TITLE:

Calibration-Free *In-Vitro* Quantification of Protein Homo-Oligomerization Using Commercial Instrumentation and Free, Open Source Brightness Analysis Software

AUTHORS & AFFILIATIONS:

Rory Nolan[1], Luis A. Alvarez[1], Samuel C. Griffiths[2], Jonathan Elegheert[2], Christian Siebold[2] and Sergi Padilla-Parra[1,2,3,4]

[1]Cellular Imaging Group, Wellcome Centre Human Genetics, University of Oxford, UK.
[2]Division of Structural Biology, Wellcome Centre Human Genetics, University of Oxford, UK.
[3]Dynamic Structural Virology Group, Biocruces Health Research Centre, Barakaldo, Spain.
[4]IKERBASQUE, Basque Foundation for Science, Bilbao, Spain.

Corresponding Author:

Sergi Padilla-Parra (spadilla@well.ox.ac.uk)

Email Addresses of Co-authors:

Rory Nolan (rory.nolan@hertford.ox.ac.uk)
Luis A. Alvarez (lalvarez@well.ox.ac.uk)
Samuel C. Griffiths (griffiths@strubi.ox.ac.uk)
Jonathan Elegheert (jelegheert@strubi.ox.ac.uk)
Christian Siebold (christian@strubi.ox.ac.uk)

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SUMMARY:

This protocol describes a calibration-free approach for quantifying protein homo-oligomerization *in vitro* based on fluorescence fluctuation spectroscopy using commercial light scanning microscopy. The correct acquisition settings and analysis methods are shown.

ABSTRACT:

Number and brightness is a calibration-free fluorescence fluctuation spectroscopy (FFS) technique for detecting protein homo-oligomerization. It can be employed using a conventional confocal microscope equipped with digital detectors. A protocol for the use of the technique *in vitro* is shown by means of a use case where number and brightness can be seen to accurately quantify the oligomeric state of mVenus-labelled FKBP12F36V before and after the addition of the dimerizing drug AP20187. The importance of using the correct microscope acquisition parameters and the correct data preprocessing and analysis methods are discussed. In particular, the importance of the choice of photobleaching correction is stressed. This inexpensive method can be employed to study protein-protein interactions in many biological contexts.

INTRODUCTION:

Protein-Protein interactions *in vitro*

Traditionally, crystallography and nuclear magnetic resonance experiments combined with cryo-electron microscopy (cryoEM) are the technologies chosen to accurately describe the three-dimensional architecture of proteins and to infer their function by scrutinizing their high resolution structural details. Proteins, however, are not static structures and can undergo a variety of conformational changes and vibrations in time and space. This is why structural information from crystallographic or CryoEM data needs to be complemented with other techniques (*e.g.,* molecular dynamics simulations and single molecule techniques): the function of a protein is related to its conformational changes and interactions, and this information is not present in a static structure. In order to probe for intra-molecular dynamics, techniques based on single molecule Forster Resonance Energy Transfer (smFRET) are very effective[1]. These approaches are able to assess different subpopulations of molecules in complex media. This is very important, as these changes are rapid and occur during the acquisition of the data (*i.e.,,* nanosecond to second range).

Two main approaches are commonly employed to detect and quantify these changes: proteins in solution and surface-immobilization. For the detection of inter-molecular interactions and in particular, the process of dimerization induced by ligands, smFRET is not always the best tool. Indeed, FRET depends not only on the distance (≈10 nm) but also on the orientation of the two dipoles (donor and acceptor, χ^2) and the overlap of the donor emission with the acceptor's absorption spectra[2], but perhaps this last condition is less important provided that the experimentalist can chose the right FRET couple. A particular disadvantage of smFRET for probing homo-dimerization comes from the labeling of the protein of interest: for hetero smFRET, dimerization can only be detected up to 50% (*i.e.,,* hetero-FRET will only be able to detect donor-acceptor and acceptor-donor homo-dimers but not donor-donor or acceptor-acceptor, which is the other 50% of the dimers). The use of fluorescence correlation spectroscopy (FCS) and derivatives (FCCS, *etc.*[3]) to ascertain protein diffusion constants and binding constants *in vitro* is another alternative. These approaches are not able to fully quantify homo-dimerization either, as in FCS one measures concentration and diffusion, and the radius and diffusion coefficient of a diffusing particle are very poorly dependent on the molecular weight; for example a 10-fold increase in the molecular weight will only imply a 2.15 fold change in the diffusion coefficient[4]. In the case of two-color FCS or FCCS, only 50% of homo-dimers will be seen for the same reason as above. The most practical and quantitative approaches to detect homo-dimerization *in vitro* and *in vivo* are homo-FRET[5] and number and brightness (N&B)[6]. Given the fact that homo-FRET requires specific instrumentation recovery of the anisotropy value (*i.e.,,* optical elements/analyzers to recover the parallel and perpendicular polarization), N&B is presented here as a favorable technique to detect protein homo-dimerization and aggregation. It can be employed both *in vitro* and *in vivo* with a commercial set-up.

Number and Brightness

N&B has been recently reviewed[7]. That review focused on the application of the technique in live cells. It is worthwhile to reproduce the mathematical formalism here as these equations will be applied to the data collected *in vitro*. First, it is necessary to define some terms and mathematical quantities:

- An entity is a set of molecules which are bound together.
- The *brightness* ε of an entity is the number of photons it emits per unit time (per frame).
- *n* is the number of entities present.
- For a given pixel over the course of an image series, $\langle I \rangle$ is its mean intensity and σ^2 is the variance in its intensity.

Then, with photon-counting detectors and assuming mobile entities and no background,

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

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

where *N* is the *apparent number* and *B* is the *apparent brightness*. This results in

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

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

Dalal *et al.*[8] showed that with analog equipment, one needs three correction terms: the *S factor*, the background *offset*, and the *readout noise* σ_0^2. Then, again assuming mobile entities,

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

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

giving

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

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

Note that the above equation for ε is different that given in Dalal *et al.*[8] and a subsequent review.[7] In Dalal *et al.* the *S* in the denominator was omitted due to a typo and this error was reproduced in the review. The equation above is the correct one. Instructions for measuring *S*, offset and σ_0^2 – together with an explanation of their meaning – are given by Dalal *et al.*[8]

The brightness ε is proportional to the oligomeric state of the diffusing entities: ε will be twice as big for dimers as it is for monomers, three times as big for trimers as it is for monomers, twice as big for hexamers as it is for trimers and so on. In this way, measuring the brightness ε, one can quantify any type of multimerization.

If there are a mixture of oligomeric states present, number and brightness is not capable of recovering the individual oligomeric states present. This is a limitation of the technique.

Detrend algorithm and nandb software

The importance of correcting for photobleaching has been previously stressed[9]. Photobleaching inevitably occurs during light microscopy experiments in time-lapse mode; both in live cells and *in vitro*. Many approaches have been described in the literature to correct for bleaching.[7] The exponential filtering technique with automatic choice of detrending parameter τ is the current best. It is integrated into the free, open source software nandb[9]. Indeed, software that requires the user to manually choose their detrending parameter can lead to incorrect results because this parameter choice will likely be arbitrary and incorrect. The *automatic* algorithm inspects the data and determines the appropriate parameter for it, without the need for user intervention[9]. Even with the best choice of smoothing parameter, detrending has its limitations and works well only with photobleaching percentages lower than 25%, as shown with simulations[9]. Interestingly, when using the automatic detrending routine, its accuracy is such that one can work with low brightness values (even $B < 1.01$), and hence low intensities, and still be precise enough to quantify homo-dimerization.

Photobleaching also causes another problem: the presence of photobleached fluorophores in a multimer complex. This makes *e.g.,* a trimer appear like a dimer when one of the three units in the trimer is non-fluorescent. Hur and Mueller[10] showed how to correct for this and this correction was also stressed in a subsequent review[7]. The nandb software includes this correction[9].

The FKBP12F36V system

FKBP12F36V is a protein which does not naturally oligomerize but is known to dimerize upon the addition of the AP20187 drug (colloquially known as the BB dimerizing ligand)[11,12]. This makes it an ideal test case for number and brightness: with labelled FKBP12F36V, a doubling of oligomeric state should be observed upon addition of BB.

PROTOCOL:

1. FKBP12F36V-mVenus Purification

1.1. Transform (DE3) pLysS cells with pET22b vector containing monomerized human FKBP12F36V[12] and N-terminal His6 and mVenus tags (vector available on request). Plate cells onto LB agar supplemented with 50 µg/mL Ampicillin and 34 µg/mL Chloramphenicol.

1.2. Transfer transformed colonies into 100 mL LB starter culture and grow for 16 - 20 hours at 37 °C with shaking.

1.3. Dilute dense starter culture (OD600 > 1) 1:100 in LB medium (2 x 500 mL batches) and grow for 2 – 3 hours to OD600 = 0.6 - 0.8 (37 °C, 200 rpm).

1.4. Cool cultures on ice. Induce with 250 µM IPTG and grow for 16 – 20 hours at 21 °C, 200 rpm.

1.5. Harvest cells by centrifugation at 2000 x g for 20 minutes.

1.6. Resuspend pellet in 40 mL of IMAC buffer A (20 mM sodium phosphate pH 7.5, 500 mM NaCl, 3 mM imidazole, 1 mM β-mercaptoethanol) supplemented with EDTA-free protease inhibitors (1 tablet per cell pellet).

1.7. Sonicate cells (500 Watts, 20 kHz, 40% amplitude, 9 s on, 11 s off for 15 min) on ice and harvest soluble material by centrifugation at 20,000 x g.

1.8. Transfer soluble lysate to a conical flask and add 2 mL of resin (see **Table of Materials**). Incubate for 1 hour with 105 rpm rotation

Note: Nickel sepharose may also be used for this IMAC step.

1.9. Harvest resin and wash with 250 mL of IMAC buffer A followed by 500 mL of IMAC buffer B (20 mM sodium phosphate pH 7.5, 500 mM NaCl, 7 mM imidazole, 1 mM β-mercaptoethanol).

Note: Increase to 50 mM imidazole if using Nickel sepharose resin.

1.10. Elute His6-tagged protein using IMAC buffer C (20 mM sodium phosphate pH 7.5, 500 mM NaCl, 300 mM imidazole, 1 mM β-mercaptoethanol).

1.11. Inject onto a size exclusion column (see **Table of Materials**) equilibrated in 10 mM HEPES pH 7.5, 150 mM NaCl, 1 mM DTT. FKBP12F36V has its peak elution at 87.71 mL on the column we used.

1.12. Assess purity via SDS-PAGE and pool and concentrate as required.

2. Preparation of Multiwell Plate Array

2.1. Thaw the purified FKBP12F36V (or labeled protein of interest) from -80 °C.

2.2. Prepare a solution of 100 nM purified FKBP12F36V (medium, 10 mM HEPES pH 7.5, 150 mM NaCl, 1 mM DTT). Sonicate and centrifuge (quick spin of 13000 rpm) to prevent the formation of aggregates.

2.3. Pipette 100-200 µL into an 8 well observation chamber with a glass bottom.

2.4. Add the BB dimerizer to final concentrations of 10, 20, 40, 80, 100, 150, 300, and 500 nM[12,13].

2.5. As a reference, prepare a solution of 100 nM of mVenus alone to evaluate potential aggregation and precipitation effects and recover a brightness value for the monomer with the same acquisition settings.

3. Calibration-free Confocal Acquisition

3.1. Start the confocal system (**Figure 1**). Any light scanning microscope confocal system equipped with digital detectors or well-characterized analog detectors[8], and capable of keeping a constant dwell time for every pixel acquired would work.

3.2. Set the excitation beam path:

3.2.1. Turn on the 514 nm laser and set it at 20 – 100 nW Power at the exit of the objective (for FKBP12F36V-mVenus).

3.2.2. Select the 63X1.4NA objective or a collar correction water immersion objective designed for FCS.

3.2.3. Turn on one HyD, APD or calibrated PMT detector. Detectors capable of photon-counting are preferable, as in this case, calculation of *S*, offset and σ_0^2 are unnecessary.

3.2.4. Select the emission window from 520 – 560 nm

3.2.5. Set the pinhole at 1 Airy unit for the corresponding emission ~545 nm.

3.2.6. Set the acquisition mode at 16 x 16 pixels

3.2.7. Set the pixel dwell time t_{dwell} such that it satisfies $t_{frame} >> \tau_D >> t_{dwell}$, where τ_D is the residence time of the diffusing protein and t_{frame} is the frame rate. This corresponded to setting the dwell time to ~13 µs.

Note: Some commercial manufacturers had scanners that were not keeping the dwell-time per pixel constant. This constancy is crucial for the method to work.

3.2.8. Set the pixel size at ~120 nm.

3.2.9. Select the *xyt* acquisition mode and select the number of frames to be acquired per acquisition and well (for example 5,000).

3.2.10. If the system is equipped with high-throughput mode, introduce the coordinates of each well and the number of acquisitions per well to automate the process.

Note: Be careful to ensure the presence of a water dispenser for if using an immersion objective.

3.2.11. If the system is equipped with a perfusion system, load the BB solution and program the perfusion to start right-after the 5000th frame to evaluate the kinetics of dimerization while acquiring *e.g.,* 10,000 images.

3.3. Add a drop of oil into the oil immersion objective / water if utilizing a collar correction water immersion objective designed for FCS.

3.4. Mount the 8 well observation chamber into the stage.

3.5. Select the correct well and focus on the solution.

Note: IMPORTANT: Avoid focusing close to the bottom glass to avoid reflection and scattering. When focusing deeper into the solution, disconnect the automated focus option.

3.6. Start the acquisition and save the resulting stack of images in TIFF format.

4. Detrend and Brightness Analysis using the R Package nandb

4.1. As a preprocessing quality check, use ImageJ[14] to take a look at the images and recover the intensity profile, as shown in **Figure 2a**. This is useful to determine whether or not too much photobleaching has occurred. If there is too much bleaching, the data is not suitable for further analysis.

Note: ImageJ can also be useful to convert images to TIFF from commercial formats. The nandb software described below can only work with TIFF files.

4.2. Download and install R and RStudio[15,16]. It is best to download and install R first, then RStudio.

Note: What follows is a description of how to use the nandb R package. Knowledge of the R language is not required to use nandb, however it will make life easier.

4.3. Install the nandb package.

4.3.1. Open RStudio and in the console, type install.packages("nandb") and wait for the installation.

4.4. Get to know nandb

4.4.1. Review the manual[17].

4.4.2. Review the built-in RStudio help for various functions. The most likely function to be used will be using will be brightness(). View the help file for this function by typing ?brightness() at the console.

4.5. Calculate brightness

4.5.1. Say one has an image file on the desktop called img001.tif (note that `nandb` only works with TIFF files). One can calculate the brightness of that image:

b <- brightness("~/Desktop/img001.tif", tau = "auto")

4.5.1.1. This assigns the brightness of the image to the variable b in R. The tau = "auto" ensures that the image is correctly detrended prior to brightness calculation. The most common thing to do from here is to calculate the mean or median brightness of the image. One can do this by typing mean(b) or median(b). One can also write the brightness image to the desktop using

ijtiff::write_tif(b, "~/Desktop/whatever_img_name")

4.5.2. Say one has folder full of images images_folder on the desktop and one needs to calculate the brightnesses of these images and write the brightness images as TIFF files. Then see ?brightness_folder(). This function processes a whole folder all at once:

brightness_folder("~/Desktop/images_folder", tau = "auto")

This is particularly good for those who have a software they prefer to R, because all of the files are processed in one single command, and then one can go on working with the output brightness TIFF images in their chosen software, be it ImageJ[14], Python or something else.

REPRESENTATIVE RESULTS:

Detrending and monomeric brightness

Once the data has been acquired, one can start the brightness calculations to determine the oligomeric state of the protein of interest in the solution. Even if the effect of bleaching in solution may not be as drastic as it can be *in vivo*, the intensity trace will still probably not have stationary mean, possibly due to photophysical effects related to the fluorophore,[18] but the reasons behind this are not fully understood. This has an impact when calculating the brightness; when trying to determine monomer–dimer transitions, this aspect is crucial. By applying the automatic detrend algorithm to the data shown in **Figure 2a**, the intensity trace is properly corrected, providing a more accurate value for the brightness of FKBP12F36V-mVenus in solution. Before addition of BB, without detrending, B = 1.026, whereas after detrending, B = 1.005 (**Figure 2b**).

Determination of FKBP12F36V -mVenus monomer-dimer transitions *in vitro*

Sequences of 20,000 images of purified FKBP12F36V -mVenus in 100 nM solution were acquired as specified in the protocol section. After 10,000 frames were acquired, the homodimerizer drug BB was added to the solution while acquiring. Each consecutive series of 5,000 frames was analyzed (**Figure 3**). The mean brightness 5 minutes after BB addition was B = 1.010, which is a 2-fold increase, indicating a FKBP12F36V dimer. The kinetics of the process is shown in **Figure 3b**; the delay between BB addition to full FKBP12F36V dimerization was approximately 2 minutes.

Identification of protein aggregates

A number of protein aggregates were detected both in a limited number of frames and also in the intensity trace (**Figure 4**). These aggregates occur naturally in solution when working with proteins and can be eliminated by sonicating and/or increasing the protein dilution. Nevertheless, in some biological problems, one needs to detect transitions between monomers and big aggregates. **Figure 4** shows an example where a FKBP12F36V protein aggregate diffused through the observation volume (images 16x16); for our previous calculations these data were discarded, however, one example is shown in **Figure 4** to show that these aggregates can be detected and characterized using the same settings and approach. At first sight, when evaluating the 5000 images, one can recover the average brightness for FKBP12F36V-mVenus treated with BB (B = 1.010). Viewing the raw brightness image, one can clearly see, though, a region of interest of approximately 8 pixels with a high value of $B \approx 1.080$. This region of interest coincides with a few frames at t = 34 – 37 s where a FKBP12F36V aggregate diffused around the observation area. The aggregate did not remain in the observation volume for long enough to accurately determine its size.

FIGURE AND TABLE LEGENDS:

Figure 1. Application of N&B to detect protein monomer-dimer transitions in solution. (a) Simplified optical path of a laser scanning microscope (LSM) equipped with a laser source (set at 514 nm in the case of mVenus labeled proteins) directed (blue arrows) toward an immersion objective (in our case a 63X1.4NA oil) illuminating a 100 nM solution of FKBP12F36V-mVenus solution. The emission fluorescence (green arrows) passes through a dichroic mirror and is directed toward a bandpass filter that cleans the emission light, and a pinhole set at 1 Airy unit situated right before a point digital detector capable of photon counting. **(b)** A confocal volume of illumination is scanned through 16 x 16 pixels illuminating single FKBP12F36V-mVenus molecules that enter and exit the Gaussian shape confocal volume producing an array of fluorescence intensity fluctuations. **(c)** Image series acquired over time.

Figure 2. Automatic detrending is needed to accurately measure a population of monomers in solution. (a) A stack of 5000 16 x 16 pixel images was acquired as described in Protocol section. The intensity of the first frame is shown together with the average time-resolved intensity profile, which shows long term fluctuations that might be related to bleaching and other solvent and/or

photophysic effects. Whatever the cause for these long-term fluctuations, they impact the brightness calculations and hence require detrending. Without automatic detrend, one gets B = 1.026, whereas after automatic detrending, B = 1.005. Also, the brightness without (left panels, second row) and with (right panels, second row) smooth filtering is shown. **(b)** The same data presented in (a) was detrended and the results in terms of intensity and brightness shown.

Figure 3. In-vitro N&B is able to resolve the transition between FKBP12F36V **monomers and dimers in real time.** The addition of the dimerizer drug BB resulted in a two-fold increase in true brightness right after 2 min (from 0.005 to 0.010 in true molecular brightness). **(a)** The intensity of the first frame is shown together with the mean intensity profile of the detrended image. The brightness without (left panels, second row) and with (right panels, second row) smooth filtering is shown. **(b)** Mean brightness is plotted (every 5000 frames) using the true molecular brightness ϵ. The dimerization occurs 2 minutes after BB addition (t = 1 min) at t = 4 min. The fitted curve is a sigmoidal function to stress the tendency toward dimerization after addition of the dimerizer drug (BB). Error bars represent the standard error of the brightness distribution at each time point.

Figure 4. In vitro N&B to resolve the size of protein aggregates in solution. (a) Time-resolved intensity detrended trace for the acquisition of FKBP12F36V-mVenus are shown together with brightness images (second row). **(b)** The time-resolved detrended intensity trace shows a maximum in intensity that is highlighted with a red dotted circle (top left panel). The first intensity frame that corresponds to this particular time (t = 34 seconds) is shown and a red arrow shows a FKBP12F36V-mVenus aggregate entering the illumination area. A closer look into the smoothed brightness image shows a region of interest that contains high oligomeric states and the rest of the image contains a mix between monomers and dimers with an average of B = 1.008.

DISCUSSION:

N&B is a technique to detect multimerization using commercial light scanning confocal microscopes equipped with digital detectors. This approach is quite attractive compared to single point FCS, FCCS and smFRET because it is calibration free and the brightness calculation is straightforward and concentration independent[6]. It is of major importance, however, to correct for bleaching and long-term intensity fluctuations before performing brightness calculations[9]; a slight increase of brightness due to bleaching and other artifacts could be misinterpreted as a change in oligomeric state (as seen in **Figure 2a**) if detrending is neglected or performed incorrectly. The use of the automatic detrend algorithm allows for accurate brightness calculations.

It is important, to follow a number of rules when acquiring the images. First, the residence time of the molecules in the confocal volume, and therefore their diffusion constant should be known in order to set the right parameters of the confocal system; the residence time of the labeled molecules needs to reside between the pixel dwell time and the frame time[7]. Choosing the right fluorophore is crucial. A bright and stable fluorophore is necessary for good signal to noise and minimal photobleaching. Laser power should be kept relatively low to avoid bleaching. Photon

counts of 1 or 2 per pixel are sufficient. Keeping counts low is also crucial to avoid detector pile-up. The technique copes with low photon budgets better than it copes with bleaching. In this protocol mVenus has been employed; which is a relatively bright fluorophore, comparable to eGFP[19]. An alternative is the use of nanoboosters that selectively target a fluorescent protein; these can be much brighter than traditional fluorophores[20].

In terms of hardware, digital photon-counting detectors make quantification easier, but N&B is possible with analog detectors, provided that one has recovered some acquisition parameters.[8]

Even though other approaches like anisotropy can detect homo-dimerization, with the advent of new software to simplify analysis, N&B stands alone as an accessible approach to detect and quantify protein interactions and oligomerization, both *in vitro* and in live cells.

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DISCLOSURES:

The authors have nothing to disclose.

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