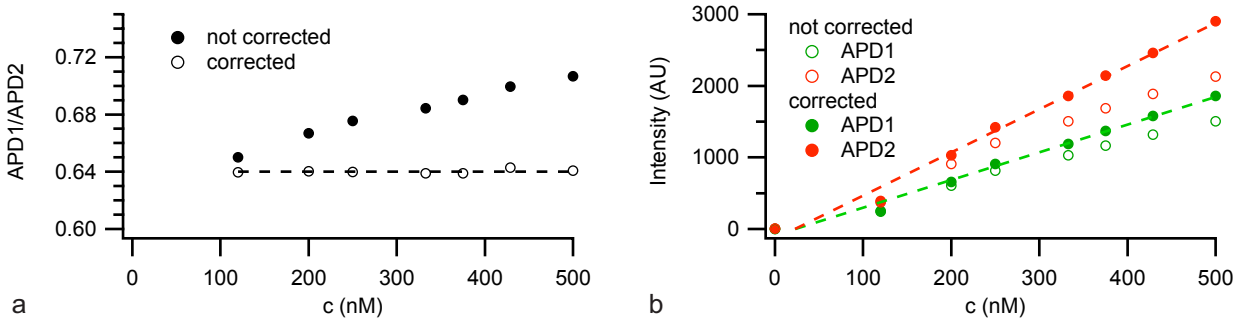

Figure S-1. Saturation effect of the APDs. a) Intensity ratio of APD1 to APD2 versus dye concentration. Without saturation correction the ratio increases due to the faster saturation of APD2. b) Fluorescence intensity increases in both APDs due to increasing dye concentration. The effect of the correction of the intensities due to APD saturation is more pronounced for higher intensities.

Giant vesicles experiments

To confirm the strong interaction of Atto633 with the membrane of nanometer-scaled liposomes, we performed a giant vesicle experiment, in which the adsorption of dyes to the membrane can be directly visualized. Giant vesicles were formed by electro-swelling (Vesicle Prep Pro, Nanion Technologies, Munich, Germany) in 309 mM D-Sorbitol solution. The same lipid composition as in the small liposome experiment was used. Giant vesicles were immobilized on a functionalized glass surface and the water-soluble dye Atto633 were added in a micromolar concentration (Figure S-2, left). The giant vesicles are visible as circular contrast areas with low fluorescence intensity, since the water-soluble dye cannot enter the lumen of the vesicles. An increased fluorescence signal in the membrane region of the vesicles reveals that Atto633 is adsorbing to the membrane. A similar experiment with Atto655 showed no dyes sticking to the lipid membrane (Figure S-2, right).

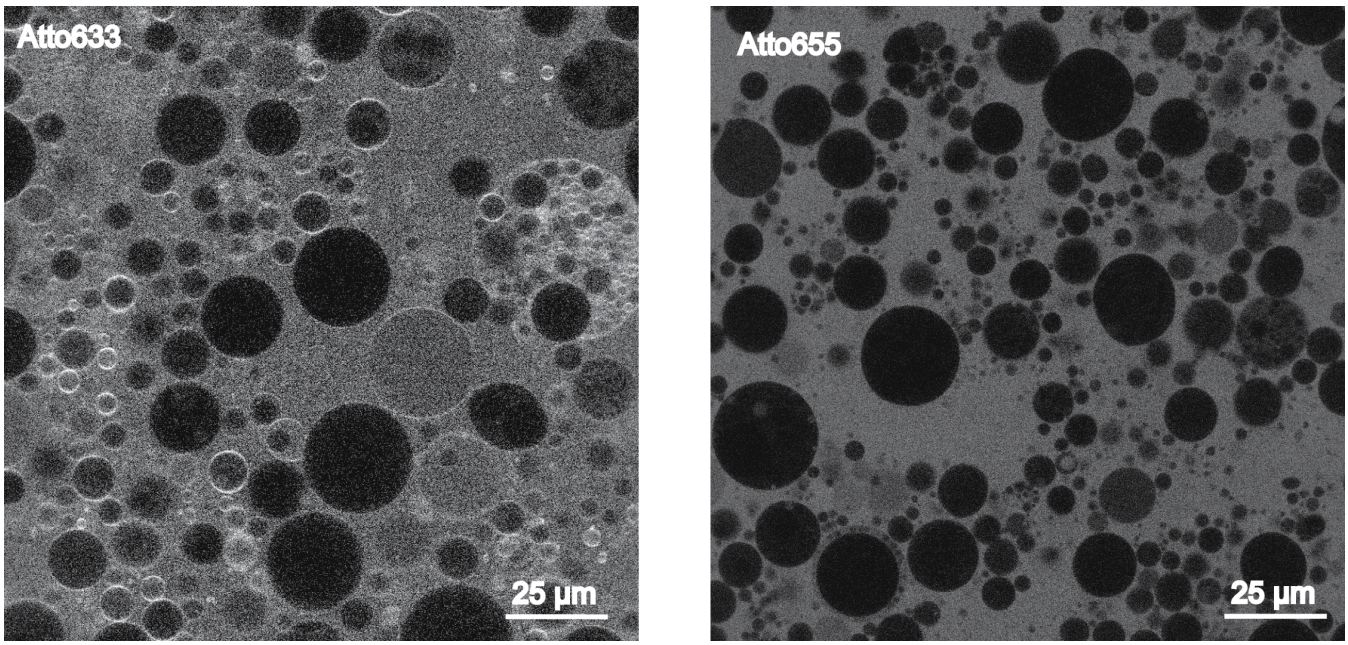

Figure S-2. Adsorbance of dyes to giant vesicles. Micrographs of negatively charged giant vesicles in solutions of fluorescent dyes Atto633 (left) and Atto655 (right). Giant vesicles are visible as circular areas with low fluorescence intensities. An increased fluorescence signal in the membrane region of the vesicles can be seen with Atto633 (left), indicating that the dye adsorbs to the vesicle membrane. This is not observable with Atto655 (right).

Bleaching effect

When fluorescence anisotropy reports only the rotational diffusion of freely rotating molecules than photobleaching has a neglectable affect on the anisotropy value, since fluorescence anisotropy is a ratiometric measurement. However, membrane bound molecules are restricted in their rotational freedom, therefore we investigated the bleaching effect by continuous illuminating of nanometer-scaled liposomes associated with Atto633. More than hundred immobilized vesicles were observed and the average fluorescence intensity and anisotropy were determined. The fluorescence intensity as well as the fluorescence anisotropy decreased with illumination time due to bleaching of dye molecules (Figure S-3). As we showed for nano-scaled liposomes (Figure 7) and giant vesicles (Figure S-2), Atto633 is strongly interacting with the vesicle membrane and not freely rotating in the vesicle lumen. The combination of the rotational restricted Atto633 dye and photo-selective excitation, leads to preferentially bleaching of dyes parallel aligned to the laser polarization and thus in turn to a decrease in the anisotropy value. Since this bleaching dependent decrease in anisotropy value could be interpreted as an increase of rotation freedom of the dye, bleaching should be avoided. We used in our study imaging condition in which the typical photobleaching was minimized to less than 2% per image scan for all fluorophores and therefore the effect of photobleaching is neglectable on the measured anisotropy.

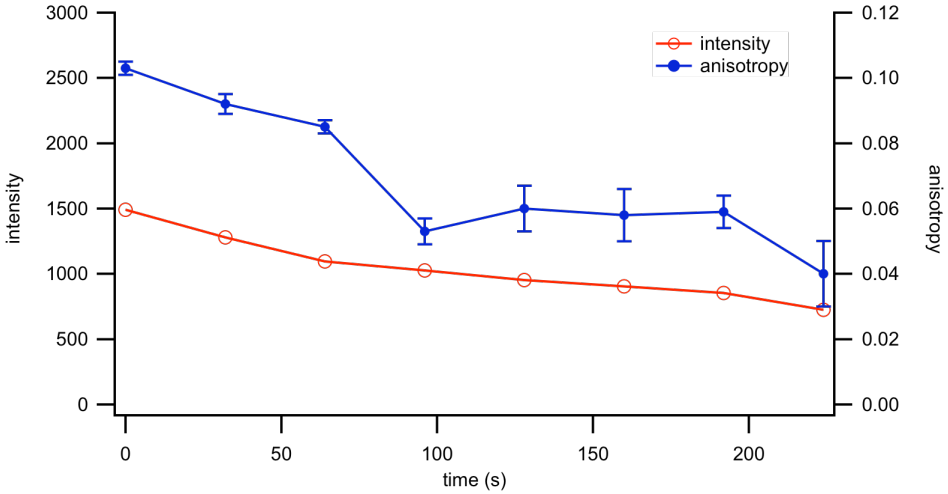

Figure S-3. Effect of bleaching on anisotropy. Hundreds of immobilized vesicles with encapsulated Atto633 were imaged over time. A decrease in Atto633 fluorescence intensity and anisotropy can be seen in the vesicle population with increasing time of illumination.

Supplementary References

(1) Naegerl, U. V.; Willig, K. I.; Hein, B.; Hell, S. W.; Bonhoeffer, T. *Proc. Natl. Acad. Sci. U. S. A.* **2008**, *105*, 18982-18987.