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Short Note

Single-Fluorophore Diffusion in a Lipid Membrane over a Subwavelength Aperture

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Abstract. We use submicrometer apertures milled in an aluminium film to study the diffusion dynamics of β-Bodipy-FL-C_5-HPC (Bodipy-PC) fluorophores in a lipid dioleoylphosphatidylcholine (DOPC) multilayer. The observation volume is limited by the aperture diameter, well below the optical wavelength. This spatial resolution improvement comes together with an enhancement of the detected fluorescence per molecule as compared to an open sample, with a significant increase up to 3.5 times.

Key words: fluorescence correlation spectroscopy, confocal microscopy, nano-apertures, lipid bilayer

Submicrometer holes milled in metallic films are attractive tools to design new optical microscope setups with subwavelength spatial resolution and single fluorophore analysis ability in a highly concentrated solution. Furthermore local surface plasmon modes can enhance the local field at the aperture's vicinity [1], eventually increasing the molecule/optical field interaction. In the experiments reported in [2–5], subwavelength apertures of diameters ranging from 30 to 400 nm were shown to act as small reaction chambers, limiting the observation volume of a standard confocal microscope to the aperture. By acting as a spatial pinhole filter in the object plane, the nanoaperture greatly improves the microscope's spatial resolution far below the optical wavelength. This allows to reduce the observation volume from a fraction of femtoliter (obtained with a standard confocal microscope) to the attoliter or zeptoliter range (10^{-18} or 10^{-21} l), gaining 3 to 6 orders of magnitude. Thus, techniques such as fluorescence correlation spectroscopy (FCS) [6], which only work while observing a small number of molecules, are readily applicable to significantly higher molecular concentrations.

The understanding of biological membrane organization requires new strategies enabling submicrometer studies in live cells [7]. To demonstrate that the diffusion of single fluorophores in a membrane can be probed at various spatial scales below the diffraction limit, we use circular nanoapertures milled in an aluminium film to study the diffusion dynamics and fluorescence emission of Bodipy-PC fluorophores in a DOPC multilayer. We report a linear dependence between the mean diffusion time and the aperture surface, which indicates that the observed diffusion is mainly 2-dimensional and limited by the aperture size. We also observe a striking enhancement of the fluorescence rate emitted per molecule as compared to an open multilayer, with a significant increase up to 3.5 times. Enhancing the fluorescence rate per molecule is especially important to obtain a signal to noise ratio greater than one, enabling viable and quick FCS measurements.

Dioleoylphosphatidylcholine lipids (Avanti Polar Lipids, Inc. Alabaster, AL, USA) were dissolved in chloroform at a concentration of 25 mg/mL and mixed with β-Bodipy-FL-C_5-HPC (Invitrogen, Carlsbad, CA, USA) at a ratio of 1:5000. The mixture was allowed to stand at room temperature for 5 min before a 100 μL droplet was deposited on the metal sample. The preparation was then evaporated in a vacuum dessicator for at least one hour. The dry lipid residue was hydrated with distilled water and equilibrated at room temperature. Finally, the sample was rinced twice with distilled water to throw away the liposomes and keep only the lipid multi-layer. The production of the nanoapertures together with our experimental setup are described in [4]. The excitation wavelength of 488 nm was provided by an Ar^+ laser. Tight focusing conditions were used to illuminate a single aperture with a beam waist of 250 nm (calibrated from FCS experiments on Rhodamine 6 G in water solution).

The mean number of molecules N in the observation volume is computed from the fluorescence intensity autocorrelation function value at the origin:

$$g^{(2)}(0) = 1 + \frac{1 + n_T}{N}\left(1 - \frac{\langle b\rangle}{\langle i\rangle}\right)^2,$$

where n_T stands for the triplet amplitude, $\langle i\rangle$ is the mean intensity and $\langle b\rangle$ the mean background. Let us point out that this formula is independent of the shape of the excitation field and the type of the diffusion statistics. For each experimental run, the triplet amplitude n_T was fitted to the experimental autocorrelation function (assuming a 2-dimensional Brownian diffusion, see Figure 1). The excitation power was set to 20 μW to avoid fluorescence saturation and photobleaching. We checked that the average number of molecules and the mean diffusion time remained constant while increasing the excitation power up to 80 μW.

Figure 1 displays the normalised autocorrelations for various aperture diameters. It is apparent that the use of submicrometer apertures allows to dramatically decrease the diffusion time from 2.4 ms for an open DOPC multilayer to 0.3 ms inside a 150 nm aperture. This indicates a strong decrease of the observation volume

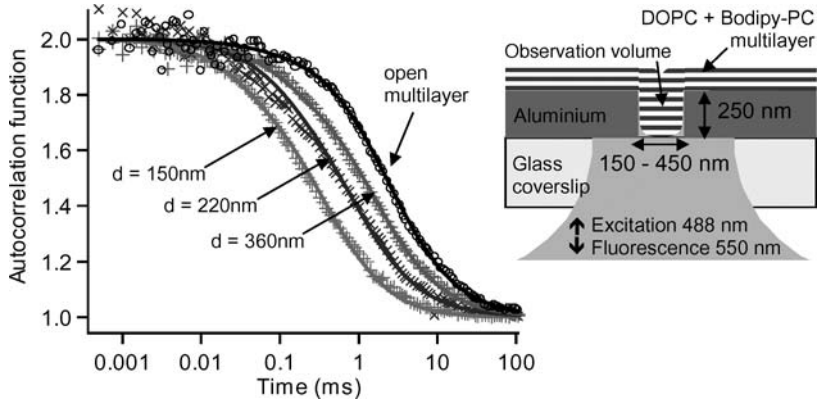

Figure 1. Normalised autocorrelation functions of Bodipy-PC probes diffusing in a DOPC multilayer membrane, for various aperture diameters d (excitation power 20 μW), including numerical fits (lines) assuming a 2-dimensional Brownian diffusion. Insert: schematic view of the sample.

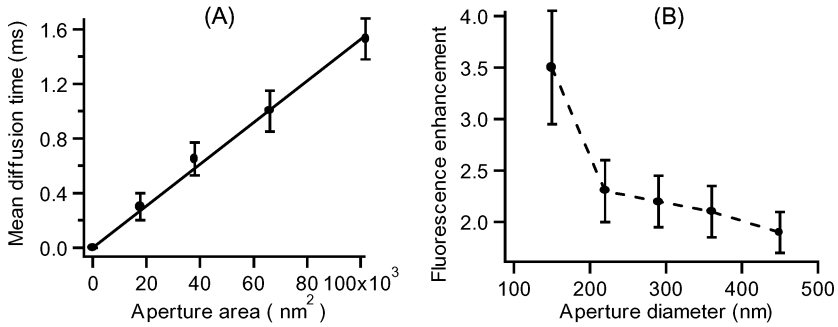

Figure 2. (A) Mean diffusion time versus the aperture surface, obtained from the autocorrelation functions displayed on Figure 1. The line is a numerical fit. (B) Enhancement of the fluorescence rate per molecule as compared to its value in open solution versus the aperture diameter (excitation power 20 μW, dashed line to ease viewing).

well below the diffraction limit. The mean diffusion time obtained from numerical fit assuming a 2-dimension diffusion is reported versus the aperture surface on Figure 2(A). The reported linearity between the mean diffusion time and the aperture surface is a relevant witness that the observed diffusion is mainly 2-dimensional and limited by the aperture size. From this data, we can give a raw estimate of the diffusion coefficient of Bodipy-PC in DOPC of about 6 μm^2/s. This appears consistent with the diffusion coefficient of 7 μm^2/s, obtained from experiments carried on giant unilamellar vesicles.

We also investigated the fluorescence rate per molecule inside the aperture as compared to an open multilayer. Relevant information is readily obtained by dividing the total average number of photocounts per second by the average number of

molecules N computed from $g^{(2)}(0)$. Figure 2(B) presents the enhancement of η as compared to its value in open solution. A significant enhancement of 3.5 is observed for the smallest hole diameter (150 nm) and a two-fold enhancement is commonly seen for aperture diameters in the 250–400 nm range. These results appear consistent with the observations reported for Rhodamine 6G in aqueous solution inside the nanoapertures [4], where an enhancement factor of 6.5 was reported for Rhodamine molecules in a 150 nm aperture. The discrepancy between the enhancement factors can be explained by the fact that the shape of the lipid multilayer within the nanoaperture may affect the detection efficiency. Namely, if the lipid multilayer does not fill the aperture and is limited to its upper side, the detected fluorescence rate will be decreased by the low transmission through the subwavelength hole.

To conclude, we have demonstrated that submicrometer apertures milled in a metallic film improve the spatial resolution of fluorophore diffusion studies into lipid membranes. Although one would expect the nonradiative process to quench the fluorescence emission at the vicinity of the metallic surface, the detected fluorescence enhancement demonstrates that the overall effect acts in favour of the radiative processes as compared to an open multilayer, thus allowing viable FCS experiments. Subwavelength apertures for single molecule spectroscopy on lipid membranes have been used independently by Samiee *et al*. [8].

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