Strong electromagnetic confinement near dielectric microspheres to enhance single-molecule fluorescence

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Abstract: Latex microspheres are used as a simple and low-cost means to achieve three axis electromagnetic confinement below the standard diffraction limit. We demonstrate their use to enhance the fluorescence fluctuation detection of single molecules. Compared to confocal microscopy with high numerical aperture, we monitor a detection volume reduction of one order of magnitude below the diffraction limit together with a 5-fold gain in the fluorescence rate per molecule. This offers new opportunities for a broad range of applications in biophotonics, plasmonics, optical data storage and ultramicroscopy.

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1. Introduction

The common strategy to detect single molecule fluorescence uses a confocal microscope with a high numerical aperture (NA) objective to maximize the collected signal and reject the scattered light. Optical diffraction limits this approach to rather large detection volumes of typically 0.5 fL ($= 0.5\ \mu\text{m}^3$) and low detection rates per molecule. Nanophotonics offer new opportunities to overcome these limits [1, 2]. A major goal is to tailor the molecular electromagnetic environment, so as to simultaneously enhance the collected fluorescence and decrease the detection volume to reduce background noise and enlarge the practicable domain of concentrations for single-molecule analysis. Among the many different nanophotonic techniques that have been implemented recently, nanometric apertures, super-critical angle collection, microfluidic channels, and stimulated emission depletion stand out as promising tools (see [3] and references therein). Despite recent progress in nanotechnologies, a fundamental limitation on the existing techniques is that they rely either on complex optical instrumentation, and/or expensive nanofabrication facilities. Therefore, simple and highly parallel optical systems allowing light manipulation at the nanoscale are currently in high demand.

Here, we propose a simple, low-cost method to focus light on a spot presenting sub-wavelength dimensions in both transverse and axial dimensions. We demonstrate that a three-axis sub-wavelength light confinement can be obtained by illuminating a single dielectric microsphere with a tightly focused Gaussian beam (Fig. 1(a)). This system is exploited to enhance the detection of single fluorescent molecules : we report a simultaneous decrease of the confocal observation volume by an order of magnitude and an enhancement of the fluorescence brightness by a factor of five. This offers new opportunities for applications in biophotonics, plasmonics, and optical data storage. It also raises a number of fundamental questions regarding the interaction between the microsphere, the focused laser beam and the molecular emission.

Our findings appear very different to the previous investigations of the electromagnetic distribution close to a dielectric microsphere. Previous studies did all consider plane wave illumination. In this case, the beam that emerges from the microsphere has a high intensity, sub-wavelength transverse dimensions and low divergence, and was termed “photonic nanojet” [4]. Photonic nanojets have been experimentally characterized in [5], and have been demonstrated to enhance nanoparticle backscattering at microwave frequencies [6], and two-photon fluorescence with floating microspheres added to the analyte solution [7]. Due to its large dimension along the optical axis (typically about 2-3 μm), a photonic nanojet generated by plane wave illumination is not suitable for single molecule fluorescence analysis, as it will not yield better optical confinement than standard confocal microscopy. Quite surprisingly, we show below that a microsphere under focused Gaussian illumination can outperform classical confocal microscopy and simultaneously significantly enhance the fluorescence from a single emitter. This concept has strong connections with solid immersion lenses (SIL) [8], but with a much simpler and cost-effective system based on isolated latex microspheres.

2. Numerical simulations

To study the interaction between a highly focused laser beam and a dielectric microsphere, we have performed three dimensional numerical simulations using the Mie theory with analytic expressions for the incident and scattered fields on a basis of multipolar wave functions [9]. The sphere is characterized by a refractive index of $n = 1.6$ and a 2 μm diameter, with a water embedding medium ($n = 1.33$). A circularly polarized Gaussian laser beam of wavelength 633 nm is focused from the glass side with NA $= 1.2$. Without the microsphere, the incident beam focuses a few microns above the glass substrate with a waist of 280 nm (Fig. 1(b)), corresponding to the normal confocal microscopy situation. With the microsphere (Fig. 1(c)), a high intensity region is introduced, with reduced dimensions in both axial and transverse directions.

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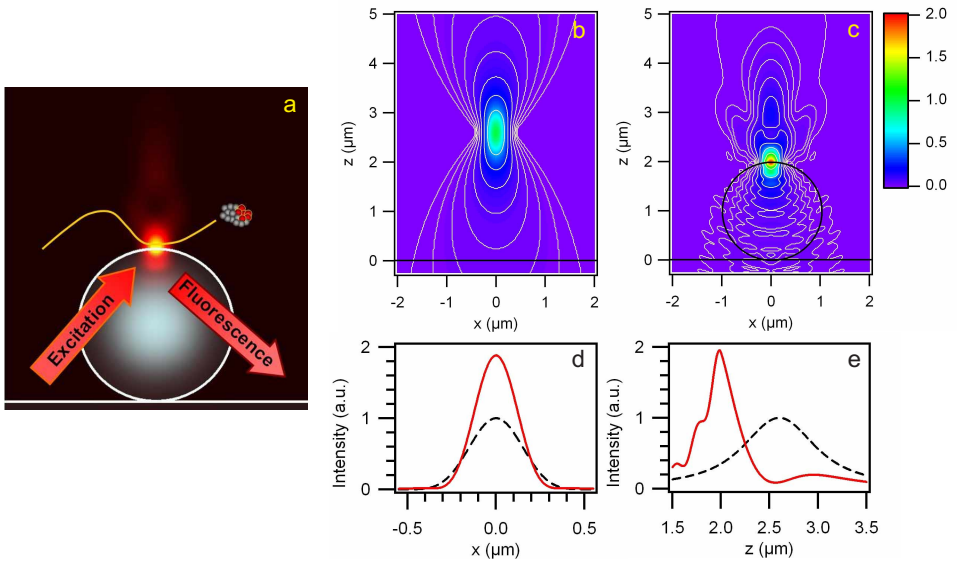

Fig. 1. (a) Microsphere configuration for single molecule detection enhancement. Numerical simulations are computed without (b) and (c) in the presence of a 2 μm sphere illuminated by a focused Gaussian beam at $\lambda = 633$ nm with NA= 1.2 (factor of 2 between adjacent contours). Cuts along the horizontal and vertical axis at best focus outside the sphere are given in (d) and (e). Dashed lines correspond to a Gaussian illumination without the sphere, solid lines are for the resulting beam with the microsphere.

The focus of the incident beam respective to the microsphere was set to the position numerically yielding the best electromagnetic confinement. A remarkable property is that focused Gaussian illumination of a microsphere can achieve strong three-axis electromagnetic confinement, with a large reduction of the beam extent along the optical axis. From Fig. 1(d) and (e), we estimate the transverse FWHM of 270 nm and axial half decay length in water of 180 nm. Both values are well below those of the unperturbed Gaussian illumination. Moreover, numerical results indicate a factor 2 intensity enhancement at the focus with the microsphere. We explain this phenomenon by the combination of two effects : (i) the microsphere overfocuses the beam, creating large transverse wave-vectors by refraction on the microsphere, and (ii) interferences between the incident beam passing outside the sphere and the transmitted and refracted light. The interferences are destructive a few hundred nanometers above the sphere upper surface, which efficiently reduces the axial extension of the spot. These processes produce an enlarged effective numerical aperture and spatial confinement along the longitudinal axis.

3. Materials and methods

To investigate the electromagnetic distribution close to a microsphere illuminated by a tightly focused laser beam, we monitored the fluorescence intensity of single molecular dyes that diffuse around the structure (Fig. 2(a)). Latex microspheres (refractive index 1.6) of calibrated diameters from 1 to 5 μm (dispersion below 0.1%) were taken as purchased from Fluka Chemie GmbH (Buchs, Switzerland), diluted in pure water and dispersed on a cleaned microscope coverslip (refractive index 1.51, thickness 150 μm). Slowly drying the water solvant overnight is a simple and efficient way to ensure electrostatic adhesion between the microsphere and the

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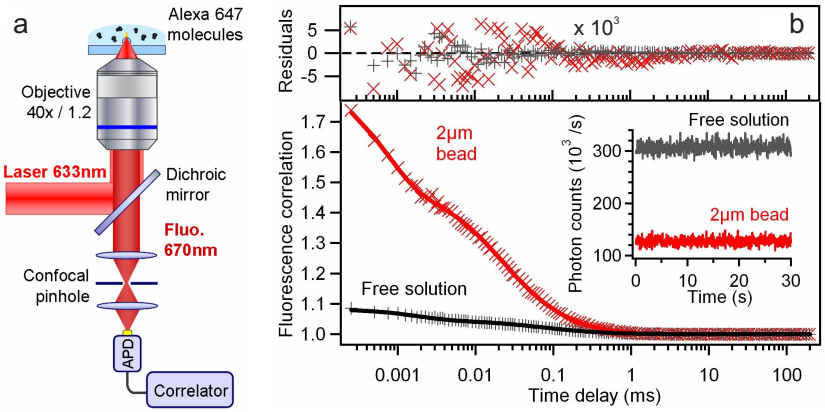

Fig. 2. (a) Schematics of the experimental set-up. (b) Correlation functions recorded in free solution and on a 2 μm sphere, using the same A647 solution (crosses: raw data ; lines: numerical fits). Analysis based on Eq. (1) yields for the free solution : $N = 21.3$, $\tau_d = 71$ μs, $n_T = 0.80$, $\tau_{b_T} = 1.9$ μs, $CRM = 14.5$ kHz, and for the 2 μm sphere : $N = 2.06$, $\tau_d = 22.6$ μs, $n_T = 0.76$, $\tau_{b_T} = 0.8$ μs, $CRM = 62.0$ kHz. The insert shows a snapshot of the raw total fluorescence signal.

glass slide. Concentration was set to isolate one single sphere per 10x10 μm^2. Our experimental setup is based on an epifluorescence microscope with a 40x, NA= 1.2 water-immersion objective (Zeiss C-Apochromat). Alexa-Fluor 647 dyes (A647) diluted in pure water are excited by a focused linearly polarized CW He-Ne laser beam at 633 nm, with a power of 40 μW. The microsphere sample is positioned at the objective focus with nanometric resolution using a 3 axis piezoelectric stage (Polytek PI P517). The fluorescence is collected via the same microscope objective, and filtered from the scattered laser light by a dichroic mirror. A 30 μm confocal pinhole conjugated to the microscope object plane rejects out-of-focus light, and defines a three-dimensional detection volume calibrated to 0.5 μm^3 for experiments on free solution. Lastly, the fluorescence is focused on avalanche photodiodes (Perkin-Elmer SPCM-AQR-13) with 670 ± 20 nm bandpass filters. To analyse the fluorescence signal, we implemented fluorescence correlation spectroscopy (FCS) [10]. FCS is a robust method that provides access to the average number of detected molecules N together with the average count rate per molecule CRM. The fluorescence intensity temporal fluctuations $F(t)$ are recorded to compute the intensity correlation : $g^{(2)}(\tau) = \langle F(t).F(t+\tau)\rangle / \langle F(t)\rangle^2$, where $\langle . \rangle$ stands for time-averaging. This operation is performed by a hardware correlator (ALV-GmbH ALV6000), each FCS measurement was obtained by averaging 10 runs of 10 s duration.

To analyse the FCS data we used a three dimensional Brownian diffusion model [10]:

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

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 40 μW excitation, the background noise was less than 2,000 counts/second, which can be safely neglected when compared to the total fluorescence signal (insert of Fig. 2).

We note that strictly speaking, the assumption of a free diffusion model is not fulfilled close to a latex microsphere. For the discussion presented here, we need to estimate the size of the

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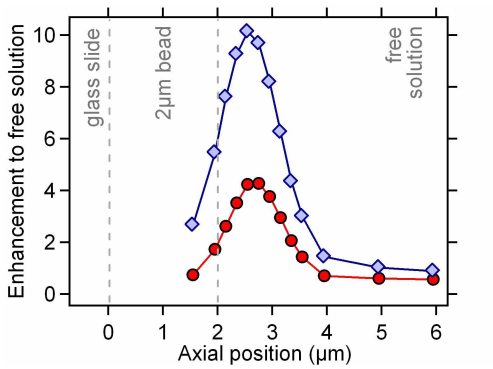

Fig. 3. Observation volume reduction (blue diamonds) and *CRM* enhancement (red circles) versus the focus position with respect to the 2 μm sphere.

observation volume and the count rate per molecule. Both are obtained from the number of detected molecules N, which is proportional to the inverse of the correlation function amplitude at origin $g^{(2)}(0)$. To fully take into account the fast photokinetics of a real dye (photoisomerization and triplet blinking), one has to consider the first channels of the correlogram (with characteristic times below 10 μs), yet the knowledge of the shape of the correlation function at larger times is not necessary. We point out that the expression of $g^{(2)}(0)$ given by Eq. (1) is independent of the shape of the excitation field and the type of diffusion statistics, and is therefore sufficient to reliably estimate N. This is 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. As shown by the residuals of the fit displayed in Fig. 2(b), the agreement between the expression in Eq. (1) and the experimental data is very good.

Determining the molecular diffusion coefficient in the case of the microsphere is a complex task, as it requires to take into account various artifacts brought by the non-Gaussian observation volume [11]. The restriction of the range available for diffusion affects the diffusion time (millisecond time range), but leaves unaffected the correlation amplitude and the fast components of the correlogram (triplet photokinetics). Still, we point out that relative measurements of variations in diffusion times are always possible, and are sufficient to assess enzymatic activity, chemical rate constants, or binding rates.

4. Experimental results

Figure 2(b) presents raw correlation functions recorded in free solution and at the optimum position on a 2 μm sphere. To avoid for possible calibration errors, the same fluorophore solution was used for both acquisitions. Numerical analysis of the FCS data provides the average number of molecules N inside the detection volume. The ratio of the total fluorescence signal by N yields the fluorescence count rate per molecule *CRM*, which accounts for spatial averaging over all molecular orientations and positions in the detection volume. Analysis of the data displayed in Fig. 2(b) reveals a 10x reduction of the number of molecules, together with a 4.3x *CRM* increase and 3.1x diffusion time reduction. This clearly assesses the gain brought by the microsphere as compared to conventional confocal microscopy : reduced observation volume and increased molecular brightness. We explain the fluorescence enhancement as resulting from two effects : (i) a local intensity enhancement due to extra focusing by the microsphere

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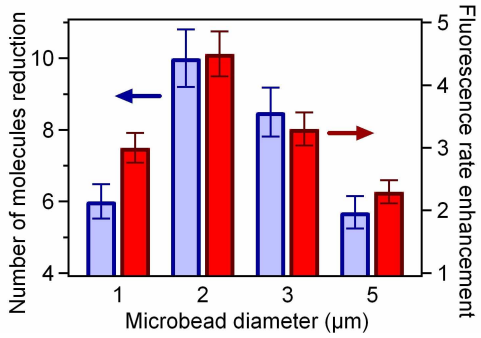

Fig. 4. *CRM* enhancement and observation volume reduction vs. diameter.

(see Fig. 1), together with (ii) an improved fluorescence detection efficiency related to a better collection of light emitted at high incidence angles [12]. Detailed analysis of this phenomenon will form the basis of a future communication.

To study the axial positioning dependence, we report a set of measurements taken while varying the focus position of the incident beam with respect to the 2 μm microsphere. The blue diamonds in Fig. 3 represent the corresponding observation volume reduction $\eta_V = (N_{sphere}/N_{sol})^{-1}$, where N_{sphere} is the number of molecules measured using the sphere and N_{sol} the free solution reference. We simultaneously display the count rate per molecule enhancement $\eta_{CRM} = CRM_{sphere}/CRM_{sol}$ (red circles). To ensure the optimum observation volume reduction and *CRM* enhancement, an accurate $\pm$150 nm axial positioning is necessary, which is readily within the reach of modern translation stages. A similar analysis was performed with spheres of different diameters. We report the best values for η_V and η_{CRM} in Fig. 4, with a clear optimum for 2 μm diameter. We also point out that factor 5 volume reductions and factor 2 fluorescence enhancements are readily obtained with diameters from 1 to 5 μm. Lastly, we considered the case of a bare glass/water interface. At the optimum position, we obtained $\eta_V = 2.1$ and $\eta_{CRM} = 1.2$, showing a modest influence from the plane interface.

5. Conclusion

We have demonstrated that a dielectric microsphere under focused Gaussian illumination can achieve strong three-axis optical confinement, and significantly enhance the fluorescence from a single emitter. This configuration outperforms the results obtained using standard confocal microscopy with a high numerical aperture objective. Since the electromagnetic enhancement is maximum at the microsphere top surface, the present technique can be straightforwardly extended to the detection of luminescent probes bound to the functionalized sphere surface or embedded in lipid membranes deposited on top of the microspheres. Furthermore, microspheres can be combined with low NA objectives to form high performance optical systems, as an alternative to the expensive and complex high NA objectives [13]. Lastly, let us point out that the values reported here are quite similar to results obtained with more complex metallic nanostructures [2, 14], where the electromagnetic enhancement can be much higher, but quenching and absorption can play a negative counterbalancing role. We thus believe that microspheres open new opportunities for low-cost and highly parallel means to develop new microscopy techniques.

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