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SHORT COMMUNICATION

Two-Photon Two-Focus Fluorescence Correlation Spectroscopy with a Tunable Distance Between the Excitation Volumes

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Abstract In the present work, a Michelson interferometer was combined with a two-photon excitation microscope to perform two-focus Fluorescence Correlation Spectroscopy. This simple and original approach allows us to tune the distance between the two excitation volumes and determine absolute diffusion constants. The technique was validated on different model systems that demonstrate the sensitivity of the approach.

Keywords Two-photon excitation · Fluorescence Correlation Spectroscopy · Michelson interferometer

Introduction

Fluorescence Correlation Spectroscopy (FCS) has emerged as a powerful tool for biological applications [1]. This technique allows characterizing the translational dynamics of fluorescent molecules or complexes in any liquid environment. By analysing the intensity fluctuations of the fluorescent species within a femtoliter volume (defined by the laser excitation), several physical parameters—diffusion time, local concentration, molecular brightness, related to the hydrodynamic and photophysical properties of these species—can be monitored [2]. FCS has been successfully applied in model systems as well as in living cells [3]. However, one concern of FCS is related to the accurate determination of the diffusion constant. Indeed, in classical FCS this parameter strongly depends on the shape of the observation volume and the experimental conditions (coverslide thickness, refractive index mismatch, optical saturation...) [4, 5]. To circumvent these limitations, several FCS based techniques were developed: standing wave FCS [6], total internal reflectance fluorescence–FCS [7], spatial correlation FCS with two laterally shifted pinholes [8], scanning FCS [9] and two-focus FCS (2fFCS) [10]. With this last method, fluorescence fluctuations from two overlapping excitation volumes are monitored and the resulting cross-correlation is used to determine absolute diffusion constants. The power of the method relies on the introduction of an external ruler, the distance between the overlapping beams generated using a Nomarski prism with two cross polarized laser beams [10].

In this context, we have developed a new 2fFCS approach using a two-photon excitation microscope coupled to a Michelson interferometer to generate two adjustable overlapping beams. This method, that requires only one laser, was validated on different model systems to illustrate its sensitivity.

Materials and methods

Sample preparation

Highly pure NCp7(11-55) peptides were prepared by chemical synthesis [11]. Peptide concentration was determined using an extinction coefficient of 5700 $M^{-1}cm^{-1}$ at 280 nm (MW=5138 Da). DNA oligonucleotides (ODN) were synthesized at a 0.2 μmol

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scale by IBA (Göttingen, Germany). Labelled oligonucleotides were modified at the 5' terminus with 5/6 TertraMethylRhodamine (TMR) or Rhodamine 6G (Rh6G) via an amino-linker with six-carbon spacer and HPLC purified. Titration curves were obtained by mixing a fixed amount of 5'TMR-AATGCC hexanucleotide with increasing amounts of NCp7(11-55) in 25 mM TRIS–HCl (pH 7.5), 30 mM NaCl, 0.2 mM $MgCl_2$ at 20°C. The mixtures (400 μ L of final volume) were then equilibrated for several minutes in a Tween-coated eight-well Lab-Tek chamber system, before measurements. DNA duplexes were obtained by mixing 5'Rh6G (14-39)cTAR with a 5-fold excess of complementary sequences in 50 mM TRIS (pH 7.8), 75 mM KCl, 7 mM $MgCl_2$. The samples (10 μ L) were heated for two minutes at 85°C and then cooled stepwise and finally diluted in the same buffer before measurement to reach a final concentration of 200 nM.

Fluorescence correlation spectroscopy and two-photon imaging

FCS measurements were performed on a home-built two-photon laser scanning system set-up based on an Olympus IX70 inverted microscope with an Olympus 60x 1.2NA water immersion objective [13]. Two-photon excitation was provided by a titanium-sapphire laser (Tsunami, Spectra Physics) and photons were detected with an Avalanche Photodiode (APD SPCM-AQR-14-FC, Perkin Elmer) connected to an on-line hardware correlator (ALV5000, ALV GmbH, Germany). To generate two overlapping excitation volumes in the sample, a Michelson interferometer was introduced in the optical path of the microscope as explained in "Results and discussion". Typical acquisition time was 10 min (20× 30 s) with an excitation power around 2.5 mW at the sample level. Imaging was carried out using two fast galvo mirrors in the descanned fluorescence collection mode. Photons were detected with an APD connected to a counter/timer PCI board (PCI6602, National Instrument).

Data analysis

Data analysis was performed by assuming two identical laterally shifted observation volumes. In this case, the autocorrelation function can be expressed in the Green's functions formalism:

$$G(\tau,\delta) = Ac \int dr_1 \int dr_2 U(r_1) \frac{1}{(4\pi\, Dt)^{\frac{3}{2}}} \times \exp\left[-\frac{(r_2 - r_1 - \delta)^2}{4Dt}\right] U(r_2) \quad (1)$$

where $U(r)$ is the molecule detection function giving the spatial probability to observe a molecule at a position r in one detection volume, D is the diffusion coefficient, c is the concentration of the fluorescent species, δ is the distance between the two beam waists and A a factor that accounts for the detection efficiency of the whole set-up. To obtain an analytical solution for Eq. 1, $U(r)$ is assumed to be a 3D-Gaussian:

$$U(r) = U_0 \exp\left[-\frac{2x^2}{\omega_0^2} - \frac{2y^2}{\omega_0^2} - \frac{2z^2}{\omega_z^2}\right] \quad (2)$$

In this case, Eq. 1 can be written as:

$$G(\tau,\delta) = \frac{1}{N}\left[\frac{1}{1+\frac{4D\tau}{\omega_0^2}}\right]^{-1}\left[\frac{1}{1+\frac{4D\tau}{\omega_z^2}}\right]^{-\frac{1}{2}} \times \exp\left[-\frac{\delta^2}{4Dt + 2\omega_0^2 + \omega_z^2}\right] \quad (3)$$

If $\delta = 0$, Eq. 3 reduces to the classical expression for a 3D diffusion model.

Results and discussion

Two overlapping excitation volumes were generated from a single laser source by adding a Michelson interferometer in the optical path of the microscope. When the two arms of the interferometer are spatially and temporally aligned, an interference pattern is generated. On the contrary, if the pulses in the two arms of the interferometer are temporally shifted, they do no more interfere. Moreover, it is possible to produce two non-collinear beams by tilting the beamsplitter. The introduction of an angle between the beams leads to a lateral shift in the focal plane of the microscope (Fig. 1a). By changing the tilt angle, the distance between the two foci can thus be easily varied. To determine the absolute distance between the two overlapping beams, shutters (S in Fig. 1a) were placed on the two arms of the interferometer. Two images were obtained by closing alternatively the shutters. The two images were fitted with a 2D gaussian function and the distance between the excitation volumes was deduced from the position of the peaks (Fig. 1b). The point spread function (PSF) of the set-up was determined from a z-scan on one fluorescent bead. The measured lateral (ω_0) and axial (ω_z) resolutions were respectively 0.34 μm and 1.1 μm (Eq. 2).

A 2fFCS measurement was first performed with a 50 nM aqueous solution of TMR. The obtained correlation curve is reported in Fig. 2a. Using an inter-foci distance of 390 *nm*, a diffusion constant of 421 ±

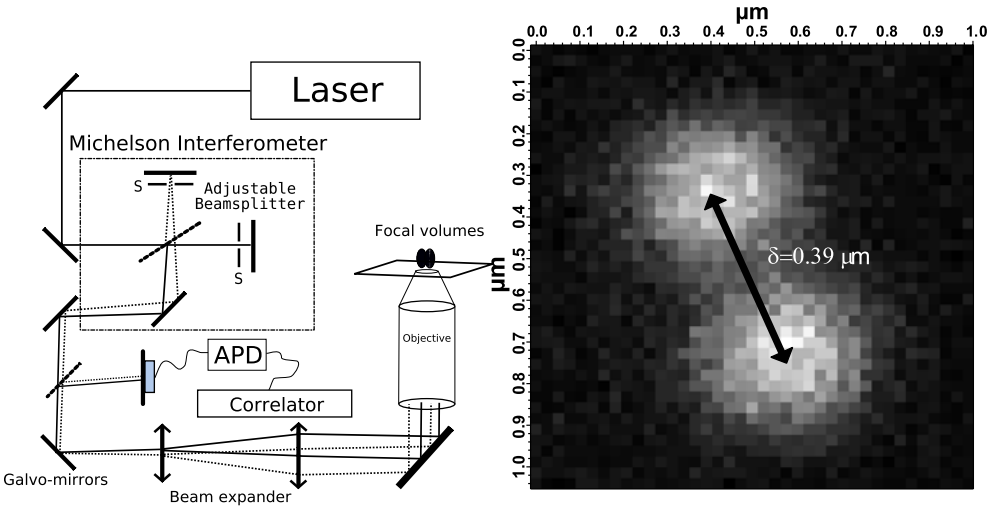

Fig. 1 **a** Scheme of the experimental set-up. **b** Image of one fluorescent bead obtained in the descanned mode by illuminating the sample with two slightly tilted beams. In this experiment, the measured distance was 0.39 µm

7 $\mu m^2 s^{-1}$ was determined, in excellent agreement with the value obtained by scanning FCS for Rh6G [9]. The inter-foci distance can be varied continuously. However, due to the exponential damping factor in Eq. 3 the distance has to be lower than 800 nm to get reliable fit (data not shown). To further highlight the accuracy of the method, 2fFCS measurements were repeated with TMR solutions of increasing viscosity, obtained by adding different amounts of sucrose [12]. As expected from the Stokes-Einstein equation, the diffusion coefficient scales linearly with the inverse of the viscosity (Fig. 2b).

2fFCS was also applied to monitor the binding of the NCp7(11-55) peptide to a 5'-TMR-AATGCC hexanucleotide. In classical FCS, a significant change in the diffusion constant can be evidenced only if the mass ratio between bound and free forms is at least five, due to the cubic root dependence of the diffusion coefficient with the molecular mass and the uncertainty on the excitation volume. In Fig. 3a, the correlation curves for free 5'-TMR-AATGCC and 5'-TMR-AATGCC bound to NCp7(11-55) were reported. The corresponding diffusion constants were respectively 216 $\mu m^2 s^{-1}$ and

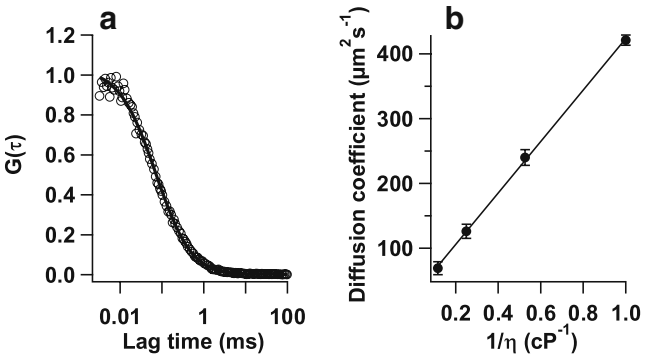

Fig. 2 **a** Correlation curve obtained with a 50 nM aqueous solution of TMR (*circles*). The fit in *solid line* provides a diffusion constant of 421 ± 7 $\mu m^2 s^{-1}$ for $\delta = 0.39$ µm. **b** Variation of the diffusion constant of TMR as a function of the inverse viscosity (calculated from the sucrose concentration [12]). Typical acquisition time per point was 10 min

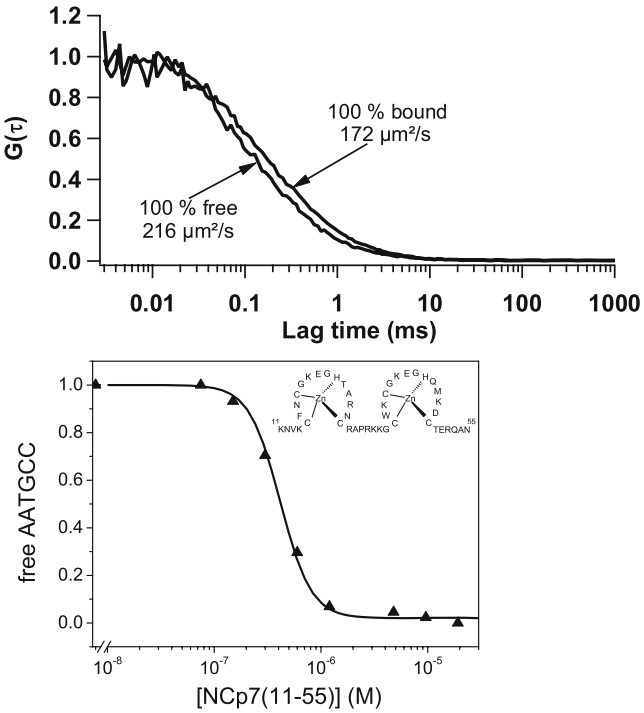

Fig. 3 **a** Correlation curves obtained for 5'-TMR-AATGCC in the absence or in the presence of a saturating concentration (20 µM) of NCp7(11-55). **b** Variation of the population of free oligonucleotide as a function of the NCp7(11-55) concentration. From the titration curve, an affinity constant $K = (2.4 \pm 0.8) \times 10^6\ M^{-1}$ was determined. Typical acquisition time per point was 10 min

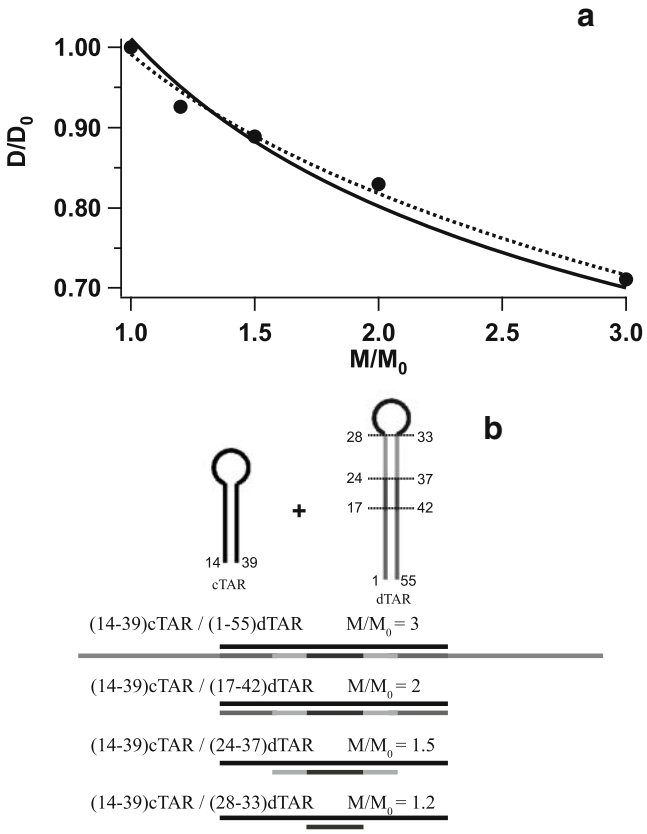

Fig. 4 **a** Variation of the diffusion constant (*black circles*) as a function of the molecular mass of the duplexes. The diffusion constant D and the molecular mass M of the duplexes were expressed as a ratio to respectively, the diffusion constant D_0 and the molecular mass M_0 of the 5'Rh6G(14-39)cTAR sequence. The *solid* and *dotted lines* correspond respectively to the fits obtained with the spherical and the rod-like models, respectively. Typical acquisition time per point was 10 min. **b** Schematic structure of the duplexes and their corresponding mass ratio

172 $\mu m^2 s^{-1}$, showing that the method can discriminate the two forms, differing by a factor 3 in their molecular mass. The brightness of the free and bound forms were identical indicating that TMR emission was not affected by NCp7(11-55). Next, the correlation curves obtained with different amounts of NCp7(11-55) were analyzed with a two population model and fixing the diffusion constants of the free and bound species. From the variation of the population of free species as a function of the NCp7(11-55) concentration (Fig. 3b), we found an affinity constant $K = (2.4 \pm 0.8) \times 10^6\ M^{-1}$, in excellent agreement with that measured from fluorescence anisotropy titrations (data not shown).

Finally, the technique was further validated by measuring the diffusion constant of 5'Rh6G(14-39)cTAR thermally annealed to complementary oligonucleotides of different molecular weights. In Fig. 4a, we reported the dependence of the diffusion constant of the different duplexes as a function of their molecular weight, both parameters being expressed relatively to the corresponding parameters of free 5'Rh6G(14-39)cTAR. The solid and dotted lines correspond to fits obtained assuming either spherical ($M_w \propto R^{\frac{1}{3}}$) or rod-like ($M_w \propto L$) shape for the complexes, respectively [13, 14]. A reasonable fit was obtained with both models, but the rod-like model was clearly the best one for duplexes with $\frac{M}{M_0} > 1.5$. This rod-like behavior for the two largest duplexes is fully consistent with the predicted dimension of their 25 bp double-stranded central part (length=90 Å and width=24 Å). Thus, the introduction of the exact distance between the two foci allows us to evidence changes as small as 20 % in the molecular mass.

In the present work an original approach, using a two-photon excitation microscope and a Michelson interferometer, was developed to perform 2fFCS. The technique can be easily implemented on any two-photon excitation microscope and allows measuring absolute diffusion constants overcoming thus one of the major limitation of classical FCS.

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