Journal of Visualized Experiments

Oligomerization dynamics of membrane receptors in living cells by Total Internal Reflection Fluorescence Microscopy and Number and Brightness analysis

--Manuscript Draft--

Article Type:	Methods Article - JoVE Produced Video
Manuscript Number:	JoVE60398R1
Full Title:	Oligomerization dynamics of membrane receptors in living cells by Total Internal Reflection Fluorescence Microscopy and Number and Brightness analysis
Section/Category:	JoVE Biology
Keywords:	Receptor clusters, N&B, Number and Brightness, moment analysis, protein oligomers, cell membrane, TIRF microscopy
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Additional Information:	
Question	Response
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Madrid, Madrid,Spain

1 **TITLE:**
2 Oligomerization Dynamics of Cell Surface Receptors in Living Cells by Total Internal Reflection
3 Fluorescence Microscopy Combined with Number and Brightness Analysis
4
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27 **KEYWORDS:**
28 Receptor clusters, N&B, Number and Brightness, moment analysis, protein oligomers, cell
29 membrane, TIRF microscopy
30
31 **SUMMARY:**

32 We describe an imaging approach for the determination of the average oligomeric state of
33 mEGFP-tagged-receptor oligomers induced by ligand binding in the plasma membrane of living
34 cells. The protocol is based on Total Internal Reflection Fluorescence (TIRF) microscopy
35 combined with Number and Brightness (N&B) analysis.
36
37 **ABSTRACT:**
38 Despite the importance and ubiquity of receptor oligomerization, few methods are applicable
39 for detecting clustering events and measuring the degree of clustering. Here, we describe an
40 imaging approach to determine the average oligomeric state of mEGFP-tagged-receptor
41 homocomplexes in the membrane of living cells. The protocol is based on Total Internal
42 Reflection Fluorescence (TIRF) microscopy combined with Number and Brightness (N&B)
43 analysis. N&B is a method similar to fluorescence-correlation spectroscopy (FCS) and photon
44 counting histogram (PCH), which are based on the statistical analysis of the fluctuations of the

45 fluorescence intensity of fluorophores diffusing in and out of an illumination volume during an
46 observation time. In particular, N&B is a simplification of PCH to obtain information on the
47 average number of proteins in oligomeric mixtures. The intensity fluctuation amplitudes are
48 described by the molecular brightness of the fluorophore and the average number of
49 fluorophores within the illumination volume. Thus, N&B considers only the first and second
50 moments of the amplitude distribution, namely, the mean intensity and the variance. This is, at
51 the same time, the strength and the weakness of the method. Because only two moments are
52 considered, N&B cannot determine the molar fraction of unknown oligomers in a mixture, but
53 it only estimates the average oligomerization state of the mixture. Nevertheless, it can be
54 applied to relatively small time series (compared to other moment methods) of images of live
55 cells on a pixel-by-pixel basis, simply by monitoring the time fluctuations of the fluorescence
56 intensity. It reduces the effective time-per-pixel to a few microseconds, allowing acquisition in
57 the time range of seconds to milliseconds, which is necessary for fast oligomerization kinetics.
58 Finally, large cell areas as well as sub-cellular compartments can be explored.
59
60 **INTRODUCTION**
61 We describe a Total Internal Reflection Fluorescence-Number and Brightness (TIRF-N&B)
62 imaging approach for determining the average oligomeric state of receptor molecules at the
63 plasma membrane of live cells, aiming at linking the receptor assembly dynamics to the
64 biological function of the proteins (**Figure 1**).
65
66 Upon extracellular ligand binding, receptors initiate the intracellular signal transduction
67 depending on their conformation, oligomerization, potential co-receptors and membrane
68 composition. Despite the importance and ubiquity of receptor oligomerization, recognized as a
69 key event in cellular signaling[1-7], few methods can detect clustering events and measure the
70 degree of clustering experimentally[8,9]. The confocal volume (x,y $\approx$ 300 nm, z $\approx$ 900 nm) is
71 insufficiently resolved for proving molecular interaction and stoichiometry, even after
72 optimization by image restoration algorithms[10]. The sub-unit composition of protein oligomers
73 cannot be resolved on a purely spatial basis even by super-resolution methods at x,y resolution
74 of 20-70 nm such as PALM[11], STORM[12], and STED[13]. Moreover, their temporal resolution (in the
75 order of minutes per image) cannot follow kinetics in the range of seconds. Single molecule
76 step-bleaching resolves the stoichiometry of protein oligomers only if they are immobile[14].
77
78 One of the most versatile methods to measure density and oligomerization of fluorescently
79 tagged proteins within single images is the spatial intensity distribution analysis (SpIDA), which
80 relies on spatial sampling. It is applicable to both chemically fixed and live cells, and allows the
81 analysis of several regions of interest of the cell simultaneously using standard fluorescence
82 microscopy[15]. Alternatively, moment methods, such as fluorescence-correlation spectroscopy
83 (FCS)[16], photon counting histogram (PCH)[17], and Number and Brightness (N&B)[18,19], are suitable
84 for quantitative oligomer measurements. These methods analyze the fluorescence intensity
85 fluctuations that can be observed in time when the fluorophores diffuse in and out of an
86 illumination volume. The amplitudes of the intensity fluctuations can be uniquely described by
87 the molecular brightness of the fluorophore (ε) and the average number of fluorophores (n)
88 within the illumination volume[17] (**Figure 2**). Typically, the diffusion coefficient of the

89 fluorophores and the average number of molecules (inversely related to the G(0) value) within
90 the illumination volume can be obtained by FCS[20]. However, since the diffusion time only scales
91 with the cubic root of the mass, FCS is not sufficiently sensitive to detect changes in molecular
92 mass[21]. In practice, single color FCS cannot detect dimerization of membrane receptors. PCH
93 resolves mixtures of different oligomers accurately. Using more than two moments of the
94 amplitude distribution, it detects molecules of different brightness that occupy the same
95 illumination volume. Scanning FCS[22] and developments, such as the interesting pair-correlation
96 of molecular brightness (pCOMB) approach[23], introduced to extend the range of applicability of
97 fluorescence correlation methods in biological systems[24], remain single point methods lacking
98 the capability of fast measurements in a large area of a cell, requiring many consecutive
99 observations at each pixel and data acquisition in the order of seconds.
100
101 N&B is a simplified version of PCH that considers only the first and second moments of the
102 amplitude of the fluorescence distribution, namely the mean intensity, <I>, and the variance, σ^2
103 (**Figure 2**)[18,19] and, because of that, it cannot determine the molar fraction of unknown
104 oligomers in a mixture, but only estimates the average oligomerization state of the mixture.
105 Nevertheless, N&B has the advantage of working with relatively smaller time series of images of
106 live cells than PCH on a pixel-by-pixel basis, simply by monitoring the fluctuations on time of the
107 fluorescence intensity. Because N&B reduces the time-per-pixel to a few microseconds, it can
108 follow fast oligomerization kinetics over large cell areas, allowing image acquisition on a time
109 scale of seconds in raster scanning microscopy (e.g., confocal, 2-photon) and milliseconds in
110 camera-based microscopy (e.g., TIRFM).
111
112 Several reports have demonstrated the capability of N&B to quantify the number of subunits in
113 protein clusters by imaging extended cell regions. Paxillin-EGFP clusters were detected at the
114 adhesion sites in CHO-K1 cells[25], and the intracellular aggregation of the pathogenic Httex1p
115 peptide was described in COS-7 cells[26]. N&B was applied for following the ligand-driven
116 oligomerization of the ErbB receptor[27], and the effect of the ligand FGF21 on Klothob (KLB) and
117 FGFR1c in HeLa cells[28]. The combination of TIRF imaging and N&B analysis was used to show
118 that dynamin-2 is primarily tetrameric throughout the entire cell membrane[29]. We applied N&B
119 to both raster scanning and TIRF images to prove ligand-driven dimerization of uPAR and FGFR1
120 cell membrane receptors[30,31].
121
122 Fluorescence correlation methods, such as N&B, FCS and PCH, are based on the notion that in
123 an open volume the occupation number of particles follows a Poisson distribution. Because
124 only the photons that the fluorophores emit can be detected, the mean value for a measured
125 fluorescence intensity versus time in a pixel of the image, $\langle I \rangle$, is the product of the average
126 number of fluorophores in the illumination volume, n, and their molecular brightness, ε[17]:
127
128 $eq.\, 1 \,\langle I \rangle = \varepsilon n$
129
130 where ε is expressed as the number of photons emitted per unit of time (conventionally per
131 second) per molecule when the molecule is at the center of the illumination volume.
132

133   Brightness is a property of each fluorophore in a given acquisition set up, while intensity is the
134   sum of all contributions from all fluorophores. In biological contests, brightness will increase
135   with the increase of the number of fluorophores that fluctuate together, giving information on
136   the oligomerization state of the fluorescently-tagged protein. The fluctuation amplitudes at a
137   given pixel is measured from the variance of the fluorescence signal, σ^2:

139    $eq.\,2\;\sigma^2 = \langle I^2 \rangle - \langle I \rangle^2$

141   Where the mean of the square of intensity, $\langle I^2 \rangle$, and the square of the mean of intensity, $\langle I \rangle^2$,
142   are computed from the individual intensity values in each pixel of each frame:

144    $eq.\,3\;\dfrac{I_1 + I_2 + I_3 + \cdots I_K}{K} = \langle I \rangle$

146    $eq.\,4\;\dfrac{I_1^2 + I_2^2 + I_3^2 \ldots I_K^2}{K} = \langle I^2 \rangle$

148   where K is the number of total frames in the time series. Experimentally, it is necessary to
149   compute for the entire image series the variance that describes the scatter of the individual
150   intensity values at each pixel of a single image around the mean intensity value. The variance
151   includes all fluctuations of different origins. In a first approximation, the variance due the
152   diffusing particles in the illumination volume, σ^2_0, can be separated from the variance due to
153   the detector shot noise, σ^2_d. The two variances are independent; thus, the total variance is
154   given by their sum:

156    $eq.\,5\;\sigma^2 = \sigma_0^2 + \sigma_d^2$

158   The variance, due to molecular fluctuations in and out of the detection volume, is linearly
159   dependent on the molecular brightness and intensity:

161    $eq.\,6\;\sigma_0^2 = \varepsilon \langle I \rangle$

163   Rearranging eq. 6 according to eq. 1:

165    $eq.\,7\;\sigma_0^2 = \varepsilon^2 n$

167   According to the typical concept in fluorescence correlation spectroscopy, equation 7 states
168   that the variance due to the number of fluctuations depends on the square of the particle
169   brightness.

171   Then, the variance due to detector fluctuations is a linear function of the detected intensity,
172   under the assumption that the detector is operated below its saturation limit[19]:

174    $eq.\,8\;\sigma_d^2 = a \langle I \rangle + c$

175

176 In the case of photon counting detectors $a=1$ and $c=0$, thus the detector variance is equal to the
177 average intensity:

178

179 $eq. 9 \; \sigma_d^2 = \langle I \rangle$

180

181 To apply these concepts to real measurements in live cells, Gratton and colleagues[18] define the
182 apparent brightness, B, for each pixel as the ratio of the variance over the average intensity:

183

184 $eq. 10 \; B = \dfrac{\sigma^2}{\langle I \rangle} = \dfrac{\sigma_0^2}{\langle I \rangle} + \dfrac{\sigma_d^2}{\langle I \rangle} = \dfrac{\varepsilon^2}{\varepsilon n} + \dfrac{\varepsilon n}{\varepsilon n} = \varepsilon + 1$

185

186 B is the parameter that is measured experimentally. In this work, time series images of FGFR1
187 receptors at the plasma membrane of HeLa cells are captured by TIRF microscopy and the
188 average apparent brightness, B, is determined by the N&B analysis. Then, after addition of
189 FGF2, consecutive time series are captured to follow the changes in the self-assembly of the
190 receptor molecules in the membrane surface after stimulation of the receptor with the
191 canonical ligand.

192

193 However, since the detector of the TIRF microscope is a EMCCD camera, the expression for the
194 apparent brightness needs to be modified as[19]:

195

196 $eq \; 11 \; B = \dfrac{\sigma^2 - \sigma_d^2}{(\langle I \rangle - offset)}$

197

198 where *offset* is the intensity offset of the detection electronics that is a characteristic of the
199 detector settings. The variance and average intensity for an analog detector are respectively
200 given by:

201

202 $eq. 12 \; \sigma^2 - \sigma_d^2 = SG\varepsilon n + G^2 \gamma \varepsilon^2 n$

203

204 $eq. 13 \; \langle I \rangle - offset = G\varepsilon n$

205

206 where G is the analog gain in digital levels (DL/photons), *S*, the digital levels per photon[19], is
207 given by the slope of an intensity versus variance plot for a light source with constant intensity
208 (no temporal fluctuations). The γ factor is related to the shape of the pixel detection volume.
209 According to Hassler et al.[32], the γ factor is equal to 0.3 for TIRF imaging working at the
210 maximum gain of the detection camera[19]. The offset, S and G parameters are characteristics of
211 the camera and the microscope. The apparent brightness, B, is obtained by rearranging eq. 11
212 according to eq. 12 and 13:

213

214 $eq \; 14 \; \dfrac{B}{S} = \dfrac{G}{S} \gamma \varepsilon + 1$

215

216 Experimentally, ε is a complex function of laser intensity and the detection efficiency of the
217 system. Nevertheless, since B/S is linearly dependent on ε, it is only important to determine the
218 relative value of ε for a given detection mode:
219

220 $$eq.\,15 \quad \frac{B}{S} = B' = \varepsilon' + 1$$

221
222 where ε' is proportional to ε. Still, a calibration is performed using an internal reference.
223
224 **PROTOCOL**
225
226 **1.   Sample preparation**
227
228 1.1.   Day 1. Seed HeLa cells in complete medium at a concentration of 100,000-200,000
229 cell/mL in glass-bottom dishes. Seed 6-8 replicate dishes.
230
231 NOTE: In this example, the medium is supplemented with 10% heat inactivated Fetal Bovine
232 Serum (FBS), 1 mM sodium pyruvate, 100 U/100 µg penicillin/streptomycin. Several replicate
233 dishes are prepared.
234
235 1.2.   Day 2-3. When cells are at sub-confluency, transfect half of the dishes with the protein
236 plasmid and the second half with reference plasmids (monomer and dimer), in serum-free
237 medium.
238
239 NOTE: Transfection is made in serum free medium supplemented with antibiotics, 0.1% Bovine
240 Serum Albumin and 25 mM HEPES buffer, without Phenol Red.
241
242 1.3.   Day 3-4. Check that the transfected cells are adherent to the bottom of the dishes and
243 the cell membrane is fluorescent. Discard dishes with overgrown cells or with very low
244 fluorescence.
245
246 NOTE: Do not let cells overgrow. Cells must be well distributed and be adhered to the glass area
247 of the dish (**Figure 1A**). Precoated glass bottom dishes can be used for favoring cell adhesion.
248 The cell culture is tested for mycoplasma contamination before any experiment. In this
249 example, cells are transfected with a (A207K)mEGFP-FGFR1 plasmid and the reference cells are
250 transfected with a GPI-(A207K)mEGFP and a GPI-(A207K)mEGFP-(A207K)mEGFP plasmids using
251 standard protocols. For live cell microscopy, an indicator-free medium is recommended; 25 mM
252 HEPES buffer is added to prevent pH changes during imaging.
253
254 **2.   TIRF imaging - alignment of the laser line and optimization of TIRF illumination**
255
256 2.1.   Four hours before experiment, activate the temperature incubator of the microscope at
257 37 °C.
258

259 2.2.   Turn on the microscope, computers and cameras and wait for the cameras to reach the
260 proper working temperature.

262 NOTE: The working temperature of the camera used in this study is -70 °C.

264 2.3.   Place a little drop of oil on the objective. Put a sample dish in place. Close the doors of
265 the incubator and let the temperature of the dish equilibrate (~10 min).

267 2.4.   Turn on the epifluorescence lamp and the 488 nm laser.

269 2.5.   Select the epifluorescence contrast mode to explore the sample, searching a cell to
270 focus from the ocular.

272 NOTE: The use of a fluorescent lamp for searching cells trough the ocular is not mandatory. A
273 suitable laser line can be used instead.

275 2.6.   Select the proper filter for collecting the green emission through the microscope camera
276 (Band Pass Ex 490/20 (500) Band Pass Em 525/50, or similar.

278 2.7.   Switch from the ocular to the camera port (camera #1 in **Figure 1**) in epifluorescence
279 mode, refine the focus and change to TIRF mode. Epifluorescence and TIRF modes might be
280 named with a different nomenclature depending on the brand of the microscope.

282 NOTE: There may be issues focusing or aligning the laser if there are no fluorescent markers at
283 the coverslip interface. To align the laser properly (essential for good TIRF), focus on the
284 coverslip. It is often very difficult to determine whether the coverslip is in focus. As a
285 suggestion, focus on the edges of the cells.

287 2.8.   Activate the auto-alignment following the instructions of the TIRF microscope.

289 NOTE: Briefly, for steps from 2.4 to 2.8, first find the cells through the ocular and focus on
290 them, then send the emission to the camera port of the TIRF microscope, re-focus the cells on
291 the microscope computer screen and activate the procedure for laser alignment. The alignment
292 consists in finding the critical angle at which illumination becomes evanescent (**Figure 3**).
293 Commercial microscopes might have slightly different alignment protocols and also be fully
294 automated; others might have a small camera for facilitating the visualization of the critical
295 angle conditions.

297 2.9.   Choose a suitable illumination depth and optimize the direction of the evanescent field
298 (**Figure 3**).

300 **3.   TIRF imaging: capture of the time series**

302 3.1.   Define a region of interest (ROI) of at least 256 x 256 pixels.

303

304 NOTE: In this set up, the capture is done with camera #2 under software that directly controls
305 only the camera (See **Figure 1** legend).

306

307 3.2.   Set the exposure to 1 ms and the EM gain to 1000 (this is the G factor in eq. 12 and 13).
308 At such a speed, it might be necessary to adjust or increase the laser power. Here laser power is
309 0.5 mW.

310

311 NOTE: Depending on the type of the camera and the limits imposed by the diffusion coefficient
312 of the protein, fluorescence intensity and background, the general criteria for setting the laser
313 power are not to saturate the detector, minimize photobleaching, and capture as fast as
314 possible at a reasonable S/N. The EM gain is always set at the maximum of the camera (see
315 Introduction).

316

317 3.3.   Run a first trial sequence under initial conditions and roughly estimate the S/N value.
318 The conditions are acceptable at S/N = 2-3 or higher, as measured in the first frame of the first
319 time series.

320

321 3.4.   Use the slider of the emission splitting system connecting camera #2 to the microscope
322 for masking a side of the image (**Figure 1B, Figure 4A-B**)

323

324 NOTE: In this set up a multichannel imaging connector is installed on camera #2 to enable the
325 acquisition of two spatially identical images simultaneously. The system is equipped with slides
326 for mounting different emission filters. One of the sliders mounts a black mask to cover a side
327 of the image. The masked area is used for the internal calibration of each time series, to
328 determine the camera parameters (eq. 12 and eq. 13). In this way there is no need for an
329 independent calibration step and, importantly, calibration is performed in parallel to the
330 capture of each time series. In the absence of this system, the camera can be calibrated
331 applying published protocols[33].

332

333 3.5.   Select the camera **file autosave** option.

334

335 3.6.   Start the acquisition of the image series. Acquire a minimum of 700 frames at a
336 minimum S/N ratio of 2.

337

338 NOTE: The number of frames that are necessary for analysis depends on the sample stability to
339 photobleaching and on the dispersion of the data. Therefore, the quality of each time series is
340 assessed during N&B analysis.

341

342 3.7.   Without taking the dish out of the microscope, add the ligand.

343

344 3.8.   Select a cell with a bright fluorescence membrane and quickly start the first time series
345 of the kinetic run.

346

347 NOTE: If the addition of the ligand is done quickly, this first capture sets the point = 0 time of
348 the ligand kinetics. The software registers the exact time of the capturing.
349
350 3.9.   Search a second cell and acquire the second time point of the kinetics.
351
352 NOTE: Point-visiting routines are available in some microscopes equipped with x,y,z motorized
353 stages. These allow the memorization of multiple positions on the cell dish, and can help in
354 keeping a more constant interval of time between image-series on different cells.
355
356 3.10.   Capture a new cell for each time point of the kinetic run.
357
358 NOTE: After capturing, a cell is partially photobleached and it cannot be re-imaged. Because of
359 that, the kinetics is obtained by combining time series of many cells, each captured at a
360 different time point.
361
362 3.11.   For each new dish, repeat the protocol from step 2.3 to 3.9.
363
364 NOTE: For reference dishes, add a volume of the vehicle (PBS supplemented with 0.01% bovine
365 serum albumin) equivalent to that used for the ligand.
366
367 **4.   Number & Brightness (N&B): quality check of the time series**
368
369 4.1.   Convert and save as .TIFF the files acquired with the camera software (.sif files in this
370 example).
371
372 4.2.   Import .TIFF files in the analysis software routine by activating the N&B graphical user
373 interface (GUI) MATLAB.
374
375 NOTE: A customized Matlab executable N&B routine is used here (N&B analysis at
376 https://www.cnic.es/en/investigacion/2/1187/tecnologia). By opening an imported .TIFF file,
377 the routine generates the average intensity image, the average intensity profile and it allows
378 inspecting the series frame-by-frame (**Supplemental Figure S1**). Other software are available
379 for N&B analysis (e.g., SimFCS software).
380
381 4.3.   Discard series for which the average intensity profile shows more than 10%
382 photobleaching, and series in which there has been an evident cell membrane distortion or
383 translation during acquisition.
384
385 4.4.   Crop frames that are evidently out-of-focus.
386
387 NOTE: A cropping tool is implemented in the routine to discards single or multiple frames
388 within the image series. This operation is allowed because frame-to-frame time is not critical
389 whereas the pixel dwell time (exposure time) is (see Discussion).
390

391   4.5.   Keep for the analysis only series with at least 500 time frames.

392

393   **5.**   **Number & Brightness (N&B): determination of the camera parameters (offset, σ and S)**

394

395   5.1.   Activate the routine **Calibrate Camera**.

396

397   5.2.   Select an area of at least 20x50 pixels in the detector noise region (**Figure 4**).

398

399   NOTE: The routine originates a histogram of the values (also defined Digital Level, DL) and it
400   returns a logarithm plot of the Frequency versus Digital Levels.

401

402   5.3.   In the log Frequency versus Digital level plot, move the linear red cursor to delimit the
403   Gaussian and the linear part of the curve.

404

405   NOTE: The red cursor divides the two sections of the curve, and activates the routine returning
406   the offset, which is the center of the Gaussian function of the camera response, the σ of the
407   Gaussian fit, and the S factor, which is the slope of the linear part of the camera response
408   (**Figure 4C-D**).

409

410   **6.**   **Number & Brightness (N&B): computation of the B-values in selected region-of-**
411   **interest (ROI)**

412

413   6.1.   Activate the **B** key.

414

415   NOTE: This action generates the average intensity image (**Figure 5**, first column) and the B-
416   image in which each individual B-value is associated to the related pixel in the image
417   (**Supplemental Figure S1**).

418

419   6.2.   Apply a minimum binning (2 2) to reduce the dispersion of the data and to generate the
420   B-I histogram (Figure 5, second column).

421

422   NOTE: The B-I histogram represents the distribution of the B-values of all pixels of the image
423   versus the pixel intensity. $Y = B/S$; $X = (\langle I \rangle - \text{offset})/S$ (**Supplemental Figure S1** and eq. 11 and
424   15).

425

426   6.3.   Inspect the B-I histogram using the iterative square cursor.

427

428   6.4.   Select a square ROI for the analysis (**Figure 5**, third column).

429

430   NOTE: The cursor synchronizes a mobile mask on the average intensity image, highlighting the
431   pixels that are selected inside the square cursor area (**Supplemental Figure S1**). By this
432   inspection, it is possible to exclude from the analysis the background and areas with very low
433   intensity.

434

435   6.5.   Generate the B-map of the selected ROI (**Figure 5**, fourth column).

437   6.6.   Save the ASCII file of the B-values associated to the selection.

439   6.7.   Import the ASCII file in a graphic software to compute the frequency distribution of the
440   data and obtain the average B-value ± S.E (**Figure 5**, fifth column).

442   NOTE: If data are homogeneous, the frequency distribution of the B—values approximates a
443   Gaussian distribution.

445   6.8.   Apply eq. 15 to derive the average brightness $\langle \varepsilon' \rangle = \langle B' \rangle - 1$ [(counts /molecule) per
446   dwell time] for each cell at each time point of the kinetic run. Normalize the data according to:

$$eq.\,16 \quad \frac{\langle \varepsilon'_t \rangle}{\langle \varepsilon'_0 \rangle} = \frac{\langle B'_t \rangle - 1}{\langle B'_0 \rangle - 1}$$

448   where $\langle B'_t \rangle$ is the average B-value measured at time "t" after ligand addition, and $\langle B'_0 \rangle$ is the
449   average B-value measured at the time t=0 (10-20 s after ligand addition).

451   NOTE: The normalization of the results allows the direct comparison of experiments that are
452   carried out in different days. It compensates for differences in the measured brightness due to
453   laser power and technical fluctuations.

455   6.9.   Plot the Normalized Average Brightness versus acquisition time to build the kinetic run
456   (**Figure 6**).

REPRESENTATIVE RESULTS

The results for two representative HeLa-mEGFP-FGFR1 cells seeded in the same culture dish are shown in **Figure 5** and supplemental **Table S1**. The two cells were captured at time 0 min (**Figure 5A, top**) and 7 min (**Figure 5A, bottom**) after addition of the FGF2 ligand.

Figure 5 also shows the results of two representative HeLa cells expressing either the pure monomer, GPI-mEGFP (**Figure 5B, top**), or the covalently linked dimeric fluorophore, GPI-mEGFP-mEGFP (**Figure 5B, bottom**), exposed at the cell membrane and captured under the same experimental conditions.

The average apparent brightness, B, in the HeLa-mEGFP-FGFR1 cells increases from 1.070 ± 0.001 S.E. to 1.141 ± 0.001 S.E., whereas the reference monomeric (**Figure 5B, top**) and dimeric (**Figure 5B, bottom**) samples return B values of 1.070 ± 0.001 S.E. and 1.141 ± 0.001 S.E respectively. Thus, by comparison, the FGFR1 receptor is present mainly in the monomeric form at the cell membrane surface at start, but it progresses towards a predominant dimeric state upon stimulation with its canonical ligand FGF2. On average then, the prevalent state of the FGFR1 molecules in the two representative cells is clearly different.

By applying the analysis to several cells in the same dish, each one captured at a different time point, the average brightness as a function of time is obtained (**Figure 6A**). The kinetic run in

478   **Figure 6A** describes a slow process of dimerization that persists for several minutes at the cell
479   surface. The FGF2-induced FGFR1 dimerization and subsequent internalization of the receptor
480   is a well-known mechanism[34]; therefore, the results are in full agreement with the present
481   notion on the FGFR1 signal transduction, and confirm the potentiality of the TIRF-N&B
482   approach for studying the oligomerization of cell membrane proteins up to the determination
483   of subtle monomer-dimer dynamics.
484
485   The normalized average brightness analysis of the results is a suitable tool for comparing the
486   effect of different ligands on the same receptor. One example is given in **Figure 6B**. The
487   protocol was repeated using the same standards and stimulating the cells with a non-canonical
488   FGFR1 ligand, NCAM-Fc (50 μg/mL). In this case, the kinetic profile reveals fast and cyclic
489   transitions of the receptor in oligomeric mixtures, which also reaches brightness values above
490   that of the dimer. A normalized average value of 3 is repeatedly observed. However, the
491   limitation of the N&B analysis (only two moments of the intensity fluctuation versus time are
492   considered) does not allow to demonstrate undoubtedly the formation of a trimeric form. The
493   same normalized average brightness could be the result of various combinations of larger
494   oligomers and monomers of the receptor. Yet, the results clearly demonstrate the
495   spatiotemporal differences of the effect of the two ligands on the same receptor.
496
497   **FIGURE AND TABLE LEGENDS**
498   **Figure 1: Overview of the experimental protocol.**
499   (**A**) Cells are plated on glass bottom dishes and transfected with the fluorescently tagged
500   receptor. (**B**) Time series images are captured on a commercial TIRF microscope equipped with
501   a TIRF 100x1.46 oil objective and incubator chamber. In this commercial setup, the software
502   does not allow the built-in EMCCD camera #1 to work at very short exposure times necessary
503   for acquiring N&B time series. This is an important point since the exposure time limits the
504   range of molecular diffusions that can be captured. The shortest the exposure time, the faster
505   the molecular diffusion that can be analyzed. Exposures ranging from 0.5 to 1 ms are
506   sufficiently fast for membrane protein diffusion. Therefore, a second EMCCD camera (#2) is
507   added to an additional port of the microscope to work directly under the camera software,
508   bypassing the microscope software. In this adapted configuration, the microscope software and
509   camera #1 are used only for TIRF alignment. TIRF time series are then acquired using camera #2
510   that runs at very short exposure times such as 1 ms and at the maximum EM gain. Camera #2
511   also has a pixel size of 124 nm that allows oversampling and binning of the images (see protocol
512   section 6.2). Other configurations to gain imaging speed are possible, depending on the
513   characteristics of different TIRF microscopes, whereas the use of sCMOS cameras is not
514   advisable, because noise is not random in the image[35]. (**C**) After capturing, time series are
515   inspected as a quality check. Series are discarded if photobleaching is higher than 10% as it can
516   be determined by plotting the average frame intensity versus frame number. Series are also
517   discarded if there has been an evident distortion of the cell membrane or translation of the cell
518   during acquisition. (**D**) The average intensity in each pixel is saved. (**E**) A B-I histogram
519   representing the apparent brightness, B, in each pixel of the image is generated. (**F**) The B-I
520   histogram is used for selecting a ROI that is above the background. (**G**) The frequency
521   distribution of the B-values is analyzed to determine the average B-value ± S.E.

523 **Figure 2: N&B principle.**
524 N&B quantifies the average oligomerization state of fluorophores by measuring the
525 fluorescence fluctuations that occur when they move in and out the illumination volume during
526 the acquisition of a time stack series of "K" images. The amplitude of the fluctuations is
527 characterized statistically by computing the ratio between variance of the fluctuating signal, σ^2,
528 and mean intensity value, $\langle I \rangle$. In the simplest scenario (**A**), when the illumination volume is
529 empty (i.e., no fluorophores), the ratio describes instrument noise. If the fluorescence signal
530 fluctuates due to mobile fluorophores (**B, C**), the "extra" variance is directly proportional to the
531 molecular brightness, ε (detected photon counts per molecule and per second), of the diffusing
532 molecules. In (**B**), there are 8 monomeric diffusing fluorophores and in (**C**), the same
533 fluorophores diffuse as 2 tetrameric oligomers. In these two cases, the average intensity is the
534 same, but the standard deviation and brightness are different (1ε, 4ε), because the amplitudes
535 of the fluctuations are different. $\varepsilon = 0$ when fluorophores are immobile or absent.
536

537 **Figure 3: TIRF microscopy.**
538 Although N&B runs equally well with raster scanning microscopes equipped with multiphoton,
539 continuous wave or pulsed single photon lasers and analogue or photon counting detectors,
540 TIRF microscopy is ideal for fast temporal imaging of molecular dynamic events occurring at or
541 near the cell surface with speeds, resolution and signal/noise ratio (S/N) that are not possible to
542 achieve by other imaging techniques. (**A**) TIRF microscopy employs the principle of total
543 internal reflection by which an angled excitation laser light excites only fluorophores that are
544 just underneath a glass-water interface of the coverslip. The laser irradiates the specimen at an
545 angle of incidence greater than or equal to the critical angle of refraction whereas the
546 excitation laser light is totally reflected. The reflection generates a very thin electromagnetic
547 field in the specimen called the evanescent wave. The resulting fluorescent light emitted by the
548 tiny illuminated section of the specimen is collected through a microscope objective placed
549 perpendicularly below to the slide. (**B**) The panel shows a representative example at a given
550 wavelength of the relative intensity of an evanescent field versus depth, which decreases
551 exponentially with increasing distance from the surface, providing high axial resolution
552 fluorescence images. The lateral resolution is set by the numerical aperture and magnification
553 of the objective and by the pixel size of the detection camera. The cell interior is not illuminated
554 and it does not contribute to the signal with intracellular autofluorescence. Multi-angle TIRF
555 microscopes allow selecting various penetration depths. They provide a scale that depends on
556 the excitation wavelength and usually goes from 70 nm up to 250 nm depth for single color TIRF
557 image. The evanescent illumination depth chosen for this protocol is 110 nm, and it is the result
558 of a compromise between the necessity of using low laser power and the intensity of the
559 fluorescence signal that decreases sharply with the decrease of the penetration depth. It is
560 important to avoid overly large evanescent fields that can illuminate many intracellular vesicles
561 and intracellular population of fluorophores. Therefore, depending on the type of sample,
562 several penetration depths should be explored, searching for the best combination: high signal-
563 to-noise ratio, low excitation power, short exposure time, short penetration depth. Once this
564 optimization is done, the penetration depth is kept constant for all controls and samples. (**C**)
565 Representative epifluorescence and TIRF images of a HeLa-GPI-mEGFP cell after software-

guided optimization of the depth and direction of the evanescent field. Multi angle TIRF microscopes also allow to optimize the direction of the evanescent field. This step is recommended for minimizing scattering (i.e., sharp increase of the intensity and less crisp image), and it can be carried out according to the instructions of the specific microscope used. In this protocol the microscope software includes an automatic optimization of the direction of the evanescent field. For manual optimization, refer to published protocols[36,37]. (**D**) Representative cell expressing the mEGFP-FGFR1 construct in epifluorescence and after optimization of the TIRF illumination.

Figure 4: Capture of the time series and calibration of the camera response to single photons.
(**A**) Example of the first frame out of 700-frame time series captured on a HeLa-mEGFP-FGFR1 cell in the Cropped Sensor Mode. The camera chip is partially masked (red rectangles) using the dual view connector installed on the TIRF microscope (**Figure 1B**). The internal calibration regions are recognized in the average intensity image (**B**) and processed (**C**) for estimating the camera parameters by plotting the log-frequency versus Digital Levels (**D**). An analog detection system, such as an EMCCD camera, detects pulses of photocurrent instead of photon counts, and the photon pulse height distribution is quasi-exponential. The first part of the distribution is due to the amplifier and analog-to-digital converter and it contributes a Gaussian readout noise (the variance introduced by the signal recording). The most populated channel (i.e., the most frequent value) of the distribution is the *offset* (eq. 13). Using a vertical cursor in a log plot, the first part of the distribution is separated from the exponential second part, above 250 DL, which represents the average camera response to a single photon (the slope is the S factor in eq. 12). The measurement of these parameters allows estimating the density of photons that are recorded during the acquisition. Counts (DL) are in pseudo color scale. Pixel size = 124 nm; Image format = 256x256 pixels, penetration depth of the evanescent field ~ 110 nm; Calibration ROI #1 = 19x256 pixels; Calibration ROI #2 = 5x256 pixels. DL = digital levels. Exposure = 1 ms and EMGain (the G factor in eq. 12, 13 and 14) = 1000. Note that cameras working in photon counting mode with background are equivalent to analog detectors with S = 1 (eq. 12) and with σ_d^2 and offset (eq. 13) measured in the same way as for an analog system[18].

Figure 5: N&B analysis of FGFR1 oligomerization.
(**A**) Representative results from two HeLa cells in the same dish expressing mEGFP-FGFR1 and captured at time 0' (top) and 7' (bottom) after addition of 20 ng/mL FGF2, and (**B**) two HeLa cells expressing the reference constructs GPI-mEGFP (top) and GPI-mEGFP-mEGFP (bottom). The entire analysis sequence shows (from left to right): Average intensity of the time series; Plot of all B-values as a function of fluorescence intensity in which the color code represents the number of pixels having the same B-value in the image and the rectangular cursors delimit the region of interest (ROI), the background (BG) and regions of very low intensity (LI). Notice that immobile fluorophores will give B-value = 1 since in the absence of fluctuations ε = 0; B-map of the chosen ROI and the associated B-distribution histogram. The B-value distribution is fitted with a Gaussian function (dashed red line) to compute the average apparent brightness, B (full red line) and the distribution statistics (supplemental Table S1) B-value = B' = B/S (eq. 15); Intensity = $(\langle I \rangle - offset)$/S; M = monomer; D = dimer; scale bars = 10 microns. Raw data time

609   series in **Supplemental Movie 1, Supplemental Movie 2, Supplemental Movie 3,** and
610   **Supplemental Movie 4**.

612   **Figure 6: Kinetics of FGFR1 oligomerization induced by ligand activation.**
613   Representative kinetic runs described by the Normalized Average Brightness (eq. 16) of HeLa-
614   mEGFP-FGFR1 cells after stimulation with 20 ng/mL FGF2 (**A**) or 50 µg/mL NCAM-Fc (**B**). Cells in
615   the same dish were captured at increasing time points and the apparent average brightness, B ±
616   S.E., was computed from the B-distribution histograms. The figure is adapted with permission
617   from Zamai et al. Journal of Cell Science (2019)[31].

619   **Figure 7: Fast Kinetics and data interpretation.**
620   Scatter plot of the all Normalized Average Brightness values obtained from replicate kinetic
621   runs in which HeLa-mEGFP-FGFR1 cells were stimulated either with NCAM-Fc (50 µg/mL) or
622   FGF2 (20 ng/mL).

624   **Supplemental Figure S1: Screen snapshot of the analysis routine in MatLab**
625   Screen snapshot of the N&B graphical user interface (GUI) MATLAB routine showing the initial
626   analysis steps: upload time series; compute average intensity image, compute intensity profile,
627   compute B-map and B-I histogram.

629   **Supplemental Table S1: Apparent Brightness Analysis**
630   Statistics and Gaussian fitting parameters of the B-distributions in **Figure 5** were carried out.
631   The least square Gaussian fits were obtained under the constrain of standard deviation > 0 with
632   the X-range automatically chosen and maximum iterations = 1000. R square: mEGFP-FGFR1
633   monomer = 0.9957, mEGFP-FGFR1 dimer 0.9940; GPI-mEGFP = 0.9985; GPI-mEGFP-mEGFP =
634   0.9970.

636   **Supplemental Movie 1.** Time series of an HeLa-mEGFP-FGFR1 cell at time = 0 minutes after
637   FGF2 stimulation (20 ng/mL in PBS supplemented with 0.01% BSA) shown in **Figure 5**. The series
638   of 800 frames are reproduced uncompressed at 7 fps. Image format = 256x256 pixels; pixel size
639   = 124 nm; The internal calibration area is identified as dark lateral bands.

641   **Supplemental Movie 2.** Time series shown in **Figure 5** of a HeLa-mEGFP-FGFR1 cell at time = 7
642   minutes after FGF2 stimulation (20 ng/mL in PBS supplemented with 0.01% BSA) The series of
643   800 frames are reproduced uncompressed at 7 fps. Image format = 256x256 pixels; pixel size =
644   124 nm; The internal calibration area is identified as dark lateral bands.

646   **Supplemental Movie 3.** Time series shown in **Figure 5** of a HeLa-GPI-mEGFP cell after adding
647   the vehicle (PBS supplemented with 0.01% BSA). The series of 1000 frames are reproduced
648   uncompressed at 7 fps. Image format = 256x256 pixels; pixel size = 124 nm; The internal
649   calibration area is identified as dark lateral bands.

651   **Supplemental Movie 4.** Time series shown in **Figure 5** of a HeLa-GPI-mEGFP-mEGFP cell after
652   adding the vehicle (PBS supplemented with 0.01% BSA). The series of 1000 frames are

653   reproduced uncompressed at 7 fps. Image format = 256x256 pixels; pixel size = 124 nm; The
654   internal calibration area is identified as dark lateral bands.

655

656   **DISCUSSION:**

657   N&B requires several precautions in the choice of the cell model and labelling strategy. It can be
658   applied only to live cells that remain stably adhered during the image capture time. Extra
659   fluctuations due to the whole cell rigid displacement might be handled with appropriate image
660   restoration approaches[38]. However, generally when a cell moves, the cell membrane also
661   deforms, and structure deformation, producing large extra variance, introduces serious
662   limitation to the analysis of membrane proteins. In this work, the fluorescent construct is
663   expressed in the HeLa cell line because the constitutive FGFR1 is negligible. The presence of the
664   constitutive protein is a condition to avoid, otherwise mixed populations of fluorescent and
665   non-fluorescent receptor oligomers would likely form. Consequently, the N&B analysis, which is
666   based only on the brightness of the fluorescently-tagged protein population, would return an
667   unreliable estimation of the average oligomeric state. This aspect is particularly important
668   when applying N&B to detect receptor dimerization events in which the brightness only
669   increases by a factor of 2, such as the FGFR1 dimerization in the presence of the FGF2 ligand. In
670   such a case, the presence of mixed dimers can completely hide the dimerization events
671   detectable by N&B. The contamination of fluorescent oligomers with non-fluorescent
672   constitutive forms is one of the potential pitfalls of any approach based on the detection of
673   fluorescence, unless the tagged protein is largely overexpressed. However, oligomerization
674   studies in conditions of protein overexpression raise concerns about their functional
675   significance. Transiently transfected cells will likely have variable levels of fluorescence (i.e.
676   protein concentration) and are useful for testing the independence of the average brightness
677   on the protein concentration, since N&B can be applied to a wide range of concentrations,
678   under the condition of linear response of the detector (see the definition of the brightness, eq.
679   1).

680

681   Another constraint is the stoichiometric labelling of the receptor using a stable fluorophore in
682   live cells. Halo-tags, fluorescent proteins (FP) or other covalent fluorescent tags are suitable
683   labels to obtain an exact number of bound fluorophores per molecule of protein in cell. To
684   reduce the complexity of the N&B analysis, we use either fluorescent proteins or halo-tags
685   yielding a 1-to-1 fluorophore-to-protein labelling. The FP of choice must be monomeric in the
686   mature form, having a negligible self-association tendency that, otherwise, might induce
687   artificial oligomerization of the FP-tagged receptors. In this protocol, we use the (A207K)mEGFP
688   because this single point mutation abolishes the mEGFP self-aggregating tendency as previously
689   shown[31,39,40]. Regardless of the labelling strategy, the fluorescently tagged protein should be
690   checked for the retention of the biological activity in the chosen cell model, as the functionality
691   of the tagged molecules is essential for linking oligomerization events to receptor signaling. In
692   our model, we proved the autophosphorylation of the fluorescent (A207K)mEGFP-FGFR1 after
693   stimulating the transfected cells with the receptor ligand FGF2[31].

694

695   N&B is based on the diffusion of molecules in the illumination volume. In digital camera
696   detection, the exposure time (i.e., the pixel dwell time in analog detection, t_{dwell}) must be

697 short enough that one and only one configuration of particles in the focal volume is captured.
698 This is to say that the probability of a fluorophore entering or exiting the illuminated volume
699 during the exposure time is very small. The exposure time must be shorter than the
700 fluorophore residence time (τ_D) which is the average time that a fluorophore spends in the
701 illuminated volume. Therefore, before starting an N&B experiment, it is necessary to have some
702 notion about the residence time (or the diffusion coefficient) of the target protein, which in a
703 first approximation, depends on subcellular localization and molecular mass. Frame rate is the
704 inverse of the time needed for the camera to acquire an image and then completely read that
705 image out, and it is often, calculated approximately from the total number of pixels and the
706 readout rate, combined with the exposure time. Even though frame rate does not need to be
707 fast, cycle time (t_{cycle}), which is the sum of the frame rate and the time to prepare the capture
708 of the next frame, might affect the analysis. These constraints can be generalized as: $t_{cycle} \gg$
709 $\tau_D \gg t_{dwell}$. If the cycle time is too fast, the molecules will not have moved appreciably from
710 one frame to the next in that time, and will appear as immobile (B = 1). However, because
711 hundreds of frames are needed for the analysis, cycling is set as fast as possible (increasing or
712 decreasing the image format in number of pixels) to avoid cell displacements and distortion of
713 the cell membrane that would add large extra variance during the series acquisition. In the
714 example of this protocol, the slowest cycle time is 22 ms, so that 500 frames can be acquired in
715 11 s.
716
717 Molecular brightness is not an absolute quantity; It depends on the molecular properties of the
718 fluorophore (cross section and quantum yield), on the experimental set up (detector and
719 optics) as well as on the excitation conditions such as the laser power. Thus, the TIRF-N&B
720 approach yields an apparent brightness and because of that it is fundamental to set up a
721 "reference cell model" that expresses a reference construct with univocal oligomeric state. The
722 reference construct carries the same fluorophore tag of the protein under study [here, the
723 (A207K)mEGFP] and it localizes in the same subcellular compartment (here, the plasma
724 membrane). In this work we use a glycosylphosphatidylinositol (GPI) anchored-(A207K)mEGFP
725 construct that we have repeatedly demonstrated to be a reliable monomeric brightness
726 standard for cell surface receptors labelled with the same fluorophore[30,31].
727
728 One major drawback is the occurrence of fluorophore photobleaching that very likely happens
729 when the same cell is repeatedly exposed to light during a kinetic run, and leads to an
730 underestimation of the oligomerization state. To prevent that, the brightness kinetics is
731 obtained by combining the average brightness of different cells each captured at a different
732 time point (**Figure 6**). This is to say that in a Petri dish each cell is a different time point of the
733 kinetics and a petri dish represents an entire kinetic run.
734
735 When the oligomerization dynamics is fast and complex, such as the FGFR1 oligomerization
736 induced by a non-canonical ligand, NCAM[31], the profile of the normalized average brightness
737 versus time can be variable and unstable (**Figure 6B**). In such a case, reproducibility cannot be
738 determined by reading replicate dishes of cells at precise time points, due to the necessity of
739 moving and focusing on a new cell each time, select a ROI, capture and inspect/discard time
740 series. Thus, reproducibility can be evaluated in terms of kinetic profile similarity and amplitude

741 of the brightness changes.

742

743 An overview of the results is presented in **Figure 7**. The scatter plot illustrates only the average
744 brightness measured in cells of the same dish, neglecting the time of each capture. In this plot
745 the major difference induced by the two ligands on the oligomerization state of the receptor
746 remain evident, although all kinetic information is removed. However, for both set of
747 experiments (FGF2- versus NCAM-induced oligomerization), the conventional approach of
748 determining the mean ± S.D. of each run in **Figure 7** would lead to misleading conclusions since
749 the FGFR1 molecules stimulated by FGF2 clearly transit into two well defined states, whereas
750 NCAM induces unstable and cyclic oligomeric FGFR1 mixtures that would not be well
751 represented by a mean ± S.D. value.

752

753 In summary, from an experimental point of view, N&B requires only access to a microscope
754 equipped with a fast acquisition module and a dedicated software. The protein of interest can
755 be tagged with a variety of monomeric fluorescent proteins or organic fluorophores, but
756 quantitative measurements require several conditions to be fulfilled: stoichiometric labelling,
757 reference brightness standard, detector calibration and no photobleaching. In this context, the
758 N&B approach is a powerful tool to decipher the spatiotemporal oligomerization of proteins in
759 live cells. Furthermore, recent advances in resampling raw data to solve the statistical
760 weighting[41] and combining fluorescence cross-correlation spectroscopy with cross-correlation
761 N&B[42] are refining and improving the applicability of the N&B approach to protein
762 oligomerization and interaction studies.

763

771

772 **DISCLOSURES:**
773 The authors have nothing to disclose.

774

775 **REFERENCES:**
776 1   Agwuegbo, U. C., Jonas, K. C. Molecular and functional insights into gonadotropin
777 hormone receptor dimerization and oligomerization. *Minerva Ginecologica.* **70** (5), 539-548
778 (2018).
779 2   Ferre, S. et al. G protein-coupled receptor oligomerization revisited: functional and
780 pharmacological perspectives. *Pharmacological Reviews.* **66** (2), 413-434 (2014).
781 3   Marsango, S., Ward, R. J., Alvarez-Curto, E., Milligan, G. Muscarinic receptor
782 oligomerization. *Neuropharmacology.* **136** (Pt C), 401-410 (2018).
783 4   Oishi, A., Cecon, E., Jockers, R. Melatonin Receptor Signaling: Impact of Receptor
784 Oligomerization on Receptor Function. *International Review of Cell and Molecular Biology.* **338**

785   59-77 (2018).

786   5   Thelen, M., Munoz, L. M., Rodriguez-Frade, J. M., Mellado, M. Chemokine receptor
787   oligomerization: functional considerations. *Current Opinion in Pharmacology.* **10** (1), 38-43
788   (2010).

789   6   Van Craenenbroeck, K. GPCR oligomerization: contribution to receptor biogenesis.
790   *Subcellular Biochemistry.* **63** 43-65 (2012).

791   7   Wnorowski, A., Jozwiak, K. Homo- and hetero-oligomerization of beta2-adrenergic
792   receptor in receptor trafficking, signaling pathways and receptor pharmacology. *Cell Signaling*
793   *Technology.* **26** (10), 2259-2265 (2014).

794   8   Fricke, F., Dietz, M. S., Heilemann, M. Single-molecule methods to study membrane
795   receptor oligomerization. *Chemphyschem.* **16** (4), 713-721 (2015).

796   9   Vidi, P. A., Ejendal, K. F., Przybyla, J. A., Watts, V. J. Fluorescent protein
797   complementation assays: new tools to study G protein-coupled receptor oligomerization and
798   GPCR-mediated signaling. *Molecular and Cellular Endocrinology.* **331** (2), 185-193 (2011).

799   10   Trussell, H. J. in *Academic Press Library in Signal Processing* Vol. 4 eds Joel Trussell et al.)
800   3-9 (Elsevier, 2014).

801   11   Betzig, E. et al. Imaging intracellular fluorescent proteins at nanometer resolution.
802   *Science.* **313** (5793), 1642-1645 (2006).

803   12   Rust, M. J., Bates, M., Zhuang, X. Sub-diffraction-limit imaging by stochastic optical
804   reconstruction microscopy (STORM). *Nature Methods.* **3** (10), 793-795 (2006).

805   13   Nagerl, U. V., Willig, K. I., Hein, B., Hell, S. W., Bonhoeffer, T. Live-cell imaging of
806   dendritic spines by STED microscopy. *Proceedings of the National Academy of Sciences of the*
807   *United States of America.* **105** (48), 18982-18987 (2008).

808   14   Tsekouras, K., Custer, T. C., Jashnsaz, H., Walter, N. G., Presse, S. A novel method to
809   accurately locate and count large numbers of steps by photobleaching. *Molecular Biology of the*
810   *Cell.* **27** (22), 3601-3615 (2016).

811   15   Godin, A. G. et al. Revealing protein oligomerization and densities in situ using spatial
812   intensity distribution analysis. *Proceedings of the National Academy of Sciences of the United*
813   *States of America.* **108** (17), 7010-7015 (2011).

814   16   Qian, H., Elson, E. L. Distribution of molecular aggregation by analysis of fluctuation
815   moments. *Proceedings of the National Academy of Sciences of the United States of America.* **87**
816   (14), 5479-5483 (1990).

817   17   Chen, Y., Muller, J. D., So, P. T., Gratton, E. The photon counting histogram in
818   fluorescence fluctuation spectroscopy. *Biophysical Journal.* **77** (1), 553-567 (1999).

819   18   Dalal, R. B., Digman, M. A., Horwitz, A. F., Vetri, V., Gratton, E. Determination of particle
820   number and brightness using a laser scanning confocal microscope operating in the analog
821   mode. *Microscopy Research and Technique.* **71** (1), 69-81 (2008).

822   19   Unruh, J. R., Gratton, E. Analysis of molecular concentration and brightness from
823   fluorescence fluctuation data with an electron multiplied CCD camera. *Biophysical Journal.* **95**
824   (11), 5385-5398 (2008).

825   20   Hess, S. T., Huang, S., Heikal, A. A., Webb, W. W. Biological and chemical applications of
826   fluorescence correlation spectroscopy: a review. *Biochemistry.* **41** (3), 697-705 (2002).

827   21   Muller, J. D., Chen, Y., Gratton, E. Fluorescence correlation spectroscopy. *Methods in*
828   *Enzymology.* **361** 69-92 (2003).

829   22   Levi, V., Ruan, Q., Kis-Petikova, K., Gratton, E. Scanning FCS, a novel method for three-
830   dimensional particle tracking. *Biochemical Society Transactions.* **31** (Pt 5), 997-1000 (2003).
831   23   Hinde, E. et al. Quantifying the dynamics of the oligomeric transcription factor STAT3 by
832   pair correlation of molecular brightness. *Nature Communications.* **7** 11047 (2016).
833   24   Waithe, D. et al. Optimized processing and analysis of conventional confocal microscopy
834   generated scanning FCS data. *Methods.* **140-141** 62-73 (2018).
835   25   Digman, M. A., Dalal, R., Horwitz, A. F., Gratton, E. Mapping the number of molecules
836   and brightness in the laser scanning microscope. *Biophysical Journal.* **94** (6), 2320-2332 (2008).
837   26   Ossato, G. et al. A two-step path to inclusion formation of huntingtin peptides revealed
838   by number and brightness analysis. *Biophysical Journal.* **98** (12), 3078-3085 (2010).
839   27   Nagy, P., Claus, J., Jovin, T. M., Arndt-Jovin, D. J. Distribution of resting and ligand-bound
840   ErbB1 and ErbB2 receptor tyrosine kinases in living cells using number and brightness analysis.
841   *Proceedings of the National Academy of Sciences of the United States of America.* **107** (38),
842   16524-16529 (2010).
843   28   Ming, A. Y. et al. Dynamics and Distribution of Klothobeta (KLB) and fibroblast growth
844   factor receptor-1 (FGFR1) in living cells reveal the fibroblast growth factor-21 (FGF21)-induced
845   receptor complex. J*ournal of Biological Chemistry.* **287** (24), 19997-20006 (2012).
846   29   Ross, J. A. et al. Oligomerization state of dynamin 2 in cell membranes using TIRF and
847   number and brightness analysis. *Biophysical Journal.* **100** (3), L15-L17 (2011).
848   30   Hellriegel, C., Caiolfa, V. R., Corti, V., Sidenius, N., Zamai, M. Number and brightness
849   image analysis reveals ATF-induced dimerization kinetics of uPAR in the cell membrane. *FASEB
850   J.* **25** (9), 2883-2897 (2011).
851   31   Zamai, M. et al. Number and brightness analysis reveals that NCAM and FGF2 elicit
852   different assembly and dynamics of FGFR1 in live cells. *Journal of Cell Science.* **132** (1) (2019).
853   32   Hassler, K. et al. Total internal reflection fluorescence correlation spectroscopy (TIR-FCS)
854   with low background and high count-rate per molecule. *Optics Express.* **13** (19), 7415-7423
855   (2005).
856   33   Di Rienzo, C., Gratton, E., Beltram, F., Cardarelli, F. From fast fluorescence imaging to
857   molecular diffusion law on live cell membranes in a commercial microscope. *Journal of
858   Visualized Experiments.* 10.3791/51994 (92), e51994 (2014).
859   34   Beenken, A., Mohammadi, M. The FGF family: biology, pathophysiology and therapy.
860   *Nature Reviews Drug Discovery.* **8** (3), 235-253 (2009).
861   35   Joubert, J., Sharma, D. Light microscopy digital imaging. *Current Protocols in Cytometry.*
862   **Chapter 2** Unit2 3 (2011).
863   36   Gell, C., Berndt, M., Enderlein, J., Diez, S. TIRF microscopy evanescent field calibration
864   using tilted fluorescent microtubules. *Journal of Microscopy.* **234** (1), 38-46 (2009).
865   37   Burghardt, T. P. Measuring incidence angle for through-the-objective total internal
866   reflection fluorescence microscopy. *Journal of Biomedical Optics.* **17** (12), 126007 (2012).
867   38   Trullo, A., Corti, V., Arza, E., Caiolfa, V. R., Zamai, M. Application limits and data
868   correction in number of molecules and brightness analysis. *Microscopy Research and
869   Technique.* **76** (11), 1135-1146 (2013).
870   39   Caiolfa, V. R. et al. Monomer-dimer dynamics and distribution of GPI-anchored uPAR are
871   determined by cell surface protein assemblies. *Journal of Cell Biology.* **179** (5), 1067-1082
872   (2007).

873   40   Campbell, R. E. et al. A monomeric red fluorescent protein. *Proceedings of the National*
874   *Academy of Sciences of the United States of America.* **99** (12), 7877-7882 (2002).
875   41   Cutrale, F. et al. Using enhanced number and brightness to measure protein
876   oligomerization dynamics in live cells. *Nature Protocols.* **14** (2), 616-638 (2019).
877   42   Dunsing, V., Chiantia, S. A Fluorescence Fluctuation Spectroscopy Assay of Protein-
878   Protein Interactions at Cell-Cell Contacts. *Journal of Visualized Experiments.* (142) (2018).
879

Figure 1

A cells adhered on glass bottom dishes

B camera 1   camera 2
camera 1 control
camera 2 control
dual view
capture time series

C K frames

D in each pixel compute frame intensity versus time

E B-I histogram   ROI

F B-map   built the B-plot and the B-map of selected ROIs

G compute the average B-value for selected ROIs

Total Internal Reflection Fluorescence Microscopy (TIRFM)

Number and Brightness (N&B)

Figure 2

Figure 3

A

B

C

D

Figure 4

A

B

Calibration ROI #1

Calibration ROI #2

C

Counts (DL)

Counts (DL)

D

Log Frequency

offset

S

Counts (DL)

Figure 5

A (Sample): Average Intensity (mEGFP-FGFR1 t = 0", mEGFP-FGFR1 t = 7'), B-I histogram, Selected ROI, B-map, B-distribution

B (Reference): Average Intensity (GPI-mEGFP, GPI-mEGFP-mEGFP), B-I histogram, Selected ROI, B-map, B-distribution

Figure 6

A

B

Figure 7

Video or Animated Figure

Click here to access/download
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1_HeLa_mEGFP-FGFR1_FGF2_0 min.mov

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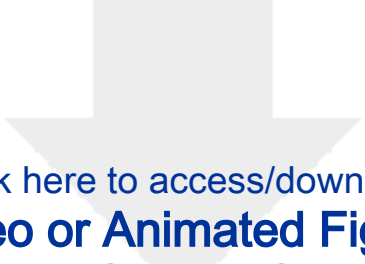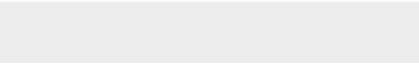
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2_HeLa_GPI-mEGFP-mEGFP.mov

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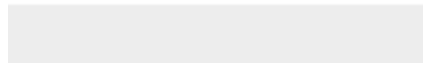
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3_HeLa_GPI-mEGFP.mov

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4_HeLa_mEGF-FGFR1_FGF2_7 min.mov
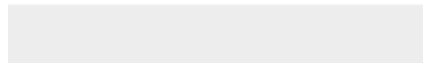
Parameter	GPI-mEGFP	GPI-mEGFP-mEGFP	mEGFP-FGFR1 (time = 0')	mEGFP-FGFR1 (time = 10')
Gaussian mean B-value	1.070	1.141	1.070	1.141
S.E. on the mean B-value	0.001	0.001	0.001	0.001
S.D. of the B-vaue distribution	0.059	0.067	0.077	0.075
95% Confidence interval of the mean B-value	1.069 to 1.071	1.139 to 1.143	1.069 to 1.072	1.139 to 1.143
S.D. of the 95% confidence interval of the B-value	0.058 to 0.060	0.065 to 0.069	0.075 to 0.079	0.073 to 0.078
Fitting constrain	S.D. > 0	S.D. > 0	S.D. > 0	S.D. > 0

S.E. standard error; S.D. standard deviation

Table of Materials

Name of Material/Equipment	Company	Catalog Number	Comments/Description
3-Colour Fast TIRF Leica AM TIRF MC inverted microscope, with smi-automatic TIRF alignment. The microscope is equipped with a diode 488 nm laser, a 100x1.46 oil TIRF objective, Ex/Em Bandpass filters at 490/20 and 525/50, temperature/CO2 incubator and Andor DU 8285 VP EMCCD camera. The microscope is operated by Leica LIF software.	Leica Microsystems, Wetzlar, Germany		
Albumin from Bovine Serum 98% minimun	Sigma-Aldrich, St. Louis, MI, USA	A7906-100G	
DMEM without Phenol Red with 25 mM HEPES	GIBCO Thermo Fisher Scientific,Waltham, MA, USA	21063029	Used serum free for microscopy
DMEM high-glucose GlutaMAX I	GIBCO Thermo Fisher Scientific,Waltham, MA, USA	10566-016	Used for complete medium
Dulbecco´s Phosphate Buffered Saline 10x (PBS)	Biowest, Nuaillé, France	X0515-500	
Emission splitting system Photometrics DV2	TeledynePhotometrics, Tucson, AZ, USA		
Fetal Bovine Serum, qualified, Brazil	GIBCO Thermo Fisher Scientific,Waltham, MA, USA	10270106	10% inactivated supplement for complete medium
Glass bottom 35-mm sterile 1.5 dishes	MatTek, Ashland, MA, USA	P35G-0.170-14-C	uncoated, glass thickness 0.17 microns
GraphPad Prism	GraphPad Software Inc., San Diego, CA, USA		
Human cervical carcinoma (HeLa), serum-free animal component (AC) cells	Millipore-Sigma ECACC, Darmstadt, Germany	CB_08011102	
iXonEM+ 897 EMCCD (back-illuminated) ANDOR camera controlled by ANDOR Solis software	Oxford Instruments, Andor TM Technology, Abingdon-on-Thames, UK		This camera, installed in an additional port of the microscope, is used for acquiring the N&B time series
Matlab Executable N&B routine	Unit of Microscopy and Dynamic Imaging, CNIC, Madrid, Spain		download at https://www.cnic.es/en/investigacion/2/1187/tecnologia
MatLab v.2018b	The MathWorks, Inc. Natick, MA, USA		download at https://www.mathworks.com/products/matlab.html
Penicillin:Streptomycin for tissue culture 100x	Biowhittaker Inc. Walkersville, MD, USA	LONZA 17-602E	supplement for medium at Penicillin/Streptomycin 100U/100µg.
pN1-mEGFP-FGFR1 expression vector	Unit of Gynecological Oncology Research, European Institute of Oncology IRCCS, Milan, Italy		Zamai et al., 2019
pN1-N-Gly-mEGFP-GPI expression vector	Unit of Microscopy and Dynamic Imaging, CNIC, Madrid, Spain		Hellriegel et al., 2011
pN1-N-Gly-mEGFP-mEGFP-GPI expression vector	Unit of Microscopy and Dynamic Imaging, CNIC, Madrid, Spain		Hellriegel et al., 2011
Recombinant FGF2	PeproTech EC, Ltd., London, UK		Ligand solution: 20ng/mL of FGF2 in PBS supplemented with 0.01%BSA.
Sodium pyruvate GIBCO	ThermoFisher Scientific	11360070	1mM supplement for medium
TransIt-LT1 Transfection Reagent	MirusBio LLC, Madison, WI, USA	MIR 2300	
Trypsin-EDTA (0.25%), phenol red	GIBCO Thermo Fisher Scientific,Waltham, MA, USA	25200056	
Type F Immersion liquid 10 mL	Leica Microsystems, Wetzlar, Germany	11513 859	
UltraPure BSA (50 mg/mL)	ThermoFisher Scientific	AM2618	0.1% supplement for medium without phenol red used for transfections

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Title of Article: Oligomerization dynamics of cell surface receptors in living cells by Total Internal Reflection Fluorescence Microscopy combined with Number and Brightness analysis

Author(s): Moreno Zamai, Antonio Trullo, Elvira Arza, Ugo Cavallaro,Valeria R. Caiolfa

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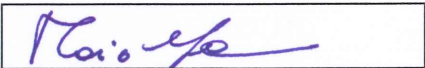
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• **Textual Overlap:** Significant portions show significant overlap with previously published work. Please re-write the text on lines 43-50, 73-75, 81-119, 140- 145, 158-165, 468-473 to avoid this overlap.
We have rephrased the lines (highlighted in blue font colour).

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The limits are fulfilled

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We think we fulfil all these requirements

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Reviewer #1:

Manuscript Summary:

This protocol describes the use of TIRF microscopy and moment analysis (N&B) for the quantification of protein multimerization in living cells. As a practical example, the dimerization of FGFR1 induced by ligand addition is described.

I found the technical approach interesting and the manuscript well-written. My major concern (see below for details) is that the protocol seems written for somebody who already has an idea of what has to be done, does not need to change or tune any condition and will use the same hardware. Nothing wrong with this but, in my understanding, the authors have here the chance to discuss the protocol in a deeper way, while also making it more approachable for new-users or users who do not work with the same hardware. We thank the reviewer for the careful and constructive revision of our manuscript. We agree with the general comment. Accordingly, we have revised the text to keep it in the length limits but, at the same time, to present a protocol less linked to the specific microscope available to us and to our model sample, with the intention of providing a guide more generally applicable by non-expert users. For this reason, we have revised the figure legends in depth. Major changes are highlighted in blue text font. Our replies to the specific comments are reported below (in blue font).

More specifically,

Major Concerns:

1- What camera is used here?
We have realized that there has been some confusion in the protocol about the use of the two cameras installed on our commercial TIRF microscope. We have carefully revised the entire TIRF section in the protocol, the legend of Figure 1 and amended the Table of Materials. The camera used for acquiring the N&B series is a iXon897 EMCCD by Andor TM Technology.

2- What are the advantages of using EMCCD vs e.g. sCMOS? Would sCMOS create trouble if one wanted to look at the moments of spatial distributions, rather than within single pixels?
EMCCD cameras have an extended multiplication register that amplifies the photoelectron signal before readout, thus, sensitivity increases two or three order of magnitude without increasing the read noise. EMCCD cameras fit well into low-light applications requiring also a moderate to high frame-rate as in case of N&B time series. More importantly, however, are the characteristics of the camera noise. Noise in scientific EMCCD and CCD cameras is generally random, following a Gaussian distribution. Read noise, photon shot noise and dark current noise follow Gaussian-type distributions. Scientific CMOS cameras have distributions of read noise across the pixels that are highly skewed outside the Gaussian distribution towards higher noise values. The skewed distribution indicates that a relevant number of pixels have a variety of noise levels and that noise is not random in the image. N&B can be applied only if the detector noise is random. The point raised by the Reviewer is important for the protocol, therefore we have included a comment in the revised legend of Figure 1 (lines 437-438).
Honestly, we are not very familiar with moments of spatial distributions, but we guess that non Gaussian noise in the images might not be optimal for the approach.

3-Does the EM value just affect the factor G or also the measurement in general (I understand that the very last question might be outside the scope though).
Yes, it does, changing the S/N. Therefore, we keep EM constant and at the highest value. We follow the method implemented by Unruh, J. R. & Gratton (2008) for EMCCD cameras in which the calibration is made a part, running a black time series alone. Since we measure the calibration parameters simultaneously to the acquisition of each time series, we could even change the EM value, but it wouldn't be recommended.

4- What is the role of pixel size (does it have to be e.g. smaller than the PSF)?

1

Yes, pixel size must be smaller than the PSF to avoid artifacts due to inhomogeneity of the illumination that causes extra variance.

5- Can a volume be calibrated so to obtain absolute concentrations?
Yes, volume can be calibrated for obtaining absolute concentrations. Due to the length limits of the text, we don't have room in this protocol to consider the computation of the average number of molecule ("N") and the analytical approach for the volume calibration.

6- What is the advantage of N&B in TIRF geometry, compared other excitation/detection schemes?
N&B runs equally well with confocal laser scanning microscopes equipped with continuous wave or pulsed single photon lasers or multiphoton microscopes, and with analogue or photon counting detectors. The inverted objective-based TIRF geometry is preferred for studying proteins at the plasma membrane and at the cell adhesion sites because the evanescent illumination. which decreases exponentially from the cover slip, doesn't penetrate the cell more than 70-250 nm adjacent to the coverslip. We have revised Figure 3 and the legend to clarify these concepts.

7- What is the influence of the pixel dwell time / frame rate?
We thank the Reviewer for this important question that we didn't discuss in the first version of the manuscript. Now we have inserted a new paragraph in the discussion dealing with these concepts (lines 628-648).

8- Using very fast acquisition, like the one described here (1 ms), wouldn´t one see the effect of fast photophysics (e.g. blinking) in fluctuations as well?
Blinking of mEGFP is in the 1-10 microsec range, our exposure time is 1 ms. In this condition blinking shouldn't affect the measurement.

9- Of course, the accumulation time is not that important, as long as it stays smaller that typical diffusion times, but what is the time between frames? This might be discussed better.
The Reviewer's comments here and in point 7 about time scales are important and, as it is said above, we have introduced a dedicated paragraph in the discussion (lines 628-648).

10- I completely missed the reason/use of the two cameras. Are two cameras needed?
We apologize again for the confusion about the two cameras. We have realized that this was a weak part of the protocol and, as it is said above, we have revised the entire TIRF section and explained the reason of our specific configuration in the legend of Figure 1 (lines 424-436). Two cameras are not needed if the proper exposure and cycle times can be obtained with the microscope built-in camera.

11- Lines 273-275: I understand the author refer to a specific software, but maybe they can explain a bit better what happens during these steps, also for non-expert users or for users working with other setups (I understand that this is rather a "style choice"). Some things can definitely be generalized though (e.g. GFP filter cube)
We have revised the entire TIRF section of the protocol to make the protocol more general and approachable by non-expert users.

12- Lines 281-282:
similar to the previous point. Why 110 nm? What happens if this number is varied?
Evanescent wave penetration is addressed in the revised legend of Figure 3 (lines 484-492).

13- What if the software does not have this button? Maybe a hint to users who have to manually change the angle and a reference to papers describing how to measure the penetration depth might be useful. Or some hint from the authors might suffice. At this regard, Figure 3 C and D should be explained and discussed in more detail.
We have revised Figure 3 legend in points C and D (lines 493-501) and added two references for angle calibration (refs Burghardt 2012 and Gell et al., 2009).

14- Is it possible to measure also at non-optimal depth?

Yes. However, non-optimal depth means that if you go too deep inside the cell you might get relevant signal from intracellular structures and vesicles and autofluorescence. If the section is too tiny, S/N might be too low when you run the series at the shortest exposure time. This point is now better discussed in the revised Figure 3 legend point B (lines 484-501).

15- What are the laser powers used (e.g. at the objective lens)?
The objective lens is specified in the Table of Materials, 100x1.46 oil and in the legend of Figure 1 (line 425). The laser power is now specified in the revised protocol (now line 280-281).
And, very important, when an user is calibrating the instrument at the beginning: what kind of signal can be expected for a certain molecule at a certain laser power?
Well, here the answer cannot be specific. We might say that one can get a rough idea of the S/N of the first frame of the first series to evaluate both, the laser power and the expression of the fluorescent construct in the chosen cell. $S/N \geq 2$ might be a starting condition. We have inserted this guideline in the protocol (lines 287-289).
I understand that one could compare the final B values to those reported here, but is there a simple way for checking at the beginning of the measurement that the setup is working in an optimized way?
The calibration of the TIRF angle is independent of the subsequent measurement. Nowadays, commercial TIRF microscopes have thorough automatic routines that are self-checking. Depth and orientation of the evanescent wave is more subjective. However, we can only give general guidelines because signal is a function of several parameters that can change a lot from one equipment to another depending on the type of laser, quality of the objective (not only magnification and NA), type and quality of filters, type and quality of the camera. At the end, as we stress in the manuscript, it is of foremost importance to have a reference sample that can be explored at different expression levels of the reference fluorescent protein construct.

16- Line 316: Can N (or the intensity) be too high (for statistics, S/N or the hardware)?
Yes it can. However, N&B is less restrictive than PCH/FCS and it covers a wider range of fluorophore concentrations. The lowest limit depends on S/N. Statistics becomes a problem for S/N < 2 (we have amended the text for this (see lines 282-286). EMCCDs are more sensitive at low-light level applications than CCD or scientific cMOS cameras. The highest limit mainly depends on the dynamic range of the camera that needs operate at the highest EM gain. Generally, the highest limit depends on the expression levels in sample. Saturation of the detector must be avoided, and in this sense, very bright clusters scattered in a dim cell area often might not be analyzed. N&B is still an approach for low-intensity signals.

17- Line 523: The authors report the widths of the B distributions. What is the physical meaning of this width? It sounds interesting but I am not sure about its use. In spatially inhomogenous samples, I imagine it says something about the inhomogeneity. But what about homogeneous samples? Is it connected to S/N? The smaller, the better?
Since we do not observe bimodal or multimodal frequency distributions, we conclude that samples are homogeneous. This means that we cannot distinguish significant areas (1000 pixels at least, as from our previous work in Hellriegel et el., 2011, supplemental figures S1-S4) that are associated with statistically different brightness. Therefore, we think that the width of the distributions mainly depends on the S/N. We work at the limit of S/N to avoid over expression of the receptor in the membrane, while we generally accept higher expression levels of the reference constructs that do not cluster and that we have repeatedly studied also in FCS. The reference samples have better S/N and narrower distribution width. In addition, we also think that the width of the distributions can be associated to minor environmental changes affecting the brightness of the fluorophore. The fitting parameters of supplemental table describe the overlap of the monomer and dimer distributions and, thus, the confidence limit of the measurements. As we showed in our previous work, the width of the distribution also depends on the number of frames in the time sequence (more frames, better statistics) and on the diffusion coefficient (supplemental figures in Hellriegel et el., 2011).

18- I was surprised to see that GFP dimers had indeed a brightness 2 times higher than the monomer. How do the authors explain it in the light of published observations regarding the presence of an offset in the linear relation between number of subunits and brightness (Slaughter et al 2008 PlosONE) or, using a

different physical model, a <100% fluorescence probability for GFP (Ulbrich et al 2007 Nature Methods, Dunsing et al 2018 Scientific Reports, Godin et al 2015 BiophysJ)?

Figure 5B in Slaughter et al 2008 PlosONE paper shows an offset due to autofluorescence. In TIRF the autofluorescence contribution to signal is minimized since the exponential decay of the evanescent wave excludes the excitation of the cell interior. Ulbrich et al 2007 Nature Methods does not apply fluctuation methods but single molecule photobleaching. The paper published by Dunsing et al in Scientific Reports 2018 is very interesting. However, the dark states are much less critical for mEGFP than for mEYFP and mCherry. Moreover, in that work N&B is applied to laser scanning confocal images at a pixel dwell time of 25 microsec, 40 times faster than our exposure time, because they track faster diffusion times. Thus, long-lived dark states might not affect our measurements of plasma membrane proteins, which we can follow with longer dwell times. Obliviously, a part from the example shown in this manuscript, we have about 10% variability on the brightness of the reference dimer, more than what we observe for the monomeric brightness (Zamai et al., 2019). Finally, although the emission probability for each fluorescent protein is considered less than 100 %, the deviation from linearity might be hidden by the relatively large distribution width of our data.

Minor Concerns:

19- Figure 1 could be explained better for non-expert users.
The legend has been re-written including more details.

20- Lines 89-94: spIDA should also be mentioned
Undoubtedly, SpIDA must be mentioned. We apologize for having neglected this very useful approach. We have added a sentence with reference (lines 79-83).

21- Line 172: I do not understand the "For that" here. The difference between epsilon and B (or the definition of B itself) do not "require" the existence of a subpopulation of immobile fluorophores. The physical reason is rather the poissonian nature of the photon detection process. But I might have just misunderstood what the authors meant here.
We have revised the text (lines 178-179).

22- Line 246: something is wrong here WHY?
We have deleted the note that did not make much sense.

23- Line 260: why both lamp and laser?
We use the lamp simply because using an excitation bandpass filter we have more light on sample. It is easier to inspect the dish quickly and select a cell with dim-medium fluorescence. We added a note that this step can also be done with laser illumination (lines 253-254).

24- New cameras work in photon counting mode. How would this affect the described approach? Photon counting mode with background is equivalent to analog mode with S=1 and with σ_d^2 and offset measured in the same way as for an analog system [Dalal et al., Micros Res Tech 2008]. We have introduced this comment in the revised Figure 4 legend (lines 520-522).

25- Line 438: The dimerization is described as a "slow process" but, for example, it seems very quick in Fig 6A. Can the process be actually followed in between cells with only monomers and cells with 100% dimers?
The process from monomers to dimers takes about 2 minutes (in terms of cell processes it is quite slow). However, we could capture intermediate states (a mixture between monomers and dimers) as in cell 4 of Fig 6A. Because of the technical constrains (search a new cell at each time point, optimize focus, activate the camera) it is impossible to collect two images from two different cells in less than one minute.

26- Figure 4B: Is there a specific reason for the shape and number of the calibration ROIs?
The shape is due only to the type of dual view adaptor that we can use. The number of pixels cannot be less than 1000 for statistics [Hellriegel et al., 2011, supplemental figures].

27- Line 470: The signal does fluctuate also in this case
The figure 2 legend is amended (lines 455-456).

28- Line 474: "equal number of molecules" might not be clear
The figure 2 legend is amended (now lines 458-460).

29- Line 505: it might useful to some reader if the authors explained how S is in practice connected to the single photon response
This has been specified in the revised text (line 202).

Reply to Reviewer #2:

Manuscript Summary:
The authors describe a protocol for measurement of membrane receptor oligomerization dynamics in live cells using N&B, TIRF microscopy and a custom software. The manuscript goes in the step-by-step experimental process from sample preparation to setting TIRF microscope and camera for acquisition. The approach for dynamics here presented uses different cells for each aggregated time-point, where each measurement consists of a number (500-700) of frames, from which one average value of Brightness is calculated. Calibration is performed on an image-to-image basis by intentionally covering part of the sensor, thus allowing for a measurement of the background parameters for each image.
We thank the reviewer for the careful and constructive revision of our manuscript.
Our replies to his individual comments are reported below (in blue font). We have modified the manuscript also to comply with Editor's requests and Reviewer # 1 comments. Accordingly, we have revised the protocol section to keep it in the length limits. At the same time to be less linked to the specific microscope available to us and to our particular biological sample, we have inserted more details in the figure legends, with the intention of providing a guide more generally applicable by non-expert readers. Major changes of the text are highlighted in blue font.

Major Concerns:
The article is well written and detailed in the experimental and imaging settings. I don't have major concerns regarding the protocol, just minor concerns regarding the specific values (for example exposure, number of frames) which are named in some of the protocol's steps.

Minor Concerns: I have a few minor concerns which I believe could help improve the manuscript.

1. line 92 of introduction, in the section describing FCS and PCH, it would be helpful for a reader to mention Scanning FCS which, while also limited in the capability of imaging large areas, can cover smaller areas larger than a single-point quite efficiently. The work of Elizabeth Hinde (Quantifying the dynamics of the oligomeric transcription factor STAT3 by pair correlation of molecular brightness. Nature Communications 2016; 7(11047).) would be a good example to reference.
As suggested, we have introduced scanning FCS. In addition to the interesting work by Hinde, we have also mentioned the original paper by Levi V., et al., 2003 that introduced the approach and a 2018 methodological reference.

2. line 249, TIRF Imaging 2.1.2 , "wait for camera to cool down" probably should be changed to "wait for camera to reach a stable temperature".
We have changed the sentence in "wait for the camera to reach the proper working temperature" and added a NOTE (lines 245-247).

3. line 274 TIRF Imaging 2.1.7 ""Contrast Method" to the " TIRF Mthod"" the authors should mention here, not only in the note below, that these two methods might have different names on different systems.
We have revised the test (lines 258-259).

4. line 286 TIRF Imaging 2.2.1 the authors could mention briefly how the exposure time is chosen for this camera. On a different note, this step of the process appears very specific for the instrument used by the authors. Is the instrument custom made or commercially available? Some details can be found in the table at the end of the manuscript but it would be helpful to know some of the essential requirements before the start of the protocol steps. In this case the step appears to require an Andor camera and Andor's Solis software, independently from the Leica instrument.
We have realized that this was a weak part of the protocol and we have revised the entire TIRF section, revised figure 1 and 3 in depth, and dedicated a new paragraph in the discussion to the topic of exposure and cycling times.

5. line 296 TIRF Imaging 2.2.3 how were exposure and gain chosen here? are they specific for the membrane receptor used in this experiment? Should exposure be dependent on the diffusion rate of the labelled receptor?

See the revised sections mentioned above. We have chosen the exposure time of 1 ms accordingly with the diffusion rate of labelled receptors in the plasma membrane. The EMgain =1000 is the highest value for the Andor camera at our disposal, and it is the value suggested by Unruh and Gratton (Unruh, J. R. & Gratton, E. Analysis of molecular concentration and brightness from fluorescence fluctuation data with an electron multiplied CCD camera. *Biophys J.* **95** (11), 5385-5398, (2008).)

6. line 322 TIRF Imaging 2.2.8 would it be more efficient to pick cells for imaging (and save corresponding positions) before adding ligand?
Many motorized stages allow for saving positions, this would ensure a more constant time between the image-series on different cells.
We have added this option in the revised text (lines 312-314).

7. line 488 Figure 3 caption: SW should be spelled out once in this paragraph for ease of reading.
Amended

8. there is a reference for N&B GUI Matlab routine (Zamai et al, 2019), however given the software is essential for this protocol it would be helpful to have a link in this manuscript (for example a webpage or cloud-storage link) where to obtain the analysis tool.
We have made our routine available and inserted a direct link for download (lines 327-328).

Figure S1