Measuring protein surface density on glass substrate using fluorescence fluctuation spectroscopy

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Abstract. Surface functionalization implies to know the surface density of proteins, which, for cellular biology, impacts on cells behavior (motility, spreading, and fate). Contradictory, the quantification of this parameter is lacking. We studied the surface density of deposited proteins on glass using the Image Correlation Spectroscopy (ICS) technique as part of the Fluorescence Fluctuation Microscopy (FFM) methods. As the confocal microscope scans the surface, the proteins deposited on it contribute to the image through the confocal point spread function (PSF). The width of the image autocorrelation is equal to the width of the PSF; its amplitude provides the average number of proteins within the PSF, proportional to the surface density. Nevertheless, the assessment of the surface density can be underestimated if the surface coverage is not uniform or overestimated if a background contributes to the fluorescence signal. Hence, we combine the ICS and photobleaching (pICS) to detect these artifacts and estimate the actual surface density. The pICS method makes it possible to have a better control of the estimation of the surface density. We noticed that the surface density of proteins increased with their initial concentration in solutions. Overall, FFM is a quantitative technique to infer the surface density of protein.

1. Introduction

Surface functionalization is carried out to control the behavior of living material when interacting with the surface. There are also methods to prepare the surface, depending on what properties one would like to alter, for example, mechanical polishing to adjust the roughness, chemical etching to pure the surface, and plasma treatment to modify the surface charge [1]. At the cellular level, surface functionalization plays a role to improve adhesion and enhance the stability of protein on the surface. The protein-surface interaction is important to modulate cell adhesion so that the cell can migrate. The adhesion proteins are located on the cell surface involved in binding other cells or with the extracellular matrix, e.g., fibronectin and laminin, in the cell adhesion process. Cell migration will depend on the density of this adhesion protein [2]. The protein adsorption is also important for biomaterials engineering. Protein adsorption is the first process following the implantation procedure before tissue is finally formed. Uncontrolled protein adsorption can trigger inflammations [3].

Some methods are previously known to study protein on a surface. Atomic force microscopy can inform about the single molecule mechanical properties of proteins bound to the surface and their
surface density by analyzing the force on a cantilever tip, but this technique took time to scan a large surface area. A quartz crystal microbalance gives an assessment of mass or protein on the surface, but it is limited to protein at low concentration. Standard fluorescence microscopy provides images of protein that are hardly quantitative because one cannot relate the quantity of fluorescence to the surface density of molecules. Accordingly, we want to develop a quantitative technique derived from fluorescence fluctuation spectroscopy technique to assess the absolute number of proteins covering the surface on a higher and lower concentration of protein solutions.

The autocorrelation is a statistical tool to analyze the spatial and temporal fluctuations of fluorescence molecules coming in and out of an excitation volume, either because of diffusion or because of a laser scan. In our case, images are generated with a laser scanning confocal microscope. As we scan an apparent homogeneous 2D sample, depending on the surface density of molecules, we observe fluctuations of the fluorescence signal. To get information about the average number of molecules within the point spread function (PSF) of the laser beam, we will apply Image Correlation Spectroscopy (ICS), a technique based on the analysis of fluorescence fluctuations of microscopy image using correlation functions [4]. Firstly, we will focus on the application of the ICS method to obtain the number of fibronectin molecules. Further, we elaborate the ICS technique to calculate the real number of superficial fibronectins and laminins by combining the ICS with photobleaching method and assuming that the dye labeling of these proteins obeys a Poissonian distribution.

2. Material and methods

2.1. Preparation of samples and substrate

Fibronectin labelled with rhodamine (FNR01 from Cytoskeleton) was reconstituted to obtain a stock solution of 1mg/mL in 20mM Tris-HCl pH 7.6, 20 mM NaCl, 0.1 mM EDTA, 15 mM BME, and 5% (w/v) sucrose. The concentrated protein was then diluted in pure water into solutions of two different concentrations: 0.4 and 4 nM. Next, 300 µl of the protein solution was put into 8-wells Nunc® Lab-Tek® II Chambered Coverglass with a bottom surface of 0.7 cm². Beforehand, the coverslip was treated with plasma to clean the surface as well as to modulate the charge. The samples were left in the dark overnight to ensure that the protein well attached to the glass surface before doing the imaging.

As for rhodamine-labelled laminin (LMN01, Cytoskeleton), the stock solution (1 mg/mL in 20mM Tris-HCl pH 7.6, 20 mM NaCl, 0.1 mM EDTA, 15 mM BME, and 5% (w/v) sucrose) was diluted in HEPES (pH 7.4) into concentrations of 1.1 and 5.7 nM. The experiment was performed using 8-wells Nunc® Lab-Tek® I Chambered Coverglass with a bottom surface of 0.8 cm² and filled with 200 µL of laminin solution.

2.2. Experimental setup and procedure

The experiment on fibronectin was performed on a Leica SP8 confocal microscope with a 63×-oil objective (NA 1.4). Before acquiring images, the focus was adjusted using a maximum intensity criterion based on the reflection of 561nm-laser on the water-glass interface. Series of 20 images of excited protein were recorded with a pixel dwell time of 5 µs, a pixel size of 50 nm, and image size of 512x512 pixels. The acquisition was carried out with low power of 516nm-laser (~5 µW) to avoid photobleaching. Between each series of image acquisition, a photobleaching sequence was performed by tuning the same laser at high power of ~50 µW. While to provide background images, a series of 20 images at the pure water-glass interface was collected with the same parameters as that of the acquisition sequence. Meanwhile, the confocal images of laminin-covered surface were acquired with a 40×-oil objective (NA 1.3) on the same microscope and parameter setting as that of the fibronectin. A series of 10 images, used as background, was recorded at 50 µm above the surface (in the presence of the solution). Raw data were analyzed using a plugin developed on ImageJ to obtain the absolute number of molecules [Arnold Fertin, 2019, personal communication].
3. Results and discussion
As the molecules are immobile, we carried out ICS to analyze the images obtained with the confocal microscope. Figure 1 shows how the autocorrelation profile is obtained from fluorescence fluctuation. The intensity of emitted fluorescence in each pixel represented the spatial information of the image from which the number of molecules can be assessed.

**Figure 1.** The series of images of molecules fluctuations detected and recorded by laser scanning confocal microscope intrinsically provides spatial information of fluorescence intensity. The autocorrelation function at $G(0)$ is inversely proportional to the average number of molecules in the detection volume.

3.1. Image Correlation Spectroscopy (ICS)
Image correlation spectroscopy is originally based on spatial correlation on an image obtained with a laser scanning microscope to determine the density of fluorescence particles [5]. The concept behind ICS is that the width of the spatial autocorrelation function of an ensemble of immobile points evenly distributed is nothing but the width of the PSF of the confocal system. In principle, the autocorrelation measures the similarity of a signal. In addition, the amplitude of the autocorrelation curve is related to the average number of particles within the focal volume (i.e., PSF) of radial extent $\omega_r$ [6]. The fluctuations of fluorescence intensity in space have an average intensity of $\langle I(x, y) \rangle$ and fluctuations $\delta I$. When the autocorrelation analysis is performed, we correlate the intensity at every pixel point on the single image with that at a shifted point in space in the same single image. This autocorrelation function is then normalized by dividing it to the square of the average intensity [4] as given by

$$G(\xi, \psi) = \frac{\langle I(x,y) \cdot I(x+\xi,y+\psi) \rangle}{\langle I(x,y) \rangle^2}$$

(1)

Series of images makes it possible to improve the signal-to-noise ratio of the spatial autocorrelation function. It is also noteworthy that any fluorescent molecules, much smaller than the focal volume, either in a single or a complex form bearing at least one fluorophore, is described as a single entity when passing through the volume. For an ensemble of non-interacting or independent molecules, the amplitude of the autocorrelation function at lag zero (i.e., the maximum of the function) is the normalized variance of the fluctuation intensity $\delta I$. Consequently, the value of $G(0)$ is the reciprocal average number of molecules within the focal volume [7] that reads

$$G(0) = 1 + \frac{\langle (\delta I)^2 \rangle}{\langle I \rangle^2} = 1 + \frac{1}{\langle N \rangle}$$

(2)

Any decrease in the surface density of the molecules would increase the Gaussian curve amplitude. As the microscope scans the surface, a less dense surface would have fewer excited molecules in the
observation area. Consequently, the magnitude of the autocorrelation function at y-intercept increases due to more fluctuations between pixels. Having the information on the average number of molecules in the focal volume, we can derive the surface density of proteins given by $N/\pi \omega_z^2$, and also the mean brightness of the molecules, or Count Rate per Molecule, given by $CRM = CR/N$, where CR is the mean intensity or Count Rate.

3.2. The surface density of molecules with Image Correlation Spectroscopy

Fibronectin and laminin are extracellular matrix proteins that interplay in processes like adhesion, proliferation, migration, and apoptosis because these proteins bind to cell surface receptors, like integrins. In our system, the positively charged fibronectin and laminin are covering and attached to a negatively charged glass substrate. Varying the concentration of the protein solution would provide a difference protein density on the surface. However, the surface density does not express the absolute number of proteins diluted in solution because of not all protein bind to the glass.

Before performing ICS, it is important to correct the raw images from the background coming from an additional presence of a stationary fluorescence source: the light scattering from the glass interface or the fluorescence of surrounding molecules in solution. Otherwise, it could bias the analysis because this constant signal will contribute to the fluorescence intensity without producing fluctuations. In other words, it makes the amplitude of the normalized autocorrelation function lower, thus overestimating the number of molecules. We noticed that the background effect was more pronounced with a lower surface density. Another issue that should be addressed with ICS is the non-uniformity of images coming from the spatial variation of the surface density or of the brightness. As a result, the autocorrelation function cannot be fitted properly. This situation can be corrected by applying a flattening where each background-corrected image is divided by its own smoothed version [8]. We perceived that the lower the concentration of the protein solution was, the better homogeneity of the surface density obtained.

![Figure 2](image_url)

**Figure 2.** Nominal concentration of protein in solution of (a) 4 nM and (c) 0.4 nM showed a noticeable fluorescence signal occupying the points of the image of the surface. Hence, the autocorrelation function generated different amplitude for the two corresponding concentrations (b and d).

The autocorrelation profile from fluorescence images of protein shows a lower amplitude when the protein solution is denser because fewer fluctuations are recorded (Figure 2). The average number of molecules in the observation volume extracted for fibronectin protein was 0.6 molecules for a nominal
concentration of 0.4 nM and 6.8 molecules for that of 4 nM. Hence the surface density of the less concentrated fibronectin is about 16 times lower compared to the denser one. This was due to the difficulty to control the concentration of protein in solution preparation.

3.3. Photobleaching Image Correlation Spectroscopy

The image correlation spectroscopy itself has already told us about the number of molecules after the background correction and flattening procedure. Combining the ICS technique with photobleaching would give additional information about the labeling of the molecules.

However, generally, in the case of fibronectin and laminin, every single protein holds a few and a different number of dyes (the average degree of labeling is 1-3 dyes/protein for fibronectin and 2-5 dyes/protein for laminin). To put it another way, all the single molecules do not have the same brightness because they bear a different number of fluorophores, thus inducing fluctuations of intensity. However, when photobleaching takes place, the number of the non-bleaching dyes held by each protein decreases, which results in a decrease of the mean brightness of the proteins and the number of molecules (because fully bleaching molecules progressively disappear). To exploit quantitatively the photobleaching for proteins with a given degree of labeling, we assume that the corresponding distribution is Poissonian and thus, is completely defined by the mean number of dyes that they bear. When the sample is photobleached, the distribution remains Poissonian but has a lower mean. By assuming that, we can relate the measured brightness (CRM) to the count rate (CR, i.e., the intensity) [9] as

\[
CRM = CRM_{\text{dye}} \left(1 + \frac{CR}{N_{\text{protein}} CRM_{\text{dye}}} \right)
\]

where \(CRM_{\text{dye}}\) is the brightness of single rhodamine tagged on a protein. In the case where \(CR \gg N_{\text{protein}} CRM_{\text{dye}}\), that is when the proteins bear many unbleached dyes (like at the beginning of the photobleaching process), the total fluorescence signal is proportional to the brightness of the proteins multiplied by the total number of proteins. At the end of the photobleaching, where the CR approaches zero, the brightness of the proteins equals that of single rhodamine.

Using the combination of photobleaching and ICS techniques (pICS) would give an apparent number of proteins, which is higher than if we use only the ICS method (Table 1). This is due to the fact that the distribution of labels contributes to the fluctuations that are otherwise interpreted as a lower number of molecules.

| Protein  | Nominal concentration (nM) | Average number of molecules |
|----------|---------------------------|----------------------------|
| Fibronectin | 0.4 | 0.62 | 0.67 |
| Fibronectin | 4.0 | 6.81 | 9.06 |
| Laminin | 1.1 | 0.58 | 0.62 |
| Laminin | 5.7 | 3.44 | 3.59 |

The correction of the underestimation of the number of molecules resulted in a higher value of the corresponding surface density. Figure 3 shows a relation between the nominal concentration of protein in solution and its surface density using the pICS method. The behavior of the relation between the high and the low concentrations was evidently consistent for both proteins. The more molecules we introduced on the surface would raise the fluctuation. Thus, it should increase the number of
molecules. Compare to the fibronectin for each higher and lower concentration, the laminin showed a lower surface density. This was expected, as the laminin is a larger protein (higher molecular weight) than the fibronectin, there would be less occupancy of the glass surface for the laminin [10].

![Figure 3](image.png)

**Figure 3.** The surface density of fibronectin and laminin protein absorbed on the glass substrate.

4. **Conclusion**

The image correlation spectroscopy (ICS) is a technique based on the spatial autocorrelation of an image from which the average surface density of molecules can be extracted, assuming that molecules have the same brightness and are homogeneously distributed in space. In the case of the molecules not bearing the same number of dyes, combining the ICS with photobleaching should make the ICS more quantitative.

5. **References**

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