

Application of fluorescence fluctuation spectroscopy to the measurement of the concentration of molecules deposited on solid substrates

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ABSTRACT

Fluorescence fluctuation spectroscopy is applied to study molecules, passing through a small observation volume, usually subjected to diffusive or convective motion in liquid phase. We suggest that such a technique could be used to measure the areal absolute concentration of fluorophores deposited on a substrate or imbedded in a thin film, with a resolution of a few micrometers. The principle is to translate the solid substrate in front of a confocal fluorescence microscope objective and to record the subsequent fluctuations of the fluorescence intensity. The validity of this concept is investigated on model substrates (fluorescent microspheres), DNA-chips, and dye-stained histidine molecules anchored on silanized glass surfaces.

Keywords: confocal microscopy; optics at surfaces; laser induced fluorescence

1. INTRODUCTION

Fluorescence correlation spectroscopy (FCS) consists in calculating the autocorrelation of the fluorescence intensity time trace emanating from the small confocal volume illuminated by a focused laser beam.¹ From the analysis of this correlation function one is able to get information about the average number of fluorescent molecules in the observed volume, which can be related to their absolute concentration in the medium. This information is in many cases of great interest and often very difficult to get from other methods. A complementary method to FCS is based on the analysis of the moments of the statistical distribution of photon counts or of the photon count histogram.²⁻⁴ The corresponding experiments have been carried out in solution, liquid membranes, or in biological cells, where the molecules are spontaneously moving due to diffusion.

In the present work we consider the case of fluorophores anchored on a solid substrate, translated with respect to the laser beam.⁵ As in the case of solutions, the concentration of fluorophores can, in principle, be extracted from these fluctuations. The aim of this work is to investigate experimentally the validity of this concept. Potential applications include the characterization of fluorescent biochips and functionalized surfaces, or the measurement of fluorescent defects in optical thin films.

An analogous approach consists in averaging the autocorrelation functions corresponding to the successive lines of the confocal scan,⁶ reviving the original scanning-FCS (S-FCS) technique initially developed by Petersen in the 1980s.⁷ Other recent versions of S-FCS, consisting in moving the laser beam in respect to the sample, are proposed to access both spatial and temporal information of biological media.^{8,9}

2. METHODS

2.1 Samples

Three kinds of samples, deposited on a glass coverslip, have been studied: 20 nm fluorescent nanospheres (FluoSpheres F-8888, Invitrogen), Rh6G labeled DNA (OliGold, Eurogentec,) and Pro-Q 488 or GFP stained histidine molecules anchored on silanized glass (in this latter case we were interested in measuring the number of histidine molecules grafted on the functionalized surface).

The nanospheres samples were prepared by evaporating a drop of a diluted suspension of nanospheres in water (at pH 10, to prevent their aggregation).

For the DNA samples, spots of single strand DNA molecules, 21 bases long, were deposited on a glass coverslip from a highly diluted solution. A small liquid chamber was placed onto it and the DNA were hybridized with the complementary strand labeled by Rh6G.

For the histidine samples the substrate was a clean glass coverslip that was first silanized, then grafted with glutaraldehyde which acted as a crosslinker with either a peptide ended by 6 histidines (Covalab) or with a histidine-tagged GFP (Qiagen)). In the first case the histidines were further stained after incubation with a solution of Pro-Q Sapphire and Hepes.

2.2 Experimental set-up

Our experimental set up consists in a home made confocal microscope built from an inverted microscope. The 488 nm radiation from an air cooled Ar+ laser was coupled to a single mode fiber which acted as a spatial filter, collimated using a telescope and then directed to the sample through a water immersion objective (UPLAPO 60× NA = 1.2, Olympus). The excitation power at the sample was about 1 μW (nanospheres) or 100μW (Rh6G labeled DNA chips and stained histidine). The emitted fluorescence was collected by the same objective, spectrally filtered, and focused, through an additional telescope, on a multimode optical fiber ($\phi = 110 \mu\text{m}$) connected to an avalanche photodiode (PerkinElmer).

A home-made data acquisition system recorded the delay time between two consecutive detected fluorescent photons while moving the sample with a piezoelectric device (Piezosystem Jena). Both the photon count trace at any arbitrary bin time and the autocorrelation function could be reconstructed.

The profile of the "molecular detection efficiency function", $W(r)$, depends on the size and shape of the focused laser beam onto the sample and on the geometry of the collection of the fluorescence. In our case, we could estimate it experimentally, either from the correlation time of the fluorescence of molecules deposited on the solid substrate knowing the translation velocity (see below) or from the line shape of the fluorescence peaks corresponding to isolated nanospheres. It can be approximated by a gaussian function, $W(r) = \exp(-2r^2/w_0^2)$, where the value of w_0 is extremely sensitive to the position of the observed sample surface with respect to the focal plane of the objective and may vary from one record to the other (see below). Its minimum value is in the range 0.3-0.4μm.

3. RESULTS

3.1 Model substrates (fluorescent nanospheres): fluorescence correlation spectroscopy

Figs. 1a and 1d show the photon count rates, I_{flu} , recorded when a fluorescent nanosphere sample is translated at constant velocity ($v=10\mu\text{m/s}$). The surface concentration of the nanospheres is low enough so that single fluorophores can be detected as isolated peaks. Their width at half maximum of about 0.3 μm corresponds to $w_0 \cong 0.25\mu\text{m}$. Several facts can account for the dispersion of the peaks height: dispersion of the brightness of the nanospheres, formation of aggregates, and position of each nanosphere with respect to the center of the focal spot.

Figs. 1c and 1d show the corresponding autocorrelation functions of the fluorescence. The autocorrelation functions show a plateau whose duration, τ_c , is about the time it takes for the focal spot to move on the sample by about its radius w_0 . Actually the autocorrelation functions $G(\tau)$ can be fairly well fitted by a gaussian function:¹⁰

$$G(\tau) = 1 + [G(0) - 1] \times \exp[-(\tau / \tau_c)^2] \quad (1)$$

The value at the plateau, $G(0)$, gives the mean effective number $\langle N \rangle$ of fluorescent particles inside the confocal volume

$$\langle N \rangle = \frac{\gamma_2}{G(0) - 1} \quad (2)$$

where the shape factor $\gamma_2 = 1/2$ for a 2-D gaussian $W(r)$. Then the surface concentration $C = \langle N \rangle / \int W(r) d^2r$ follows:

$$C = \frac{1}{[G(0) - 1] \pi w_0^2} \quad (3)$$

with $w_0 \sim v \tau_c$ where v is the velocity imparted to the sample.¹⁰ From Fig. 1c we extract $G(0) = 5.9$ and $w_0 = \tau_c / v \sim 0.35 \mu\text{m}$, thus Eq. 3 gives $C = 0.53$ particles/ μm^2 , which is the order of magnitude found by directly counting the particles on a wide field image. By contrast the autocorrelation function shown on Fig. 1d corresponds to $G(0)=26$ with the same value of τ_c as Fig. 1c, which indicates that C is much smaller: $C = 0.12$ particles/ μm^2 . At first sight this seems consistent with the record of Fig. 1b where the number of fluorescence peaks seems smaller. However a more careful inspection of Fig. 1b shows that this is not the case: the change of vertical scale due to the huge peak at $t \sim 13.5$ s hides the smaller peaks whose number is about as large as in Fig. 1a. This illustrates the fact that the number $\langle N \rangle$ given by Eq. 2 is an effective number averaged over the distribution of brightness of the particles. We shall come back on this below.

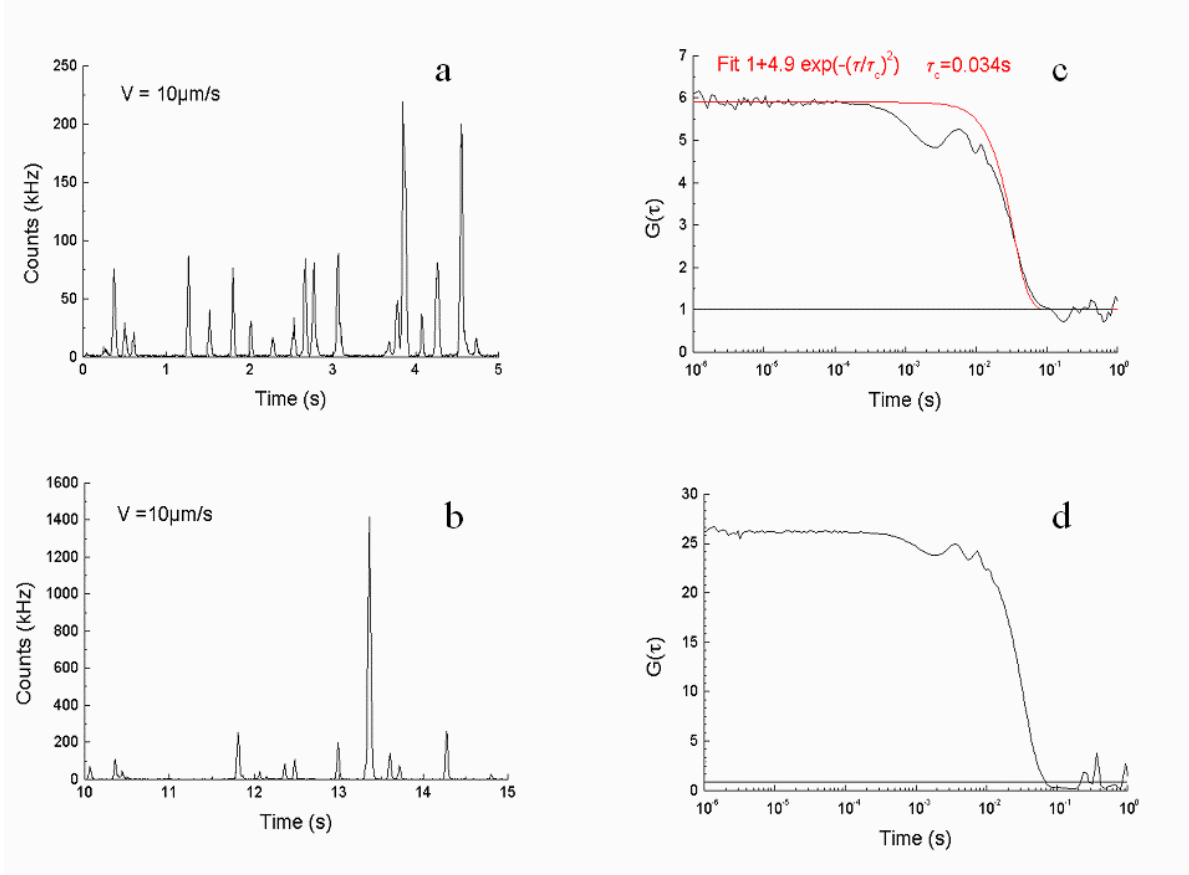


Figure 1: Fluorescence data obtained by translating a substrate with a highly diluted deposit of fluorescent nanospheres. a) and b): records corresponding to two different regions of the sample; c) and d): autocorrelation functions. Note the change of vertical scales. The autocorrelation curve can be well fitted by a gaussian function (see smooth curve on c)) except some oscillatory behavior which is fairly reproducible which is due to some mechanical oscillations of our experimental set up.

On Fig. 2 we show the effect of a focusing defect on the autocorrelation function; One of the two plotted curves is the same as Fig. 1c, where the microscope objective was carefully focused on the sample surface. The second curve is the autocorrelation function of a fluorescent record of the same region of the sample, but with the objective moved axially by $1.5 \mu\text{m}$. We see that the defocusing yields a longer correlation time and a smaller amplitude of $G(0)$, which is consistent with the fact that this increases the diameter of the focal spot and the size of the observation volume and thus $\langle N \rangle$.

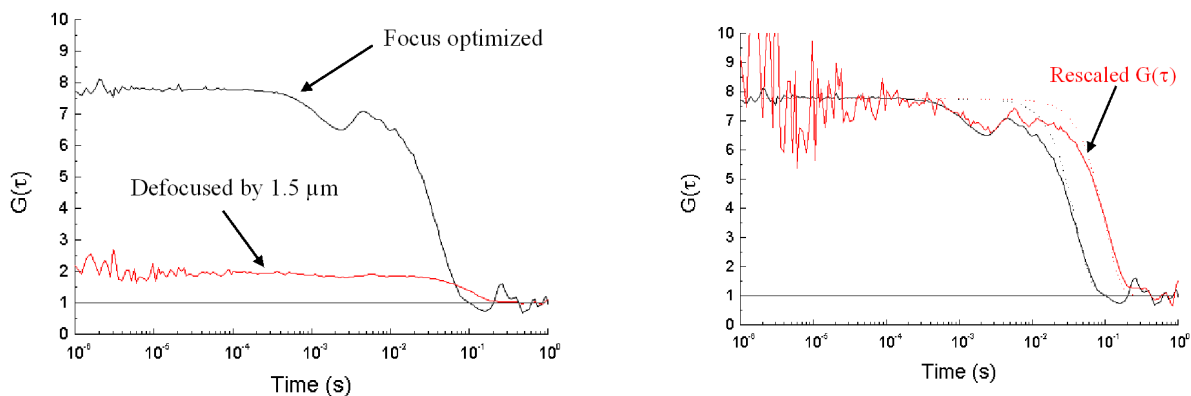


Figure 2: Effect of the focusing on the autocorrelation function: comparison of the autocorrelation function computed from two fluorescence records corresponding to the same region of the sample.

3.2 Samples with molecular fluorophores deposited on surfaces: photon counting statistics

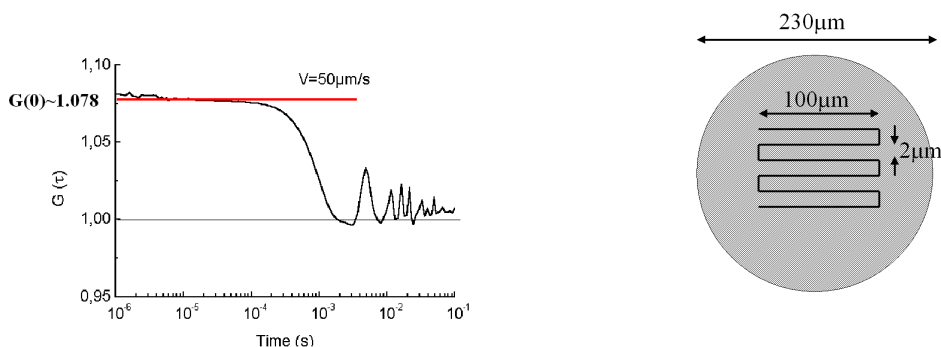


Figure 3: Autocorrelation function of the fluorescence intensity recorded when translating a sample of Rh6G-DNA. The autocorrelation time is smaller than in Figs. 1-2 due to the larger scanning velocity ($50\mu\text{m/s}$). It is also affected by mechanical vibrations which cause the observed oscillations. The autocorrelation is computed from the whole fluorescence photon count trace recorded over a path of about $600\mu\text{m}$ at the surface of the sample (6 lines of $100\mu\text{m}$ length spaced by $2\mu\text{m}$ according to the scheme depicted on the right, not on scale).

In the situation corresponding to Fig. 1 the fluorophores are so diluted that they can be counted one by one. A more interesting situation occurs when the concentration is such that the individual particles cannot be resolved anymore. This is often the case when we consider molecular fluorophores deposited on solid surfaces.

For instance Fig. 3 is an example of the autocorrelation function of the fluorescence recorded in conditions similar to Fig. 1 with Rh6G-DNA hybridized with complementary strands anchored on the glass surface. The value of the autocorrelation function extrapolated to zero time $G(0)$ is fairly small, which would indicate a rather large concentration corresponding to $\langle N \rangle = 0.5/0.078 = 6.4$ molecules in the observation volume, according to Eq. 2. However, this value of $\langle N \rangle$ is averaged over the $600\mu\text{m}$ long path across the sample. Such a large value was chosen because a large sample of photon counts is needed to obtain the autocorrelation function with a good signal/noise ratio. This is a severe drawback of the method if the sample is inhomogeneous: it is actually often the case. However to determine $\langle N \rangle$ we do not need the whole $G(\tau)$, but $G(0)$. It turns out that $\langle N \rangle$, and thus C , can also be obtained from the two first moments of the statistical distribution of photon counts.³ Let k be the number of photons counted during each bin time

δt (typically δt is chosen about 0.1 ms). From the mean, $\langle k \rangle$ and the variance, $\langle \Delta k^2 \rangle$, of k estimated during time intervals of duration $\Delta t \gg \delta t$, one can derive the relation:⁴

$$\langle N \rangle = \gamma_2 \frac{\langle k \rangle^2}{\langle \Delta k^2 \rangle - \langle k \rangle} \quad (4)$$

We have checked on our data, and it can be shown theoretically, that this value of $\langle N \rangle$ is identical to that of Eq. (2) where $G(0)$ is calculated over the same time interval Δt (Δt typically ranges from 0.1 to 0.5 s).⁴ In our conditions we have found empirically that $\langle N \rangle$ is independent on the choice of δt , as long as $\delta t < \tau_c$. This shows that calculating $\langle N \rangle$ with the photon statistics avoids calculating the autocorrelation function and fitting it to determine $G(0)$. Moreover, and this is the main advantage is this method, it is not necessary to record a very large amount of photon counts to get an estimation of $\langle N \rangle$ with an acceptable statistical uncertainty (The statistical uncertainty on $\langle N \rangle$ can be estimated using the expressions of the variances of the first two factorial cumulants, $\kappa_{[1]}$ and $\kappa_{[2]}$, given by Müller). This allows the determination of $\langle N \rangle$ over a small part of the sample (in practice about 5-20 μm path), and the mapping of the concentration over the sample. Examples of such results are shown on Figs. 4 -6.

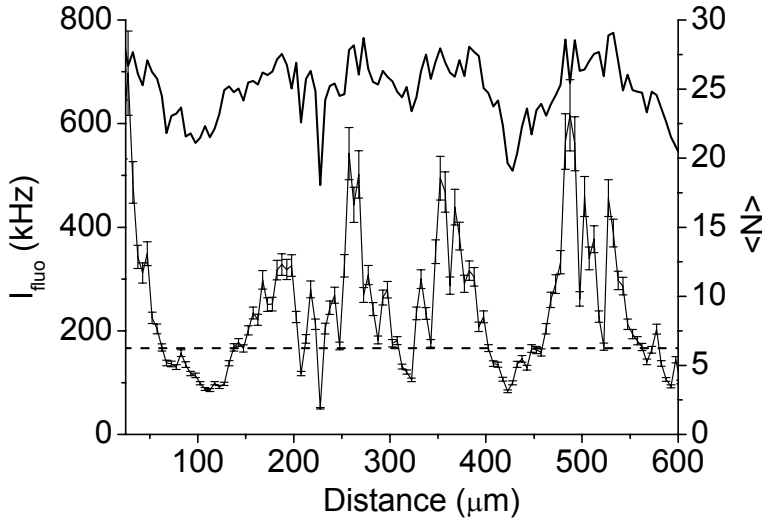


Figure 4: Scan of fluorescent DNA molecules deposited on a glass coverslip (same scan as Fig. 3). The solid line is the local count rate, averaged over distances of 5 μm ($\Delta t=0.1\text{s}$, scan speed=50 $\mu\text{m/s}$). The solid curve with error bars is the local number of fluorescent molecules at the focal spot estimated from the fluctuations of the fluorescence using Eq. (4). Error bars are statistical uncertainties estimated according to Müller (Ref[3]). The horizontal dashed line is the number of molecules given by Eq. (2) with the autocorrelation function calculated over the whole scan (see Fig. 3).

We see that in all cases there is an obvious and strong correlation between the photon count rate, I_{fluo} , and the effective, locally averaged, number of fluorescent molecules, $\langle N \rangle$, deduced from the short time scale fluctuations of I_{fluo} . The statistical uncertainty on $\langle N \rangle$ and I_{fluo} can be estimated using the expressions of the variances of the first two factorial cumulants, $\kappa_{[1]}$ and $\kappa_{[2]}$, given by Müller.³ The statistical uncertainty on I_{fluo} (displayed on Figs. 5-6) is negligible in our case. To improve the accuracy of $\langle N \rangle$ one could be tempted to increase the time interval Δt , *i.e.* the number of photons, used to calculate $\langle N \rangle$. Keeping the same scan speed would degrade the spatial resolution, given by $v\Delta t$. In addition, if the concentration is spatially inhomogeneous over a length scale smaller than the resolution, the value of $\langle N \rangle$ calculated with Eq. (4) would be underestimated because $\langle \Delta k^2 \rangle$ would be biased towards larger values. Alternatively, one could increase Δt and decrease the scan speed to keep the same resolution. However, this is limited

by the photobleaching of the molecules. For instance, a relatively stable molecule such as Rh6G has a lifetime of about 25 ms at 100 μW ($w_0 = 300$ nm), that precludes scan speeds slower than 10 $\mu\text{m/s}$. In the situation corresponding to Fig. 4 a compromise was found by scanning the sample at a speed of 50 $\mu\text{m/s}$ and calculating $\langle N \rangle$ every 0.1 s, that corresponds to a resolution of 5 μm .

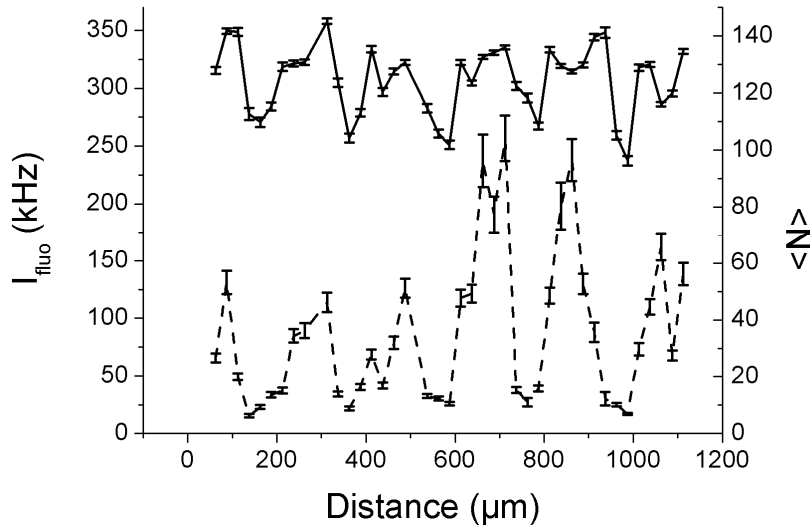


Figure 5: Scan of fluorescent Pro-Q Sapphire stained histidine molecules deposited on a glass coverslip. The solid line is the local count rate, averaged over distances of 12.5 μm ($\Delta t = 0.25$ s, scan speed = 50 $\mu\text{m/s}$). The dashed curve is the local number of fluorescent molecules at the focal spot estimated from the fluctuations of the fluorescence using Eq. (4). Error bars are statistical uncertainties estimated according to Müller (Ref[3]).

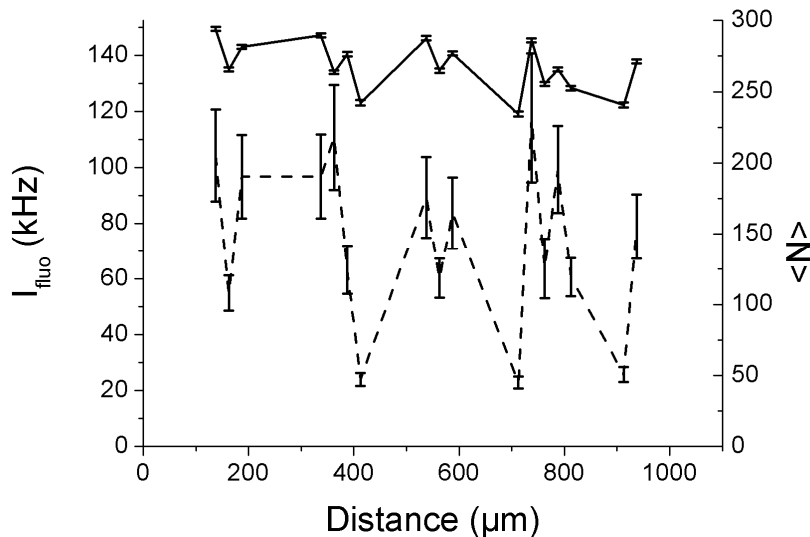


Figure 6: Scan of fluorescent GFP-stained histidine molecules deposited on a glass coverslip. The solid line is the local count rate, averaged over distances of 25 μm ($\Delta t = 0.5$ s, scan speed = 50 $\mu\text{m/s}$). The dashed curve is the local number of fluorescent molecules at the focal spot estimated from the fluctuations of the fluorescence using Eq. (4). Error bars are statistical uncertainties estimated according to Müller (Ref[3]).

4. DISCUSSION

However the comparison between the fluorescence intensity and mean effective number of fluorophores recorded at different positions on a sample shows that the relationship between I_{flu} and $\langle N \rangle$ is neither linear (the variations of I_{flu} are not proportional to those of $\langle N \rangle$) nor single valued (two regions of the substrate having the same fluorescence intensity may have different $\langle N \rangle$). Several facts may explain this discrepancy:

- i. Existence of a background signal not related to the fluorescence. However we have checked that the photon count outside the region of the glass substrate where the molecules are spotted is 1-2kHz, much less than I_{flu} .
- ii. When there are molecules of different brightness, Eq. (4) gives an effective number, $\langle N_{eff} \rangle$, which can be associated to an effective brightness, $\varepsilon_{eff} = I_{flu} / \langle N_{eff} \rangle$, while the actual mean number of molecules $\langle N \rangle_{act}$ is related to the mean of the brightness distribution $\langle \varepsilon \rangle = I_{flu} / \langle N \rangle_{act}$. Following Petersen, ε_{eff} can be expressed as a function of the mean, $\langle \varepsilon \rangle$ and the variance, $\langle \Delta \varepsilon^2 \rangle$, of the brightness distribution:⁷

$$\varepsilon_{eff} = \langle \varepsilon \rangle (1 + \langle \Delta \varepsilon^2 \rangle / \langle \varepsilon \rangle^2) \quad (5)$$

and then

$$\langle N \rangle_{act} = \langle N_{eff} \rangle (1 + \langle \Delta \varepsilon^2 \rangle / \langle \varepsilon \rangle^2) \quad (6)$$

A number of facts can affect the fluorescence quantum yield which may change from one molecule to the other and result in a distribution of brightness (dependence of the fluorescence emission on the orientation of the fluorescent molecules with respect to the surface, quenching of the fluorescence by other molecules depending of their relative positions (e.g. quenching of Rhodamine by DNA Guanosine) and by the surface, effect of surface roughness).¹¹ As a result, when $\langle \Delta \varepsilon^2 \rangle^{1/2}$ is much smaller than $\langle \varepsilon \rangle$, Eq.(5) shows immediately that $\varepsilon_{eff} = \langle \varepsilon \rangle$ and thus $\langle N_{eff} \rangle = \langle N \rangle_{act}$. However, when $\langle \Delta \varepsilon^2 \rangle^{1/2}$ is larger than $\langle \varepsilon \rangle$ then ε_{eff} is larger than $\langle \varepsilon \rangle$ and $\langle N_{eff} \rangle$ is smaller than the actual number of molecules $\langle N \rangle_{act}$ by a factor which may depend on the position if the statistical distribution of ε is position-dependent. An example of such a situation is shown on Fig. 1 where it can be seen that when there are a few very bright particles the estimation of the mean number of particles (given by $[G(0)-1]^{-1}$, Fig. 1d) is biased by a large factor.

- iii. If the spatial distribution of the fluorophores over the distance $\Delta x = v \Delta t$ is not locally poissonian then Eq.(4) underestimates the actual value of $\langle N \rangle$ due to the enhanced fluctuations of the fluorescence signal which result.
- iv. Other non-poissonian causes of fluctuations of the fluorescence signal, like the mechanical vibrations seen on Figs.1-3 have the same effects.

5. CONCLUSION

In conclusion, we report a novel method, based on fluctuation analysis, that complements the information given by direct fluorescence measurements. It can map the absolute concentration of fluorescent particles deposited on a substrate with a typical resolution of a few μm , and estimate the corresponding statistical uncertainties (Performing fluorescence correlation spectroscopy at the same resolution would provide a noisy and useless temporal information). The measurement is biased when the distribution of molecular brightness is broad. If the signal-to-noise ratio is high enough the analysis of the higher moments of the fluorescence signal should in principle point out these effects. Anyway the method gives a lower bound of the actual concentration. Finally, it must be emphasized that the method is neither sensitive to the intensity of the laser nor to the sensitivity of the photon detector and that it does not need a calibration, except the width of the ‘‘molecular detection efficiency function’’, w_0 , which can be calculated from the width, τ_c , of the autocorrelation function. Thus the statistical analysis of photon counts can provide important information when studying fluorophores deposited on a surface, as encountered in biochips or in the characterization of surface functionalization.

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