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Dr. Antonio Virgilio Failla

Manuscript Revision

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Dear editor,
we are contacting you to submit our revision on the manuscript entitled:

" Visualizing adhesion formation in cells by means of advanced spinning disk-total internal reflection fluorescence microscopy." authors : B. Zobiak[1] (b.zobiak@uke.de), Antonio Virgilio Failla[1] (a.failla@uke.de)
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we are much grateful to you and the referees for the concerns and corrections. All the remarks and criticisms give us the opportunity to strongly improve the quality and clarity of our manuscript. We added, as requested, more details to the presented protocol and we modified several scientific statements in order to avoid misinterpretation regarding the goals of this manuscript and its scientific relevance. In the following, all the manuscript modifications that are necessary to finalize this revision will be described in details. We will first start answering the reviewers according to the order of appearance, and afterwards we will present the corrections required by the editor:

Reviewer number one:

1) "I find myself asking what purpose this manuscript really serves. The authors have already published an article describing this microscope system. Invoking superlatives regarding their technology which are not tested, even qualitatively, does not really convince this referee."

Although comments questioning the novelty of the article can be disregarded, we are grateful to the reviewer because he/she gives us the chance to clarify better the purpose of this manuscript. We added the following sentence at the end of the introduction:

"Since this microscope is commercially available the goal of this manuscript is to describe in details and provide open source tools and protocols for image acquisition, registration and visualization associated with SD-TIRF microscopy." (lines 63-66).

2) Many many people have studied adhesion dynamics with either spinning disk or TIRF. The true requirement for the combination of these approaches is never directly demonstrated.

We believe that, in this manuscript, we display once more that spinning disk TIRF microscopy can be considered as a powerful approach to simultaneously localize biologically relevant processes at the cell membrane and track them further into the cell body: TIRF is well known to offer high resolution localization of the cell membrane [AL Mattheyses et al., 2010, J Cell Sci, doi:10.1242/jcs.056218; JG Burchfield et al., 2010, Traffic, doi:10.1111/j.1600-0854.2010.01039.x] and Spinning Disk offers one of the best compromise between imaging speed and optical resolution in a live cell imaging experiment [J Oreopoulos et al., 2014, Methods Cell Biol, doi: 10.1016/B978-0-12-420138-5.00009-4; JM Murray et al., 2007, J Microsc, doi: 10.1111/j.1365-2818.2007.01861.x].

In order to clarify this point, we modified the first sentence in the abstract from:

"In living cells, many processes, such as endocytosis or adhesion formation, take place between the plasma membrane and the intracellular space."

To:

"In living cells, processes such as adhesion formation involve extensive structural changes in the plasma membrane and the cell interior." (lines 29-30).

We also added the new references 8-11 to the introduction:

"In detail, TIRF microscopy permits to specifically isolate and localize the plasma membrane[8,9], while SD microscopy is one of the most sensitive and fast live imaging techniques for the visualization and tracking of subcellular organelles in the cytoplasm[10,11]." (lines 57-60).

3) The pros and cons of a dual-camera system, as opposed to a single camera with an emission splitter, can be debated.

In order to clarify this point we added the following sentences:

"Additionally, these microscopes had only one detector, reducing further the speed for multi-channel acquisitions."

To:

"Additionally, previous SD-TIRF microscopes had only one detector per imaging mode, reducing further the speed for multi-channel acquisitions. Technically, in those systems a split-view configuration could be implemented that allows simultaneous dual-color acquisition with a single camera. This, however, would permit imaging of only half of the field of view." (lines 320-324).

4) What exactly are the experimental questions that can't otherwise be addressed? Other reviews and methods papers exist for imaging adhesion dynamics (e.g. see PMID: 20971702).

As already pointed out at point 2) of the same reviewer, we did not provide any unique biological results, but a unique tool to improve and ease the visualization of dynamic process between the cell membrane and the cell interior.

5) There doesn't really seem anything special about the sample preparation for the studies presented.

The goal of this work was not to implement a novel sample preparation protocol, but this assay was scarcely and imprecisely described in the literature. For clarifying this point, we modified the following sentence from:

“This specific assay has rarely been described in the literature[12,13] and adhesion formation has mostly been investigated in e.g. migrating cells[14,15].”

To:

“This specific assay has rarely and not clearly been described in the literature[17,18]. Moreover, adhesion formation has mostly been investigated in e.g. migrating cells[19,20]. Thus, we needed to adapt this methodology (cell line, coating, medium, composition) in order to carry out the experiments described in this paper.” (lines 237-241).

6) As for the image acquisition, this report employs a very particular type of microscope that will not be available to the vast majority of individuals. Thus, I keep asking myself, what is the purpose of this manuscript.

We answered to this question, addressing point 1) of the same reviewer, asserting also the availability of this microscope.

7) Line 29: Why mention endocytosis at all, if it is not covered in this manuscript.

Following the reviewer’s suggestion, endocytosis will be not listed in the abstract anymore and only mentioned in the introduction.

8) Line 29-30: "…between the plasma membrane and the intracellular space..." is very confusing. Why not just say, " associated with the adherent cell surface" or something?

We already answer this question changing the text in the abstract as described in point 1) reviewer number 1.

9) Line 56-57: It is totally inaccurate to say that things like adhesion dynamics "cannot be successfully recorded by the above mentioned super-resolution techniques" - there are many many examples in the literature. You can parse and equivocate all you want, but this is not very scientific, or useful.

We thank the reviewer for this observation, thus we changed the following sentences from:

“However, these approaches still have limited applicability for live imaging experiments, in which large volumes need to be visualized with high acquisition speed. A variety of processes regulated via the plasma membrane, e.g. endo-/exocytosis, adhesion, migration or signaling, occur with high speed within large cellular volumes and cannot be successfully recorded by the above mentioned super-resolution techniques.”

To:

“However, these approaches still have limited applicability for live imaging experiments in which large volumes need to be visualized with multiple frames per second acquisition speed. Varieties of highly dynamic processes regulated via the plasma membrane, e.g. endo-/exocytosis, adhesion, migration or signaling, occur with high speed within large cellular volumes.” (lines 52-56).

Reviewer number two:

1) The authors describe a new way to integrate SD and TIRF imaging in one setup. They are right that one problem with this approach has been switching time between modes. They authors claim that their setup is fast. If I understand this correctly, switching between SD and TIRF is accomplished by moving the W1 disk out of the way for TIRF. If this is correct, that

cannot possibly be very fast. I think it is essential that an actual number is given for how long it takes to switch between modalities. [I now see that 0.5 s is given as switching time at the very end. This is really not particularly fast. So, this issue with SD/TIRF has not been solved and the authors should be more upfront about that. The statement that 'only the microscope presented here (Figure 1) meets the criteria to perform live imaging SD-TIRF' seems a bit of an overstatement].

We agree with the reviewer that not only our SD-TIRF set up can perform SD-TIRF microscopy, thus we modified the following sentences:

"The combination of both imaging techniques in a single setup has already been realized in the past[8,9], however, only the microscope presented here (Figure 1) meets the criteria to perform live imaging SD-TIRF experiments of the aforementioned processes."

was changed to:

"The combination of both imaging techniques in a single setup has already been realized in the past[12,13], however, the microscope presented here (Figure 1) finally meets the criteria to perform live imaging SD-TIRF experiments of the aforementioned processes at 3 frames per second speed." (lines 60-63)

Moreover this sentence:

"In the TIRF configuration, the SD unit is bypassed so that the same two cameras can be used for detection allowing fast switching between the two imaging modalities. This feature enables dual-channel simultaneous acquisition, thus 4 channels SD-TIRF imaging at unprecedented speed and accuracy can be performed."

was changed to:

"In TIRF configuration, the SD unit is moved out of the light path within circa 0.5 s so that the same two cameras can be used for detection, allowing faster switching between the two imaging modalities compared to circa 1 s that was reported in the past[13]. This feature enables dual-channel simultaneous acquisition, thus 4 channels SD-TIRF imaging at previously unmatched speed and accuracy can be performed." (lines 78-83).

And:

"The few SD-TIRF microscopes that were described previously[8,9], mainly lack of sufficiently high imaging speed."

was changed to:

"The few SD-TIRF microscopes that were described previously[12,13], mainly lack of sufficiently high imaging speed to follow cellular processes in 3D in which a temporal resolution of less than 2 s per image stack is often necessary. The presented setup can achieve imaging rates up to 0.78 image stacks per second, and rates of 3.5 s per large image stack in live experiments investigating 3D vesicle dynamics have been demonstrated[7]." (lines 315-320).

2) Also, is there any concern that this will cause long-term damage to the mechanics moving the disk if that has to occur for each image pair?

According to a communication with Yokogawa, 100,000 switching cycles are within the device' warranty. The system is in use since 2015 and we did not experience any issues with the mechanics so far.

3) How precisely is switching accomplished?

The only information that was provided by Yokogawa is a scheme of the unit that demonstrated at which position the disk moves in and out, and that the dichroic that is located between the two disks remains in the position. See the provided document "CSU-W1.pdf", our model is described as "T2" (dual camera option).

4) In general, there is really very little technical description of the instrument other than the schematic figure. I think this is an issue.

In order to provide more information, we changed the sentence:

"The emitted light is filtered by various band-pass filters placed in front of the two EM-CCD cameras (Photometrix Evolve)."

to these sentences:

"The emitted light is split by a dichroic mirror (561 nm long-pass or 514 nm long-pass) and filtered by various band-pass filters (55 nm wide centered at 525 nm, 54 nm wide centered at 609 nm for green and red fluorescence, respectively) placed in front of the two EM-CCD cameras. Please note that more technical details about the setup are listed in Zobiak et al.[7]." (lines 74-78).

5) My main concern is that the protocol is very centered on using a specific microscope setup for which little detail is given. So, it is unclear how and what can be adopted by others.

The answers of point 1) reviewer 1 and point 4) reviewer 2 already helped to take away this concern. However, to make clearer that the presented protocol can be useful for the scientific community, we added or modified the following sentences:

"Load the *.nd file, select the SD-data in the first step, and the TIRF-data in the second step."

was changed to:

"Load the image dataset, select the SD-series in the first step, and the TIRF-series in the second step." (lines 207-208).

"Note: Data import is possible from various file types, for example TIFF-series or platform-dependent file types such as *.nd. The file type cannot be recognized only if it was not exported by the acquisition software as independent, compression-less TIFF format." (lines 212-214 were added).

"Hence, this optical configuration is favourable for implementing any kind of TIRF microscopy (*e.g.* variable or fixed angle illumination) into existing SD-microscopes that allow bypassing the SD unit." (lines 336-338 were added).

6) The cell culture part is fairly generic (which is fine), but maybe state somewhere that methods may need to be adapted for other cell types etc. In many cases, one might not want to trypsinize and reseed cells just before imaging.

In order to clarify this point, the following sentences were added:

"These cell lines were chosen for their pronounced cytoskeleton and higher robustness in live imaging experiments opposed to, for example, primary cells. Those might not withstand imaging in very sensitive condition as they are after trypsin treatment." (lines 233-236).

7) It seems the protocol is geared toward observing adhesion formation. The title states that, but a little explanation of the biological relevance (why SD and TIRF together?) would be important.

The answers to point 1) and 2) reviewer one can be used to clarify this point.

8) Does ascorbic acid really help with phototoxicity? Does it inhibit bleaching? A reference should be provided here.

In order to clarify this point, the following sentences were added:

"AA is an anti-oxidizing agent that can reduce phototoxic effects during live imaging[14]. We have tested it successfully in this assay, *i.e.* more cells appeared healthy under the conditions applied than without AA addition. However, the pH of the medium was lowered by 0.17 pH units." (lines 126-129).

The following reference number 14 was added: [Wäldchen et al., 2015, Sci Rep, doi: 10.1038/srep15348.]

9) DMEM has all kinds of fluorescent stuff. Depending on the fluorescent filters used (filter characteristics should be stated somewhere) this may not be a big problem, but we find that Phenolred-free and flavin-reduced imaging media are better. The more background fluorescence in the sample, the more Poisson noise, i.e. worse SNR.

We are very thankful and we included the reviewer's suggestion in the protocol section:

"Note: Use fluorescence-optimized cell culture medium if possible, such as phenolred-free and (ribo-) flavin-reduced medium." (lines 125-126).

10) Laser power should be stated in mW that actually come out of the objective. Relative power statements (20%) are generally useless for others as they cannot be compared with other instruments.

We follow the reviewer's indication, adding the requested information in the text:
"The data presented here was acquired with 200 ms exposure, gain level 500 and 20 % laser power."

was changed to:

"Note: The data presented here was acquired with 200 ms exposure, gain level 500 and 20 % laser power that equals excitation intensities of 0.5 W/cm² for 488 nm and 1 W/cm² for 561 nm, respectively." (lines 157-159).

"The acquisition rate of 30s/frame, enabling multi point acquisition, appears to be ideal, while the intensity level of the excitation laser (about 20 %) needs to be taken under critical consideration."

was changed to:

"The interval of 30 s/timepoint, enabling multi point acquisition, appears to be ideal, while the radiation intensity of the excitation laser (between 0.5-1 W/cm²) needs to be taken under critical consideration." (lines 262-264).

Reviewer number three:

1) FIJI plugin - "SD_TIRF helper" described in the manuscript is not available and hence authors need to provide link for downloading the plugin.

The file "SD-TIRF_helper_JoVE.ijm" was uploaded along with all other files and should have been made available for the reviewers. In case the editors cannot make the file accessible, it was published online via Github (http://www.github.com/bzobiak/ImageJ).

2) Microscope calibration and registration is a critical step for validating setup. Hence the authors should elaborate on the protocol for bead registration during post processing.

The protocol sections 2.3 and 3.1 have been extended accordingly:

"2.3. Find the fluorescent beads with the ocular or on the computer screen, activate one TIRF channel and set the angle to a value that allows TIRF illumination. Activate the auto-focus and adjust the focus if necessary.

Note: A few microliter of the beads suspension (see point 1.3.2.) can be added to obtain freely floating beads in the medium. These non-adherent beads should disappear beyond the critical angle, ensuring a correct TIRF illumination."

was changed to:

"2.3. Find the fluorescent beads with epi-fluorescent illumination at the ocular or on the computer screen, then activate one TIRF channel and set the illumination angle to a value that denotes TIRF illumination. Activate the auto-focus by pushing the button at the microscope panel and adjust the focus with the offset wheel. Acquire a 2-color dataset, *i.e.* TIRF-488 and TIRF-561, for subsequent bead-based image registration (see point 3.1).

Optional: To ensure TIRF illumination, add a few microliter of the freely floating fluorescent multi-color beads suspension (see point 1.3.2). Activate the live view of a TIRF channel and increase the illumination angle. The non-adherent beads will disappear beyond the critical angle, ensuring a correct TIRF illumination[8]." (lines 169-178).

3) XZ views in Figure 2A,C should be distinctly marked as SD and TIRF images for the ease of understanding. In general XZ views are not clear in Fig 2A and Fig2C.

According to the reviewer's suggestions, the channel names were included in the images and a line that depicts the position for the XZ projection was drawn. The Figure 2 caption was updated with the following text:

"The XZ views in A and D are the orthogonal projections extracted from the dashed white lines drawn therein (bottom = substrate)." (lines 300-302).

4) Figure 2B should be represented as kymograph.

In order to implement this modification, Figure 2B was shortened to three time points and a kymograph for the entire image dataset added as Figure 2C. The previous Figure 2C is now Figure 2D. Moreover, we added this text to the Figure 2 caption:

"C. Kymograph of the dashed yellow line drawn in A.". (line 297).

5) Authors need to elaborate on post processing of the images.

We agree to the reviewer and we enlarged section 3 dedicated to the image post processing:

“3.1. In order to generate a registration-free hyperstack in FIJI[10], a macro named “SD-TIRF helper” has been written that can be applied to 2-4 channel timelapse datasets. The macro can be run from the plugins menu.”

was changed to:

“3.1. In order to generate a registration-free hyperstack in FIJI[15], a macro named “SD-TIRF_helper” has been written that can be applied to 2-4 channel SD-TIRF timelapse datasets. Save the file “SD-TIRF_helper_JoVE.ijm” in the FIJI sub-folder “macros” and run the macro by clicking on the menu command “Plugins>Macros>Run…” (lines 194-197).

“3.1.1. If the channel registration option has been selected, decide if a new registration correction should be created or an existing landmark file should be used.

Note: The turboreg plugin[11] will be applied to fluorescence beads reference images. The plugin needs to be installed by FIJI software according to the general guidelines for plugin installations.”

was changed to:

“3.1.1. If the color channels need registration correction, select the option and create a new bead-based registration reference (landmark file) or use an existing file that was created before.

Note: The turboreg plugin[16] will be applied to fluorescence beads reference images. Install the plugin in FIJI software according to general guidelines for plugin installations.” (lines 199-204).

“3.1.2. Import the data with the bio-formats importer and choose hyperstack as a viewing option. Load the *.nd file, select the SD-data in the first step, and the TIRF-data in the second step. FIJI will display the data sorted by channel and stage position, *i.e.* normally all SD-channels and all TIRF-channels show up as one hyperstack for every stage position that has been selected.”

was changed to:

“3.1.2. Import the data with the bio-formats importer and choose hyperstack as a viewing option. Load the image dataset, select the SD-series in the first step, and the TIRF-series in the second step. FIJI will display the data sorted by channel and stage position, *i.e.* normally all SD-channels and all TIRF-channels show up as one hyperstack for every stage position that has been selected.

Note: Data import is possible from various file types, for example TIFF-series or platform-dependent file types such as *.nd. The file type cannot be recognized only if it was not exported by the acquisition software as independent, compression-less TIFF format.” (lines 206-214).

“3.1.4. The SD- and TIRF datasets will be merged into a single SD-TIRF hyperstack. For the TIRF channels, a number of z-planes with zero intensity values will be added on top that matches with the number of z-planes in the SD dataset. This is important for the visualization of the final multi-dimensional hyperstack”

was changed to:

“3.1.4. Select the desired color look-up table (LUT) for every SD- and TIRF channel and merge them into a single, multi-dimensional hyperstack.

Note: During processing of the TIRF channels, a number of z-planes with zero intensity values are added on top of the bottom plane that matches with the number of z-planes in the SD dataset. This step is important for the visualization of the final hyperstack. This methodology is correct, since the depth

of the TIRF illumination (less than 200nm[7]) is smaller than the z-step size of the SD stack (400nm).” (lines 219-226).

We also added the following text in the Figure 2 caption:

“Images were linearly contrast-enhanced and median-filtered with a 3x3 kernel.” (lines 301-302).

6) Authors need to be careful in mentioning names of super resolution techniques- e.g- STED (Stimulated emission depletion) instead of Simulated emission depletion microscope (Pg3).

We corrected this typo according to the reviewer’s suggestion (line 48).

7) Please mention the specific model of EM-CCD cameras. Also mention more on the method of autofocus used.

The exact model (Photometrix Evolve) is stated in the table of materials as requested by the editor. The usage of the auto-focus was specified in more detail in the protocol section 2.2.3, i.e.:

“Set the z-stack only for the spinning-disk channels to 10 µm with 0.4 µm spacing. The lowest plane should be the focus position of the auto-focus, i.e. the bottom-offset should be set to “0”. All TIRF channels should be recorded as a single plane.”

was changed to:

“Set the z-stack for the spinning-disk channels to 10 µm with 0.4 µm spacing. De-activate z-stacks for the TIRF channels. Set the bottom-offset to “0”, *i.e.* the lowest plane will be the focus position of the hardware auto-focus” (lines 161-163)”

And:

“Activate the auto-focus by pushing the button at the microscope panel and adjust the focus with the offset wheel” (lines 171-172).

8) Authors need to comment specific sensitivity achieved using SD-TIRF Vs TIRF alone.

We thank the referee for pointing this out. Indeed, the sensitivity was not meant to be any different between the two modes. Thus, we changed this sentence:

“SD-imaging proofed to be the method of choice here as it allowed visualizing this process with high sensitivity and spatial resolution.“

To the following:

“SD-imaging proofed to be the method of choice here, as it allowed visualizing this process with high sensitivity, spatial resolution and from a complete perspective. In a previous report[17], TIRF alone could only let speculate about the origin of peripheral adhesions, whereas SD-TIRF imaging clearly revealed its association with filopodia (Figure 2B, time point 17 min, white arrowhead).” (lines 254-260).

The caption of Figure 2B was changed accordingly from:

“The magnified view of the boxed region in A (45 minutes frame) depicts cell spreading and the transition from nascent to focal adhesions as well as stress fiber formation (see frames at 37 and 42 minutes).”

to:

"The magnified view of the boxed region in A (frame at 45 minutes) depicts cell spreading and the transition from nascent to focal adhesions as well as filopodia-associated adhesions (white arrowhead) and stress fiber formation (see frame at 27 minutes, yellow arrowhead)." (lines 294-297).

Editorial comments:

Changes to be made by the Author(s) regarding the written manuscript:

1) Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

The manuscript was proofread and corrected for grammar or spelling issues to the best of our knowledge.

2) Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."

Figure 1 has been modified from a publication that was published as open-source with a CC-BY license (https://creativecommons.org/licenses/by/4.0/legalcode). The signed license is provided as PDF file and allows general reproduction.

3) Figure 2: Please change "" symbol to the time unit "min".

Figure 2 was changed according to the suggestion of the editor.

4) JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. For example: Visitron systems, VisiView, Nikon, Yokogawa, Roper Scientific, Photometrix Evolve, OptiMEM, TurboFect, TetraSpeck, etc.

All commercial language was removed from the protocol and generic terms used instead. Manufacturers are now mentioned in the table of materials and reagents. ImageJ and FIJI are kept, being open source and developed by worldwide contributors.

5) Please revise the protocol to contain only action items that direct the reader to do something (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note." Please include all safety procedures and use of hoods, etc. However, notes should be used sparingly and actions should be described in the imperative tense wherever possible.

The protocol part was carefully revised and imperative tense used wherever possible. Subjunctive phrases were re-written. The following changes were made:

"Note: Ensure that cells are handled in a laminar flow hood throughout this protocol." (line 96 was added).

"The lowest plane should be the focus position of the auto-focus, *i.e.* the bottom-offset should be set to "0". All TIRF channels should be recorded as a single plane."

was changed to:

“De-activate z-stacks for the TIRF channels. Set the bottom-offset to “0”, *i.e.* the lowest plane will be the focus position of the hardware auto-focus” (lines 161-163).

“A few microliter of the beads suspension (see point 1.3.2.) can be added to obtain freely floating beads in the medium. These non-adherent beads should disappear beyond the critical angle, ensuring a correct TIRF illumination.”

was changed to:

“Optional: To ensure TIRF illumination, add a few microliter of the freely floating fluorescent multi-color beads suspension (see point 1.3.2.). Activate the live view of a TIRF channel and increase the illumination angle. The non-adherent beads will disappear beyond the critical angle, ensuring a correct TIRF illumination[8].” (lines 175-178).

“Afterwards, 1-2 positions can be discarded.”

was changed to:

“Afterwards, discard 1-2 positions.” (lines 188).

“The macro can be run from the plugins menu.”

was changed to:

“Save the file “SD-TIRF_helper_JoVE.ijm” in the FIJI sub-folder “macros” and run the macro by clicking on the menu command “Plugins>Macros>Run…”.” (lines 196-197).

“If the channel registration option has been selected, decide if a new registration correction should be created or an existing landmark file should be used.”

was changed to:

“If the color channels need registration correction, select the option and create a new bead-based registration reference (landmark file) or use an existing file that was created before” (lines 199-201).

“The plugin needs to be installed by FIJI software according to the general guidelines for plugin installations.”

was changed to:

“Install the plugin in FIJI software according to general guidelines for plugin installations.” (lines 203-204).

6) Please add more details 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. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. Some examples:
1.1: Please provide the composition of full growth medium.

The composition of the medium is available in the table of materials.

1.3.1: Please specify the size of the glass bottom dish.

The following sentence was changed from:

“Prepare a 10 µg/µL solution of fibronectin in PBS to coat the glass surface of a glass bottom dish.”

to:

“Prepare a 10 µg/µL solution of fibronectin in PBS to coat the glass surface of a 35 mm glass bottom dish. Use only high quality 0.17 mm glass coverslips for optimal TIRF performance and avoid plastic bottom dishes.” (lines 110-112).

> 1.3.4: Please specify the reaction temperature for the detachment. Please specify the temperature of the incubator.

The following text:

“Wash the cells with PBS, add 250 µL Trypsin-EDTA and wait until the cells are fully detached. Resuspend the cells carefully in 1 mL AA-DMEM with a pipette and add it to 4 mL AA-DMEM in a 15 mL Falcon tube. Place the cell suspension with a slightly opened lid in an incubator in the vicinity of the microscope”

was changed to:

“Wash the cells with 2mL PBS, add 250 µL Trypsin-EDTA and wait until the cells are fully detached (2-3 min in a 37 °C incubator). Resuspend the cells carefully in 1 mL pre-warmed AA-medium with a pipette and add it to 4 mL AA-medium in a 15 mL cell culture tube. Place the cell suspension with a slightly opened lid in an incubator set to 37 °C and 5 % CO_2 in the vicinity of the microscope.” (lines 131-135).

> 2.2, 2.3, 2.6, 3.1: Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc.) to your protocol steps.

2.2: The acquisition settings were described in more detail:

“Set the time-interval to 30 s and the duration to 60-90 min. Activate the auto-focus function for every time point.”

was changed to:

“Activate the auto-focussing function of the hardware-based auto-focus for every time point (value “1”).” (lines 150-151).

“All TIRF channels should be recorded as a single plane.”

was changed to :

“De-activate z-stacks for the TIRF channels.” (lines 161-162).

“Activate the multi-point function.”

was changed to:

“Activate the multi-point function “stage positions”.” (line 165).

2.3: The application of fluorescent beads for image registration and TIRF illumination adjustment was described in more detail. Please see the answer to reviewer number three point 2.

2.6: The sentence has been changed from:

“Start recording the data.”

to:

“Start data acquisition by clicking on the “Sequence” button.” (line 190).

3.1: Post-processing using the provided macro was described in more detail. Please see the answer to reviewer number three point 5.

7) Discussion: As we are a methods journal, please also discuss critical steps within the protocol.

More critical steps were discussed throughout the protocol as also hinted by other reviewer comments, for example the usage of high quality glass coverslips (1.3.1), ascorbic acid and phenolred-free media (1.3.3), environmental control (2.1), data import (3.1.2), data processing (3.1.4).

Please do not hesitate to contact me if more questions arise.

Yours sincerely,

Dr .Antonio Virgilio Failla

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