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Detecting protein aggregation and interaction in live cells: A guide to number and brightness

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ABSTRACT

The possibility to detect and quantify protein-protein interactions with good spatial and temporal resolutions in live cells is crucial in biology. Number and brightness is a powerful approach to detect both protein aggregation/desegregation dynamics and stoichiometry in live cells. Importantly, this technique can be applied in commercial set ups: both camera based and laser scanning microscopes. It provides pixel-by-pixel information on protein oligomeric states. If performed with two colours, the technique can retrieve the stoichiometry of the reaction under study. In this review, we discuss the strengths and weaknesses of the technique, stressing which are the correct acquisition parameters for a given microscope, the main challenges in analysis, and the limitations of the technique.

Contents

1. Introduction: fluorescence microscopy and detecting protein interactions

To better understand different biological functions within the cell (e.g. receptor dynamics, signal transduction or chromatin dynamics) it is of principal importance to describe how proteins interact with each other. Traditionally, *in vitro* biophysical assays

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have been used to characterize protein interactions, but recently, a myriad of different fluorescence microscopy techniques have been applied for this purpose *in vitro* and in live cells. Within this family, we review number and brightness [1] which belongs to the field of fluorescence fluctuation spectroscopy (FFS). Number and brightness (N&B) applies moment analysis [2,3] to measure the average number and brightness of labelled entities in each pixel of a stack of fluorescence microscopy images. These quantities give information on the concentration of these entities and their oligomeric state. These images may be acquired with a simple laser scanning microscope (LSM, with either digital or analog detectors) or a camera-based fluorescence microscope, for example, a total internal reflection (TIRF) microscope [4,5]. One advantage of fluorescence fluctuation approaches is that they require relatively low concentrations of labelled protein (in the range of nM, [6]), therefore issues related to protein over-expression [6] can be avoided. An caveat of N&B is that it functions correctly for diffusive entities only and therefore cases with a subpopulation of immobile particles cannot be well-quantified. As with many fluorescence microscopy-based approaches, the rate of photobleaching will affect quantification and, if not properly addressed, will confound the final results [7].

2. Number and brightness in the current biological context: the CRISPR era

The quantification of protein interactions at endogenous levels of proteins is essential to unveil the biological function at the molecular level in a living cell. Conventionally, immunostaining and over-expression of fluorescently-tagged proteins have been used to quantify protein interactions. Both of these methods have their caveats: immunostaining with primary or secondary antibodies can lack specificity [8] and hence must be performed and interpreted with great care, and over-expression can cause changes in cell function and induce artificial protein interactions [9] by forcing protein contacts through crowding. Several strategies have been devised to overcome these issues in single cells, e.g. recombinant antibody-like proteins [10] and conditional tag knocking strategies [11]. Genome editing, which allows manipulation of the genome *in vivo* to insert a tag sequence of a fluorescent protein into the gene of interest, offers great promise to solve these problems. CRISPR-Cas9 [12–14] permits fluorescent labelling of endogenous proteins and is most likely the best system to quantify protein interactions at endogenous expression levels in live cells. CRISPR-Cas9 endogenously labelled samples have been imaged using laser scanning confocal microscopy [15], and super-resolution techniques (PALM and dSTORM) have also been applied [16–18]. We anticipate that FFS approaches – N&B in particular – will become very attractive for CRISPR-Cas9 engineered cell lines expressing endogenous levels of labelled proteins, because they work well with dim (lowly-expressing) samples.

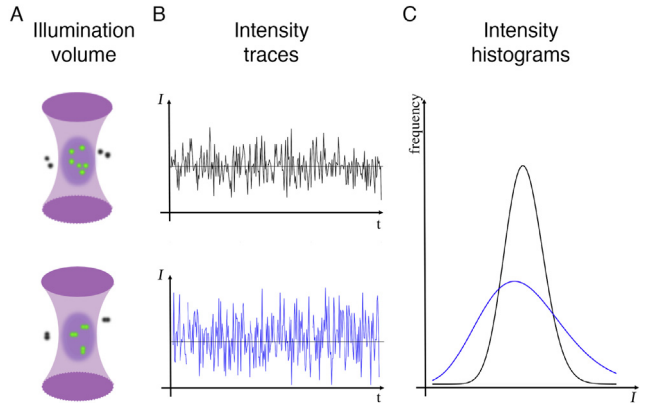

Fig. 1. Consider a system in which monomeric proteins undergo dimerization. A. Row 1: prior to dimerization, an average of 6 monomers are excited in the observation volume. Row 2: after dimerization, an average of 3 dimers are excited in the observation volume. We have the same concentration of fluorophores and therefore the same intensity average, however after dimerization, we have a higher variance in intensity. This is because now the fluorophores are entering and leaving the observation volume two at a time. B&C. This constant mean and increase in variance is seen in the intensity traces and the intensity histograms (monomers in black, dimers in blue). The widening of the histogram in the dimeric (blue) case shows the increase in variance. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3. Number and brightness, theory and analysis

Before discussing the strengths and weaknesses of N&B in live cells, we introduce the mathematical concepts first described by Digman et al. [1] (Fig. 1). Define an *entity* as a set of molecules which are chemically bound and the brightness ϵ of an entity as the mean number of photon detector counts it gives per unit time when in the illumination volume. For an image series where the i^{th} slice in the stack is the image acquired at time $t = i$, for a given pixel position (x, y), we define $\langle I \rangle$ as the mean intensity of that pixel over the image series and σ^2 as the variance in that intensity. Define n as the mean number of entities in the illumination volume corresponding to that pixel. If we are in photon-counting mode with zero background and all entities are mobile, we have

$$N = \frac{\langle I \rangle^2}{\sigma^2} = \frac{\epsilon n}{1 + \epsilon}$$

$$B = \frac{\sigma^2}{\langle I \rangle} = 1 + \epsilon$$

where N and B are referred to as the "apparent number" and "apparent brightness" respectively. This gives

$$n = \frac{\langle I \rangle^2}{\sigma^2 - \langle I \rangle}$$

$$\epsilon = \frac{\sigma^2}{\langle I \rangle} - 1$$

These relations are derived using a moment analysis technique which was originally applied to molecules in solution by Qian and Elson [19]. Dalal et al. [20] showed that with a scanning confocal microscope in analog mode, we must use three correction terms: 1. The proportionality constant S, which is the conversion factor between photons detected and the number of counts returned by the analog electronics. That is, if the analog electronics give c_a counts, then this corresponds (on average) to $S \times c_a$ photons detected. 2. The offset (bias) due to the analog electronics in the level of the background. 3. The readout noise σ_0^2 is the variance in this background signal. Then, if all entities are mobile, we have

$$N = \frac{(\langle I \rangle - \text{offset})^2}{\sigma^2 - \sigma_0^2} = \frac{\epsilon n}{1 + \epsilon}$$

$$B = \frac{\sigma^2 - \sigma_0^2}{\langle I \rangle - \text{offset}} = S(1 + \epsilon)$$

which give

$$n = \frac{(\langle I \rangle - \text{offset})^2}{\sigma^2 - \sigma_0^2 - S(\langle I \rangle - \text{offset})}$$