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Fluorescence correlation spectroscopy on 2D optical antennas for single molecule studies in living cells

Pau Santos Vives

Supervised by Dr. Bruno Castro and Prof. María García Parajo, ICFO

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Fluorescence correlation spectroscopy on 2D optical antennas for single molecule studies in living cells

Pau Santos Vives
Single Molecule Biophotonics Group, ICFO – Institut de Ciencies Fotoniques, Mediterranean Technology Park, 08860 Castelldefels (Barcelona), Spain

Abstract. The intricate molecular events that contribute for the non-homogenous and dynamic nature of the cell membrane occur at spatial and time scales which are not readily accessible by conventional microscopy techniques. This thesis explores the combination of photonic antenna devices and fluorescence correlation spectroscopy (FCS) to achieve simultaneously a high spatial (~10 nm) and temporal (~1 µs) resolution that allow the study of the molecular dynamics of a living cell membrane in a non-invasive way. In the first part of this work, confocal microscopy and optical antenna experiments of single fluorescent dyes diffusing in water were performed to characterize and validate the experimental approach. In the second part, preliminary results on the diffusion of lipids in living cell membranes using an antenna-in-box are reported. Due to its geometry and nanophotonic properties, the antenna-in-box provides simultaneous background suppression, volume reduction and fluorescence enhancement, which in combination with FCS seems to be an ideal tool to unravel the molecular processes that govern cell membrane organization and function.

Keywords: Nanoantenna, nanophotonics, single molecule fluorescence, cell membrane organization, cell membrane dynamics.

1. Introduction
Recent research has demonstrated that the cell membrane is not homogeneous and that its diffusing molecules not always follow Brownian motion [1, 2, 3]. The heterogeneous organization of the cell membrane is crucial for cellular processes such as signalling pathways, cellular adhesion and endocytic uptake [4, 5, 6, 7]. Unfortunately, due to the spatial (from few to hundreds nm) and temporal (~ µs) scales of the cell membrane processes, knowledge about the molecular mechanisms governing these events are still largely unknown. Actually, it has been show that molecular sub-compartmentalization occurs in a nanometric scale, below the diffraction limit of light [2, 8].

Several far-field optical techniques have been recently developed to break the diffraction limit. These are called super-resolution microscopy techniques. Stimulated emission depletion (STED) microscopy is an example of such technique. It consists on illuminating the sample with two simultaneous beams; one of them, the STED beam, is shaped like a doughnut with a wavelength to de-excite the fluorescent molecules by stimulated emission. With this technique 20-70 nm of spatial resolution can be achieved [9, 10]. In a non-biological sample, a resolution down to 2.4 nm has been reported [11]. However, STED microscopy has some drawbacks. Its resolution depends on the excitation power of the STED beam, resulting in photobleaching and potential cell damage. Moreover, being a scanning technique, STED microscopy is too slow to give information about the dynamics occurring in cell membrane.

Another way to overcome the spatial resolution set by diffraction is to use stochastic optical reconstruction microscopy (STORM) [12] or photo-activated localization microscopy (PALM) [13, 14]. These techniques are based in the stochastic nature of the fluorescence process. The main idea behind them is to limit sample excitation to just a few molecules at the same time. The fluorescent species should be separated enough to be able to differentiate them taking into account the Abbe’s criterion. After that, using computational algorithms and approximating the point spread function as a Gaussian profile it is possible to localize the molecule position and reconstruct the image with an accuracy in the order of tens of nm [12, 13, 14]. Nevertheless, these techniques work with reconstructed images and due to the stochastic principle they are not useful to follow the molecular dynamics.

Another super-resolution technique is near-field scanning optical microscope (NSOM) [2, 15]. By scanning the sample with an evanescent field created at the exit of a tip ended with a sub-wavelength aperture, a sub-diffraction image is obtained. Image resolution is determined by the aperture size: smaller the aperture size, better the resolution. However, the amount of light exiting the aperture decreases considerably. On the one hand, this technique can achieve 10 nm of spatial resolution, can
be used with different wavelengths and it is non-invasive. On the other hand, it is a difficult experimental technique, the tip is fragile and should be positioned close to the sample increasing the risk of breakage and the evanescent field provides low intensity field implying low fluorescence signal.

Even though all these methods provide enough spatial resolution to study intricate molecular processes that occur at the cell, they are unable to provide information about the temporal resolution of these molecular events. This information can be obtained using among others, single particle tracking (SPT) [16] or fluorescence correlation spectroscopy (FCS) [17, 18, 19].

On SPT, a small subset of the biomolecules under study is labelled with a fluorescent dye. Using a fast charge coupled device (CCD) camera it is possible to follow and record the position of the fluorescent molecule in time (i.e., its trajectory). The localization of the particle in each frame is then calculated using similar computational tools as in PALM and STORM, being the localization accuracy dependent on the detected number of photons. The major constrain of SPT relies on the need of having only a small number of molecules fluorescently labelled, so that they can be individually identified within the diffraction limit resolution. FCS is a statistical analysis technique based on similarity of the intensity fluctuations of the fluorescence signal of the sample over time. From a mathematical point of view it consists on the autocorrelation of the photon detection events, i.e., the intensity signal is compared with itself shifted in time. The autocorrelation curve, is defined as $G(\tau) = < \delta I(t) \times \delta I(t+\tau) > / < I(t) >^2$, where $\tau$ is the lag time, $I$ is the fluorescence intensity, $\delta I$ are the fluctuations in the intensity signal and the bracket means the average [17]. The fluorescence signal from a sample of diffusing molecules follows a Poisson distribution. There is mean value of the intensity signal but this value could vary randomly in each time instant. The important data in FCS analysis is not the intensity signal, but actually the fluctuations of the intensity of the signal. The main cause for a fluctuation in the intensity in a sample of diffusing molecules comes from the diffusion of molecules in or out of the illumination volume. For this reason FCS can be used to determine the diffusion coefficient of the molecule, after the precise determination of the illumination volume. The number of molecules inside of the illumination volume at the same time has to be low so that the diffusion of a single molecule in and out of the illumination volume results on a significant signal intensity fluctuation, not being included on the average intensity. Since the confocal illumination spot is about 1fL, to perform a proper FCS analysis the concentration should be in the order of few nanomolar. With the proper conditions, from FCS it is possible to determine all the parameters that can induce a fluctuation in the fluorescent intensity.

FCS and SPT are very powerful tools due to their temporal resolution. However, due to the fact that both techniques require a small number of molecules inside the illumination volume, they are not suitable to study molecular processes at the high physiological concentrations found on cells.

An elegant solution to reduce the number of molecules inside the illumination volume while keeping sample concentration constant relies on the use of photonic antennas. Optical antennas can confine the light in a volume smaller than the wavelength, breaking the diffraction limit. The definition of the optical antenna is “a device that converts freely propagating optical radiation into localized energy, and vice versa [20]”. Optical antennas have several applications but they are particularly interesting for nano-imaging because they can confine the optical field in a subwavelength volume and at the same time they can enhance the radiation properties of the quantum emitter (fluorescent molecule, quantum dot). The fluorescence emission can be modified by its photonic environment, namely the excitation intensity, the quantum efficiency of the emitter and its radiation pattern [21, 22, 23]. Surface plasmons play an interesting role to enhance the fluorescence signal from a quantum emitter. Thus, optical antennas can confine the field in a small volume, offering a resolution below the diffraction limit and simultaneously enhance the fluorescence emission.

To study the dynamic and heterogeneous nature of live cell membranes, a technique that simultaneously provides a high temporal and spatial resolution, while at the same time being non-invasive is required. In this thesis, the combination of optical antennas and fluorescence correlation spectroscopy to study the dynamics of cellular processes in living cells is explored. By combining optical antennas with FCS, simultaneous high temporal and spatial resolutions are achieved in a non-invasive way, which seems to be an ideal approach to study the molecular dynamics of a living cell.

2. Experimental methods
2.1 Experimental setup
All experiments were performed on a commercial time-resolved confocal microscope MicroTime 200 (PicoQuant GmbH, Germany)\cite{24} with a 63x, 1.2 NA water immersion objective, using as excitation sources either a LDH-470 or a LDH-640 pulsed diode laser (470 and 640 nm, respectively). The photon detection was performed by two avalanche photodiodes (Appendix). Note that a halfwave plate is used to select the direction of the polarized excitation light. A 488/561/635 nm band pass dichroic mirror reflects the excitation light and transmits the fluorescence light to the detectors. Data acquisition and analysis was done with SymphoTime 32 software \cite{24}.
For the antenna experiments, the metallic nanostructure is placed on top of the objective, in the focus plane of the confocal microscope.

2.2 Antenna-in-Box
The optical antennas used in this work were antenna-in-boxes \cite{25} (figure 1.a). Nanoantennas were milled by FIB on 50-nm-thick gold films deposited by thermal evaporation. Adhesion between the gold film and the glass coverslip substrate was ensured by a 3-nm-thick titanium layer deposited by electron-beam evaporation. Briefly, the antenna consists in two dimers inside a rectangular box. When the antenna is excited with linear polarized light parallel to the dimer axis a hotspot is created between the two dimers (figure 1.b). The hotspot confines high power in a small volume. The rectangular box also plays an important role. Since the box dimension are below the diffraction limit it blocks part of the photons coming from the excitation beam, avoiding some background, and thus, increasing the signal to noise ratio \cite{25}.

![Figure 1. Photonic antenna used in this work (antenna-in-box). (a) Scanning electron microscope image of an antenna-in-box (b) Finite-difference time-domain simulation of the intensity enhancement in the plane xz when the antenna-in-box is excited with light polarized along the long axis of the box.](image)

2.3 Fluorescence Correlation Spectroscopy
From FCS analysis it is possible to determine all the parameters that induce fluctuations in the fluorescent signal. For free diffusing particles without triplet state, the main parameters that can be found from the autocorrelation curve are the number of particles and the diffusion time (the average time that a particle needs to cross the illumination volume). The fluctuations in the intensity are caused by a molecule diffusing in and out of the illumination volume, and thus the diffusion time can be derived from the decay time of the autocorrelation curve. The number of particles inside the observation volume is the inverse of the amplitude of the autocorrelation function (at lag time 0).
Conceptually, as the number of molecules increases, the relative effect of a single molecule on the total fluorescence intensity decreases \cite{19, 26, 27}.
In this thesis it is assumed that the illumination volume is approximated to 3D Gaussian (solution) or 2D Gaussian (cell membrane) profile. Although this assumption is correct for confocal illumination
but not for the antenna configuration, we will use it to simplify the analysis. The autocorrelation functions for free diffusing particles diffusing in a 3D and a 2D Gaussian profile is given respectively by [17]:

\[ G(\tau) = \frac{1}{N} \left(1 + \frac{\tau}{\tau_d}\right)^{-1} \left(1 + \frac{\tau}{k^2 \tau_d}\right)^{-1/2} \]

Equation 1

\[ G(\tau) = \frac{1}{N} \left(1 + \frac{\tau}{\tau_d}\right)^{-1} \]

Equation 2

Where \( N \) is the number of particles inside the illumination volume, \( \tau_d \) is the diffusion time, \( k \) is the ratio between axial and radial dimensions and \( \tau \) is the lag time.

3. Results

3.1. Confocal FCS of a single dye in solution

In the first part of the results section are presented FCS results of the fluorescent dye Atto488 (Atto-Tec GmbH, Germany) in aqueous solution obtained using confocal microscope FCS. In these experiments, the effects of the fluorescent dye concentration, excitation power and medium viscosity on the autocorrelation curves was studied.

3.1.1 Effect of dye concentration on the autocorrelation curves

The amplitude (i.e. the value of the autocorrelation at lag time 0) of the autocorrelation curve is the inverse of the number of particles inside the excitation volume. For a given set up, the excitation volume is constant (it depends on the wavelength, numerical aperture and the pinhole) and thus for different concentrations there will be a different number of molecules inside the excitation volume. In figure 2.a it can be seen that the amplitude of the autocorrelation curves decrease as the dye concentration increases. As expected, dye concentration does not affect its diffusion time \( (\tau_d=0.06068 \, \text{ms}) \), as the normalized autocorrelation curves clearly show (figure 2.b).

A linear relationship between the concentration and the number of particles is expected. To check that this is true in these experiments the values of the fitted number of particles inside the illumination volume were represented as a function of the concentration of Atto 488 (figure 3).
3.1.2 Effect of the laser excitation power on the autocorrelation curves

In this experiment, the concentration of the dye solution is kept constant while the excitation power is varied. The experimental results (figure 4.a) show that the autocorrelation curves are better defined as the power increases and simultaneously the number of particles increase. The fitted curves also show the same diffusion time, 0.06283 ms. These results show that the illumination volume and the diffusion coefficient are not affected by the excitation power. In the fluorescence process, the molecule absorbs a photon, the photon energy is used to excite the electron to a higher energy level. The electron suffers non-radiative relaxations and then is relaxed emitting a photon. The excitation power is proportional to the number of photons emitted by the laser. This explains the fact that the number of particles increases with the excitation power. The probability that a molecule is excited increases as the density of photons increase. Of course, this only happens if we are under saturation conditions, as in the case of the experiments shown in figure 4.b. The fact that the curves look nicer at higher powers can be explained in the same way. As the excitation power increases the radiative pathway is facilitated and thus more photons are emitted and detected, increasing the number of points that are used to calculate the autocorrelation curve.

3.1.3 Effect of medium viscosity on the autocorrelation curves

![Graph showing the effect of laser excitation power on autocorrelation curves](image)

Figure 4. Effect of the excitation power on the autocorrelation curves. (a) Autocorrelation curves at different excitation power for Atto 488. Each colour corresponds to a different excitation power as it is specified in the legend. Points are the experimental data and the lines represent the fitting of equation (1) to the data. (b) Number of particles inside the excitation volume as a function of the laser power. There should be a limit where the number of particles remains constant even if the power is increasing, but it was not possible to reach this limit with our experimental setup.
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While the amplitude of the autocorrelation curve is related with the number of particles inside the illumination volume, the decay of the curve is related with the time the molecule diffuses through the illumination volume. To be more specific, the diffusion time (i.e. the time that a particle needs to cross the illumination volume) is the time that the autocorrelation curve needs to get its half maximum value. It is well known in fluids theory that diffusion coefficient of a spherical particle in an aqueous solution is inversely proportional to the viscosity (Einstein-Stokes Equation)[28]:

$$D = \frac{k_B T}{6\pi \eta r}$$

Equation 3

where $D$ is the diffusion coefficient, $k_B$ is the Boltzmann’s constant, $T$ is the temperature, $\eta$ is the viscosity of the medium and $r$ is the radius of the diffusing particle.

In figure 5 are presented the normalized FCS curves of Atto488 in aqueous solution containing different sucrose concentrations. Experimentally it has been found that the viscosity of the medium is exponentially proportional to the concentration of sucrose in % of mass [29]. The results clearly show the influence of the viscosity in the diffusion time, as the % of sucrose increases the diffusion time increases (figure 5).

Figure 5. Effect of the medium viscosity on the autocorrelation curves Normalized autocorrelation curves for Atto 488 at 5nM. Different colours correspond to different % of sucrose in the solution. Symbols are the experimental data and the lines represent the fitting of equation (1) to the data. As the % of sucrose increases the diffusion time increases.

To quantitatively assess the relationship between medium viscosity and the diffusion time, the fitted diffusion times were plotted as a function of the % of sucrose (figure 6). From the Einstein-Stokes equation (Equation 3). It is known that the diffusion coefficient is inversely proportional to the viscosity. Thus the diffusion time should be directly proportional to the medium viscosity. Conversely, medium viscosity is exponentially proportional to % of sucrose [29] and therefore the diffusion time of the dye should be exponentially proportional to the % of sucrose. Indeed, fitting an exponential equation to the experimental data a good agreement was obtained, as clearly shown in figure 6.

$$y = 0.059e^{0.0269x} \quad R^2 = 0.995$$

% sucrose

\[0,2\]
\[0,15\]
\[0,1\]
\[0,05\]

\[0\]
\[10\]
\[20\]
\[30\]
\[40\]
\[50\]

\[\tau [ms]\]

\[\%\text{ sucrose}\]
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Figure 6. Exponential relation between diffusion times and % of sucrose. Diffusion times obtained from the fit of equation 1 to the experimental data as a function of the % of sucrose. Points are the average of diffusion times between three different dyes concentration (1 nM, 5nM and 10 nM. Data not Shown). Black line is the exponential fitting to the equation \( y = a e^{b x} \).

3.2 FCS of a single dye in solution using the antenna-in-box

To characterize and validate the use of FCS in combination with optical antennas to study the dynamics of membrane molecules in living cells, experiments with an aqueous solution of Atto655 (Atto-Tec GmbH, Germany) were first performed.

When the antenna is excited with linear polarized light along the dimers direction we expect to have a small hot spot with high intensity due to the plasmon coupling (figure 1.b).

For the FCS analysis I first measured with the box aperture (without dimers). From the experimental data analysis it is possible to retrieve the diffusion times, number of particles and then the count rate per molecule (brightness). With these parameters it is possible to analyse the antenna-in-box data considering two different species [25] corresponding to molecules diffusing in the hotspot and inside the box aperture:

\[
G(\tau) = \frac{N^0 Q^0 G_0(\tau) + N_0 Q_0^2 G_{d0}(\tau)}{(N^0 Q^0 + N_0 Q_0)^2} \quad \text{Equation 4}
\]

where \( N_0, Q_0 \) and \( G_{d0}(\tau) \) are the fixed number of particles, brightness and autocorrelation curve from the box contribution (to be more specific, \( N_0 \) is the 0.7*number of particles determined from the box experiments. This factor 0.7 is to take into account the volume of the dimers). These parameters are fixed by previous experiments in the box alone. \( N^*, Q^* \) and \( G_d^* \) are the corresponding parameters from the hotspot.

In figure 7.a are represented the autocorrelation curves of the experimental data obtained for the antenna-in-box and the box alone for the parallel and perpendicular polarizations. From our experimental set up we are not able to get the autocorrelation curve at shorter times. To appreciate better the difference between the parallel excitation polarization (V) or perpendicular polarization (H), an extrapolation of the autocorrelation curves to shorter times using the parameters from the experimental data is presented. Figure 7.b shows the normalized autocorrelation functions. Clearly, the curve for the antenna-in-box excited with the correct polarization decays faster than the others, indicating a volume reduction. Note also, that the box aperture also works as a sub wavelength aperture, breaking the diffraction limit. The main results are summarized in table 1.

![Figure 7](image)

Figure 7. FCS on antenna-in-box (a) Experimental autocorrelation curves and simulated curves according to equation (1) (box alone) and equation (4) (antenna-in-box) with the parameters from the experimental data. (b) Normalized autocorrelation curves for the antenna-in-box, box alone and confocal. V and H denote the two perpendicular excitation polarizations used in the experiments. For the nanostructures experiments I used 5µM of Atto 655 at 10µW at 640nm. For the confocal experiments I used 2.5nM at 22mW.

<table>
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<th>Table 1. Parameters from FCS analysis. Antenna parameters are the hotspot contribution.</th>
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<td>Parameter</td>
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### 3.3 Antenna-in-Box Living Cell

After the characterization of the experimental approach, preliminary experiments on live Chinese hamster ovary (CHO) cells were carried. During measurements the cells were kept at 37 °C and on an atmosphere of 5% CO₂. As a proof-of-principle the dynamics of phosphoglycerolipid-\( \text{Atto647N} \)-1,2-dipalmitoyl-\( \text{sn} \)-glycero-3-phosphoethanolamine (Atto 655-PE) was measured. This fluorescent lipid is known to follow a 2D free diffusion with a diffusion coefficient of \( D = 0.49 \pm 0.04 \ \mu\text{m}^2\ \text{s}^{-1} \) [30].

Figure 8.a shows a cell on top of an array of antennas-in-box. The image is a reconstruction as a function of the intensity, as more white more intensity. The white spots correspond to the antenna enhancement. Due to the slow diffusion coefficient of PE in cell membrane (around 4 times slower than Atto 655 in solution) a maximum excitation of 1\( \mu \)W was used to avoid photobleaching. In these conditions we expect to have a low intensity contribution from box background and then see bursts of intensity in the photon counting time trace when a fluorescent molecule diffuses through the hotspot (figure 8.b). As is expected, there are bursts only when the excitation light is polarized in the correct direction. The problem that we have at the moment is that we cannot see enough number of bursts to perform the autocorrelation analysis.

Figure 8. FCS measurements using antenna-in-box on live cell membranes. (a) Confocal microscopy image of a CHO cell labelled with 200nM of Atto655-PE on top of an array of antenna-in-box. (b) Photon counts as a function of time measured at the indicated antenna on a). Pol V and Pol H correspond respectively to the polarization parallel and perpendicular to the antenna.

### 4. Discussion

Cellular processes occur at high temporal and spatial scales, making it challenging to get access to the molecular mechanisms and dynamics controlling them. It has been demonstrated that FCS is a powerful technique when used in the proper conditions. It is non-invasive and due to its temporal resolution it is an ideal technique to study the molecular dynamics in the living cell surface. However, an intrinsic limitation of FCS is that only a small number of molecules should be inside the observation volume at the same time. Since the molecular concentration in the living cell membrane is in the order of micromolar it is mandatory to overcome this concentration-imposed limit. This can be achieved by reducing the illumination volume well below the diffraction limit. Optical antennas can reduce the illumination volume and at the same time enhance the fluorescence signal. Here we took advantage of the volume reduction, brightness enhancement and the background suppression of the
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antenna-in-box. Its characteristic shape offers an incredible signal to noise ratio. The experimental results in solution demonstrated that the antennas work as expected. The time resolution of the analysis software is around $10^{-3} \sim 10^{-4}$ ms. Even the diffusion time of Atto655 through the hotspot in solution is smaller than the software resolution, there is a clear difference in the experimental data between the antenna-in-box in the correct and the incorrect polarization of the excitation light, the box alone and the confocal volume. The obtained results by extrapolating the experimental data are the expected. On the other hand the diffusion in the cell membrane is slower than in solution and temporal resolution of the analysis software is enough.

The living cell experiments on optical antennas at the moment were not completely successful. The problem was that there were no enough fluorescent molecules diffusing through the hotspot. There were bursts in the intensity signal, but they were insufficient to calculate the autocorrelation curve. Since the bursts only appear when the optical antenna is excited with the correct polarization, the hypothesis that the antenna is not working as expected is discarded. The other hypotheses are that there was not enough labelled lipids, that the membrane lipids were not diffusing properly or that the excitation intensity of the hotspot was not reaching the membrane.

1- First hypothesis: since the intensity signal just shows a few numbers of bursts, this might mean that there is a labelling problem so that not enough fluorescent molecules are attached at the membrane. The antenna-in-box is designed to work with high concentrations due to its small hotspot illumination are of the order of 75 nm$^2$ (depending on the gap size). For low concentrations the mean number of molecules in the hotspot are much lower than one. This could explain the low number of bursts.

2- The second hypothesis is that the molecules are not diffusing freely. The contact between the cell membrane and the nanostructure could cause stacking effects in the most of fluorescent molecules. If this hypothesis is correct, the bursts correspond to the little amount of molecules that are able to freely diffuse.

3- The third hypothesis is that the excitation intensity of the hotspot is not reaching the cell membrane. The cell membrane is not completely flat and static over the nanostructure. It is in constant movement, oscillating in the vertical direction, perpendicular to the surface (20 nm per 2 sec [30]). As the intensity in the hotspot decays exponentially in the vertical axis it is possible that the most part of the time the cell membrane is too far from the nanostructure and the fluorescent molecules are not excited by the hotspot.

5. Conclusions and future perspectives

The final goal of this project is to study the molecular dynamics in the cell membrane and their organization. Before reaching this goal it is mandatory to be sure that the experimental methods are working as expected to avoid artefacts or non-reliable results. For this reason the first experiments were performed with PE whose dynamics in the cell membrane are well known. Future experiments should be focussed in solving the experimental problems found and checking the validity of the hypothesis commented above in the following way:

1- Labelling problem: Use different concentrations of fluorescent dyes and measure the intensity signal in confocal and in antennas. If the signal does not change in the same proportion for confocal and antennas measurements, there is a problem to label the lower membrane. If they change in the same proportion it is enough to choose the labelling according to have a fluorescent molecular concentration in the order of micromolar.

2- Diffusion problem: since the diffusion coefficient of PE is well known, looking at the burst width it is possible to determine the experimental diffusion time. If the theoretical time and the experimental time are the same, there is no diffusion problem.

3- Excitation intensity of the hotspot not reaching the cell membrane: for larger gap size the hotspot is bigger, so this hypothesis can be checked using antennas with different gap size. Simulations should be also important in here.

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Appendix

A 1. Schematics of the experimental setup used.