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Two-detector number and brightness analysis reveals spatio-temporal oligomerization of proteins in living cells

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ABSTRACT

Number and brightness analysis (N&B) is a useful tool for the simultaneous visualization of protein oligomers and their localization, with single-molecule sensitivity. N&B determines particle brightness (fluorescence intensity per particle) and maps the spatial distribution of fluorescently labeled proteins by performing statistical analyses of the image series obtained using laser scanning microscopy. The brightness map reveals presence of the oligomers of the targeted protein and their distribution in living cells. However, even when corrections are applied, conventional N&B is affected by afterpulsing, shot noise, thermal noise, dead time, and overestimation of particle brightness when the concentration of the fluorescent particles changes during measurement.

The drawbacks of conventional N&B can be circumvented by using two detectors, a novel approach that we henceforth call two-detector number and brightness analysis (TD-N&B), and introducing a linear regression of fluorescence intensity. This statistically eliminates the effect of noise from the detectors, and ensures that the correct particle brightness is obtained. Our method was theoretically assessed by numerical simulations and experimentally validated using a dilution series of purified enhanced green fluorescent protein (EGFP), EGFP tandem oligomers in cell lysate, and EGFP tandem oligomers in living cells. Furthermore, this method was used to characterize the complex process of ligand-induced glucocorticoid receptor dimerization and their translocation to the cell nucleus in live cells. Our method can be applied to other oligomer-forming proteins in cell signaling, or to aggregations of proteins such as those that cause neurodegenerative diseases.

1. Introduction

Upon stimulation with specific ligands, many receptors change their localization and oligomeric states. It is, therefore, important to characterize protein localization and oligomerization in live cells.

Fluorescence microscopy coupled with fluorescence fluctuation spectroscopy (FFS) is suitable for monitoring the localization and oligomerization of proteins in living cells. Fluorescence Correlation Spectroscopy (FCS) [1,2], the longest used FFS, can be used to determine the number of particles in the measurement volume, the diffusion coefficient, and the particle brightness (fluorescence intensity per one particle), which can be used to deduce the oligomeric state of the target particles. However, it is difficult to detect long-distance changes in protein localization using FCS because there is usually only one point of measurement. Imaging-based FFS techniques have been developed to overcome this drawback. They include image correlation spectroscopy (ICS) [3], raster image correlation spectroscopy (RICS) [4,5], temporal image correlation spectroscopy (TICS) [6], and imaging FCS [7]. ICS determines the radius of the particles, the number of particles in a field of view, and the particle brightness. RICS can measure tiny and fast-diffusing particles/molecules, including organic fluorescent dyes, and small proteins. However, the spatial resolution of RICS is always lower than the resolution of the original image, owing to the correlation calculation between adjacent pixels. For example, when we get original images as 512×512 and the correlation is calculated between adjacent 16×16 pixels, the spatial resolution of the result becomes 32×32. TICS is useful for measuring relatively slow-diffusing particles such as membrane proteins, but is not suitable for fast-diffusing particles because of the low frame rate of confocal imaging. Imaging FCS, which is similar to TICS, uses an electron multiplying charge-coupled device (EMCCD) or a complementary metal oxide semiconductor (CMOS) for image acquisition.

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Number and brightness analysis (N&B) is another FFS technique. It produces a map of the number of particles and the particle brightness [8]. Spatial resolution in N&B is the same as that achieved using a laser scanning microscope (LSM). N&B can be applied to fast-diffusing particles, such as diffusing protein in living cells, compared to TICS and Imaging FCS. For TICS and Imaging FCS, frame time (time taken to obtain a frame and lag time of each frame) is important and required to be much shorter than the characteristic decay time of the fluctuating particles (residence time of particles in measurement volume at a pixel) for calculation of correlation in the temporal domain. This restricts their application to measuring slow-diffusing particles, such as membrane proteins. On the other hand, for N&B, frame time is not required to be shorter than the characteristic decay time of the fluctuating particles. However, pixel dwell time (time taken to obtain a pixel value) is required to be much shorter than the characteristic decay time of the fluctuating particles for N&B [8]. N&B does not analyze the temporal intensity fluctuation during crossing of the particles through the measurement volume, but analyze the variance of instantaneous intensity depending on number of particles in measurement volume during shorter time than characteristic decay time (corresponding to diffusion time in FCS). Therefore the lag time of N&B is not effective but only pixel dwell time is effective, and N&B can be applied to relatively fast-diffusing particles such as diffusing protein in living cells. Nevertheless, N&B does not determine the characteristic decay time or diffusion coefficient unlike TICS and Imaging FCS. N&B has been used in several studies, including a distribution analysis of the oligomeric receptor protein ErbB1, before and after stimulation with epidermal growth factor (EGF) [9]. This study revealed the ligand-independent dimerization of ErbB1 when the density of ErbB1 is sufficiently high and the conversion of ErbB1 into its higher oligomeric state after the stimulation. Another example of the use of N&B is for the investigation of the formation of huntingtin peptide inclusions in degenerative neurological disorders [10]. This study revealed a time-dependent formation of inclusions and a two-step pathway to inclusion formation. Yet another example of the use of N&B is for the investigation of dynamin-2 oligomer formation in HIV-1 infection [11]. This study revealed that the low oligomeric state of dynamin-2 was associated with the HIV envelope protein. N&B is useful for investigating the diffusing particles in living cells and for resolving the particles spatially. Currently, it is impossible to predict when and where oligomers or inclusions will form. N&B enables simultaneous measurements to be carried out over relatively wide fields in living cells. Therefore, it is expected that N&B will be used widely for the study of oligomer or aggregation formation. However, the accuracy of conventional N&B is not adequate and the results are easily affected by sample concentration, necessitating correction for detector dead time [12,13] and correction for noise. Furthermore, conventional N&B overestimates particle brightness when the concentration of the fluorescent particles is changed (Supplementary Note S1) or fluorescence intensity is decreased by photobleaching during measurement [14].

In this study, we developed an improved N&B, called two-detector N&B (TD-N&B), which overcomes the problems associated with conventional N&B without any mathematical corrections. We achieved improvements by eliminating noise effects from detectors, by incorporating a two-detector optical system, and by introducing linear regression to compensate for the change in fluorescence intensity. Furthermore, we demonstrated the feasibility of our method quantitatively by analyzing enhanced green fluorescent protein (EGFP) dilution series, and by investigating EGFP tandem oligomers in cell lysate and living cells. Finally, we monitored the dimerization of EGFP-tagged glucocorticoid receptors (EGFP-GR), as a cell signaling process. The nuclear translocation and dimerization of GR were visualized from a time series of the brightness map of EGFP-GR, which was obtained by TD-N&B. This is the first step towards using quantitative imaging to elucidate dynamic GR mechanisms. Moreover, our method can be applied to other oligomer-forming proteins, or to aggregation of proteins, such as those that cause neurodegenerative diseases, which are difficult to visualize in living cells or whole organisms using biochemical methods and molecular biology.

2. Methods

2.1. Theory

2.1.1. Two-detector number and brightness analysis (TD-N&B)

The experimental setup of the TD-N&B system is shown in Fig. 1A. Two image series were obtained simultaneously using a LSM and two avalanche photodiodes (APDs) operating in the photon counting mode (Fig. 1B). We used the APDs to detect fluorescence, but it is also possible to use a detector operating in the analog mode or pseudo-photon counting mode with calibration for image acquisition [15]. The calibration includes: the offset; the S factor, which converts photon counts into digital data, and the read-out variance of the detector. Regardless of the operating mode, the pixel dwell time must be short, compared to the characteristic decay time of the particles in the measurement volume to detect the fluctuation in the number of particles, which is a necessary condition for image acquisition. In our condition, pixel dwell time was 12.61 μs. The characteristic decay time of EGFP monomer in solution measured by FCS was 90 μs. The characteristic decay time of EGFP tandem oligomers in cell lysate and living cells and EGFP-GR in living cells were longer than 90 μs, and the pixel dwell time is 10 times shorter than those. The TD-N&B system analyzes the time sequence of the count rate (photon counts per second), which is corresponding to fluorescence intensity at the same pixel in the two-image series. A schematic diagram of count rate as a function of time at one pixel is shown in Fig. 1C. TD-N&B determines the particle brightness and the number of particles at every pixel using a linear component and a fluctuation component of the count rate.

The time series of the count rate at a pixel detected by the two avalanche photodiodes APD_1 and APD_2 are given by:

$$I_1(t) = \varepsilon_1 N(t) + D_1(t), \tag{1}$$

$$I_2(t) = \varepsilon_2 N(t) + D_2(t), \tag{2}$$

where $I_1(t)$ and $I_2(t)$ are the count rates detected by APD_1 and APD_2, respectively, and ε_1 and ε_2 are the particle brightness values detected by APD_1 and APD_2, respectively (defined as photon counts detected per second per particle). The ratio of ε_1 to ε_2 depends on the performance of the half mirror and the quantum efficiencies of the APDs. $N(t)$ is the number of particles in the measurement volume (the confocal volume of the LSM). $D_1(t)$ and $D_2(t)$ are the noise from APD_1 and APD_2, respectively.

$I_1(t)$, $I_2(t)$, $N(t)$, $D_1(t)$, and $D_2(t)$ can be separated into two components, linear and fluctuating, as follows (Fig. 1C):

$$I_1(t) = I_{l1}(t) + \delta I_1(t), \tag{3}$$

$$I_2(t) = I_{l2}(t) + \delta I_2(t), \tag{4}$$

$$N(t) = N_l(t) + \delta N(t), \tag{5}$$

$$D_1(t) = D_{l1}(t) + \delta D_1(t), \tag{6}$$

$$D_2(t) = D_{l2}(t) + \delta D_2(t). \tag{7}$$

where, subscript l represents the linear component and δ signifies the fluctuations. The linear component with offset can be obtained