Reply to the Editorial Comments:

• Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammatical errors.

• **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).

• **Protocol Detail:** Please note that your protocol will be used to generate the script for the video, and must contain everything that you would like shown in the video. **Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc) to your protocol steps.** There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.
1) 3.2, 3.3: Mention explicit details of software actions.
We provide a link for downloading the software

• **Protocol Highlight:** Please ensure that the highlighting is continuous and does not exceed 2.75 pages.
The limits are fulfilled

• **Discussion:** JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please ensure that the discussion covers the following in detail and in paragraph form (3-6 paragraphs): 1) modifications and troubleshooting, 2) limitations of the technique, 3) significance with respect to existing methods, 4) future applications and 5) critical steps within the protocol.
We think we fulfil all these requirements

• **References:** Please spell out journal names.
For the reference list we have applied in EndNote the style dowloadable for JoVE web site, according to the link provided by your editorial office.

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We include in the submission of the revised manuscript the explicit permission of Journal Cell Science to partially reproduce some illustration. We have amended the figure legends accordingly.

From: **permissions** permissions@biologists.com
Subject: RE: Zamai et al Journal of Cell Science(2019), 132 doi:10.1242/jcs.220624
Date: 12 July 2019 at 15:10
To: Caiolfa Valeria caiolfa.valeria@hsr.it, permissions permissions@biologists.com
Cc: Moreno Zamai mzamai@cnic.es

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We wish you the best of luck with your manuscript.

Kind regards

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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)?

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).