Studying protein clustering by number and brightness analysis using photon-counting confocal microscopy

Peter Nagy, Department of Biophysics and Cell Biology, University of Debrecen, Hungary

Introduction

Protein clustering plays a pivotal role in the regulation of several physiological and pathological processes. Although methods abound which have the potential to measure protein associations, only relatively few of them are capable of quantifying protein clusters in terms of the number of proteins/cluster or the fraction of associating proteins [1]. Such approaches often require sophisticated instrumentation and involve complex mathematics. Number and brightness (N&B) analysis, introduced by Digman et al. in 2008 [2], seems to be an exception to this rule since its theoretical background is easy to understand and no special, dedicated instrument is required to implement the technique.

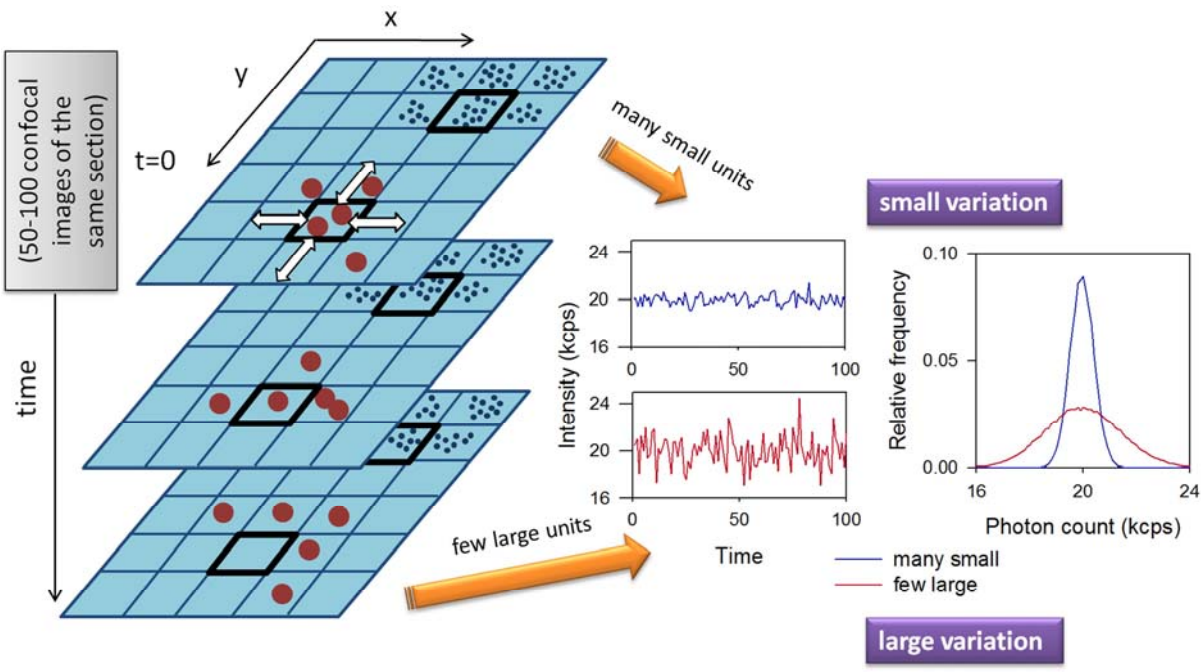

Fig. 1 Principle of N&B analysis
Left: The fluctuation in the number of detected photons is measured in a time series in confocal microscopy. If the number of particles/pixel is low, the relative variation in the occupation number will be large.
Right: An average detected photon count rate of 20,000 photons/s (20 kcps=20 kilo-counts per second) was simulated by many dim particles (blue curves) and few bright particles (red curves).
Note: 20 kcps corresponds to 2 photons/dwell time if the dwell time is adjusted to 10 μs.

Terms, definitions:

- (pixel) dwell time, integration time: in a confocal system the duration of time in which photons are collected from a single pixel. Usually it is in the μs range.
- frame time: the time in which a whole image frame is recorded. It is equal to the number of pixels multiplied by the dwell time.
- repetition time: the time in which the same pixel is resampled (recorded again) when taking multiple images of the same section. It is approximately equal to the frame time, but for technical reasons the repetition time is somewhat longer than the frame time (because the scanner needs to return from the position of the last pixel to that of the first one).
- molecular brightness (ϵ): the number of *detected* photons emitted by a single diffusing unit (monomer, dimer, etc.) during the pixel dwell time. It is smaller than the number of emitted photons since most emitted photons are undetected: photons do not enter the objective (importance of high numerical aperture), photons are lost in the optics and PMTs have low quantum efficiency: only 10-20% of photons hitting the photocathode of a PMT are detected. The molecular brightness is determined by the laser intensity, the extinction coefficient and the fluorescent quantum yield of the fluorophore and by the efficiency of the instrument in detecting photons. It must be noted that there is an alternative definition of ϵ according to which it is the total number of detected photons/diffusing unit in *unit* time. However, the former definition will be used throughout this chapter.
- occupation number: the number of molecules in a single pixel.

Theory of N&B analysis

Let us assume that a fluorescently labeled protein undergoes diffusion in a cell or in the cell membrane (Fig. 1). There is a requirement for how mobile the molecule has to be, i.e. how large its diffusion constant needs to be for the N&B method, but this point will be addressed later. Due to molecules moving into and out of a pixel the number of molecules in a pixel at any given time will fluctuate (as long as the pixel dwell time is short enough so that averaging does not dampen the fluctuations). The number of molecules in a pixel can be described by the Poisson distribution:

$$P(N) = \frac{\lambda^N}{N!} e^{-\lambda} = \frac{\langle N \rangle^N}{N!} e^{-\langle N \rangle} \qquad (1)$$

where *P(N)* is the probability of finding *N* molecules in the pixel and λ is the parameter of the Poisson distribution. $\langle N \rangle$ stands for the time average of *N*, i.e. the expected number of molecules in a pixel. λ turns out to be equal to $\langle N \rangle$ and to the variance (σ^2) of the Poisson

distribution. Therefore, the relative fluctuation in the occupation number is given by

$$\frac{\sigma_{occ}}{\langle N \rangle} = \frac{\sqrt{\langle N \rangle}}{\langle N \rangle} = \frac{1}{\sqrt{\langle N \rangle}} \qquad (2)$$

which implies that the higher the occupation number, the lower its relative variation is. The above equation also suggests that the relative fluctuation of the occupation number holds information about the average number of molecules in a pixel, i.e. $\langle N \rangle$.

Now let us consider more rigorously the variance of the detected number of photons (σ^2) in a pixel during the pixel dwell time. Two factors contribute to this variance: (1) the fluctuation in the occupation number (σ_N^2) and (2) the Poissonian nature of photon detection also known as shot noise (σ_D^2). σ^2 can be partitioned into two components, σ_N^2 and σ_D^2, according to the law of total variance (a similar principle is applied when partitioning the sum of squares (*SS*) in ANOVA into within-groups and between-groups *SS*):

$$\sigma^2 = \sigma_N^2 + \sigma_D^2 \qquad (3)$$

where σ_D^2 can be considered to be the conditional variance of the photon number given *N* molecules in the pixel. Let us define apparent brightness (*B*) for every pixel as the ratio of the variance of fluorescence intensity (photon number) to the average intensity. The average intensity and the variance are defined as follows (ϵ designates the molecular brightness):

$$\langle I \rangle = \frac{\sum_i \varepsilon N_i}{n} = \varepsilon \langle N \rangle, \quad \sigma = \frac{\sum_i \left(PC_i - \langle PC \rangle \right)^2}{n} \qquad (4)$$

where *n* is the number of samplings (slices) and *PC* is photon count. Therefore, *B* is

$$B = \frac{\sigma^2}{\langle I \rangle} = \frac{\sigma^2}{\varepsilon \langle N \rangle} = \frac{\sigma_N^2 + \sigma_D^2}{\varepsilon \langle N \rangle} \qquad (5)$$

Let us first consider σ_N^2. The occupation number (*N*) fluctuates according to the Poisson distribution. ϵ number of photons are detected from every fluorophore during the dwell time, so we need to determine the variance of $N\epsilon$, where *N* is distributed according to the Poisson distribution and ϵ is constant. If a random variable is multiplied by a constant, its variance is multiplied by the square of the constant. Therefore

$$\sigma_N^2 = Var(N\varepsilon) = \varepsilon^2 Var(N) = \varepsilon^2 \langle N \rangle \qquad (6)$$

where *Var()* designates the variance operator. In the above equation we also applied the fact that the variance of the Poisson distribution is equal to its mean, $\langle N \rangle$.

In-depth derivation of equation (3):

Let us denote by $poiss_Y(x)$ that x is distributed according the Poisson distribution with a mean value of Y. In a N&B experiment the number of particles (N) in a pixel is distributed according to the Poisson distribution. On average $N\epsilon$ photons are detected in a single pixel during the dwell time, but due to the random nature of photon detection this variable will also be distributed according to the Poisson distribution. Therefore, the following transformed random variable describes the distribution of photon counts (*PC*):

$$PC = poiss_{\langle N\rangle\varepsilon}\left(poiss_{\langle N\rangle}\left(N\right)\varepsilon\right) \quad \text{(i)}$$

According to the law of total variance the variance of a random variable X can be described according to the following equation:

$$Var\left(X\right) = Var\left(E\left(X\middle|Y\right)\right) + E\left(Var\left(X\middle|Y\right)\right) \quad \text{(ii)}$$

where *Var()* and *E()* represent the variance and expected value, respectively, and $E(X|Y)$ and $Var(X|Y)$ are the conditional expected value and variance, respectively, of X conditioned on the random variable Y. Therefore

$$Var\left(PC\right) = Var\left(E\left(PC\middle|N\right)\right) + E\left(Var\left(PC\middle|N\right)\right) \quad \text{(iii)}$$

The expected value of *PC* given N number of fluorophores is

$$E\left(PC\middle|N\right) = \varepsilon N \quad \text{(iv)}$$

The variance of *PC* given N fluorophores is

$$Var\left(PC\middle|N\right) = \varepsilon N \quad \text{(v)}$$

due to the properties of the Poisson distribution.

Since

$$Var\left(\varepsilon N\right) = \varepsilon^2 Var\left(N\right) = \varepsilon^2\left\langle N\right\rangle \quad \text{(vi)}$$

and

$$E\left(\varepsilon N\right) = \varepsilon\left\langle N\right\rangle \quad \text{(vii)}$$

the variance of *PC* is

$$Var\left(PC\right) = \varepsilon^2\left\langle N\right\rangle + \varepsilon\left\langle N\right\rangle \quad \text{(viii)}$$

The first and second terms in the above equation are designated by σ_N^2 and σ_D^2, respectively, in equation (3).

Next, let us derive σ_D^2. If there are N molecules in a pixel (N is a constant, not a random variable in this case), the number of detected photons will still fluctuate according to the Poisson distribution due to the random nature of photon detection. The mean of this Poisson distribution is $\langle N\rangle\epsilon$. Since in this case the Poisson distribution is not transformed, its variance is equal to its mean. Therefore

$$\sigma_D^2 = \varepsilon\left\langle N\right\rangle \quad \textit{(7)}$$

By substituting the last two equations into (5) we obtain an expression relating the *apparent* brightness to the *molecular* brightness:

$$B = \frac{\sigma_N^2 + \sigma_D^2}{\varepsilon \langle N \rangle} = \frac{\varepsilon^2 \langle N \rangle + \varepsilon \langle N \rangle}{\varepsilon \langle N \rangle} = \varepsilon + 1 \qquad (8)$$

Similarly, we can define the *apparent* number of molecules as the ratio between the square of the mean intensity and the variance:

$$N_A = \frac{\left(\varepsilon \langle N \rangle\right)^2}{\varepsilon^2 \langle N \rangle + \varepsilon \langle N \rangle} = \frac{\varepsilon \langle N \rangle}{\varepsilon + 1} \qquad (9)$$

Both B and N_A are easily derived from the mean and the variance of a pixel intensity and the molecular parameters (ϵ and $\langle N \rangle$) can be calculated. The method described above can be used for the characterization of homoclusters, but its extension, not discussed here, can be applied for the analysis of the stoichiometry of heteroclusters as well [3].

Practical considerations, requirements and limitations

In a typical N&B experiment a confocal section is recorded 50-100-times and the parameters B and N_A are calculated for every pixel yielding thousands of values whose distribution is usually displayed on 1-D or 2-D histograms (Fig. 2). The most valuable information obtained from a N&B experiment is the molecular brightness (ϵ). If the molecular brightness in an experiment turns out to be twice larger than the molecular brightness of a monomer, then dimers have been detected. It follows that for measurements in which molecules are to be enumerated, the molecular brightness of the monomer (e.g.

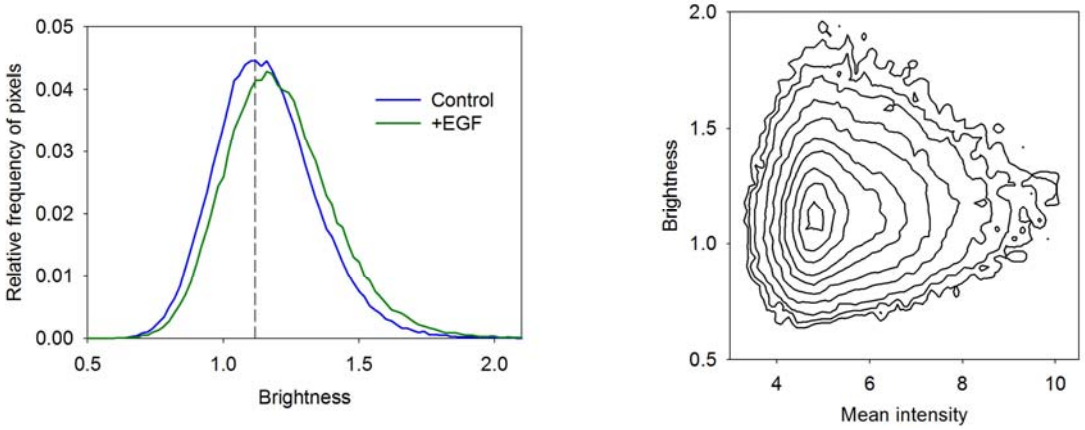

Fig. 2 Representation of N&B experiments
Left: One-dimensional histogram of pixelwise values of apparent brightness in cells expressing ErbB1-GFP. Control (serum-starved) and EGF-stimulated cells were analyzed ($Br_{control}$=1.16; Br_{EGF}=1.22). The vertical dashed reference line corresponds to the brightness of monomeric GFP (Br_{GFP}=1.118). Note that a small shift in the histogram corresponds to significant change in clustering.
Right: Two-dimensional histogram (contour plot) of the control sample from the previous graph. The distribution of brightness vs. mean intensity is displayed.

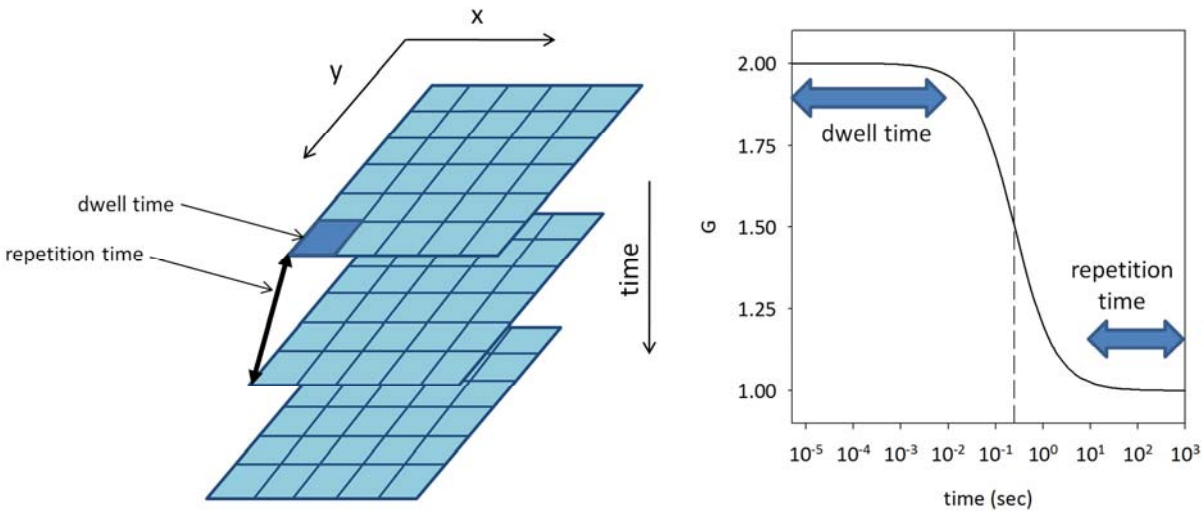

Fig. 3 The principle of choosing the dwell time and the repetition time in N&B experiments. A 3D stack of images (3rd dimension is time) is recorded (left). The autocorrelation function (G) of the number of molecules in a single pixel is shown on the right. The dashed reference line indicates the diffusional correlation time (0.25 sec) assuming a diffusion coefficient of 10^{-10} cm^2/sec and radius of the confocal detection volume of 100 nm.

monomeric GFP) has to be determined in a separate experiment.

A central assumption in a N&B experiment is that the variance of photon count is proportional to the mean intensity according to equations (6)-(7). This assumption is violated if photon counts are saturated at high laser intensities. Therefore, it is mandatory to show that the experiment is carried out in such a range of laser intensity in which the apparent brightness is linearly proportional to laser intensity.

Another important requirement for a successful N&B experiment is that the pixel dwell time has to be short enough so that fluctuations in occupation number are not averaged by long acquisition times. This corresponds to the time range in which particle numbers are highly autocorrelated (Fig. 3). The autocorrelation function of particle numbers in 2D diffusion is:

$$G(t) = 1 + \frac{1}{N_D} \frac{1}{1 + \frac{t}{\tau_D}} \qquad (10)$$

where N_D is the number of diffusing fluorophores in the confocal detection volume and τ_D is the diffusional correlation time:

$$\tau_D = \frac{\omega^2}{4D} \qquad (11)$$

where *D* is the diffusion coefficient and ω is the radius of the confocal detection volume. In addition consecutive samplings of the same pixel have to be independent of each other so that particle numbers can be assumed to be distributed according to the Poisson distribution. Therefore, the frame time has to be in such a time range where particle numbers are not autocorrelated (Fig. 3).

In N&B experiments the determination of apparent and molecular brightness is based on the analysis of fluorescence fluctuations related to diffusion. If a pixel contains only immobile molecules, then the variance component due to diffusion will be zero. In this case

$$B = \frac{\sigma_D^2}{\varepsilon \langle N \rangle} = \frac{\varepsilon \langle N \rangle}{\varepsilon \langle N \rangle} = 1 \qquad (12)$$

The above equation implies that the N&B method cannot be used for the analysis of immobile molecules since the apparent brightness of these fluorophores is always unity independent of their stoichiometry. Any molecule whose diffusion is not fast enough so that it moves in and out of a pixel during the repetition time is considered immobile in this respect.

The presence of a mixture of mobile and immobile molecules presents a problem in N&B experiments [4]. The number of immobile molecules will not fluctuate (i.e. their σ_N^2 contribution will be zero). Therefore, the measured apparent brightness in the presence of a mixture of mobile and immobile molecules (B^*) will be as follows:

$$B^* = \frac{\sigma_{n,mobile}^2 + \sigma_{d,mobile}^2 + \sigma_{d,immobile}^2}{k_{mobile} + k_{immobile}} = \frac{\sigma_{n,mobile}^2 + \sigma_{d,total}^2}{k_{total}} = \frac{f_M \sigma_{n,total}^2 + \sigma_{d,total}^2}{k_{total}} = \\ = \frac{f_M n_{total} \varepsilon^2 + n_{total} \varepsilon}{n_{total} \varepsilon} = 1 + f_M \varepsilon \qquad (13)$$

where f_M is the fraction of mobile molecules. The apparent brightness (*B*) of the mobile fraction can be calculated according to the following equation:

$$B = 1 + \frac{B^* - 1}{f_M} \qquad (14)$$

Photobleaching and sample drift result in a gradual, slow change in pixel intensities during taking the time series in N&B experiments. Stage sinking presents a special problem when imaging membrane proteins since even a tiny change in the location of the focal plane will move the membrane completely out of focus. Therefore, the average intensity of single

frames has to be plotted against time and if slow (i.e. low frequency) changes are present, they have to be eliminated by applying a high-pass filter [2].

The application of N&B analysis is the most straightforward on confocal microscopes equipped with photon counting detectors. Some microscopes (e.g. the Olympus FV-1000 you are going to use in your experiments on the course) have pseudo-photon counting detectors whose output digital levels correspond to photon counts multiplied by an unknown, detector-specific parameter (*S*). Factor S can be determined by illuminating an immobile sample in which σ_N=0. According to equation (12) the apparent brightness calculated for such a sample should be one. However, if the photon counts are multiplied by *S*, equation (12) takes the following form:

$$B = \frac{S^2\sigma_D^2}{S\varepsilon\langle N\rangle} = \frac{S^2\varepsilon\langle N\rangle}{S\varepsilon\langle N\rangle} = S \qquad (15)$$

Therefore, if variance is plotted as a function of the average intensity, the slope of the line yields factor *S* [5]. Images have to be divided by *S* before the N&B analysis.

N&B analysis can also be applied in confocal systems equipped with analog (non-photon counting) detectors. The principles which need to be taken into account are only briefly described here. For an exhaustive explanation the reader is referred to the original publication [5]. In the case of an analog detector the pixel variance is increased by the read-out noise (σ_0^2) and by factor *S* according to the following equation:

$$\sigma_{measured}^2 = S^2\left(\sigma_N^2 + \sigma_D^2\right) + \sigma_0^2 \qquad (16)$$

The average intensity is increased by the detector offset and factor *S*:

$$\langle I_{measured}\rangle = S\langle I\rangle + I_{offset} \qquad (17)$$

Both σ_0^2 and I_{offset} have to be subtracted from equation (5) to obtain the apparent brightness:

$$B = \frac{\sigma_{measured}^2 - \sigma_0^2}{\langle I_{measured}\rangle - I_{offset}} = \frac{\left[S^2\left(\sigma_N^2 + \sigma_D^2\right) + \sigma_0^2\right] - \sigma_0^2}{\left[S\langle I\rangle + I_{offset}\right] - I_{offset}} = \frac{S^2\left(\sigma_N^2 + \sigma_D^2\right)}{S\langle I\rangle} = \frac{S^2\left(\varepsilon^2\langle N\rangle + \varepsilon\langle N\rangle\right)}{S\varepsilon\langle N\rangle} = S(\varepsilon + 1) \qquad (18)$$

The three parameters (*S*, σ_0, I_{offset}) have to be determined separately as described in the original publication [5]. It was concluded that the dynamic range and the precision of

measuring the brightness at low intensities with analog detectors is inferior to photon counting devices.

Programs to evaluate N&B experiments

Any image analysis program capable of performing arbitrary calculations with pixel intensities can be used for evaluating time series in N&B experiments. On the course we are going to use a custom-written Matlab program. A dedicated software solution (Globals for Images or SimFCS) for the analysis of RICS (raster image correlation spectroscopy [6]) and N&B data is available from the Laboratory of Fluorescence Dynamics (University of California, Irvine; http://www.lfd.uci.edu/globals/). The program can be freely downloaded but a license has to be purchased after a grace period of 30 days.

Experimental protocol

CHO cells stably transfected with ErbB1-GFP will be used for the experiments. The cell line, designated F1-10, has been described previously [7] and expresses ~50,000 ErbB1-GFP/cell determined by flow cytometric calibration using QifiKit (Dako, Glustrup, Denmark).

In the experiment the epidermal growth factor (EGF)-induced dimerization of EGF receptor (EGFR or ErbB1) will be investigated. It is expected that EGF will induce dimerization or larger-order cluster formation of EGFR. The results of these experiments have been recently published [8].

A. <u>Image acquisition</u>

1. Start up the microscope. Select the 60× oil immersion objective. Adjust the excitation and detection wavelengths and the dichroic mirrors for GFP. Adjust image size to 256×256 pixels. Adjust the pixel dwell time to 10 μs, the number of frames to be collected to 100. The time series in the N&B experiments will therefore contain 100 sections. Make sure that photon counting mode is selected. Turn off frame averaging.
2. Measure a sample for the determination of factor *S*. Put an immobile sample into the microscope (e.g. fluorescent beads suspended in ethanol, dropped onto a coverslip and allowed to dry or fluorescent beads in gel). Contrast stretch the acquired image

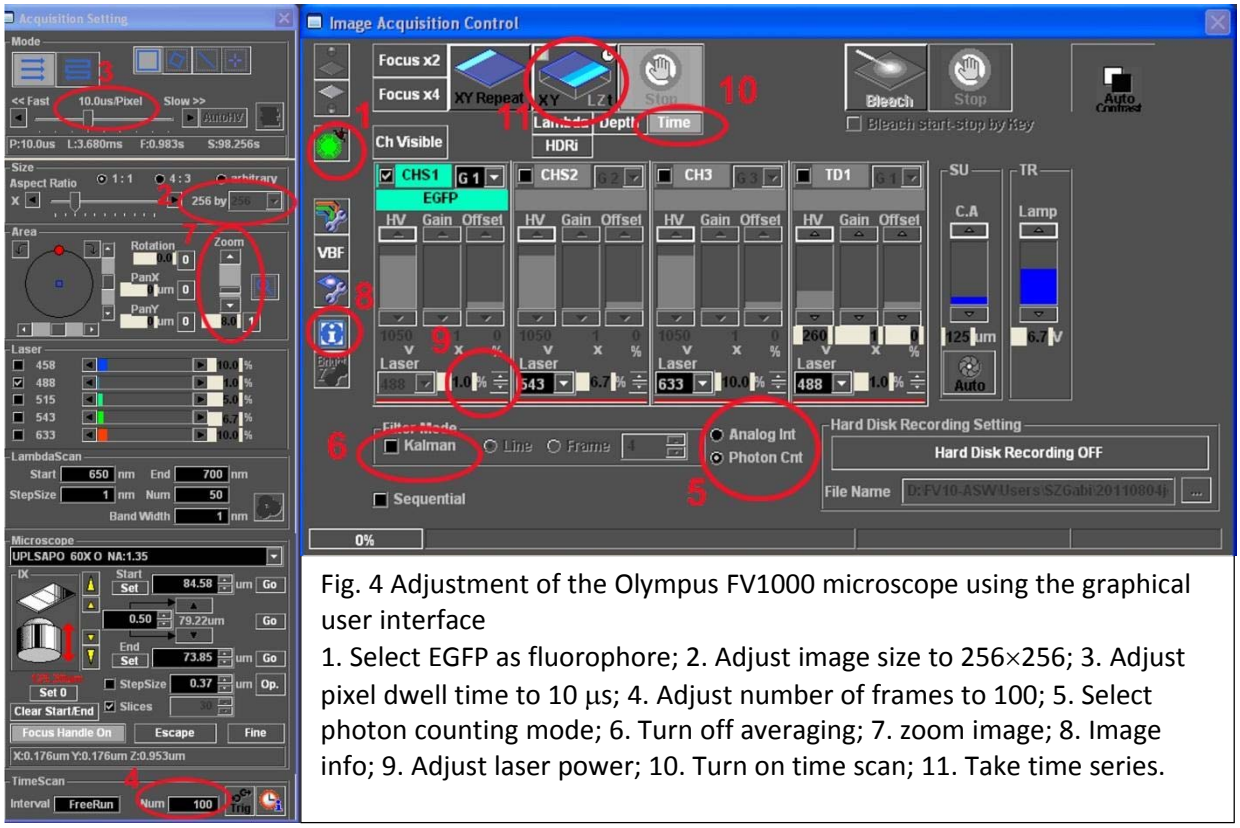

Fig. 4 Adjustment of the Olympus FV1000 microscope using the graphical user interface
1. Select EGFP as fluorophore; 2. Adjust image size to 256×256; 3. Adjust pixel dwell time to 10 μs; 4. Adjust number of frames to 100; 5. Select photon counting mode; 6. Turn off averaging; 7. zoom image; 8. Image info; 9. Adjust laser power; 10. Turn on time scan; 11. Take time series.

so that low pixel intensities are visible. By zooming the image adjust the pixel size to ~100 nm. Check the pixel size with the "Info" button. Adjust laser power so that (a) the photon count is not higher that 10-20 photons/pixel; (b) there is no significant bleaching. Take a time series of the fluorescent beads.

3. Measure a sample for the determination of the molecular brightness of monomeric GFP. Put a drop of sufficiently diluted (1-2 μM) soluble monomeric GFP onto a coverslip. Increase the zoom until the pixel size is approximately 100 nm. Take a time series.
4. Measure the association state of soluble GFP expressed in cells. Wild-type GFP is known to dimerize so the molecular brightness is expected to be ~2× higher than that of monomeric GFP [9]. CHO cells transiently transfected with GFP (pmaxGFP, Lonza, Cologne, Germany) will be used for this purpose. Select a cell showing GFP fluorescence. Increase the zoom until the pixel size is approximately 100 nm. Take a time series.
5. Put starved F1-10 cells in 100 μl Tyrode's buffer (supplemented with 10 mM glucose and 0.1% BSA) grown in 8-well chambers into the microscope. Select a cell showing ErbB1-GFP fluorescence. Adjust the focal plane to the bottom surface of the cell adjacent to the coverslip. Adjust the pixel size to the same value as in the previous step. Take a time series. Check for possible bleaching, stage drift or cell movement during the experiment. If any of the above processes is significant, repeat the time series.
6. Add 100 μl of 200 nM EGF slowly to the chamber. Since the chamber contains 100 μl buffer, EGF will be diluted 2× resulting in a final concentration of 100 nM.
7. Incubate the cells in the microscope for 2-3 min. Check if the cells moved out of the focal plane. If yes, readjust the focal plane. Take a time series.

B. Image analysis

1. Start Matlab on a machine where DipImage is installed. DipImage is a scientific image analysis library, available free of charge to non-profit institutions from the Delft University of Technology (http://www.diplib.org/).
2. Type 'dipimage' into the Matlab command prompt.

3. Start the N&B toolbox by entering ‘nb_tools’ into the Matlab command prompt (after changing the current folder to the location of the N&B toolbox if it is not in the search path of Matlab).

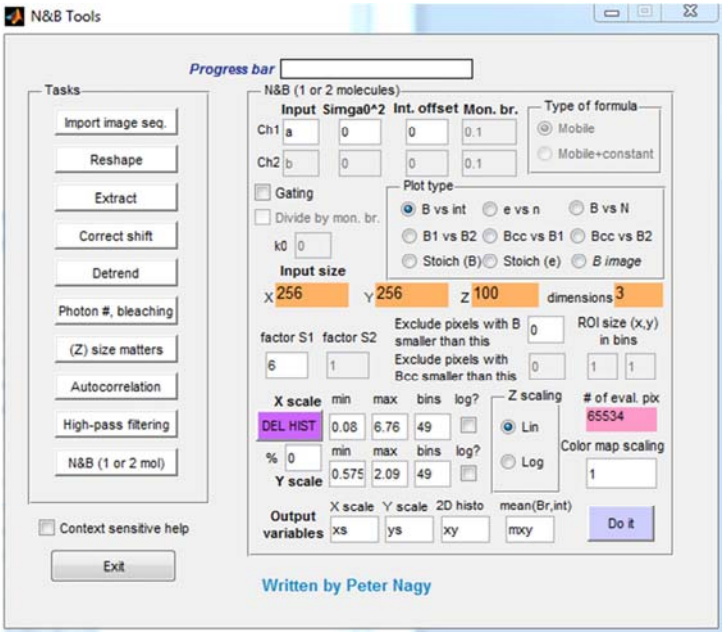

Fig. 5 The N&B toolbox in Matlab
All the usual tasks required for analyzing a N&B experiment is available in the program. These tasks can be selected on the left. Context sensitive help is available for some of the features by hovering the pointer above a button or a text box.

4. Analyze the sample recorded for determining factor *S*.
   i. Load the image sequence by pressing ‘Import image seq.’ and follow the instructions.
   ii. Press ‘Extract’ and extract the central part of the image eliminating the border pixels where inhomogeneous illumination is common.
   iii. Check for the presence of slow trends in the fluorescence intensity (caused by bleaching, cell movement or stage drift) and for the proportionality of variance and mean intensity by pressing the button ‘Photon #, bleaching’ (Fig. 6). This step should be carried out with every sample.
   iv. If low frequency (slow) changes are present in the fluorescence intensity, perform high-pass filtering (in order to remove slow trends). Press ‘High-pass filtering’, select the input and output images and the cut-off frequency (high-pass threshold). Check the result in the ‘Photon #, bleaching’ panel.
   v. Press the ‘N&B (1 or 2 mol)’ button. Select ‘B vs int’ (brightness vs. mean intensity) for plot type. Enter the variable holding the image sequence into the ‘Ch1’ box. Press ‘Do it’. The brightness vs. mean intensity plot will be displayed in

a separate window. The mean brightness can be found in the Matlab window. Change the value of 'factor S1' (factor *S* for channel 1) and repeat the analysis as long as the calculated brightness is not one. Record this value of factor *S* for further use in the remaining part of the analysis.

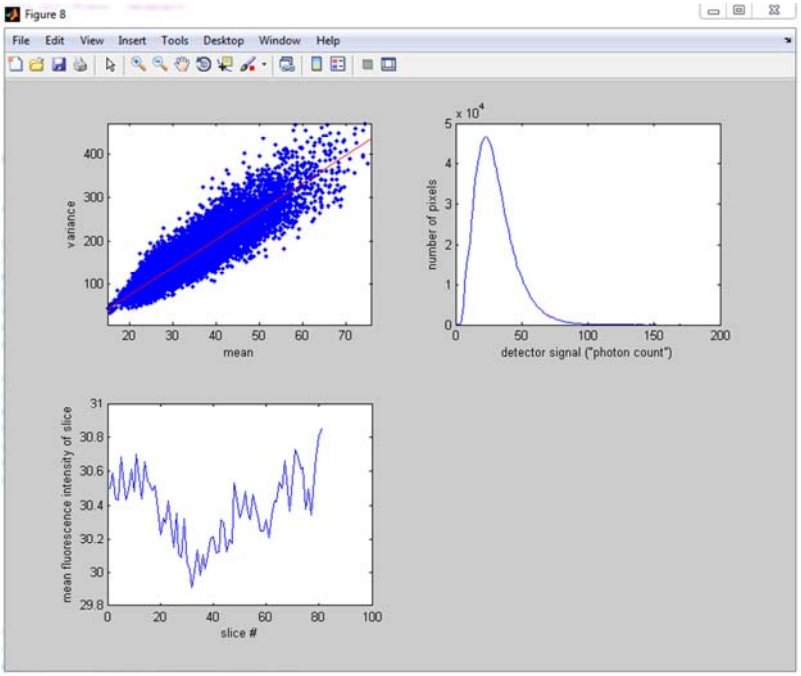

Fig. 6 Checking for the proportionality of variance and mean intensity and for the presence photobleaching, drift or movement using the N&B toolbox.
Graphs like those on the left are acceptable.

5. Determination of the molecular brightness of monomeric GFP.
   i. Load the image sequence recorded for the determination of the brightness of monomeric GFP.
   ii. Extract the central part of the image sequence, then press 'N&B (1 or 2 mol)'.
   iii. Enter the variable for the image sequence and the value of factor *S*.
   iv. Take a note of the mean brightness value.
6. Determination of the molecular brightness of GFP expressed in CHO cells.
   i. Load the image sequence of GFP-transfected CHO cells and extract the central part.
   ii. After entering the value of factor *S* in the 'N&B (1 or 2 mol)' panel analyze the molecular brightness of expressed GFP.
7. Determine the brightness of ErbB1-GFP in serum-starved and in EGF-stimulated cells.
   i. Load the image sequences into separate variables. Extract their central parts.
   ii. Analyze the brightness of ErbB1-GFP in both cases.

iii. If the low frequency part of a 2-D histogram is to be emphasized, change ‘Z scaling’ to ‘log’.

iv. If some kind of heterogeneity is present in the 2-D histograms, pixels corresponding to a given part of the 2-D histogram can be highlighted by selecting ‘Gating’ and moving the gate to a certain part of the 2-D histogram followed by pressing ‘Do it’.

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