Two-photon fluorescence correlation spectroscopy with high count rates and low background using dielectric microspheres

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Abstract: Two-photon excitation fluorescence is a powerful technique commonly used for biological imaging. However, the low absorption cross section of this non-linear process is a critical issue for performing biomolecular spectroscopy at the single molecule level. Enhancing the two-photon fluorescence signal would greatly improve the effectiveness of this technique, yet current methods struggle with medium enhancement factors and/or high background noise. Here, we show that the two-photon fluorescence signal from single Alexa Fluor 488 molecules can be enhanced up to 10 times by using a 3 μm diameter latex sphere while adding almost no photoluminescence background. We report a full characterization of the two-photon fluorescence enhancement by a single microsphere using fluorescence correlation spectroscopy. This opens new routes to enhance non-linear optical signals and extend biophotonic applications.

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1. Introduction

Two-photon excitation of molecular fluorescence has generated a large interest among the scientific community since its first demonstration in 1990 [1]. Two-photon excitation applied to scanning fluorescence microscopy is an elegant method to obtain intrinsic three-dimensional resolution, thereby increasing the image contrast and limiting the out-of-focus photodam-

age [2]. This technique enables deep-tissue imaging of living organisms, with typical penetration depths of several hundreds of micrometers [3]. Two-photon excitation of molecular fluorescence has also opened promising opportunities to extend the application of fluorescence correlation spectroscopy (FCS) [4–8]. FCS is an attractive method to determine molecular concentrations, diffusion coefficients, chemical kinetics, and fluorescence photophysics [9–11]. It was envisioned that many problems encountered in FCS measurements such as light scattering, autofluorescence, and photodamage can be reduced under two-photon excitation [4–6].

An essential limitation in two-photon fluorescence is that the two-photon absorption cross sections of common dyes are fairly low (of the order of 10^{-50} cm⁴·s/photon) [12, 13]. This makes difficult any experiment dealing with two-photon excitation of single fluorescent molecules [14, 15]. Typical two-photon fluorescence detection rates hardly reach a few thousands of counts per second and per molecule [7, 8]. Consequently, many experiments are performed close to the fluorescence saturation regime, which can lead to measurement artifacts and dye photobleaching. The relatively low fluorescence count rate per molecule also limits the applicability of two-photon fluorescence correlation spectroscopy, where the fluorescence count rate per molecule is the most crucial parameter to determine the signal-to-noise ratio (see [16] and references therein).

Enhancing the two-fluorescence signal would open new opportunities for biophotonic applications dealing with single molecule analysis or FCS. To meet this goal, much attention is currently devoted to plasmonic structures to locally enhance the electromagnetic field intensity and efficiently perform multi-photon excitation of fluorescent molecules [17–19]. However, the high photoluminescence background generated by the plasmonic structure itself puts a critical limit to the signal-to-noise ratio while detecting a single molecule [20]. Moreover, the practical implementation of plasmonic substrates is also limited by the damage threshold the metal structure can withstand without strong photodamage of the nanostructures. There is thus a challenge to develop nanophotonic structures to enhance the two-photon fluorescence detection rate of single molecules while maintaining a low background noise.

We show here that dielectric microspheres form a viable alternative to plasmonic nanostructures to enhance the detected two-photon fluorescence signal per molecule. Under normal plane wave illumination, a single dielectric microsphere has been demonstrated to focus light in a beam termed “photonic nanojet” that has a high intensity, sub-wavelength transverse dimensions and a low divergence [21–23]. This configuration has already been used to enhance the backscattering of light by metal nanoparticles [24], and to design a simple and low-cost alternative to conventional complex microscope objectives [25]. The application of microsphere focusing to non-linear optics was previously investigated in a report employing silica microspheres added to a rhodamine B dye solution placed in a flow cell [26]. A two-photon fluorescence enhancement of 30% was found by measuring the spectrally integrated signal of the solution dye with and without microspheres. Because of the spatial averaging of the phenomenon over a large area, it is not possible with this method to quantify the two-photon fluorescence gain brought by a single microsphere. The reported 30% increase in two-photon fluorescence signal is thus an underestimate of the gain brought by a single microsphere [26].

In this paper, we report the demonstration and characterization of two-photon fluorescence enhancement in the vicinity of a single dielectric microsphere. When a single 3 μm diameter latex microsphere is set close to the focus of a 1.2 NA microscope objective, we show that the two-photon fluorescence signal from single Alexa Fluor 488 molecules can be enhanced up to 10 times. This high enhancement factor comes with almost no added photoluminescence background noise. These results take advantage of the special focusing when the microsphere is illuminated by a tightly focused Gaussian beam (see Fig. 1, panels a and b). In that configuration, the microsphere over-focuses the incoming light in a spot with subwavelength dimensions

Fig. 1. (a) and (b) Schematics of the experimental setup. (c) fluorescence intensity auto-correlation functions obtained in solution (blue) and with a 3 μm latex sphere (red). (d) Summary of the reduction in the number of observed molecules N for the different sizes of microspheres as compared to the case with no microsphere.

in both the transverse and longitudinal directions, creating high local intensities and overcoming the diffraction limit [27,28]. A detailed picture of this phenomenon is given in [27], which explains this effect by the interplay of two contributions: (i) a focusing of the incident field by the microsphere and (ii) interferences between the incident field and the field scattered by the sphere. We stress that this configuration exceeds the focusing properties of a classical microscope objective with a high numerical aperture as well as a microsphere illuminated by a plane wave [22,26].

To fully characterize the two-photon fluorescence enhancement, we analyse the temporal fluctuations of the two-photon fluorescence intensity using a standard fluorescence correlation spectroscopy method [9, 10, 28]. The FCS analysis quantifies the average number of molecules N diffusing through the analysis volume. An essential limit inherent to the FCS method is that the FCS data is spatially averaged over all the possible molecular orientations and positions inside the confocal volume that is analysed. There is no sensitivity to individual molecular trajectories or dipole orientations. Instead, the FCS method provides global figures to characterize the (spatially averaged) fluorescence that is emitted from the confocal observation volume. The knowledge of N is a key to measure the average fluorescence count rate per molecule (*i.e.* the number of photons detected for a single molecule per second). Accessing to the fluores-

cence rate per molecule with and without the microsphere provides an elegant way to quantify the two-photon fluorescence enhancement brought by a single microsphere. We explain this fluorescence enhancement by a local enhancement of the excitation intensity due to the ultra-focusing of light together with a modification of the emitter's radiation pattern, directing more energy towards the detectors. A procedure that we have recently developed [29,30] allows us to determine the respective weight of the excitation and emission contributions in the two-photon fluorescence enhancement factor.

2. Materials and methods

2.1. Microspheres samples

Latex microspheres (refractive index 1.59) of well calibrated diameters 1, 1.5, 2, 3 and 5 μm were taken as purchased from Fluka Chemie GmbH (dispersion <0.1%). After dilution in pure water, microspheres are dispersed on a cleaned microscope coverslip. The concentration was set so that isolated spheres within 10x10 μm^2 are found.

2.2. Fluorescence correlation spectroscopy experimental setup and measurements

Our experimental setup is based on an inverted microscope with a NA=1.2 water-immersion objective [Fig. 1(a)]. For FCS measurements, a 50 μL droplet of Alexa Fluor 488 dye (A488, Invitrogen, Carlsbad CA, with absorption / emission peaks at 495 and 519 nm) diluted in pure water is deposited on top of the microsphere sample. For all the measurements reported here, the dye concentration is kept constant. We emphasize that the axial positioning of the microsphere from the incident focused beam is very important and an accurate ± 200 nm axial positioning is necessary. This positioning is ensured by 3 axis piezoelectric stage with nanometric resolution so as to reach the highest detected count rate per molecule. Two-photon excitation is performed by a tunable pulsed Ti:Sapphire laser delivering 150 fs pulses at 80 MHz repetition rate, with the wavelength set at 920 nm (Chameleon, Coherent, Santa Clara, CA). The backward-emitted fluorescence is filtered from the scattered laser light by a dichroic mirror and detected by two avalanche photodiodes (PerkinElmer SPCM-AQR-14, Boston MA) with 525 ± 25 nm band-pass filter. Due to the non-linear nature of the two-photon fluorescence process, no confocal pinhole is needed to reject the out-of-focus light. This makes the alignment easier compared to the one photon excitation. The fluorescence intensity $F(t)$ is analyzed by a hardware correlator (ALV-GmbH ALV6000, Langen, Germany) to compute the temporal correlation function $G^{(2)}(\tau) = \langle F(t) \cdot F(t + \tau) \rangle / \langle F(t) \rangle^2$, where $\langle \rangle$ stands for time-averaging and τ is the delay lag time. Numerical analysis of the FCS data according to an analytical model established for Brownian three-dimensional diffusion [10,28] provides the average number of molecule N in the detected volume:

$$G^{(2)}(\tau) = 1 + \frac{1}{N} \frac{[1 + n_T \exp(-\tau/\tau_{b_T})]}{(1 + \tau/\tau_d) \sqrt{1 + s^2 \tau/\tau_d}}, \quad (1)$$

where N stands for the average number of molecules, n_T the amplitude of the dark state population, τ_{b_T} the dark state blinking time, τ_d the mean diffusion time and s the ratio of transversal to axial dimensions of the analysis volume, calibrated to $s = 0.2$ for free solution. For the readers unfamiliar with FCS, let us simply emphasize that the autocorrelation function amplitude at zero lag time $G^{(2)}(0)$ is inversely proportional to N . This property is valid independently of the shape of the excitation field and the type of diffusion statistics [10,28], as a consequence of the Poissonian nature of the probability to find a molecule in the observation volume at a given time. This procedure holds for a stationary system and a dilute solution where the spatial correlation length of concentration fluctuations is much smaller than the detection volume,

which is clearly the case for our study. Finally, the fluorescence count rate per molecule CRM is deduced from the knowledge of the average fluorescence intensity and the number of emitters: $CRM = \langle F \rangle / N$.

3. Experimental results

We start our investigations by conducting two-photon FCS measurements in order to determine the number of molecules N and the CRM while varying the excitation powers for each sphere diameter and for the reference solution. Typical temporal correlation functions $G^{(2)}(\tau)$ are presented in Fig. 1(c), they are obtained with and without a single 3 μm diameter sphere for the same A488 solution. For that specific experiment with 10 mW average excitation power, the total detected signal in solution without the microsphere is about 60 kHz. When the microsphere is brought into the laser focus, the total fluorescence signal becomes 72 kHz, and the photoluminescence background (generated by the microsphere itself) is less than 2 kHz. A signal-to-noise ratio higher than 36 can readily be reached while using the microsphere. This shows that the photoluminescence background from the microsphere remains negligible, in clear contrast to metal nanostructures that can generate a huge amount of photoluminescence [20]. The relatively small gain on the overall fluorescence intensity does not preclude to a larger gain in the fluorescence rate per molecule, as less molecules contribute to the total fluorescence intensity in the case of the microsphere.

As can be seen in Fig. 1(c), the autocorrelation function amplitude at zero lag time $G^{(2)}(0)$ is higher in the presence of the microsphere, which means that the number of detected molecules is reduced compared to the open solution case. Please note that with our standard definition of $G^{(2)}$, the normalized level showing no correlations is 1. Therefore, the relevant amplitude of $G^{(2)}(0)$ representing the contribution from diffusing molecules is to be considered starting from this level. Since the 1.2 NA objective used for our experiments provides a state of the art limited diffraction volume, the lower number of molecules detected with the microsphere indicates that a sub-diffraction observation volume is reached with microsphere. To evaluate the observation volume reductions, we compute the ratio $\eta_V = N_{sol}/N_{sphere}$, where N_{sol} and N_{sphere} are respectively the number of molecules detected in free solution and with the microsphere. Figure 1(d) summarizes the observation volume reductions obtained for the different diameters of spheres. A 8-fold volume reduction is achieved with the 3 μm sphere, allowing FCS measurements to be conducted at higher concentrations [31]. In common FCS experiments, the analyte concentration is set to a few nanomolar, in order to isolate a few tens of molecules in the analyzed volume. This concentration is however too low, as many biologic processes occur at micromolar concentrations [32]. Therefore, the observation volume reduction brought by the microsphere is an important effect to extend the application range of FCS towards higher analyte concentrations.

Additionally, the FCS measurements allow the determination of the $CRM = \langle F \rangle / N$ for different excitation powers with and without the microspheres. Figure 2(a) summarizes our results. Higher $CRMs$ at all excitation powers are obtained with the different microsphere diameters as compared to the reference solution. For a given excitation power, the ratio of the $CRMs$ with and without the microsphere directly yields the fluorescence enhancement factor. We also observe that without the microsphere, the $CRMs$ for A488 molecules saturate at values of typically 5 kHz for average excitation powers above 30 mW, whereas $CRMs$ of about 10 kHz can be readily achieved with different sizes of microspheres at much lower excitation powers. The microspheres improve the detected count rate per molecule, they also allow the use of a smaller observation volume and lower excitation power, which are beneficial to reduce molecular photodamage. We also would like to point out that the signal to noise ratio in FCS is proportional to the fluorescence count rate per molecule times the square root of the total experiment acqui-

Fig. 2. (a) Evolution of the CRM versus the excitation intensity (dots) and numerical fit (solid lines) according to Eq. (2) for the different microsphere diameters and in open solution case. (b) Fluorescence enhancement factor η_F in the low excitation regime for the different sphere diameters.

sition time [16]. Therefore, the 10-fold fluorescence enhancement allows a 100-fold reduction of the total experiment duration time.

We analyse the CRM data on Fig. 2(a) by using a standard expression of the fluorescence rate for two-photon excitation [10]:

$$CRM = \frac{AI_e^2}{1 + I_e^2/I_s^2} \quad (2)$$

where I_e is the input excitation intensity, $A = \kappa\phi\sigma_{2P}$ is a constant proportional to the setup collection efficiency κ , the dye's quantum yield $\phi = k_{rad}/k_{tot}$ and the dye's effective two-photon absorption cross section σ_{2P} (k_{rad} is the radiative emission rate and k_{tot} the total decay rate). $I_s = \sqrt{k_{tot}/\sigma_{2P}}$ is the saturation intensity. We stress that I_e is the incident excitation intensity at the waist of the laser beam focused by the 1.2NA objective. I_e must not be confused with the local intensity actually sensed by the molecule, as the microsphere will further enhance this local intensity [see a further discussion below and Fig. 3(a)].

In the limit of weak excitation ($I_e \ll I_s$), the CRM is reduced to $CRM_{low} = AI_e^2$ which indicates that the fluorescence rate per molecule is proportional to the square of the incoming laser power. Another method to quantify the fluorescence enhancement $\eta_F = CRM_{sphere}/CRM_{sol}$ in the low excitation regime is thus deduced from the numerical fitting using Eq. (2) as the ratio $\eta_{F,low} = A_{sphere}/A_{sol}$. Results are presented in Fig. 2(b). As can be seen, a fluorescence enhancement up to 6-fold is readily achieved with all sphere diameters. The relative uncertainty on the fluorescence enhancement is 10%, and is mostly due to repeatability errors from sphere to sphere.

We would like to stress that a ten times two-photon fluorescence gain is reached with the 3 μm latex sphere. This is a significant enhancement that comes without the cost of any photoluminescence noise. This accurate measurement also clearly exceeds the 30% increase in two-photon fluorescence deduced from a much simpler experimental configuration [26].

As already observed in the case of one-photon fluorescence [28], there is an optimum diameter for the microsphere to provide the largest fluorescence enhancement. For latex spheres immersed in water, the optimum diameter is about 3λ , where λ is the wavelength of light in vacuum. It is interesting to note that this optimal diameter corresponds to the best over-focusing

Fig. 3. Excitation (a) and collection (b) contributions to the fluorescence enhancement for the different microsphere diameters.

ability of a microsphere illuminated by a tightly focused Gaussian beam. Therefore, this simple guideline to determine the optimum microsphere diameter holds for both one- and two-photon excitation of fluorescence. We also point out that the numerical aperture of the microscope objective has only a moderate influence on the values indicated here, as long as the NA remains above a typical value of 0.80 [27].

Recording the *CRM* evolution for increasing excitation powers allows to determine the physical origin of the fluorescence enhancement, and quantifies the respective contributions of the excitation and emission gains in the global fluorescence enhancement factor [29,30]. The two free parameters in the fit of the *CRM* using Eq. (2) (namely A and I_s) are sufficient for that purpose, as we will show hereafter. In the saturation regime ($I_e \gg I_s$), the fluorescence signal does not depend anymore on the excitation intensity, and the *CRM* is reduced to $CRM_{sat} = AI_s^2 = \kappa k_{rad}$. The latter equation states that in the fluorescence saturation regime, the detected fluorescence rate per molecule is proportional to the dye's emission rate k_{rad} and to the setup collection efficiency κ [10, 30]. Let us stress that this fluorescence saturation regime does not have to be physically reached experimentally, fitting the *CRM* data with Eq. (2) for lower excitation powers to extract the parameters A and I_s is enough. It was already shown in [30] that the microsphere does not significantly modify the dye's radiative rate k_{rad} , as the purely dielectric nature of the sphere does not noticeably modify the local photonic density of states [18]. The radiative rate k_{rad} is thus assumed unchanged by the presence of the microsphere, and the ratio of the *CRMs* in the fluorescence saturation regime thus gives the increase in the fluorescence collection efficiency $\eta_{F,sat} = (AI_s^2)_{sphere} / (AI_s^2)_{sol} = \kappa_{sphere} / \kappa_{sol}$. In the saturation regime, all the fluorescence enhancement is due to the gain in collection efficiency $\eta_{F,sat} = \eta_{\kappa}$. In the low excitation regime, the fluorescence enhancement η_F can be rewritten as $\eta_{F,low} = \eta_{\kappa} \eta_{I_e}^2$, where η_{I_e} is the excitation intensity enhancement factor. The knowledge of $\eta_{F,low}$ and η_{κ} gives finally access to $\eta_{I_e} = \sqrt{\eta_{F,low} / \eta_{\kappa}}$. We emphasize again that due to the non-absorbant nature of the sphere (dielectric material), the quantum yield modification is negligible.

Figure 3 summarizes the value of η_{I_e} and η_{κ} obtained for each microsphere diameter. The 10 fold fluorescence gain obtained with the 3 μm sphere appears as a combination of an excitation intensity enhancement by a factor 2.3 times a collection efficiency increase of 1.9. We point out that the results are consistent with those obtained under one photon fluorescence excitation [30] (taking into account the spatial scaling factor introduced by the difference excitation wavelength

used here). This fluorescence characterization procedure reveals the respective weight of the excitation and collection contributions to the total fluorescence enhancement.

4. Conclusion

In this report, we combine a single latex microsphere with a standard two-photon fluorescence microscope, and obtain two-photon fluorescence rates per molecule that are enhanced by about one order of magnitude as compared to the same solution without the microsphere. Simultaneously with the fluorescence gain, a 8-fold reduction of the confocal analysis volume is observed, which clearly overcomes the classical limit set by the far-field diffraction of light and enables FCS experiments to be conducted at higher analyte concentrations. The microspheres improve the detected count rate per molecule, they also allow the use of a smaller observation volume and lower excitation power, which are beneficial to reduce molecular photodamage. A characterization procedure based on fluorescence correlation spectroscopy determines the gains in excitation intensity and collection efficiency that both contribute to the two-photon fluorescence enhancement. Since the signal to noise ratio in FCS is proportional to the fluorescence count rate per molecule times the square root of the total experiment acquisition time [16], the 10-fold two-photon fluorescence enhancement allows a 100-fold reduction of the total experiment duration. We stress that this significant enhancement comes without adding any significant photoluminescence background from the structure itself, as this can be a major drawback while using plasmonic metal structures.

Microspheres can also be used in dual-color fluorescence cross correlation spectroscopy for monitoring fast enzymatic cleavage reaction that requires short integration time [7, 8]. The insert of a single microsphere at the end of an optical fiber appears as a promising way to achieve single molecule sensitivity with two-photon fluorescence correlation spectroscopy and standard fluorescent dyes [33, 34]. Such two-photon optical-fiber-microsphere probe can serve as a minimally-invasive endoscopic diagnostic tool to detect specific molecules in body fluids. We also believe that this technique could open new possibilities to enhance non-linear signals such as second harmonic generation and coherent anti-Stokes Raman scattering.

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