Manuscript submitted to:

AIMS Biophysics

Volume 2, Issue 1, 1–7

DOI:10.3934/biophy.2015.1.1

Received 12 February 2015, Accepted 10 March 2015, Published 16 March 2015

Research article

Determining complex aggregate distributions of macromolecules using photobleaching image correlation microscopy

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Abstract: Aggregation of cell surface receptors is believed to be linked to their biological function. In this paper, we disclose the explicit formulation for macromolecular aggregation distribution by means of photobleaching Image Correlation Spectroscopy (pbICS).

Keywords: Photobleaching ICS, Aggregation Distribution, Spatial Autocorrelation

1. Introduction

The oligomeric state of cell surface receptors is believed to be linked to their biological functioning. Ligand-induced receptor oligomerisation appears to be a common mode of activation of tyrosine kinase receptors, such as the epidermal growth factor receptor. Large receptor clusters with as many as 20 receptors per cluster are implicated in the sensitive responses of bacteria to chemical gradients. Determining the precise nature of oligomeric states of cell surface receptors is a challenging biophysical problem. Although methods for water-soluble proteins are well established, methods for determining the complex oligomeric states of membrane proteins in cells are still on the rise.

Current methods of determining the brightness or average oligomeric state of macromolecules include fluorescence correlation spectroscopy [1], image correlation spectroscopy [2], photon counting histogram [3], spatial intensity distribution analysis [4], number and brightness [5], Forster resonance energy transfer [6] and fluorescence anisotropy [7]. These methods measure and analyse the fluctuations of fluorescence or the statistical properties of the fluorescence signals to yield estimates of average brightness or oligomeric state.

All of these methods require a brightness standard to relate the measurement to a reference oligomeric state or brightness standard. Moreover as is often the case for biophysical methods an average brightness or oligomeric state is extracted which precludes an understanding of complex aggregation or oligomeric distributions.

Single molecule step-wise photobleaching is one method that can extract oligomeric state information without a reference standard. The approach is based on the statistical bleaching properties of fluorophores. When a monomer bleaches there is a change in the intensity of emission from the monomer level to a background level. If there are two fluorophores in the region of interest, such as a dimeric protein, then after one bleach event the intensity will drop to the level of the monomer signal and then after a successive bleaching event the intensity will drop again to the level of the background signal. Identification of oligomers is possible without the requirement of a brightness standard and distributions from single oligomers are obtained which can be analysed to gain further information.

We have recently developed an analogous ensemble approach based on analysis of fluorescence images of labelled molecules obtained with standard (confocal) microscopy. The approach is called photobleaching image correlation microscopy and based upon the principle that the survival probability of an aggregate of molecules during photobleaching is dependent on the number of molecules in the aggregate. Consequently, a plot of the cluster density as a function of intensity remaining (not photobleached) after photobleaching provides a two-

parameter estimate of aggregation or brightness. In our earlier paper we presented formulae for extracting the brightness or oligomeric state for a simple homogenous oligomer model.

In this report, we provide a more detailed derivation and present a list of explicit analytical formulae for complex distributions of molecules which are more likely to be encountered in real systems. We trust these formulae will find use for researchers interested in determining the oligomeric state distributions of molecules in complex environments such as biological cells.

2. Materials and Method

As photobleaching image correlation spectroscopy is a relatively new technique we will recall the salient features of the method for pedagogical purposes.

We begin by considering that our molecules of interest are labelled with an appropriate fluorophore (examples include GFP, or a dye such as fluorescein) and that the labelled molecules are presented in an appropriate form for imaging i.e. on a surface of an intact cell. For pbICS fixed cells are best because the method is suited for situations when molecules are not moving on the time-scale of the bleaching process.

By means of fluorescence microscopy one collects an image of the labelled molecules from the cells. A convenient form of microscopy is confocal microscopy because it allows a defined depth of focus and the zoom feature allows the image to be over sampled. This image is stored in memory. The process of image recording and saving is repeated until most of the original fluorescence is depleted.

The analysis of the images is done using a technique called image correlation spectroscopy.

2.1. Spatial Autocorrelation of Images- Image Correlation Spectroscopy

In an ergodic ensemble, the ensemble average of an stochastic process is equivalent to the spatial or temporal average. It empowers us to treat spatial and temporal averages correspondingly as $\langle I(t)\rangle = \langle I(x)\rangle$. To apply this hypothesis in image analysis, the image should be homogeneous [8].

As Petersen et al. [2] showed, we can write 2D spatial autocorrelation for an image as:

$$G(\eta,\sigma) = \langle I(x,y)\, I(x+\eta, y+\sigma)\rangle \tag{1a}$$

$$G(\eta,\sigma) = \frac{1}{MN}\sum_{i=1}^{M}\sum_{j=1}^{N} I(i,j)I(i+\eta, j+\sigma) \tag{1b}$$

where M and N are the number of discrete points (pixels), η and σ represent spatial-lags for x and y, respectively. It is pragmatically important to express the autocorrelation of fluctuation function, $\delta I(x,y) = I(x,y) - \langle I(x,y)\rangle$.

$$g(\eta,\sigma) = \frac{\langle \delta I(x,y)\, \delta I(x+\eta, y+\sigma)\rangle}{\langle I(x,y)\rangle^2} \tag{2a}$$

$$g(\eta,\sigma) = \frac{G(\eta,\sigma)}{\langle I(x,y)\rangle^2} - 1 \tag{2b}$$

where $\langle I(x,y)\rangle^2$ is normalising factor.

Practically, nobody use the above-mentioned equation to calculate autocorrelation of an image. Instead, one can exploit fast Fourier transformation along with the Weiner-Khinchin theorem to obtain the autocorrelation of the image more efficiently.

$$g(0,0) = \frac{FT^{-1}\{|FT\{I(x,y)\}|^2\}}{\langle I(x,y)\rangle^2} - 1 \tag{3}$$

where FT represents Fourier transformation and $|FT\{I(x,y)\}|^2$ is the power spectrum of the image. In practice, the maximum gray-value (zero-lag) of autocorrelation image can be used to calculate $g(0,0)$ as

$$g(0,0) = \frac{\text{The maximum gray-value of the autocorrelation image}}{MN \times (\text{The mean intensity of the image})^2} - 1 \tag{4}$$

2.2. General Theory of pbICS

Following statistical mechanics derivation for the fractional fluctuation of a random process in an ensemble [9] yields that the fractional fluctuation is proportional to the inverse number of particles in the observation volume:

$$\frac{\langle I - \langle I \rangle \rangle^2}{\langle I \rangle^2} = \frac{1}{\langle N \rangle} \tag{5}$$

where we have $\delta I = I - \langle I \rangle$

$$g(0,0) = \frac{\langle \delta I \rangle^2}{\langle I \rangle^2} = \frac{1}{\langle N \rangle} \tag{6}$$

then the autocorrelation function at zero-lag can provide us with the density of fluorescent molecules.

On the other hand, Elson and Magda [10] previously showed that there is direct connection between the detected intensity resulting from fluorescent fluctuation and the number of molecules of different species in illuminated volume.

$$I(t) = g \sum_j \epsilon_j Q_j \int L(\mathbf{r}) C_j(\mathbf{r}, t) d^3\mathbf{r} \tag{7}$$

where $I(t)$ is detected intensity, $L(\mathbf{r})$ displays laser intensity at point $\mathbf{r}$, $C_j(\mathbf{r}, t)$ demonstrates the concentration of the jth fluorescent components at position $\mathbf{r}$ and time t, ϵ_j and Q_j are molar extinction coefficient and fluorescent quantum yield of the jth component, respectively and g is an instrumental factor. Concentration fluctuation results in fluctuations in detected intensity:

$$\delta I(t) = g \sum_j \epsilon_j Q_j \int L(\mathbf{r}) \delta C_j(\mathbf{r}, t) d^3\mathbf{r} \tag{8}$$

with some manipulation described by Elson and Magda [10], one can reach to:

$$g(0,0) = \frac{\langle \delta I(t)^2 \rangle}{\langle I(t) \rangle^2} = \frac{\sum_j (\epsilon_j \, Q_j)^2 \langle C_j \rangle}{V \left(\sum_j \epsilon_j \, Q_j \langle C_j \rangle \right)^2} \tag{9}$$

where $\langle C_j \rangle$ is the mean concentration of the jth species. Therefore the detected intensity fluctuation is proportional to the concentration of fluorescent molecules. Assuming that the molar extinction coefficient of a cluster linearly related to the molar extinction coefficient of its constituent monomers (i.e. $\epsilon_j = n_j \, \epsilon$) and that quantum yield is unaffected by aggregation ($Q_j = Q$) [11], then one can write [12]:

$$g(0,0) = \frac{\sum_j n_j^2 c_j}{V \left(\sum_j n_j c_j \right)^2} \tag{10}$$

where n_j is the number of monomers in the jth species and c_j is the mean concentration ($= \langle C_j \rangle$)

Since the photobleaching is a random process then the number of non-photobleached monomers in a specific species can fluctuate through sample, then we should use average number of monomers as:

$$g(0,0) = \frac{\sum_j \langle n_j^2 \rangle \, c_j}{V \left(\sum_j \langle n_j \rangle \, c_j \right)^2} \tag{11}$$

Finding average number of non-bleached monomers for jth species is connected to the historical problem in statistical mechanics [13]. The problem is similar to the classical problem of boxes containing white and black

balls that solved by Newton for the first time. There are j boxes, each containing P white balls and Q black balls. We are interested to find the probability of finding i white balls by drawing one ball from each of the j boxes. We call the probability $w_j(i)$.

For drawing a single white ball from a box, the probability is obviously $p = P/(P+Q)$, where it is $q = 1-p$ for a black ball. Generally, $p^i q^{j-i}$ is the probability of drawing i white balls from i specified boxes and $j-i$ black balls from other $j-i$ boxes. Including $j!/(j-i)!i!$ ways to select i boxes from the j boxes, the answer will be

$$w_j(i) = \frac{j!}{(j-i)!i!}\, p^i q^{j-i} \tag{12}$$

since $p+q=1$ and $(a+b)^n = \sum_{k=0}^{n} \frac{n!}{(n-k)!k!}\, a^k b^{n-k}$ (binomial expansion) then $\sum_{i=0}^{j} w_j(i) = (p+q)^j = 1$. It means that $w_j(i)$ works as a probability density and we can use it to find mean value of variable as $\langle i \rangle = \sum_{i=0}^{j} i\, w_j(i)$.

We consider the relation

$$(px+q)^j = \sum_{i=0}^{j} w_j(i) x^i \tag{13}$$

where x is an arbitrary variable. First and second derivatives with respect to x give

$$jp(px+q)^{j-1} = \sum_{i=0}^{j} i\, w_j(i) x^{i-1} \tag{14a}$$

$$j(j-1)p^2(px+q)^{j-2} = \sum_{i=0}^{j} i(i-1) w_j(i) x^{i-2} \tag{14b}$$

If we let $x=1$ in these equations, the right hand sides give mean value of variables

$$jp = \sum_{i=0}^{j} i\, w_j(i) = \langle i \rangle \tag{15a}$$

$$j(j-1)p^2 = \sum_{i=0}^{j} i(i-1) w_j(i) = \langle i^2 \rangle - \langle i \rangle \tag{15b}$$

In our case, after starting photobleaching process the number of non-bleached monomers will vary

$$\langle n_j \rangle = \langle i \rangle = \sum_{i=0}^{j} w_j(i)\, i \tag{16a}$$

$$\langle n_j^2 \rangle = \langle i^2 \rangle = \sum_{i=0}^{j} w_j(i)\, i^2 \tag{16b}$$

where n_j for any j-mers with i non-bleached monomers equals $n\, i$ with $n=1$ for a monomer. By applying equation (15), one can obtain

$$\langle n_j \rangle = j\, p, \tag{17a}$$

$$\langle n_j^2 \rangle = (j\, p)^2 + j\, p(1-p) \tag{17b}$$

then the equation (11) can be rewritten as

$$g(0,0) = \frac{\sum_j \left[(j\, p)^2 + j\, p(1-p)\right] c_j}{V\left(\sum_j j\, p\, c_j\right)^2} \tag{18}$$

By taking $\langle N \rangle / V$ as cluster density, CD, and using equation (6) and (18) we will have

$$CD(p) = \frac{\left(\sum_j j\, c_j\right)^2 p}{\sum_j [j\, c_j + j(j-1)\, c_j\, p]} \tag{19}$$

That is the cluster density of a mixture of homogeneous j-mers as a function of fraction of remained fluorescence p. In other words, p is the probability of finding fluorescent components that can be calculated via dividing the mean intensity value of an image after bleach by the mean intensity value of the image before photobleaching. Ciccotosto and et al. [12] also introduced an explicit equation for a single j-mer that can be obtained by dropping summations in equation (19).

$$CD_j(p) = \frac{j\, c_j\, p}{1 + (j-1)p}, \qquad j = 1, 2, ... \tag{20}$$

3. Results and Discussion

3.1. pbICS Explicit Aggregation Distribution

By using equation (19), we can obtain cluster densities of different mixtures of j-mers. For monomers with concentration of c_1, dimers with concentration of c_2 and so on, we have

$$\begin{aligned}
CD(p)_{\{1\}} &= c_1 p \\
CD(p)_{\{1,2\}} &= \frac{(c_1 + 2c_2)^2 p}{(c_1 + 2c_2) + 2c_2 p} \\
CD(p)_{\{1,2,4\}} &= \frac{(c_1 + 2c_2 + 4c_4)^2 p}{(c_1 + 2c_2 + 4c_4) + (2c_2 + 12c_4)p} \\
CD(p)_{\{1,2,4,6\}} &= \frac{(c_1 + 2c_2 + 4c_4 + 6c_6)^2 p}{(c_1 + 2c_2 + 4c_4 + 6c_6) + (2c_2 + 12c_4 + 30c_6)p} \\
CD(p)_{\{1,2,4,6,8\}} &= \frac{(c_1 + 2c_2 + 4c_4 + 6c_6 + 8c_8)^2 p}{(c_1 + 2c_2 + 4c_4 + 6c_6 + 8c_8) + (2c_2 + 12c_4 + 30c_6 + 56c_8)p}
\end{aligned} \tag{21a}$$

where $CD(p)_{\{1\}}$ is the cluster density of *monomers* with the mean concentration of c_1, $CD(p)_{\{1,2,4,6,8\}}$ demonstrates the cluster density of a mixture of $monomers - dimers - tetramers - hexamers - octamers$ with mean concentrations of $c_1 - c_2 - c_4 - c_6 - c_8$ respectively and p displays fraction of remained fluorescence. As expected, monomer density decays linearly with fraction of molecules remaining (not photobleached). However the pbICS curves from oligomers decay in a non-monotonic fashion.

For a specific ensemble, it is possible to fit Equation (21) for obtaining c_js with the general initial condition of

$$CD_{max}(p = 1) = \frac{(\sum_j j\, c_j)^2}{\sum_j j^2\, c_j}. \tag{22}$$

As a second method, it is noted that pbICS curves can also be fitted to the form of Equation (20) for finding average j with initial condition of $c_j = CD_{max}(p = 1)$, where $\langle j \rangle$ is called the average brightness. Then the relation between $\langle j \rangle$ extracted from the latter method and c_js from the first approach can be formulated by

$$\langle j \rangle = \frac{\sum_j j^2 c_j}{\sum_j j c_j} = \frac{\sum_j j c_j}{CD_{max}} \tag{23}$$

Subsequently we can modify the Equation (20) to

$$\frac{CD(p)}{CD_{max}} = \frac{\langle j \rangle\, p}{1 + (\langle j \rangle - 1)p}. \tag{24}$$

3.2. Linear approximation

In some instances bleaching to completion may be difficult to achieve in practise and high doses of light exposure can be harmful to living cells. An alternative is to use gentle bleaching with a reduced power level such as obtained with replicate imaging under normal conditions. In this case one only partially bleaches the sample by perhaps 10% or so. With a reduced range of p values (now 1 to 0.9 instead of 1 to 0.1 that means 10% photobleaching instead of 90%) one cannot use the full non-linear Equation (19). Instead we propose to fit the data with a linear equation, which is essentially the tangent to the full function near $p = 1$. For the homogenous model (or alternatively average brightness model), the linear form of the function is given by

$$\frac{CD(p)}{CD_{max}} = \left(1 - \frac{1}{\langle j \rangle}\right) + \frac{1}{\langle j \rangle} p, \qquad (p \to 1) \tag{25}$$

where $\langle j \rangle$ can then be obtained as $\langle j \rangle = 1/\text{gradient}$ or $j = 1/(1 - \text{intercept})$. Figure 1 reveals the full pbICS curves for $j = 1.5$ and $j = 4.5$ and the linear extrapolations near $p = 1$.

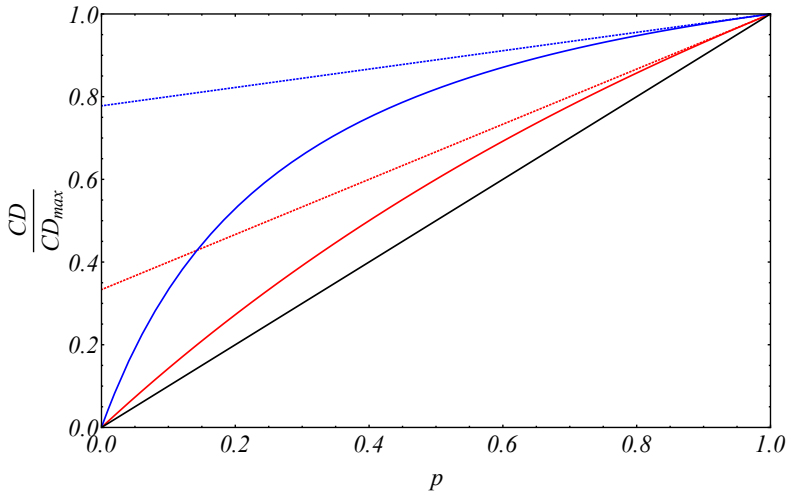

Figure 1. The graph of Equation (24) and its linear approximation from Equation (25). The black line represents $\langle j \rangle = 1$. The red curve and its linear approximation, red doted line, display $\langle j \rangle = 1.5$ and the blue curve and dotted line depict $\langle j \rangle = 4.5$.

4. Conclusion

We have elaborated upon the theoretical development of photobleaching image correlation microscopy. Formulae relating the pbICS curves to the oligomeric distribution were presented in closed form for the first time. In addition, a simple linear fitting method was introduced for small extents of photo-bleaching which should in turn find application in live cell applications.

5. Conflict of Interest

Authors report no conflicts of interest in this work.

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