

Using enhanced Number and Brightness to measure protein oligomerization dynamics in live cells

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1 **KEYWORDS** oligomer, multimer, aggregate, aggregation, spectroscopy, ephrin, micro-contact
2 printing, total internal reflection microscopy, TIRF, confocal microscopy, Number and Brightness,
3 N&B, enhanced Number and Brightness, enhanced N&B, eN&B,

4 **EDITORIAL SUMMARY** This Protocol describes enhanced Number and Brightness (eN&B), an
5 approach that uses fluorescence fluctuation spectroscopy data to directly measure the
6 oligomerisation state and dynamics of fluorescently-tagged proteins in living cells.

7 **TWEET** Detecting protein oligomerisation states and dynamics in live cells using enhanced
8 Number and Brightness (eN&B).

9 **COVER TEASER** Detecting oligomerisation dynamics in live cells

10 **Up to three primary research articles where the protocol has been used and/or developed:**

11 **1.** Ojosnegros, S. et al. Eph-ephrin signaling modulated by polymerization and condensation of
12 receptors. *Proceedings of the National Academy of Sciences of the United States of America*
13 *114*, 13188-13193, doi:10.1073/pnas.1713564114 (2017).

14 **2.** Hortigüela, V. *et al.* Nanopatterns of Surface-Bound EphrinB1 Produce Multivalent Ligand-
15 Receptor Interactions That Tune EphB2 Receptor Clustering. *Nano letters* **18**, 629-637,
16 doi:10.1021/acs.nanolett.7b04904 (2018).

17

18 **Abstract**

19 Protein dimerization and oligomerization are essential to most cellular functions, yet it remains
20 a challenge to measure the size of these oligomers in live cells, especially when their size
21 changes over time and space. A commonly used approach to study protein aggregates in cells is
22 Number and Brightness (N&B), a fluorescence microscopy method that is capable of measuring
23 the apparent average number of molecules and their oligomerisation (brightness) in each pixel
24 from a series of fluorescence microscopy images. We have recently expanded this approach in
25 order to allow resampling of the raw data to resolve the statistical weighting of coexisting
26 species within each pixel. This feature makes eN&B optimal to capture the temporal aspects of
27 protein oligomerization, when a distribution of oligomers shifts towards a larger central size
28 over time. In this Protocol, we demonstrate the application of eN&B by quantifying receptor
29 clustering dynamics using EMCCD based total internal reflection microscopy (TIRF) imaging.
30 TIRF provides a superior signal-to-noise ratio, but we also provide guidelines on how to
31 implement eN&B in confocal microscopes. For every time point, eN&B requires the acquisition
32 of 200 frames and it will take few seconds up to two minutes to complete a single time-point.
33 We provide an eN&B (and standard N&B) Matlab software package amenable to any standard
34 confocal or TIRF microscope (<http://bioimaging.usc.edu>). The software requires a high RAM
35 computer to run (64Gb) and includes a photobleaching detrending algorithm, which allows to
36 extend the live imaging for more than an hour.

37 **Introduction**

38 The physiological function of proteins often involves the controlled assembly into multimeric
39 complexes¹⁻³. Protein multimerization or clustering mediates signal transduction in several
40 classes of receptors including tyrosine kinase receptors^{4,5}, bacterial chemotactic receptors⁶, or

1 neurotransmitter receptors⁷ among many others. The clustering of membrane proteins
2 regulates the strength of cell adhesion in both integrins and cadherins, as well as the formation
3 of higher order structures such as focal adhesions^{8,9}. Viral capsids are typically large multimeric
4 structures assembled by the self-association of many copies of a few different proteins¹⁰. In
5 addition, large structural cellular components are assembled by homo-polymerization of
6 monomers into fibrils or more complex conformations^{11,12}. For instance, endocytosis and
7 vesicle transport occur after the formation of pits coated by clathrin homo-polymers¹³.

8 In many cellular functions, the stoichiometry of the protein aggregates can tune their activity.
9 For instance, oligomers of different sizes can modulate transcription factor affinity for DNA
10 binding sites or the association with different proteins¹⁴⁻¹⁸. In addition, the uncontrolled self-
11 assembly of proteins can lead to the formation of non-physiological toxic aggregates, such as
12 fibrils or plaques of Tau or α -synuclein in Alzheimer's and Parkinson's diseases respectively<sup>19-
13 27</sup>. Thus, understanding both the normal function and the pathologic disorders derived from
14 protein self-assembly requires better tools for analyzing the diversity of molecular species
15 assembled during protein aggregation. A wide variety of experimental questions require assays
16 to interrogate the nanoscale organization of protein assemblies. These assays should be
17 capable not only of measuring the stoichiometry of active protein complexes, but also powerful
18 enough to resolve the dynamics of their aggregation in live cells over time²⁸. Several imaging
19 techniques can provide quantitative information of the oligomeric state of a protein complex;
20 however, most of them are limited in one of three experimental goals: (i) obtaining the
21 complete temporal sequence of the oligomerization process; (ii) providing the dynamic range
22 required to measure a broad spectrum of oligomeric sizes; (iii) recovering spatial information.
23 The Number and Brightness (N&B) method uses fluorescence fluctuation spectroscopy data to
24 directly measure the average oligomeric state of proteins in living cells, thereby satisfying all
25 three experimental goals²⁹. Here we describe a detailed protocol for our recently developed
26 approach to perform a statistically enhanced N&B version (eN&B)²⁸. This analysis advances the
27 standard N&B by providing not only the average oligomeric value but the distribution of
28 oligomers for every pixel in an image during long acquisition periods.

29

30 **N&B, basic principles and theory**

31 A challenging question in fluorescence microscopy is how to measure the average number of
32 molecules in an image and how to measure their oligomerization state or brightness. Let us
33 consider an example with two sequences of time-lapse frames containing either four scattered
34 fluorescent monomers or one tetramer. If the intensity changes are analyzed within a pixel,
35 utilizing a simple average of the fluorescence intensities, this will produce indistinguishable
36 results between the two examples (Figure 1). N&B instead utilizes first and second moments of
37 the intensity distribution³⁰, allowing for the discrimination between different oligomerization
38 states (brightness) of molecules. Larger oligomers will show an increased variance resulting
39 from fluctuations of wider amplitude than monomers, ensuing from diffusing aggregates
40 moving in and out of the focal volume. In general terms, the larger the variance, the fewer
41 molecules contribute to the average. Moreover, the brightness analysis can be done
42 simultaneously in all the pixels of an image, procuring oligomerization maps of entire cells on a

1 pixel-by-pixel basis. All things considered, N&B is the ideal method to study oligomerization in
2 proteins which aggregation is spatially heterogeneous.

3
4 The original N&B theory was developed by Qian and Elson for measurements of
5 molecules in solution^{31,32} and was adapted for live-cell studies by Enrico Gratton's laboratory²⁹.
6 N&B is a moment analysis capable of measuring the apparent average number of molecules
7 and their oligomerization state (brightness) in each pixel from a series of fluorescence
8 microscopy images. The ratio of the square of the average (first moment) intensity ($\langle k \rangle^2$) to
9 the variance (second moment, σ^2) is proportional to the average apparent Number of particles
10 (N). The apparent Brightness, B, which represents the molecular oligomerization level, is
11 calculated as the ratio of variance (σ^2) to average intensity ($\langle k \rangle$).

$$12 \quad N = \frac{\langle k \rangle^2}{\sigma^2}$$

$$14 \quad B = \frac{\sigma^2}{\langle k \rangle}$$

15
16 The pixel volume covered by images obtained with optical microscopes working on total
17 internal reflection microscopy (TIRF) mode (assuming an illumination height of 100-200 nm) at
18 maximum resolution is in the range of 0.0011 μm^3 -0.0022 μm^3 , respectively. Depending on the
19 protein size, considering physiological concentrations, this volume can harbor tens to hundreds
20 of proteins assembled into different oligomeric states. In standard N&B all the molecular
21 diversity is summarized in a single average oligomerization value per pixel ranging from the
22 monomer to roughly 100-mer species. The ability to determine oligomerization heterogeneity
23 is limited mainly by the diffusion rate of the proteins and by the capability of the acquisition
24 device to rapidly sample in time and across a wide dynamic range of fluorescence intensity.

26 **eN&B: Statistical enhancement**

27 In standard N&B, for every time-point, F consecutive frames are acquired for the analysis of the
28 fluorescence fluctuations and the calculation of a single oligomerization value in the sequence.
29 A minimum of F=25 is advised to achieve enough statistical robustness, although F=200 should
30 be used for deeper analysis²⁹. If the oligomer population is relatively homogeneous, the
31 average oligomer size obtained with standard N&B may be an optimal representation of the
32 general oligomerization state of the protein. However, in some cases, a single average value
33 may not represent the diversity of protein complexes assembled in a single pixel. For this
34 reason, we have developed enhanced N&B (eN&B). eN&B sub-samples the entire dataset F
35 using analysis window of length w=100, shifting the window one frame at a time in a circular
36 way until the entire dataset is covered. This statistical resampling results in a distribution of
37 oligomeric values per every pixel.

1 The Number and Brightness values are recorded per every shift, ensuring the same statistical
 2 weight is given to each frame. Hence for each pixel (i,j) using eN&B we obtain an array of F
 3 values of Brightness B. Each Brightness arises from a sliding window defined as follows:

$$4 \quad B_e = \begin{cases} \frac{\sigma^2}{\langle k \rangle}_n^{n+w} & \text{if } n < F - w \\ \frac{\sigma^2}{\langle k \rangle}_n^F + \frac{\sigma^2}{\langle k \rangle}_1^{w-(F-n)} & \text{if } n > F - w \end{cases}$$

5 where e goes from 1 to F. $\frac{\sigma^2}{\langle k \rangle}_n^{n+w}$ is the B arising from the window of length w starting from
 6 position n and ending in $n+w$ while $\frac{\sigma^2}{\langle k \rangle}_n^F + \frac{\sigma^2}{\langle k \rangle}_1^{w-(F-n)}$ joins frames from position n to last
 7 frame F and from position 1 until reaching the number w .

8
 9 Similarly, we obtain corresponding F values of apparent Number N.
 10

$$11 \quad N_e = \begin{cases} \frac{\langle k \rangle^2}{\sigma^2}_n^{n+w} & \text{if } n < F - w \\ \frac{\langle k \rangle^2}{\sigma^2}_n^F + \frac{\langle k \rangle^2}{\sigma^2}_1^{w-(F-n)} & \text{if } n > F - w \end{cases}$$

12
 13 The trajectory of the sliding window follows the time sequence of the dataset; therefore the
 14 statistical resampling of eN&B works as a consecutive N&B measurement with time delay equal
 15 to the frame rate. When this process is repeated for different T points (see next section), we
 16 obtain a multidimensional matrix of data containing information from x, y pixel position,
 17 distribution of apparent Number and apparent Brightness in each pixel, and time.

18
 19 **Simulations.** The power of eN&B analysis depends on multiple factors, most significantly the
 20 dynamic range of oligomer sizes, their change in aggregation and their relative abundance, as
 21 well as their absolute concentration within a given measurement point. A simulation including
 22 two opposed, complex oligomer populations highlights the benefit of the resolving power of
 23 eN&B over standard N&B. We simulated two scenarios: one with monomers gradually forming
 24 oligomers over time (Figure 2a), and one with different oligomers co-existing in solution (Figure
 25 2b). In the first scenario, eN&B shows the clear advantage of capturing individual
 26 oligomerization state (Figure 2c); in the second scenario, the spread of eN&B delivers an
 27 approximation to the actual distribution of oligomer population (Figure 2d).

28
 29 **Photobleaching compensation and time expansion.** On the short term time dimension,
 30 camera-based N&B generally works in the msec to sec range^{33 34}, which is limited by hardware
 31 capabilities of modern microscope cameras. However, in order to time-resolve the formation of
 32 high-order aggregates or processes running with slower, larger dynamics, the acquisition of

1 images may require longer exposure than that offered by conventional N&B. When attempting
2 to time-resolve long oligomerization processes through N&B analyses, the effect of
3 photobleaching interferes with the measurements. To overcome this, we have implemented
4 boxcar filtering algorithms³⁵ to detrend the decay of fluorescence intensity during the multiple
5 light exposures in the sequential acquisition, while at the same time keeping the fluctuations
6 intact^{23,36-39}. These algorithms are implemented in our eN&B software and allow to extend the
7 data acquisition up to 10 to 15 sequential time points or even more, depending on the
8 brightness of the original sample and the frame rate (Supplementary Fig. 1). The original work
9 by Hellriegel et al. shows that even with 50% bleaching (i.e. the final frame average intensity is
10 50% of the original frame intensity), boxcar filter helps to recover the correct brightness
11 estimation³⁵. Photobleaching can be modelled by an exponential decay³⁷:

$$f(t) = e^{-\alpha t}$$

12

13 where the exponential coefficient, α , indicates the extent of photobleaching, and the effect of
14 boxcar filtering. Large box sizes do not sufficiently correct for the overestimation of B, whereas
15 smaller sizes yield to underestimate the brightness. The optimal boxcar size is dependent on
16 bleaching speed. A smaller boxcar size should be used with faster bleaching (larger α) to
17 optimally recover brightness. In a recent work⁴⁰, exponential filtering detrending permitted to
18 time-resolve the transition of monomer to dimers of a FKBP1-tagged fluorescent protein and
19 corrected images with up to 25% of bleaching^{40,41}. In both cases, boxcar and exponential
20 filtering, selecting the right window size was crucial to correct the bleaching without discarding
21 the actual fluctuation. A boxcar window of 10 frames was chosen in our software because, as
22 described in the original work³⁵, in a biological context, this range will not affect the higher
23 frequency fluorescence fluctuation of fast-diffusing species.

24

25 **Applications of the method**

26 The oligomerization of a large number of proteins has been revealed through N&B analysis.
27 Examples demonstrate the applicability of N&B to a broad variety of protein families, with
28 localization at all major cellular compartments. In the cytosol, N&B has been used to resolve
29 the oligomerization dynamics of focal adhesion components such as paxillin and actin^{8-10,29,42,43},
30 and the assembly of viral matrix proteins^{8-10,29,42,43}. A number of membrane proteins have been
31 subjected to N&B analysis, such as Annexins or uPAR^{36,44}. N&B has also been applied to the
32 study of signaling pathways, including p75, LRRK2⁴⁵⁻⁴⁷, ErbB1 and ErbB2 receptor tyrosine
33 kinases⁴⁸, and proteins involved in membrane – lipid – dynamics such as dynamin 2⁴⁹⁻⁵³. In the
34 nucleus, N&B has revealed the ligand-induced aggregation of transcription factors and has
35 been used to discriminate between different oligomer subpopulations^{20,54,55}. In addition, N&B
36 has been used to study how DNA repair proteins bind to the DNA following the recruitment of
37 double strand break factors⁵⁶. N&B has also been applied to the study of pathogenic
38 aggregation of peptides causing neurodegenerative diseases, such as huntingtin or alpha
39 synuclein^{24,38,57}. Fluorescently tagged molecules other than proteins can also be studied by
40 N&B, examples of which include the aggregation of DNA after lipofection⁵⁸.

1 In our work, we used eN&B to study the oligomerization of the EphB2 receptor during 1 h time-
2 lapse measurements following receptor activation. The Eph receptor is a membrane-tethered
3 protein which forms large aggregates upon interaction with its cognate ligand, ephrin⁵⁹.
4 Despite playing a crucial role in neural development, tissue patterning and regeneration, the
5 dynamics of Eph receptor clustering was poorly understood^{4,60,61}. We performed eN&B analysis
6 of fluorescently-tagged Eph to yield data on the receptor's oligomerisation-state over time. The
7 quality of eN&B data allowed mathematical modelling of receptor clustering and the
8 proposition of a new mechanism for Eph signalling, termed polymerization-condensation²⁸. In
9 our experimental setup, Eph-expressing cells were stimulated with the ephrin ligand presented
10 in four different spatial configurations, namely, ligands in solution, micro-printed ligand dimers,
11 micro-printed ligand clusters, and nanopatterned clusters⁶². eN&B analysis was able to capture
12 sensible variations between the different modes of ligand presentation and retrieved
13 characteristic oligomerization dynamics for each mode.

14

15 **Comparison with related methods**

16 Several different methods have been developed that can be used to study the oligomerisation
17 states/dynamics of proteins *in vivo*. In this section, we will briefly highlight the key alternative
18 approaches and their advantages and disadvantages compared to eN&B.

19 **Spectroscopy methods.** Spectroscopy methods include N&B and a broad collection of
20 techniques that measure the fluorescence intensity of molecules as they diffuse in and out of
21 the focal volume (for a comprehensive review, see Ref. ⁶³). Arguably the most popular
22 spectroscopy application is fluorescence correlation spectroscopy (FCS), which is widely used to
23 efficiently measure the diffusion coefficients of fluorescent molecules and the variation in
24 those coefficients due to the presence of different molecular species (i.e. bound or unbound
25 pairs, oligomers etc.). FCS can also be adapted to measure the oligomerization of proteins,
26 provided that proper calibrations are performed^{64,65}. FCS typically works on single-pixels (with
27 few exceptions⁶⁶), and it may therefore be challenging to capture the full diversity of oligomeric
28 states using this approach.

29 **Photon counting histogram (PCH).** PCH was originally developed by Chen et al.⁶⁷ and is the first
30 method that can be used to extract molecular Number and Brightness information from
31 fluorescence fluctuation data. PCH is capable of resolving heterogeneous molecular
32 populations⁶⁸ and it has been applied to resolve mixed oligomer populations of membrane
33 receptors⁶⁹. The information attainable by PCH is robust and complete, however, it is limited
34 to single-point detection. It also requires longer data acquisition as well as data analysis time
35 compared to N&B.

36 **Fluorescence Resonance Energy Transfer (FRET) imaging.** FRET imaging is based on the
37 detection of variations in the fluorescence intensity of a protein due to energy transfer to an
38 acceptor protein located in close (nanometer range) proximity. This is very sensitive approach
39 for detecting the interaction of protein pairs, or in qualitative terms, the formation of
40 oligomers. FRET imaging includes a diverse collection of approaches such as sensitized
41 emission, acceptor photobleaching or anisotropy-based homoFRET^{12,60}. These approaches
42 show different capability to quantify the stoichiometry of a narrow oligomeric range⁷⁰. The

1 most sensitive FRET versions, which include single-molecule detection ^{71,72} and fluorescence
2 lifetime imaging microscopy (FLIM) ⁷³, can be used to quantify a larger range of oligomeric
3 states, but data acquisition is relatively slow (in the order of minutes) and is better suited to
4 capture the dynamics of slow assembly processes such as amyloid aggregation.

5 **Other methods.** Super resolution microscopy and single-molecule detection can also be used
6 to estimate the number of proteins contained in a complex by counting fluorophore
7 photobleaching steps ^{74,75}. Intensity-based methods can quantify local concentration of
8 proteins but cannot extract the oligomer size-distribution.

9

10 **Limitations of eN&B**

11 The camera-based eN&B technique is dependent on the system capabilities to acquire short
12 exposure images while maintaining high collection efficiency (sensor Quantum Yield) and
13 collection rate with low noise. These characteristics will determine the highest protein diffusion
14 rate that can be imaged using this technique ⁷⁶. The protein diffusion rate will also determine
15 the ideal time resolution of the consecutive eN&B measurements. Fast-diffusing proteins will
16 require short exposure times resulting in a very fast 100-200 frame acquisition. For slow-
17 diffusing proteins the camera exposure time will be longer and therefore capturing 200 frames
18 will take a substantial amount of time. Even if a second time point was to be captured right
19 after the first one, there would be a minimum lapse in the order of minutes, between the start
20 of the two consecutive time-points. In extreme cases, where the protein binding kinetics is fast,
21 the amount of clustering occurring during a single F=200 acquisition may be substantial. In
22 most cases, however, the characteristic acquisition time will be faster than in standard FCS or
23 FLIM applications.

24 The characteristic diffusion rate for proteins inside cells, considering different sizes and cell
25 compartments ranges between $30 - 0.03 \mu\text{m}^2 \text{s}^{-1}$ ⁷⁷. This range can be captured approximately
26 with an exposure time range of 1s to 0.05ms. Most cameras will be able to deal with the slow
27 side without issue, which typically corresponds to protein diffusion rates within membranes.
28 However, acquiring 200 frames at 1s/frame will expose the cell to considerable amount of light,
29 and photobleaching will have to be assessed carefully. The fastest diffusion rates that can be
30 captured by eN&B will be limited by the both by shortest exposure time and acquisition speed
31 of the camera, which, at the time this protocol is written, for most brands top around 0.5ms
32 and 100 frames s^{-1} respectively. If using confocal scanning microscopes, the single pixel dwell
33 time will be considerably faster, in the order of μs . However, point-scanning systems will trade
34 off in the time to scan through an entire image, which will be considerably longer, as well as
35 the sensitivity of the detectors, which peaks at a quantum efficiency (QE) of 45% (GaAsP),
36 compared to currently available 95% QE for high sensitivity cameras (EMCCDs and bsi-
37 scCMOS). When working with fast diffusing species (small peptides in the cytoplasm), two
38 things need to be considered. First, if the photon budget is low, a short exposure time will not
39 be sufficient to collect enough photons to reach an optimal signal-to-noise ratio. Second, the
40 relative diffusion rate of GFP should be taken into account when fusing this to small peptides.
41 The diffusion rate of GFP in the eukaryotic cytoplasm is about $27 \mu\text{m}^2 \text{s}^{-1}$ ^{78,77} and molecular
42 species diffusing faster than GFP may be slowed down when fused.

1 We have not performed a formal analysis of the optimal range of oligomers that can be
2 measured with eN&B. However, theoretical and experimental measurements with the Eph
3 receptor suggests that eN&B can discriminate oligomers within the 1-to-40-mer range without
4 saturating the intensity signal. Mathematical estimations show that expanding that range up to
5 100-mer improves the fitting between imaging data and mathematical models²⁸, which
6 suggests that eN&B might be applied to an even broader range of species. However, these
7 evidences are theoretical, and a formal study about the oligomer range is missing in the N&B
8 field.

9 The camera exposure time should be set up to meet the log-linear region of the
10 autocorrelation curve obtained during FCS measurements of the monomer so that all proteins
11 moving in and out of the focal volume are captured by the camera (see Experimental Design
12 “Protein diffusion and camera exposure calibration”). However, the diffusion coefficient of the
13 protein may decrease with the size of the oligomer^{79,80}, which implies that given a certain
14 threshold size, the time the camera collects photons will be an oversampling of the actual
15 aggregate dynamics and an artefactual reduction in the number of oscillations (i.e. the larger
16 aggregates may need longer time than the one set up as the camera exposition time, to come
17 in and out of the focal volume). We use, as a rule of thumb, a cut-off of 40-mers as the upper
18 limit for oligomer detection. However, a mathematical fitting of the empirical data from EphB2
19 oligomerization suggested that establishing 100-mer as the upper detection limit would result
20 in minimum information loss and better fit of the equations compared to the analysis using a
21 40-mer upper limit²⁸. This broad range of detection may be even wider for membrane proteins
22 which diffusion is not affected by the size of the oligomer⁸⁰.

23 Other important parameters to consider in calibrating and designing the experiments in this
24 protocol are the linearity of the signal output and the dynamic range of the detector. Given a
25 specific setting configuration for the acquisition device (in this case a camera) the measured
26 intensities need to scale linearly with the input photons. The detector’s dynamic range will set
27 the limit in capturing the larger intensity fluctuations. If lower molecular brightness labels are
28 used, the number of frames can be increased to reduce statistical noise and spreading of the
29 standard deviation of the Brightness values²⁹

30 The statistical resampling leading to eN&B tackles the limitation of N&B in providing any
31 information additional to a weighted mean aggregate size per pixel. eN&B cannot discriminate
32 perfectly the relative concentrations per oligomer size, but it adds more statistical
33 representability to the estimations than the standard N&B (Figure 2). The resampling in eN&B
34 is analogous to the standard analysis done on time series to produce frequency spectra where
35 an ideal spectrum with discrete and separated tonal frequencies is used to create a synthetic
36 signal^{81,82}. The result is a spectrum with side bands and aliasing that does not really reproduce
37 the original discrete tones. In order to faithfully reproduce the original spectrum, very long
38 samples with high sampling rates and a completely ergodic series would be necessary. On the
39 other hand, when applied to time series with a broadband (continuous) spectrum, the
40 resampling recovers reasonably well the spectrum even with sub-optimal sampling parameters.
41 Therefore eN&B is an optimal algorithm to resolve the oligomerization of proteins over time.
42 During most polymerization processes, a broadband distribution of oligomers sequentially
43 moves to a higher central size and dispersity. eN&B will not unmix oligomers when perfectly

1 overlapping in time and space or extract the distribution that exactly mirrors the real one,
2 partially because sampling rates and series are limited by technology. However the
3 distributions will be centered on the dominant oligomers, and oligomers far from them will be
4 gradually underrepresented at some rate. This was found to be better than a single average of
5 the entire population (as in N&B), and delivered results more consistent with theoretical-
6 mathematical models²⁸.

7

8 **Microscope setup**

9 N&B has been implemented successfully on multiple types of fluorescence microscopes, both
10 single point scanning systems and full field camera based systems. In particular, single point
11 scanning systems with analog^{20,33,56,83} and photon counting modes were used, both in confocal
12 (single photon)^{9,24,38,44-46,50,55} and two-photon mode^{8,36,51}. EMCCD cameras have been used only
13 in TIRF microscopes^{10,33,34,42,52}. Other systems have been used for Fluorescence Correlation
14 Spectroscopy (FCS) analysis, hence, in principle, can be used for N&B and eN&B. Such systems
15 are Selective Plane Illumination Microscopes⁸⁴ and Spinning Disk confocal microscopes^{66,85}.
16 Enhanced versions of such systems, such as Lattice Light Sheet or 2p-Spinning Disk, are likely to
17 enable eN&B.

18 Here we describe a detailed protocol to perform eN&B on an EMCCD camera based TIRF
19 microscope because of the superior signal-to-noise ratio offered by such system. TIRF
20 illumination is restricted to a 100-200 nm region immediately adjacent to the glass-water
21 interface. While the plasma membrane is the ideal compartment for TIRF microscopy, it can
22 also be employed to study cytosolic proteins; it is possible to reach 1–2 μm deep into the
23 sample when imaging slightly below the critical TIRF angle (oblique incidence geometry). This
24 range allows to image actin, tubulin, or even nuclear proteins, although it is important to keep
25 in mind that there will be a contribution from out of focus fluorophores^{86,87}.

26 Following the calibration strategies described in the original Number and Brightness paper²⁹,
27 the eN&B method and software can also be used to analyze data obtained from
28 confocal^{9,24,38,44-46,50,55} and two-photon microscope set-ups in both photon-counting^{8,36,51} and
29 analog mode^{20,33,56,83} as well as light sheet systems⁸⁴.

30

31 **Experimental Design**

32 **Cell culture preparation.** For all the experiments, glass bottom dishes (MatTek Corporation,
33 USA) compatible with confocal and TIRF microscopes are firstly coated with a cell adhesive
34 polypeptide. When using a TIRF microscope we recommend to keep the plate brands and
35 model constant for all the experiments. Different brands and models may have different
36 thickness which affects the TIRF angle and the objective working distance.

37 The dishes were incubated under the cell culture hood with 300 mL of Poly-L-lysine (PLL)
38 (Sigma-Aldrich, USA) diluted at 0.05% (w/v) in PBS for 90 min at room temperature and then
39 rinsed 3 times with PBS and Milli-Q water. Controls should be carried out to make sure the cell
40 adhesive coating does not affect the protein under study. We found out that Laminin can
41 activate the Eph receptor efficiently.

1 At this point, coated dishes can be air-dried and kept at 4°C for a maximum of 24 hours before
2 the next step. In the example described here, EphrinB1-Fc (R&D Systems Inc., USA) was
3 selected as ligand for the EphB2 receptor and was presented to the cells either in soluble form
4 (Step 11) or immobilized on the substrate through a printing procedure. Control (mock)
5 stimulation were performed on PLL-coated glasses and on glasses with Fc fragment (Jackson
6 ImmunoResearch, UK) printed onto a PLL coating.

7

8 **Replicates and Controls**

9 Several controls can help to place the brightness measurements in the context of protein
10 dynamics. Positive controls should use antibodies or molecules to induce the oligomerization of
11 the protein of interest with high efficiency. In a negative control experiment the oligomer
12 should stay unassembled during the entire time-lapse recording. This can be achieved by
13 imaging the cells in the absence of any induction or by using inhibitory drugs. Mutant proteins,
14 such as negative dominants, can be used to calibrate the sensitivity of the method to different
15 oligomerization kinetics. Photobleaching can be quantified by integrating the fluorescence
16 from a single cell at the beginning and the end of either a single timepoint or the entire time-
17 lapse. A correct experimental design should also include sufficient replicas to obtain statistically
18 significant data to compare the different controls or samples.

19

20 **Instrument calibration.** Ideally, image acquisition settings are determined once at the
21 beginning of an experimental project (Steps 1-10) and maintained constant throughout the
22 experimental procedure for consistency^{33,34}. The sections below describe steps to determine
23 key camera and illumination settings affecting the fluctuations extracted in eN&B.

24

25 **Camera noise calibration.** It is important to optimize the camera's dark count and signal-to-
26 noise ratio at multiple pixel read-out rates and with several EM gain settings, respectively (Step
27 12). This serves to optimize the image acquisition conditions in a trade-off between speed and
28 instrumental background noise. Camera dark current can be measured with the shutter closed
29 and 500 millisecond exposure time (for a description of the choice of exposure see
30 Experimental Design "Protein diffusion and camera exposure calibration"). Recordings of 200
31 frames should be obtained at several pixel-transfer rates. The dark count histograms obtained
32 are analyzed regarding its mean and standard deviation values, as well as the uniformity across
33 the EMCCD chip. This calibration step aids in identifying excessive differences in pixel noise, hot
34 pixels and unusual noise patterns which might affect the analysis and hence should be
35 excluded.

36

37 **Gain.** We recommend optimizing the EM gain to maximize the signal-to-noise ratio of the
38 images (Step 7). This can be done one time and the result can be used as the standard gain

1 value for a particular microscope (even for other applications). This calibration can be done by
2 imaging fluorescent proteins or fluorescently-labeled antibodies adsorbed to a clean glass
3 surface and minimizing the signal's coefficient of variation (see Box 1 for a step-by-step
4 procedure on gain optimization).

5

6 **Pixel size.** In our lab, imaging is performed with a Nikon NSTORM system equipped with a 100x
7 Apo TIRF NA 1.49 oil-immersion objective and a 1.5x tube lens engaged (Step 6). However
8 other TIRF and confocal microscopes can be used provided that high NA objectives are used.
9 The pixel size is determined using the following equation:

10
$$P_{si} = \frac{P_{sc}}{(M * r_l)}$$

11 Where P_{si} is the pixel size on the image, P_{sc} is the pixel size on the camera sensor, M is the
12 objective magnification and r_l is the relay lens. For our setup, we obtain a final pixel size of 106
13 nm. A small pixel size is essential for measuring signal fluctuations correctly.

14

15 **Laser.** Illuminating laser power should be determined using two empirical criteria (Step 5). The
16 first is to ensure that the fluorescence intensity attributable to the fluorescent construct (in our
17 case Eph-mRuby) is solidly in the middle of the camera's dynamic range (i.e. peak value
18 ~35,000 digital levels on 16-bit images), while ensuring there are no saturated pixels. The
19 second criterion is to minimize photobleaching during a single 200-frame acquisition such that
20 the final average intensity within an imaged cell shows no more than ~5% reduction in
21 fluorescence as compared to the average intensity in the first image.

22

23 **TIRF angle.** We routinely use the commercial Nikon set up for stochastic optical reconstruction
24 microscopy (the NSTORM microscope for our experiments. This setup comprises a robust
25 optical design for focusing the illumination laser light onto the back focal plane of the objective
26 to produce TIR. As a result, the position of the TIR focusing lens can be adjusted once and
27 repeatedly used in the same setting to obtain a similar evanescent field over several imaging
28 sessions. In our example, we imaged cells expressing Eph-mRuby that were strongly adhered to
29 the glass surface and well spread, allowing us to select a field of view showing isolated, non-
30 overlapping cells. The TIR lens position can be adjusted to optimize visualization of: 1)
31 disappearance of intracellular vesicles that transport the labelled membrane protein and 2)
32 increase of detected fluorescence arising from a local field enhancement near the critical angle
33 for the water-glass interface. It is important to ensure that the intensity counts are consistent
34 between experiments with different TIR lens positions. This optimization process is rapid and
35 can easily be performed for each experiment in microscope systems showing lower robustness
36 in TIR lens positioning (Step 10).

37

1 **Camera readout mode.** The commercial NSTORM microscope we utilize is equipped with an
2 Andor iXon 897 EMCCD camera capable of either 10 MHz readout rate at 14-bit or 1 MHz at 16-
3 bit. We use the slower 1 MHz rate to access the larger 16-bit range and obtain the larger
4 dynamic range during acquisition while minimizing readout noise (Step 7). The same rationale
5 should be followed for systems equipped with a different camera. The 10-fold slower camera
6 readout rate is not problematic due to the relatively long 500 ms exposure time our
7 measurements required.

8

9 **Analog Number and Brightness calibration.** Fluorescence microscopes are affected by
10 instrumental noise. As such, the analysis requires a calibration step which is instrument
11 dependent, particularly for analog mode. Previous work⁸³ addresses the problem and serves as
12 a base for the calibration approach described here. A set of dark images contains the
13 information required for eN&B calibration. Two components can be discerned in the intensity
14 distribution of these dark sets (Figure 3a): one Gaussian part and one exponential part (linear
15 part on log scale). The center of the Gaussian component represents the offset of the system
16 while its standard deviation is the readout noise (Figure 3b). The exponential component is
17 used to obtain the conversion factor from intensities measured and photons, extracting the
18 slope S of the curve (Figure 3c). The calibration should be performed separately for every
19 experiment, as subtle variations are observed on a day-to-day usage of the instrument. We
20 provide a software packaged with an automated fitting tool for this purpose
21 (<http://bioimaging.usc.edu>) (Figure 3d) (Step 19).

22

23 **Monomer brightness calibration.** The brightness of single monomers needs to be estimated
24 from samples where the protein exists in its free form in a monomeric state. In our case study
25 with the Eph receptor, we imaged cells that were seeded for 24 h on PLL coated plates, and had
26 no exposure whatsoever to any cognate ligand. For any other membrane receptors, a similar
27 procedure must be performed, avoiding serum components or coatings that may bind or
28 interfere with the oligomerization of the protein. Excessive overexpression may also trigger
29 self-aggregation of proteins and must be also avoided. For other proteins it is important to
30 identify where the protein is found in a monomeric state²⁹. If obtaining a monomeric
31 population of the protein of interest is not possible, a variant with truncated or mutated
32 oligomerization interfaces can be generated, as long as the diffusion rate is similar to the native
33 protein.

34 Based on the brightness value of the monomer, the brightness of the different oligomers (i-
35 mer) can be calculated as follows:

$$36 \quad B_{imer}(i) = 1 + i(B_{monomer} - 1)$$

37 where B_{imer} is the brightness of the i-mer, i is the size of oligomer and $B_{monomer}$ is the
38 measured brightness for the monomer. It is important to note that some fluorescent proteins
39 are known to self-aggregate or work as dimers⁸⁸. In order to overcome fluorescent protein-

1 induced dimerization artifacts, several monomeric fluorescent proteins have been
2 described^{89,90,88}.

3

4 **Protein diffusion and camera exposure calibration.** It is essential to determine the ideal
5 camera exposure rate so that fluctuations are accurately captured between frames (Steps 8
6 and 9). This parameter is related to the diffusion rate of the protein of interest, and can be
7 defined using the autocorrelation function (ACF) from FCS analysis (Figure 4). The details on the
8 protein mobility coefficient may be biologically relevant in addition to the oligomerization
9 dynamics resolved by eN&B. In the interest of space, readers are directed to a number of
10 excellent review and method articles of FCS⁹¹⁻⁹³.

11 An important factor to consider is the fluorescence density of the sample, as FCS works
12 optimally when a low concentration of protein is present. Molecular crowding saturates the
13 focal volume and reduces the amplitude of the ACF. In FCS, this can be avoided by selecting
14 low-expressing cells or controlling the level of fluorescence by employing a photoactivatable
15 GFP (paGFP), where a subset of tagged proteins can be activated prior to FCS measurements.
16 Photoactivatable proteins yield robust FCS results⁹⁴ but are not a strict requirement. It is
17 important to note that they cannot be used for eN&B because the dark species would
18 artificially reduce the brightness B value. The diffusion measurements and eN&B should thus
19 be carried out using the same standard (monomeric) fluorescent protein.

20 Most confocal microscopes have the capability of performing both FCS and N&B
21 measurements. A number of commercial platforms now provide FCS modules, including Zeiss
22 and Olympus, which will automatically compute the ACF curve and provide mobility
23 coefficients. Alternatively, raw data can be analyzed through a number of ImageJ plugins
24 (<https://imagej.nih.gov/ij/download.html>)⁹⁵ or SimFCS (Gratton Lab, University of California
25 Irvine: <https://www.lfd.uci.edu/globals>). Using a paGFP-tagged EphB2 we previously used
26 established protocols^{28,94} to determine that the most appropriate camera exposure time is 500
27 ms when using the Zen FCS module of a Zeiss 780 platform (see Box 2 for a detailed
28 procedure).

29 Once the protein mobility coefficient δ and the focal volume waist (ω_0) are known for the
30 protein of interest (Box2), these together with the following guidelines can help the reader to
31 choose the optimal acquisition parameters. The average time a protein remains in a focal
32 volume (pixel), also known as residence time, can be computed as $\omega_0^2/4\delta$ ⁴⁰. For camera-based
33 microscopes, the time to take a single whole frame, t_{frame} , depends mainly on the camera
34 technical specifications, such as readout rate, number of pixels per frame and exposure (or
35 dwell) time, t_{dwell} , needed to collect the protein fluorescence signal. When analyzing proteins
36 with small mobility coefficients (0.03-0.04 $\mu\text{m}^2/\text{s}$) with a fast camera (10 MHz, 512x512 pixels)
37 readout time is approximately 26 ms, t_{dwell} (500 ms) is almost equivalent to t_{frame} (526 ms).
38 Therefore, in these systems, to capture fluctuations (particles moving in and out of the focal
39 volume) the exposure time is selected in such a way that $t_{\text{frame}} > \omega_0^2/4\delta$ to allow the proteins to
40 scatter through several pixels. If the sample is bright enough, the t_{frame} can be increased simply
41 by pausing between every acquisition¹. This avoids averaging out fluctuations and may increase
42 the statistical significance of the fluctuations. For laser-scanning microscopes, the dwell time

1 t_{dwell} is the time to collect the fluorescence signal at a single pixel and t_{frame} depends then on the
2 number of pixels p as $t_{\text{frame}} \geq p t_{\text{dwell}}$. In these microscopes, t_{dwell} should be shorter than $\omega_0^2/4\delta$
3 to avoid averaging out the fluctuations, i.e. to reduce the probability of a particle entering or
4 exiting the focal volume; and t_{frame} should be long enough to observe particle fluctuations (t_{frame}
5 $> \omega_0^2/4\delta > p t_{\text{dwell}}$). Therefore, t_{dwell} and t_{frame} can readily be selected for a particular microscope
6 configuration if δ is approximately known.

7
8 **Acquisition framework.** Once the cells are ready to be imaged, the acquisition starts by
9 capturing 200 sequential frames with an exposure time per frame that is proportional to the
10 diffusion rate of the protein, as determined by FCS, Raster image correlation spectroscopy
11 (RICS)⁹⁶ or equivalent (Steps 13-18). The camera exposure time will determine the interval
12 between time-points: since the 200 frames are treated as a single time-point, the longer the
13 exposure time, the longer will take to capture the 200 frames. For short exposure times, many
14 positions can be recorded at approximately the same time, for longer exposure times, the time
15 to return to the same position may make the intervals between time-points too large in order
16 to properly resolve the dynamics of the desired protein. Switching between positions needs to
17 be done manually unless a custom macro is set up for every specific microscope system. If
18 automatization is not possible, time annotation must be done manually at the beginning of
19 every time point acquisition. This requires the presence of the researcher for the entire
20 duration of the acquisition. For continuous imaging we advise dividing the acquisition into
21 contiguous badges of 200 frames and treat them as individual time points.

22
23 **eN&B Analysis.** We developed a user-friendly software package to perform eN&B which makes
24 use of an intuitive interface (Steps 19-27). The software and an example of the dataset
25 analyzed during this study are available at <http://bioimaging.usc.edu> (see also [Supplementary](#)
26 [Video 1](#)). [Our software can be used to](#) extract brightness values from fluorescence fluctuations
27 for time-lapse image sequences. The code currently requires data to be organised as multiple
28 multilayer stacks of images acquired at different time points, where a single file contains a
29 sequence of images (see Experimental Design “Acquisition Framework” and Figure 5a). The
30 software can perform two types of analysis, (i) full statistical resampling, which performs
31 windowed-frame analysis on each of the time-point image sequences, providing a distribution
32 of oligomerization states for each pixel, or (ii) a single-value analysis where only the mean
33 value of oligomerization is reported. The full statistical resampling (i) performs eN&B analysis
34 enhancing the statistical resolution of the method at the expense of a longer computational
35 time. The single-value analysis (ii) can be used to perform a rapid overview analysis of the
36 experiment.

37 The software uses LOCI Bio-Formats⁹⁷ to load microscopy data (Nikon proprietary file format in
38 our case). In an effort to simplify adoption of the technique, we have created a Tiff file
39 importer. The user can convert proprietary file formats (e.g. Olympus, Zeiss or Leica) to Tiff
40 sequences prior to performing analysis. If Tiff file sequences are used, the number of frames
41 per time point and the total number of time-points need to be specified at the software
42 interface.

1 An image of the time-series is then prompted to the user with the purpose of selecting an ROI
2 in the field of view (Figure 5b). This allows for selective analysis of specific cells and including
3 part of the background for reference during analysis. In the resulting scatter plot, each pixel of
4 the image is represented in terms of intensity and Brightness (Figure 5c, d). The portion in the
5 ROI related to background will generally provide a cluster at lower intensity values, while the
6 sample will be shifted toward higher intensities. Manually selecting the boundary between
7 these clusters is necessary for ensuring correct calculation of oligomerization levels.

8

9 **Output data.** The eN&B software produces a series of images, plots and datasheets containing
10 the measurements from the brightness analysis (Figure 6):

- 11 • Raw 16-bit TIFF grayscale images of the selected cell for every time point after
12 photobleaching detrending. Only the first of the 200 frame series is shown (Figure 6a).
- 13 • The oligomerization maps show color-coded images of the cells with every pixel color
14 coded (jet) on a scale according to the average oligomer size present in each pixel.
15 Different oligomer binning options are presented to enhance oligomer populations
16 contained in a narrow range of sizes (Figure 6b). Each binning option corresponds to
17 differently equalized colormaps focusing on smaller, medium-sized or larger oligomers,
18 or just evenly representing them and saved as 16-bit Tiff and png.
- 19 • i-mer plots display the time evolution of up to 40-mer oligomers (Figure 6c). These
20 values are provided for multiple tolerances (sigma) around the value of the monomer.
- 21 • The abundance distribution of oligomers accumulated for all pixels in the image per
22 every time point for the single-value analysis (Figure 6d) or the statistically-enhanced
23 (Figure 6e). Of note, these distributions are not normalized by the total amount of
24 pixels, therefore the integral of the distribution grows with the cell size. Raw data is
25 also provided to handle data independently.
- 26 • Excel files containing image-histogram sum and percentage data from eN&B analysis
27 are provided. The values include the total number of pixels inside the selected ROI that
28 are at a specific oligomerization level per time-point. These values are provided for
29 multiple tolerances (sigma) around the value of monomer. Different files are provided
30 for the quantification of the monomer to 40-mer range or the monomer to 100-mer
31 range.
- 32 • The full eN&B file can be saved as a Matlab (.mat) file and includes the oligomerization
33 distribution of every pixel for every time point.

34

35

36 **Materials**

37 **BIOLOGICAL MATERIALS**

- 38 • Cells expressing fluorescent proteins: in the example described in this protocol, we
39 used the HEK293T:EphB2_mRuby cell line, which was generated by lentivirus
40 transfection (ViraPower Lentiviral Packaging Mix, Thermo Fisher) of the plasmid
41 pLenti.CMV:EphB2_mRuby. The plasmid pCDNA3_EphB2_mRuby was used as a source

1 plasmid to excise the fusion construct. The cloning protocol is detailed in the original
2 publication²⁸. The plasmids pLenti.CMV:EphB2 mRuby, and paGFP-EphB2 are available
3 upon request. Alternative generic genetic constructs for expression of fluorescent
4 proteins, including mCherry or paGFP, can be obtained via Addgene.

- 5 • HEK293T cells were purchased directly to the distributor to avoid misidentification or
6 cross-contamination (Sigma-Aldrich, cat. no. 85120602, ATCC® CRL-3216™)
 - 7 ○ Caution: regularly check your cells to avoid mycoplasma contamination and
8 perform genetic tests and sequencing to ensure the cells are not cross-
9 contaminated with different cell lines.

10

11 REAGENTS

- 12 • In-Fusion® HD Cloning Kit (Clontech, cat.no. 639606)
- 13 • ViraPower Lentiviral Packaging Mix (Thermo Fisher, cat.no. K497500)
 - 14 ○ Caution: virus production must be carried out in a bio-safety level 2 laboratory
15 or higher.
- 16 • DMEM (Sigma-Aldrich, cat. no. D6546-500ML)
- 17 • DMEM without phenol red (Thermo Fisher, cat. no. 21063-029)
- 18 • Fetal Bovine Serum (FBS - Hyclone, Cultek, S.L.U., cat. no. CH30160.03)
- 19 • Trypsin 0.05% (wt/vol) (Thermo Fisher, cat. no. 25300-054)
- 20 • PBS, pH7.4 (Sigma-Aldrich, cat. no. P4417-100TAB)
- 21 • Lipofectamine 2000 (Thermo Fisher, cat. no. 11668-019)
- 22 • Opti Mem (Thermo Fisher, cat. no. 31985-047)
- 23 • Poly-L-lysine (Sigma-Aldrich, cat. no. P8920-100ML)
- 24 • Mili-Q water
- 25 • NaOH (sigma, cat. no. s0899)
 - 26 ○ CAUTION: Causes severe skin burns and eye damage. Wear protective gloves
27 and safety glasses.
- 28 • Fluorescent protein label, Atto 488 NHS ester (Atto-tec, product AD488-31)

29

30 EQUIPMENT

- 31 - 35 mm Glass bottom dishes (MatTek, cat. no.)
- 32 - LabTek glass bottom chamber slides, 1-well to 8-well (Lab-Tek™, Thermo Fisher)
- 33 - Engraved dishes (Zell-Kontakt).
- 34 - Acuderm Biopsy Punch 12 mm (Medex, cat. no. ACD-P1250)
- 35 - Vaccum Dessicator (Dynalon Labware, cat. no. 243025)
- 36 - Ultrasonic Bath (JP Selecta S.A., cat. no. 3000512)
- 37 - Shaker (Heidolph, cat. no. 543-42210-00)
- 38 - Table top centrifuge (Eppendorf, 5424R).
- 39 - Plate centrifuge (Eppendorf, 5810R)
- 40 - Rotator (Sturart, Rotator SB3)

- 1 - Zeiss LSM 780 laser scanning confocal microscope equipped with Avalanche
- 2 photodiodes of the Confocor 3 (Zeiss, Jena) equipped with a 405 nm laser line and a
- 3 water 63x/1.4 NA objective (Zeiss, Jena)
- 4 - ZEN Software FCS and RICS modules (Zeiss, Jena)
- 5 - STORM microscope system (Nikon Instruments, NSTORM) equipped with an EMCCD
- 6 camera (Andor iXon3 897), a 100x/1.4 NA objective and a 1.5x lens tube.

7

8 **Software**

- 9 - SimFCS (www.lfd.uci.edu);
- 10 - Fiji (including Bio-Formats up-to-date plugin with ND2 files reader)<https://fiji.sc/> ;
- 11 <https://loci.wisc.edu/software/bio-formats>
- 12 - Matlab (The MathWorks, 2015 or newer)
- 13 (<https://www.mathworks.com/products/matlab.html>;

14

15

16

17 **Procedure**

18 **Setting up the microscope •TIMING 1 h to reach desired temperatures**

- 19 1. Warm up the microscope 1h before starting the experiment to allow the temperature
- 20 to stabilize, matching the sample optimal temperature (i.e. 37°C).

21 **?TROUBLESHOOTING**

- 22 2. Turn on the CO₂ and set up the controller at 5%

23 **?TROUBLESHOOTING**

- 24 3. Turn on the camera ~30 min before starting the experiment so that it reaches the
- 25 optimal working temperature (i.e. -70°C for an eMCCD ANDOR).
- 26 ▲ **CRITICAL STEP** The camera read out is very sensitive to temperature oscillations.
- 27 4. Optional Step: Create a logbook text document on your PC. Clearly describe positions
- 28 and time-points in the document (Table 1). This step is not necessary if your
- 29 microscope allows automatic configuration of the imaging conditions.
- 30 5. Activate the relevant laser lines, allowing power sources to stabilize prior to image
- 31 acquisition (in the example using our HEK293T:EphB2 mRuby cell line, we use the 561
- 32 nm laser). Set the laser power to a previously determined power density, which
- 33 minimizes photobleaching (see Experimental Design “*Instrument Calibration*”).
- 34 6. Ensure the light path is correctly specified:
 - 35 • Dichroic mirrors and emission filters appropriate for the lasers and fluorophore
 - 36 being utilized, respectively.
 - 37 • Additional magnification optics required to obtain the desired pixel size (106 nm
 - 38 here, see Experimental Design “*Instrument Calibration*”).

- 1 7. Set the camera gain, readout to previously determined values (see Experimental Design
2 "Gain" and "Camera readout mode").
- 3 8. Specify the number of F frames per position and time point (i.e. F=200)
- 4 9. Set the desired frame exposure time according to FCS measurements (see
5 Experimental Design "Protein diffusion and camera exposure calibration").
- 6 10. Determine the optimal TIRF setting and proper illumination power. The objective here
7 is to obtain high signal, low background images with low photobleaching rates.
- 8 **?TROUBLESHOOTING**

9 **Sample preparation •TIMING 30 – 45 min**

- 10 11. Prepare the cells for imaging
 - 11 i. Directly place the glass bottom MatTek or Labtek imaging chamber
12 containing the cells of interest in the microscope.
 - 13 ii. Choose a field of view containing 1 to 4 isolated cells. Record the
14 position using the microscope software.
 - 15 iii. Repeat Step ii until enough suitable positions have been recorded and
16 select the best ones for imaging. **?TROUBLESHOOTING**
 - 17 iv. Gently pipette the ligand into the culture whilst avoiding moving the
18 stage.

19 **Imaging •TIMING 1 h**

- 20 12. Acquire a dark image sequence as follows: close the camera shutter and deactivate the
21 lasers without turning them off. Acquire 200 frames with the shutter closed. Save this
22 file and name it (i.e. dark_initial).
- 23 13. Focus the microscope on the cells in the first position, name the position file (i.e.
24 Position1t1) (Figure 5a), start recording the 200 frames series and write down the
25 starting time in the logbook.
26 **?TROUBLESHOOTING**
- 27 14. Immediately after finishing the first acquisition, move to the next position, refocus
28 manually if necessary, name the file (i.e. Position2t1), start image acquisition and write
29 down the starting time in the logbook.
- 30 15. Repeat Step 14 for as many positions as desired.
- 31 16. Switch back to the first position, name the file accordingly (i.e. Position1t2), start image
32 acquisition and write down the starting time in the logbook. Repeat this step for all
33 other positions.
- 34 17. Repeat Steps 14-16 for the duration of the entire imaging session.
35 **?TROUBLESHOOTING**
- 36 18. Close the camera shutter, record a dark frame series (see Step 12) and name it (i.e.
37 dark_final).

38 **Image analysis •TIMING 15 min per cell analyzed on N&B mode, 2 h per cell on eN&B mode**

- 39 19. Run the eN&B calibration, either as executable or as Matlab code
40 (<http://bioimaging.usc.edu>): Load the sequence of single-tiff files containing the dark
41 images. Record the parameters of the fit (Figure 3) for S factor, Sigma0, Offset and
42 their precision

- 1 20. Run the eN&B GUI matlab code, either as executable or as Matlab code
2 (<http://bioimaging.usc.edu>) with the following parameters:
3 • Under Settings insert the numbers obtained from calibration from Step 19 and
4 choose whether to show all figures or just to save them
5 ▲ **CRITICAL STEP** Image plotting is RAM memory expensive. If all images are shown
6 upon calculation and less than 64GB RAM is available in the system, it is possible
7 that the workstation runs out of memory before completion of the analysis.
8 • For fast N&B analysis use the standard code
9 • For a statistically enhanced analysis use the eN&B code.
10 21. Select the files from an entire time series from a single position

11 **?TROUBLESHOOTING**

- 12 22. Select a cell of interest by creating an ROI (Figure 5b). We suggest a physical size of the
13 cell area to be analyzed larger than 64x64 pixels.
14 ▲ **CRITICAL STEP** An optimal ROI should include a portion of the background outside
15 the cell to provide a reference during analysis.
16 23. Double click on the cell ROI to complete the selection.
17 24. In the brightness scatter plot, establish the signal/noise threshold by marking the edge
18 with the right end of the rectangle. If the selection of the ROI is performed correctly
19 the plot should show easily distinguishable clusters (Figure 5d). The threshold should
20 be placed at the lowest values of the right-most cluster, representing the cell.
21 **?TROUBLESHOOTING**
22 25. Double click on the corner of the ROI to trigger the analysis (Figure 5d). If all images are
23 being shown in Step 20, the process can be observed until completion.
24 26. A file-save window will prompt requesting a root-name for saving the bulk files (images
25 and excel files with raw data). The software saves by default the images in tiff and png
26 formats. If further editing is required, save the individual images by clicking “saving as”
27 on the relevant window (i.e .fig or .ems extensions).
28 27. Use the logbook data to specify the specific time-points values in the excel data (Table
29 1).

30
31 **●TIMING**

32 Steps 1-10, setting up the microscope: 1 h depending on the time needed by the camera and
33 incubator to reach working temperature.

34 Step 11, sample preparation: 15 min to place the cells on the microscope and find regions of
35 interest. Different samples may require different timing.

36 Steps 12-18, imaging: 1 h or the specific time-course of the experiment.

37 Steps 19-27, image analysis: 15 min per cell on standard N&B mode or additional 15 min if plot
38 editing is required. 2h per cell when running software on eN&B mode.

39
40 **?TROUBLESHOOTING**

1 Troubleshooting guidance can be found in **Table 2**.

2

3 **Anticipated results**

4 The protocol detailed here can be used to image and quantify the oligomerization dynamics of
5 proteins. Fluorescently-tagged proteins are directly observed using a TIRF microscope during
6 serial image acquisitions. Imaging is carried out at the maximal resolution allowed by the
7 microscope set-up. The Number and Brightness (oligomerization) of the proteins in each pixel is
8 a function of the variance and intensity of the fluorescence fluctuations. The brightness values
9 for all pixels in a cell can be visualized as a color-coded oligomerization (brightness) value
10 overlaid with a cell image (Figure 6b). Since our eN&B version includes algorithms minimizing
11 the impact of photobleaching, the method allows to resolve brightness maps during long time-
12 lapse imaging.

13 In addition to the oligomerization maps, the software can be used to retrieve more
14 quantitative plots, which we termed i-mer plots (Figure 6c). These plots display the evolution of
15 the relative concentration of the different oligomers over time, displaying each oligomer
16 species (up to 40-mer) as an independent curve. The value for each oligomer at a given time
17 point results from the addition of the relative abundance of each oligomerization value
18 (monomer, dimer etc.) from every pixel. In our experiments, the i-mer plots revealed a
19 strikingly organized sequence of events, where progressively larger oligomers take over the
20 smaller ones following a strict growth trajectory. Different ligand stimulations, such as soluble,
21 surface immobilized or multivalent, substantially changed the trajectory of the different
22 curves^{28, 62}. For example, the slope of monomer depletion may reflect the speed of the
23 oligomerization process. This plot is therefore the best tool to quantitative assess the clustering
24 dynamics of a protein of interest.

25 The data contained in the i-mer plot can be also be presented from a population point of view.
26 The relative abundance distribution of all oligomers at every time point is presented in plots
27 like the one depicted in Figure 6d. This plot complements the i-mer plot, because the shape of
28 every curve gives an overview of the diversity of the oligomer population present in the cell at
29 every time point.

30 eN&B uses a resampling method (what we call the enhancement) which procures per every
31 time point an oligomerization distribution per every pixel, instead of a single value retrieved by
32 the standard N&B. This data obtained using eN&B is too complicated to be represented in
33 simple understandable plots without averaging the information. The plot in Figure 6e gives a
34 rough idea of the amount of information generated by the method. The eN&B data is better
35 suited for additional mathematical or statistical analysis, rather than for graphic
36 representation. The software generates matrixes containing all numerical values for that
37 purpose. For every sample the relative abundance of every oligomer of every pixel in the image
38 is included in an excel file or a Matlab matrix. This data is amenable for further mathematical
39 analysis. The software allows to run the analysis on a standard N&B mode, without performing
40 the statistical enhancement. In this case the software will generate a single value per every
41 pixel, corresponding with the modal value of the oligomerization distribution. Producing

1 standard N&B data is roughly 200 times faster than eN&B data, and it is useful for exploratory
2 analysis or qualitative observations.

3 We demonstrate our eN&B using transgenic cells expressing a fluorescently-tagged EphB2
4 receptor. The cells were presented with ephrin ligands to induce receptor clustering during one
5 hour time-course. The analysis was performed every 5 minutes to provide a detailed time-
6 course of Eph clustering. The oligomerization maps show Eph aggregation across the entire cell
7 surface in a progressive manner (Figure 6c, d). Oligomerization runs uninterrupted during the
8 entire time of observation. The i-mer plot shows a characteristic pattern which is repeated
9 across many experiments. Monomers and low order oligomers can be seen to decay in the first
10 15 minutes after ligand addition. Thereafter, oligomers of progressively larger size increase
11 their abundance in a strikingly coordinated pattern. An interesting feature of the EphB2
12 receptor is that clustering keeps running beyond the point of monomer depletion, which
13 suggest that oligomers condense (coalesce) into larger ones. This type of behavior might not
14 apply for other proteins and would need to be confirmed on a case by case basis. The shape of
15 the oligomer distribution evolves over time as well (Figure 6d). At early time points narrow
16 distributions center around small oligomer values. Over time, the center of the distribution
17 shifts toward larger oligomers. The width of the distribution expands quickly over time,
18 sometimes leading to long-tailed distributions, which reflects the growing diversity of the
19 oligomer population also over time. The fast expansion of the distribution shape correlates
20 with oligomer condensation. When using the enhanced version of the analysis it is advisable to
21 analyze the same sample using the standard N&B in parallel. In a qualitative way the results
22 should be similar and the analysis runs faster. Plotted eN&B data, such as in Figure 6e, can be
23 challenging to read. For numerical analysis, the enhanced data contained in the Matlab
24 matrixes provides a more complete and faithful description of the oligomer population.

25

26

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4

5 **Author contributions**

6 S.O., J.J.O., A.S. and C.M. performed experiments. S.O., D.R., C.C. and F.C. analyzed the results
7 and designed algorithms. V.H., E. L. and E.M. designed the micro-printing protocol. S.M.
8 performed FCS analysis. M.L., E.M, A.R and S.E.F. contributed to the experimental design. S.O.,
9 F.C., J.J.O., C.C., D.R. and S.E.F. wrote the manuscript.

10

11 **Competing financial interests**

12 The authors declare no competing financial interests.

13

14 **Data/code availability statement**

15 The data collected for this study was done using our custom-made algorithms available at
16 <http://bioimaging.usc.edu>. The data analysis for this study was done using our custom-made
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2

3 **FIGURE LEGENDS**

4 **Figure 1. Enhanced Number and Brightness exploits fluorescence fluctuation analysis to**
5 **extract the oligomerization state of proteins. a)** During live-imaging, fluorescent molecules in
6 different oligomerization states diffuse in and out of the focal volume. A sequence of F images
7 ($25 \leq F \leq 200$) can capture the fluorescence fluctuations derived from the movement of proteins
8 into and out of the focal volume. As the size of the protein aggregate increases, the amplitude
9 of the fluctuations increases over time while the period will decrease (see the red Eph receptor
10 fluorescence fluctuation after ephrin stimulation and the control fluorescence from
11 unstimulated cells). Kcps, kilo counts/s. **b)** For every time point (t_1, t_2, t_3) N&B provides the
12 mean oligomerization value for every pixel. eN&B retrieves a distribution of species present in
13 the same pixel, thus revealing a wider diversity of the oligomer population. **c)** Detrending
14 algorithms correct the effect of photobleaching without affecting the fluorescence fluctuations.
15 The image shows the acquisition of 100 frames from 3 different time points (initial, mid, final)
16 from a total of 1h imaging the EphB2 receptor. The average intensity (blue) decays
17 exponentially, but the two representative pixels after correction (red and black) display intact
18 fluctuations.

19 **Figure 2. Comparison of eN&B and N&B analysis of data from simulations of oligomers freely**
20 **diffusing in a liquid solution.** The simulations were done with 1000 acquisition frames and an
21 eN&B window of 50 frames. In order to assess the power of analysis of eN&B compared to
22 standard N&B, two opposed instances cover a scenario where **a)** monomers form oligomers
23 over time and **b)** multiple oligomeric states co-exist in parallel. The grey circles represent the
24 size of the oligomer present at a given time. **c-d)** N&B and eN&B plots of the examples in **a** and
25 **b** respectively. The single bar in the graph represents the mean oligomer value retrieved by
26 standard N&B. The solid line in the graph represents the oligomer size-distribution produced by
27 eN&B. mer, oligomer size. A.u., arbitrary units.

28 **Figure 3. Analog Calibration for eN&B analysis. a)** Log scale Intensity distribution of a series of
29 dark images, acquired in absence of sample, excitation and light, after allowing the instrument
30 to temperature-stabilize (see section Experimental design “*Analog Number and Brightness*
31 *calibration*”). **b)** The Gaussian component of the intensities histogram is used to obtain the
32 offset (center) and readout noise (sigma). **c)** The exponential component slope fit provides the
33 conversion factor S of intensity to photons. Fitting precision and parameters are reported next
34 to each of the plots. **d)** Calibration is performed automatically by this tool, providing a final fit
35 overview and the factor values needed for calibration of the system in the algorithm.

36 **Figure 4. Typical ACF curve obtained from FCS analysis.** The EphB2 receptor fused to a
37 photoactivatable GFP (paGFP) was analyzed using FCS. The Normalized intensity correlation (G)
38 shows a linear decay at a certain period range. The inverse of that period is the optimal camera
39 exposure time.

40 **Figure 5. eN&B software interface and data loading. a)** Files are selected from the folder and
41 sorted in a time-sequential manner using the move up or move down buttons. Every file

1 comprises a series of 200 acquisitions corresponding to a single time point. **b)** A region of
 2 interest is selected on the grayscale raw image from the initial time to start eN&B analysis **c)**
 3 Outline of the eN&B graphical interface. **d)** Selection of signal background threshold. If ROI
 4 selection is performed correctly two clusters should be visible (cyan arrows) corresponding to
 5 the cell (right) and background (left). The edge is determined by the right edge of the selection
 6 tool (magenta arrow).

7 **Figure 6. eN&B software produces a comprehensive output of oligomerization data . a)**
 8 Photobleaching correction algorithms provide greyscale images at every time point while
 9 keeping the fluctuations. The white scale bar size is 5 μm , for reference only, not from the
 10 actual software. **b)** The oligomerization maps plot the oligomerization value of every pixel
 11 color-coded (according to the color bar showing the corresponding i-mer size from 1 to 40) on
 12 top of the original cell image. Different binning combinations can be chosen to adapt the plots
 13 to a narrow or wide range of oligomeric species. The white scale bar size is 5 μm , for reference
 14 only, not from the actual software. **c)** The i-mer plot displays the evolution of the proportion of
 15 each aggregate (i-mer) over time. The i-mer values are color-coded according to the color scale
 16 bar. **d)** Time evolution of the i-mer distribution. For every time-point, the software calculates
 17 the distribution of the relative abundance of every oligomerization value present in the cell.
 18 The shape of the distribution over time changes according to the increase or reduction of the
 19 species diversity. N&B provides a single distribution per every time point. **e)** eN&B retrieves a
 20 distribution per every pixel and time point.

21

22 **Table 1. Example of a customized logbook for time-lapse registration**

plate 1, 0.2uM ephrinb1
position1t1=9.21 position2t1=11.36
position1t2=13.52 position2t2=16.13
(...)
position1t10=50.07 position2t10=52.27

23

24

25 **Table 2. Troubleshooting Table**

Step	Problem	Possible reason	Solution
1	Cells show unexpected behavior, apoptosis, poor attachment, etc.	The temperature at the sample illumination point is not 37°C	Most microscope incubators show very heterogeneous temperature pattern across the chamber, which may not match the display settings in the temperature controller. It is advisable to use a precise

			temperature probe placed in the sample illumination point and tune the temperature controller settings to reach real 37°C
2	My microscope does not have CO ₂ chamber and the medium acidifies quickly	The pH needs to be buffered	Switch to L15 media when a CO ₂ chamber is not present. It buffers in atmospheric conditions unlike DMEM and other medias
10	The cells look more blurry than usual, or the signal is not as sharp	The TIRF angle needs to be adjusted, as a result of change of objective or the use of a different brand plate with different thickness.	For consistency it is preferred to use the same plate type and brand as well as the same objective
11	The cells look dim or too bright (saturated pixels)	The microscope set up is not optimal	The parameters that allow you to obtain a better signal are the illumination power, camera gain, and exposure time. Increasing the illumination power will result in higher levels of photobleaching but will increase the signal efficiently. Increasing the exposure time will also result in higher levels of photobleaching though the signal increase will be smaller and will affect the brightness analysis. Increasing the camera gain will not affect photobleaching but it will increase the noise.
11(iii)	Finding suitable cells takes too long	Low cells were seeded confluence The cells change shape and move	Increasing the cell confluence will increase the chance to find suitable cells for imaging, and also the probability of having more than one cell in the same field of view. The software can analyze the cells individually, so having more than one cell in the same field of view will accelerate data acquisition. Try different coating (i.e. PLL, laminin, gelatine)
13	The cell images are dark	The camera shutter is closed	Ensure to re-open the camera shutter after acquiring the last dark frame
17	The cells move The recorded positions seem to change between time-points	Cell motion (due to migration, movements from filopodia or similar projections, cell spreading etc.) may be unavoidable and it interferes with brightness measurements. The plate is drifting	If the microscope allows recording several positions simultaneously, we recommend to capture as many cells as possible. Review the movies at the end of the process to discard the motile cells. The selection of a proper adhesive coating will improve the results. Make sure you allow at least one hour for the microscope to reach and stabilize at the desired temperature. Excessive immersion liquid will also increase the probability of

			drifting. Make sure the stage holds the plate tightly.
25	The software crashes before finishing the analysis	The computer is not powerful enough for eN&B	Find a computer with minimum 64GB RAM memory available, enough hard drive space available.
25	The software gives an error message	The error message usually contains information regarding the problem. Possible reasons include insufficient memory for visualizing or saving the data, noise and background signal have not been separated properly, file type being loaded is incompatible	Try unchecking the "Show all images" checkbox in the eN&B GUI Settings. Try repeating the noise and background selection with a higher threshold. Make sure images are loaded either as Nikon .nd2 files or as single TIFF files.
26	The oligomerization maps are almost empty	Noise separation was too stringent	Repeat the analysis selecting a wider area of signal pixels.

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4 **Box 1. Determination of the EM gain setting that maximizes the signal magnitude to signal**
5 **fluctuation.**

6 This Box describes how to calibrate the gain of an EMCCD camera to maximize signal
7 amplification without amplifying the noise. Gain calibration needs to be done once and it is a
8 characteristic value for the camera (useful for eN&B and any other technique too).

9

10 **Procedure:**

- 11 1. Treat a LabTek chambered glass slide with 1 M NaOH for 10-15 minutes and let
12 it air dry.
- 13 2. Dilute a fluorescently labelled protein/antibody in PBS to approximately 0.1-1
14 ng/ml.
- 15 CRITICAL STEP Prepare a sample of fluorescently labelled proteins/antibodies
16 using a fluorophore in the same spectral range as will be utilized in the
17 experimental project (i.e. ATTO 488 for GFP).
- 18 3. Incubate the diluted solution onto the glass surface for 2-5 minutes.
- 19 4. Wash thoroughly with PBS.
- 20 5. Image the prepared sample to ensure that individual fluorescent
21 proteins/antibodies can be visualized as isolated bright spots on a dark
22 background.

1 CRITICAL STEP If too many/too few individual spots are visualized,
2 repeat sample preparation and adjust the protein/antibody dilution or
3 the incubation time.

- 4 6. Record several hundred frames, imaging the single molecules over a wide
5 range of EM gain settings to image the single molecules as they gradually
6 photobleach.

7 CRITICAL STEP We suggest as starting range: 10 – 1000 gain in log-scale
8 intervals (*i.e.* 10, 30, 100, 300, 1000). Recommended exposure time: 30-50 ms.

- 9 7. Perform single-particle tracking of the spots by fitting each of them with a 2D
10 Gaussian function with constant offset.

11 CRITICAL STEP Several open source softwares are available to do this, such as
12 u-track⁹⁸ or ThunderSTORM⁹⁹. Note the latter will require that localizations in
13 each frame be linked together to obtain a fluorescence trajectory for each
14 single protein/antibody imaged.

- 15 8. Remove the contribution from background fluorescence prior to fitting.

16 CRITICAL STEP If the software used does not provide this quantity
17 (background count) as an output, obtain it by subtracting the number
18 of background photons/counts from the integrated number of
19 photons/counts.

- 20 9. For each EM gain setting, first calculate the mean and standard deviation of the
21 background subtracted photon/count values for each single-molecule
22 fluorescence trajectory. Second, calculate the ratio of mean to standard
23 deviation for the fluorescence trajectory. The maximal value of this ratio is the
24 optimized EM gain setting.

- 25 10. If camera settings allow different pixel transfer rates, repeat steps 6-9 at
26 different readout speeds (*i.e.* 1MHz, 5MHz, 10MHz).

27 - END OF BOX 1 -

28
29
30 **Box 2. Applying FCS to quantify protein mobility coefficient and define camera exposure**
31 **settings**

32 This Box describes a protocol to carry out FCS using photoactivatable proteins. This approach
33 allows to extract protein diffusion coefficients (δ) efficiently. δ values are required to calibrate
34 the camera exposure time during eN&B imaging. The detailed original protocol can be found in
35 Ref.⁹⁴.

36 **Procedure:**

37 CRITICAL: The focal volume waist (ω_0) and structural parameter (S) must first be calibrated
38 using a fluorophore with a known diffusion coefficient such as eGFP, FITC or certain
39 Alexa/ATTO probes of the relevant emission channel (Steps 1-3).

- 1 1. Place a drop of ~1 nM solution of Atto488 in water onto a glass coverslip directly over
2 the water $\times 63/1.4$ NA objective of a Zeiss 780 (Zeiss, Jena). At 25 °C this small molecule
3 has a known diffusion coefficient of $400 \mu\text{m}^2 \text{s}^{-1}$.
- 4 2. Within the Zen FCS module (Zeiss, Jena), acquire data fluorescence counts of the
5 Atto488 standard at a single point on the 488 nm laser line for 25 s with 4 repeats. The
6 physical conditions (temperature, molarity, buffers) must be consistent to the
7 conditions for the known diffusion coefficient of the fluorophore in use.
- 8 3. The Atto488 should contain a single species freely difusing. Within the “Fit” tab, fit the
9 acquired data to the single free diffusion component model provided within the FCS
10 module with a fixed diffusion coefficient of $400 \mu\text{m}^2 \text{s}^{-1}$, and variable volume ω_0 and S.
11 The software will compute these two parameters based on the Atto488 standard, which
12 are essential when determining and unknown diffusion coefficient. Record the
13 calculated volume ω_0 and S parameters. A detailed step-by-step protocol provided with
14 the Zeiss FCS module and is a freely available online resource from Zeiss
15 ([https://www.zeiss.com/content/dam/Microscopy/Downloads/Pdf/FAQs/zen2010-](https://www.zeiss.com/content/dam/Microscopy/Downloads/Pdf/FAQs/zen2010-lsm780_basic_fcs_experiments.pdf)
16 [lsm780_basic_fcs_experiments.pdf](https://www.zeiss.com/content/dam/Microscopy/Downloads/Pdf/FAQs/zen2010-lsm780_basic_fcs_experiments.pdf)).
- 17 4. Seed cells onto glass bottom chamber slides and transiently transfect them with a
18 paGFP-tagged EphB2 and membrane-mCherry vector (Addgene:53750) using
19 Lipofectamine 2000 according to the manufacturer’s guidelines. The amount of DNA
20 transfected and the DNA:lipid ratio should be optimised based on the cell line used.
21 CRITICAL STEP When using a paGFP fused protein it is recommended to co-transfect
22 with a constitutively fluorescent marker to identify points of interest in the cell.
- 23 4. Mount the cells on the microscope and identify transfected cells based on the
24 expression of membrane-mCherry.
- 25 5. To activate the paGFP, draw a region-of-interest along the membrane using the
26 membrane-mCherry as a guide. Activate the paGFP using the 405 nm laser line, with
27 the laser power and dwell line optimized for each cell and level of transfection:. The
28 GFP signal should be visible, but the fluorescence still sparse.
29 CRITICAL STEP: Activating a small defined region will reduce phototoxicity and
30 experimental time.
- 31 6. Within the Zen FCS module, select the point of acquisition with the crosshairs options
32 using the mCherry reference to identify the membrane. In our setup, the FCS
33 measurement of EphB2-paGFP was carried out with the 488 nm laser line by acquiring
34 4 x 25 s cycles of data collection. Multiple cycles were collected as the membrane may
35 ruffle in and out of focus slightly, so the best of the four cycles was analyzed.
36 CRITICAL STEP: Measuring small structures such as membranes can be difficult,
37 therefore the application of scanning-FCS may be desirable.
- 38 7. Fit the FCS data to a previously published 2-species model¹⁰⁰. This provides the ACF and
39 in our case resulted in the identification of two rates of motion at 0.03 and $0.04 \mu\text{m}^2 \text{s}^{-1}$
40 at regions with and without cell-to-cell contact, respectively.

41 - END OF BOX 2 -

42

43 SUPPLEMENTARY INFORMATION

- 1 - **Supplementary Video:** Screen recording of the analysis of a single cell using eN&B software
- 2 - **Supplementary Figure 1:** Example of photobleaching detrending on cells that were not
- 3 stimulated with the ligand shows consistent brightness correction