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Dear editor,
we are contacting you to submit following your invitation our manuscript entitled:

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We would highly appreciate if you would proof if this as an article suitable for publication in JoVE: The corresponding author is Antonio Virgilio Failla.

This paper aims to contribute to the development of light microscopy applied to biological systems. Although both spinning disk (SD) and total internal reflection fluorescence (TIRF) microscopy are already well known since long time, their combination has not been, according to our knowledge, widely utilized in live cell imaging. We present a simple (that makes use of ordinary optics) but innovative setup that permits the combination of SD and TIRF microscopy. This is the first microscope proved to perform, live SD-TIRF experiments at high acquisition rates and represents in our opinion a unique tool for specific imaging of the plasma membrane. A series of experiments and original protocols to calibrate the microscope, prepare the sample as well as display the acquired data will be presented. The data shown aim to demonstrate the potential and performance of our setup. Please feel free to contact us any time for further information:

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Yours sincerely,

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TITLE:

Visualizing Adhesion Formation in Cells by Means of Advanced Spinning Disk-Total Internal Reflection Fluorescence Microscopy

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KEYWORDS:

Spinning disk, TIRF, fluorescence microscopy, multiple imaging, integrated microscopy, three-dimensional microscopy, illumination design, spreading, adhesion

SUMMARY:

An advanced microscope that permit fast and high-resolution imaging of both, the isolated plasma membrane and the surrounding intracellular volume, will be presented. The integration of spinning disk and total internal reflection fluorescence microscopy in one setup allows live imaging experiments at high acquisition rates up to 3.5 s per image stack.

ABSTRACT:

In living cells, processes such as adhesion formation involve extensive structural changes in the plasma membrane and the cell interior. In order to visualize these highly dynamic events, two complementary light microscopy techniques that allow fast imaging of live samples were combined: spinning disk microscopy (SD) for fast and high-resolution volume recording and total internal reflection fluorescence (TIRF) microscopy for precise localization and visualization of the plasma membrane. A comprehensive and complete imaging protocol will be shown for guiding through sample preparation, microscope calibration, image formation and acquisition, resulting in multi-color SD-TIRF live imaging series with high spatio-temporal resolution. All necessary image post-processing steps to generate multi-dimensional live imaging datasets, *i.e.* registration and combination of the individual channels, are provided in a self-written macro for the open source software ImageJ. The imaging of fluorescent proteins during initiation and maturation of adhesion complexes, as well as the formation of the actin cytoskeletal network, was used as a proof of principle for this novel approach. The combination of high resolution 3D microscopy and TIRF provided a detailed description of these complex processes within the cellular environment and, at the same time, precise localization of the membrane-associated molecules detected with a high signal-to-background ratio.

INTRODUCTION:

Our days, light microscopy techniques providing high/super resolution imaging in fixed and living specimen are developing rapidly. Super-resolution techniques such as stimulated emission depletion (STED), structured illumination microscopy (SIM) and photo-activation localization microscopy (PALM) or direct stochastic optical reconstruction microscopy (STORM), respectively, are commercially available and enable imaging of subcellular structures showing details almost on the molecular scale¹⁻⁶. 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. Recently, in order to fill up this gap, an integrated microscopy technique was proposed called spinning disk-TIRF (SD-TIRF)⁷. 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}. 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. 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.

The setup is based on an inverted microscope connected to two scan units via independent ports – the left port is linked to the SD unit and the back port to scanner unit for TIRF and photo-activation/-bleaching experiments. Up to 6 lasers (405/445/488/515/561/640 nm) can be used for excitation. For excitation and detection of the fluorescence signal, either a 100x/NA1.45 oil or 60x/NA1.49 oil TIRF objective, respectively, have been employed. 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. 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 past¹³. This feature enables dual-channel simultaneous acquisition, thus 4 channels SD-TIRF imaging at previously unmatched speed and accuracy can be performed. Moreover, alignment between SD and TIRF images is unnecessary. Image alignment between the two cameras, however, has to be checked before starting the experiment and corrected if necessary. In the following protocol, a registration correction routine was implemented in a self-written ImageJ macro. Moreover, the macro was mainly designed to allow a simultaneous visualization of SD- and TIRF datasets despite their different dimensionality. The acquisition software itself did not provide these features.

PROTOCOL:

1. Preparation of cells

1.1. Two days prior to the experiment, seed 3×10^5 HeLa or NIH3T3 cells in 2 mL of full growth medium per well of a 6-well cell-culture plate. Ensure that cells are handled in a laminar flow hood throughout this protocol.

1.2. One day prior to the experiment, prepare the transfection reagents according to the manufacturer's recommendations or an empirically determined protocol, *e.g.*:

1.2.1. Dilute 1 μg of RFP-Lifeact and 1 μg of YFP-Vinculin in a total of 200 μL reduced serum medium. Vortex the transfection reagent briefly, add 4 μL to the DNA and vortex again. Incubate the transfection mix for 15-20 min at room temperature.

1.2.2. Add the transfection mix dropwise directly to the cells. Mix by shaking the plate and place it back into the incubator.

1.3. On the day of the experiment, prepare the sample for live imaging:

1.3.1. Prepare a 10 $\mu\text{g}/\mu\text{L}$ 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. Leave the solution on the glass surface for 30 min at room temperature, then remove it and let the dish air-dry.

1.3.2 Dilute a 0.1 μm multi-fluorescent beads solution to a density of 1.8×10^9 particles per mL in distilled water and add the solution for 30-60 s to the fibronectin-coated glass surface. Immediately remove the solution and let the dish air-dry.

Note: This step is necessary only if the TIRF plane should be found before seeding cells and/or to acquire a 2-color reference image for bead-based image registration.

1.3.3. Prepare a 0.1 M ascorbic acid (AA) solution and dilute it to a final concentration of 0.1 mM in growth medium (AA-medium). Place the solution in a 37 °C water bath.

Note: Use fluorescence-optimized cell culture medium if possible, such as phenolred-free and (ribo-) flavin-reduced medium. AA is an anti-oxidizing agent that can reduce phototoxic effects during live imaging¹⁴. 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.

1.3.4. 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% CO₂ in the vicinity of the microscope.

1.3.5. Add 1 mL pre-warmed AA-medium to the glass bottom dish and place it in the holder of the pre-heated microscope (see next paragraph).

2. Live imaging

2.1. Start the environmental control of the microscope to achieve a stable 37 °C, 5% CO₂ and humid atmosphere.

Note: Here, a small stage top incubation chamber has been used that allowed stable settings within about 15min. Larger incubators will need more time to achieve stable conditions.

2.2. Fix all acquisition settings at the microscope before the cell suspension is applied:

2.2.1. Set the time-interval to 30 s and the duration to 60-90 min. Activate the auto-focusing function of the hardware-based auto-focus for every time point (value "1").

2.2.2. Adjust the camera exposure and gain, as well as the laser power for every channel. High gain levels, low exposure time and low laser power are recommendable to reduce photo-toxicity.

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.

2.2.3. 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.

2.2.4. Activate the multi-point function "stage positions".

Note: Up to 3 positions can be recorded in a 30 s time interval.

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).

2.3.1. Optional: To ensure TIRF illumination, add a few microliters of the freely floating fluorescent multicolor 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 illumination⁸.

2.4. Mix the cell suspension again by inverting the closed tube 2-3 times, and apply 1 mL of the cells to the imaging dish.

2.5. Quickly find double-transfected cells with low level epi-fluorescent illumination. Center the cells in the live camera preview using bright field illumination and mark the position. Find another 1-2 points of interest and save them to the positions list.

Note: At the beginning, the cells easily can detach due to stage movement, hence set 4-5 positions and re-check all before starting image acquisition. Afterwards, discard 1-2 positions.

2.6. Start data acquisition by clicking on the “Sequence” button.

3. Image post-processing in ImageJ

3.1. In order to generate a registration-free hyperstack in FIJI¹⁵, 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...”.

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 plugin¹⁶ will be applied to fluorescence beads reference images. Install the plugin in FIJI software according to general guidelines for plugin installations.

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.

3.1.3. Apply the registration correction to the respective channels by loading the pre-determined landmarks file.

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 200nm⁷) is smaller than the z-step size of the SD stack (400nm).

REPRESENTATIVE RESULTS:

In order to show the potential of SD-TIRF imaging, an assay was developed that should reveal the spatio-temporal organization of cell-matrix adhesion complexes and their interaction with the cytoskeleton during cellular adhesion. Therefore, adherent HeLa or, alternatively, NIH3T3 cells were transfected with YFP-Vinculin and RFP-Lifeact for 18-24h, trypsinized and seeded onto fibronectin-coated glass bottom dishes. 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. At the microscope, YFP/RFP-expressing cells were selected and the adhesion process observed during a 60min time-lapse (Figure 2 and Movies 1 and 2). 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.

As expected, cells were round-shaped at the beginning and only weakly adherent, whereas membrane protrusions were sensing the environment and making contact with the substrate. Cell-matrix contacts strengthened quickly upon formation of so-called nascent adhesions^{20,21} (Figure 2A, TIRF-488 channel, time points 0-4.5 min). The latter are spot-like, Vinculin-positive structures at the ventral side of the cell. The structures were clearly visible in the TIRF images. In the beginning of adhesion formation, actin was evenly distributed in the cell and did not localize to these early complexes (Figure 2A, SD-561 channel). Over the course of time, adhesion complexes enlarged and matured to focal adhesions (Figure 2C). These elongated structures were predominantly apparent at the periphery of the cell (Figures 2A+B) and resulted from forces that were exerted by acto-myosin fibers. These fibers started to connect to the adhesion complexes, thereby pulling them towards the cell center and inducing the strengthening of cell-matrix adhesions as well as the bundling of actin fibers²¹. Apparently, the cell also flattened as a result of actin network formation (Figure 2A, XZ view). 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 report¹⁷, 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). Indeed, actin fibers emerging centripetally from focal adhesions became visible after 27 min (Figure 2B, yellow arrowhead).

However, the acquisition settings of these experiments, *i.e.* acquisition speed, excitation intensity and detector gain, need to be carefully evaluated. 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. Figure 2D displays a cell at a different position in the same experiment that failed to attach to the

substrate. It might be possible that phototoxic effects affected the biology of the cell here, which finally resulted in membrane bubbling after circa 60 min, probably resembling apoptosis (Movie 2). This made again clear how sensitive cells can react to phototoxicity and that it is important to find a good balance between the amount of light put in and the information being taken out. Reducing the laser power, the number of images in a z-stack or increasing the gain might be the correct strategy for reducing phototoxicity. All these settings, however, should be adjusted at a level that still allows achieving enough resolution and signal to noise ratio, enabling to extract quantitative information from the recorded time lapse.

FIGURE AND TABLE LEGENDS:

Figure 1: Schematic drawing of the SD-TIRF setup. **A.** SD imaging mode: the 6 different laser lines (405/445/488/515/561/640nm, green lines) are coupled into the confocal scanning unit (CSU), passing through the SD (position 'IN') and an empty filter cube in the microscope body (MIC). Fluorescence emission from the specimen (SPEC) is projected through the pinholes of the SD and split by different dichroic mirrors, *e.g.* for green/red or yellow/cyan simultaneous acquisition, onto two different EM-CCD cameras (C1 and C2). Fluorescence filters are placed in front of the cameras (not shown). **B.** TIRF imaging mode: the laser lines are coupled into the TIRF scanner (TIRF). A multi-line beam splitter in the MIC directs the beam to the specimen and the emission light bypasses the SD ('OUT') for maximized transmission. Fluorescence is detected by the same cameras and emission filters described in A. This figure has been modified from Zobiak *et al.*⁷

Figure 2: Representative results of cell spreading and adhesion formation using SD-TIRF microscopy. **A.** Double-transfected HeLa cell expressing RFP-Lifeact (red, SD-561 channel) and YFP-Vinculin (green, TIRF-488 channel) were trypsinized and re-seeded onto fibronectin-coated glass coverslips. Adhesion formation becomes readily visible, starting with small nascent adhesions (Vinculin-positive spots) at 0 minutes that develop into larger focal adhesions after circa 5 minutes. Cortical actin is apparent after circa 10 minutes and extends at the periphery of the cells (see frames at 12 and 24.5 minutes). **B.** 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). **C.** Kymograph of the dashed yellow line drawn in A. **D.** (Photo-) Toxic effects on cells or otherwise unhealthy cells can let them fail to attach to the substrate. Imaging conditions have to be critically evaluated in order to exclude phototoxicity. Scale bar = 5 μ m (in A and D) and 2 μ m (in B and C). The XZ views in A and D are the orthogonal projections extracted from the dashed white lines drawn therein (bottom = substrate). Images were linearly contrast-enhanced and median-filtered with a 3x3 kernel.

Movie 1: 3D-reconstruction of the timelapse sequence in Fig. 2A. The image sequence was rendered with 3D imaging software. Duration: 42.5 min. Acquisition rate: 2 dual-channel SD-TIRF stacks per minute (56 frames in total) were acquired.

Movie 2: Movie of the timelapse sequence in Fig. 2C. Duration: 60 min. Acquisition rate: 2 dual-channel SD-TIRF stacks per minute (56 frames in total) were acquired.

DISCUSSION:

In this paper was presented the first successful implementation of SD and TIRF microscopy in a configuration suitable for performing live cell imaging experiments, *i.e.* high acquisition rates such as 2 SD-TIRF image stacks per minute at 3 different stage positions, corresponding to a total of 168 frames (circa 3 frames per second), were acquired. 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 demonstrated⁷. 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 also allows simultaneous dual-color acquisition with a single camera. This, however, would permit imaging of only half of the field of view. Other methods to produce multi-dimensional datasets with high spatio-temporal resolution such as widefield imaging combined with deconvolution or 3D super-resolution microscopy, *i.e.* 3D SIM or lattice light sheet microscopy^{22,23}, might be valid alternatives. However, deconvolution-based imaging can easily introduce image artifacts in low signal-to-noise ratio acquisitions (as it is often the case during live imaging applications), and super-resolution 3D live imaging is still a technically elaborative and challenging task. In the presented realization of an advanced SD-TIRF setup⁷, advantage was taken of a SD unit that allows moving the dual-disk in and out of the detection path. This configuration provides two major benefits: first, the same two cameras can be used to detect the SD and TIRF signal, which results in a high-precision overlap of these two channels. Second, the pinholes of the spinning disk do not block any emission light when operating in TIRF acquisition mode (for more details, see Figure 1B), thus increasing the collection efficiency important for high-sensitive live imaging. Hence, this optical configuration is favorable for implementing any kind of TIRF microscopy (*e.g.* variable or fixed angle illumination) into existing SD-microscopes that allow bypassing the SD unit. Furthermore, the used TIRF scanner can be run in so-called time-sharing mode, where two TIRF channels can be recorded simultaneously (as shown in Zobiak *et al.*⁷), speeding further up the acquisition of multi-channel data.

One of the biggest advantages of employing TIRF is that, with this methodology, it is possible to localize with highest precision the signal coming from the cell membrane during a life cell imaging experiment. Indeed, while a methodology like SIM provides a better z-sectioning and thus better isolation of the cell membrane in fixed samples, in live cell imaging experiments the exponential increasing/decreasing of the fluorescence signal from organelles approaching/leaving the TIRF interface allowed more specific and precise localization of the cell membrane. The localization precision, although not still quantified, promises to be many folds smaller than 150-200 nm, *i.e.* the spatial range of the evanescence field.

Presently a limitation of the method is the time necessary to remove the spinning disk unit from the light path and start the TIRF acquisition, *i.e.* 0.5 s. This delay limits the minimum time interval between two consecutive acquisitions. Technically, it should be possible to reduce this

delay through bypassing the disk with *e.g.* galvo-mirrors and thus decreasing the overall acquisition per SD-TIRF cycle. Also, newer generation cameras might allow reduced exposure times at high signal-to-noise ratio. Hence, the overall performance of this setup can be still relevantly improved by upgrading the hardware components. From the imaging point of view, however, there are several other ways to minimize the acquisition time (in descending order): avoid multiple positions, reduce the z-stack height, increase the z-spacing, minimize exposure time (increase gain and possibly use binning), shorten the distance between acquisition positions, activate the auto-focusing only every n -th time point ($n > 1$). Despite actual limitations, if all those parameters are carefully evaluated, it is possible to use SD-TIRF imaging for tracking and localizing fast moving cellular vesicles, as presented in Zobiak *et al.*⁷.

Moreover, in this paper a protocol that describes the acquisition routine of a single-channel SD-TIRF dataset was presented. Using the provided macro, raw data can be exported in a single TIFF-file containing all SD- and TIRF channels. Image registration is *per se* not necessary; however, it is important that both cameras are precisely aligned with respect to each other. Remaining pixel shifts (translation and rotation) can be detected and corrected within the provided macro. The correction algorithm makes use of a multi-channel reference image of fluorescent beads that has to be recorded just before starting the experiment. In the resulting file, the TIRF plane is set at lowest level followed by a number of zero intensity planes that are matching the dimensionality of the SD-channel. Therefore, it is important to acquire the data, as outlined in the protocol, where the imaged TIRF plane resembles the lower end of the z-stack set for the SD channel.

Finally the system could be potentially extended by a SIM module or the recently introduced multi-angle TIRFM^{24,25} to further increase the spatial resolution. However, increasing of spatial resolution can be achieved, according to the current state of the art, only at the cost of a slower imaging speed. For all the live cell imaging experiments in which it is crucial to localize structures at the plasma membrane albeit maintaining high spatial resolution of the remaining cellular volume, the here described SD-TIRF setup is an easy to integrate, readily available solution.

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DISCLOSURES:

The authors have nothing to disclose.

REFERENCES:

1. Hell, S.W., Wichmann, J. Breaking the diffraction resolution limit by stimulated emission: stimulated-emission-depletion fluorescence microscopy. *Optics Letters*. **19** (11), 780 (1994).
2. Gustafsson, M.G.L. Surpassing the lateral resolution limit by a factor of two using

structured illumination microscopy. *Journal of Microscopy*. **198** (2), 82–87, (2000).

3. Betzig, E. *et al.* Imaging intracellular fluorescent proteins at nanometer resolution. *Science (New York, N.Y.)*. **313** (5793), 1642–5 (2006).
4. Hess, S.T., Girirajan, T.P.K., Mason, M.D. Ultra-high resolution imaging by fluorescence photoactivation localization microscopy. *Biophysical Journal*. **91** (11), 4258–4272 (2006).
5. Rust, M.J., Bates, M., Zhuang, X. Sub-diffraction-limit imaging by stochastic optical reconstruction microscopy (STORM). *Nature methods*. **3** (10), 793–796 (2006).
6. van de Linde, S. *et al.* Direct stochastic optical reconstruction microscopy with standard fluorescent probes. *Nature protocols*. **6** (7), 991–1009 (2011).
7. Zobiak, B., Failla, A.V. Advanced spinning disk-TIRF microscopy for faster imaging of the cell interior and the plasma membrane. *Journal of Microscopy*. **269** (3), 282–290 (2018).
8. Mattheyses, A. L., Simon, S.M., Rappoport, J.Z. Imaging with total internal reflection fluorescence microscopy for the cell biologist. *Journal of Cell Science*. **123** (21), 3621–3628 (2010).
9. Burchfield, J.G., Lopez, J.A., Vallotton, P., William, E. Exocytotic vesicle behaviour assessed by total internal reflection fluorescence microscopy. *Traffic*. **11** (4), 429–439 (2010).
10. Oreopoulos, J., Berman, R., Browne, M. Spinning-disk confocal microscopy. present technology and future trends. *Methods in Cell Biology*. **123**, 153–175 (2014).
11. Murray, J.M., Appleton, P.L., Swedlow, J.R., Waters, J.C. Evaluating performance in three-dimensional fluorescence microscopy. *Journal of Microscopy*. **228** (3), 390–405 (2007).
12. Trache, A., Lim, S.-M. Integrated microscopy for real-time imaging of mechanotransduction studies in live cells. *Journal of Biomedical Optics*. **14** (3), 034024 (2009).
13. Stehbens, S., Pemble, H., Murrow, L., Wittmann, T. Imaging intracellular protein dynamics by spinning disk confocal microscopy. *Methods in Enzymology*. **504**, 293–313 (2012).
14. Waldchen, S., Lehmann, J., Klein, T., Van De Linde, S., Sauer, M. Light-induced cell damage in live-cell super-resolution microscopy. *Scientific Reports*. **5**, 1–12 (2015).
15. Schindelin, J. *et al.* Fiji: an open-source platform for biological-image analysis. *Nature Methods*. **9** (7), 676–682 (2012).
16. Thévenaz, P., Ruttimann, U.E., Unser, M. A pyramid approach to subpixel registration based on intensity. *IEEE Transactions on Image Processing*. **7** (1), 27–41 (1998).
17. Partridge, M.A. Initiation of attachment and generation of mature focal adhesions by integrin-containing filopodia in cell spreading. *Molecular Biology of the Cell*. **17** (10), 4299–4311 (2006).
18. Dubin-Thaler, B.J., Giannone, G., Döbereiner, H.G., Sheetz, M.P. Nanometer analysis of cell spreading on matrix-coated surfaces reveals two distinct cell states and STEPs. *Biophysical Journal*. **86** (3), 1794–1806 (2004).
19. Choi, C.K., Vicente-Manzanares, M., Zareno, J., Whitmore, L.A., Mogilner, A., Horwitz, A.R. Actin and α -actinin orchestrate the assembly and maturation of nascent adhesions in a myosin II motor-independent manner. *Nature Cell Biology*. **10** (9), 1039–1050 (2008).
20. Bachir, A.I., Zareno, J., Moissoglu, K., Plow, E.F., Gratton, E., Horwitz, A.R. Integrin-associated complexes form hierarchically with variable stoichiometry in nascent

441 adhesions. *Current Biology*. **24** (16), 1845–1853 (2014).

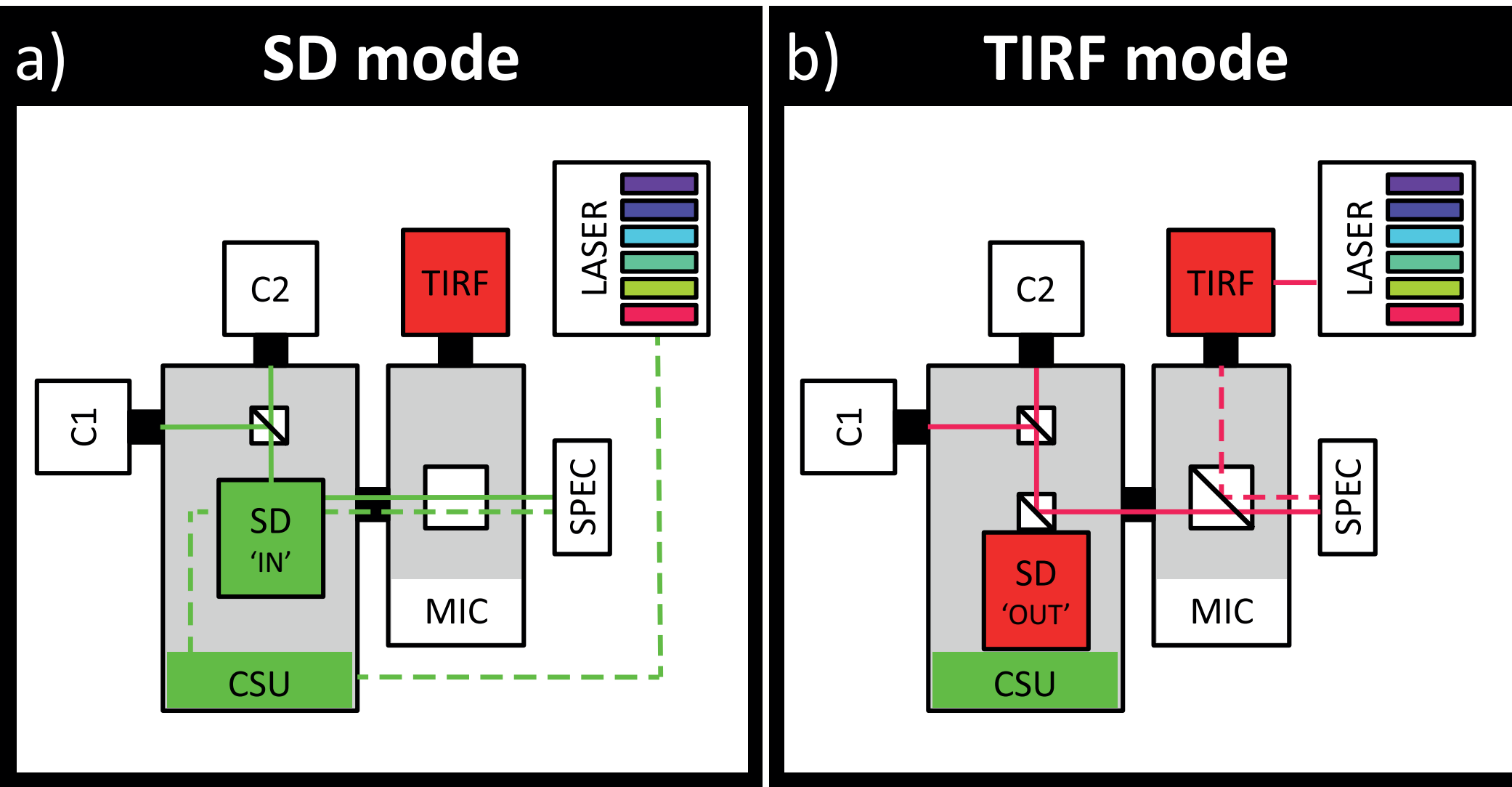
442 21. Sun, Z., Guo, S.S., Fässler, R. Integrin-mediated mechanotransduction. *Journal of Cell*
443 *Biology*. **215** (4), 445–456 (2016).

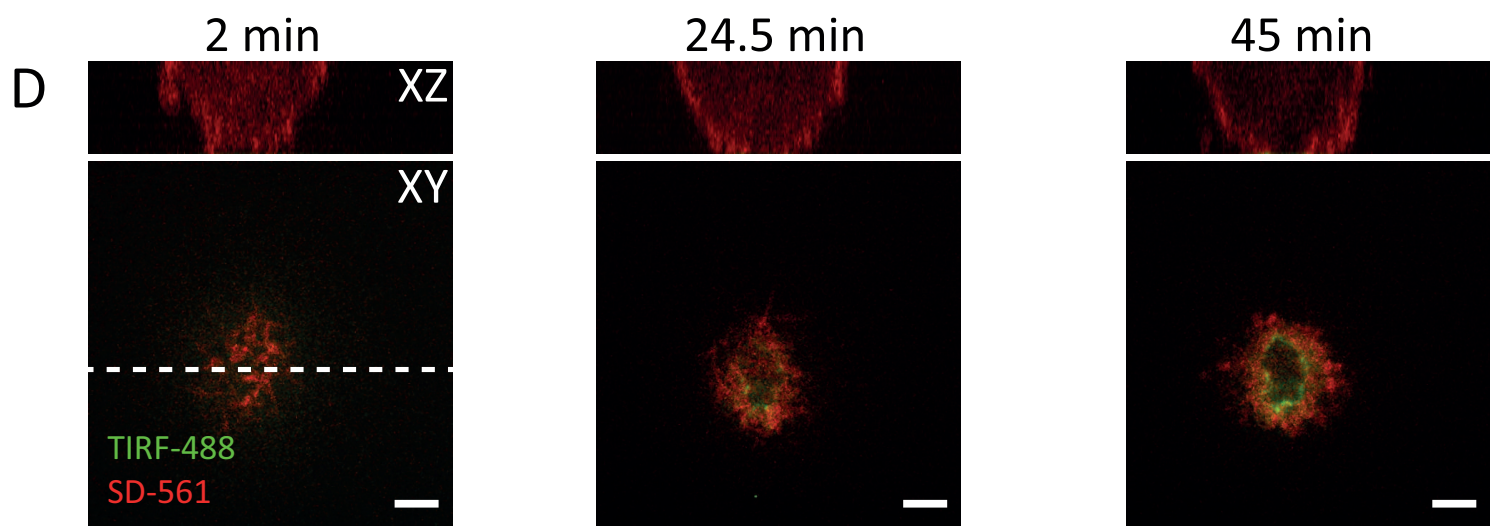
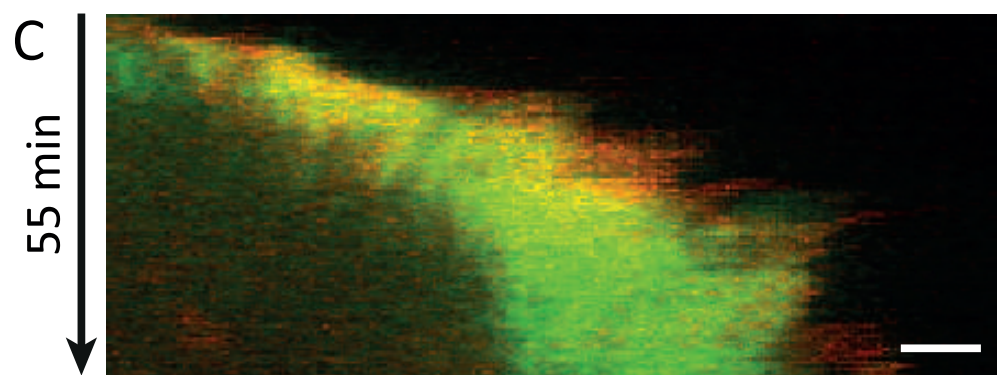
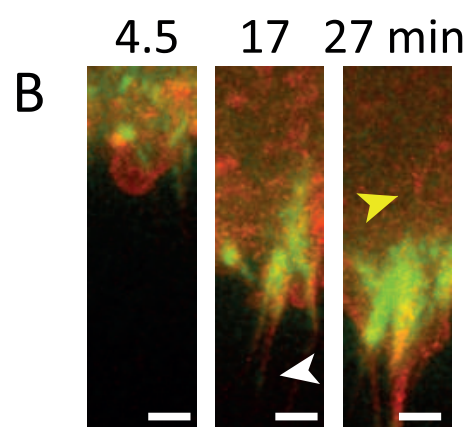
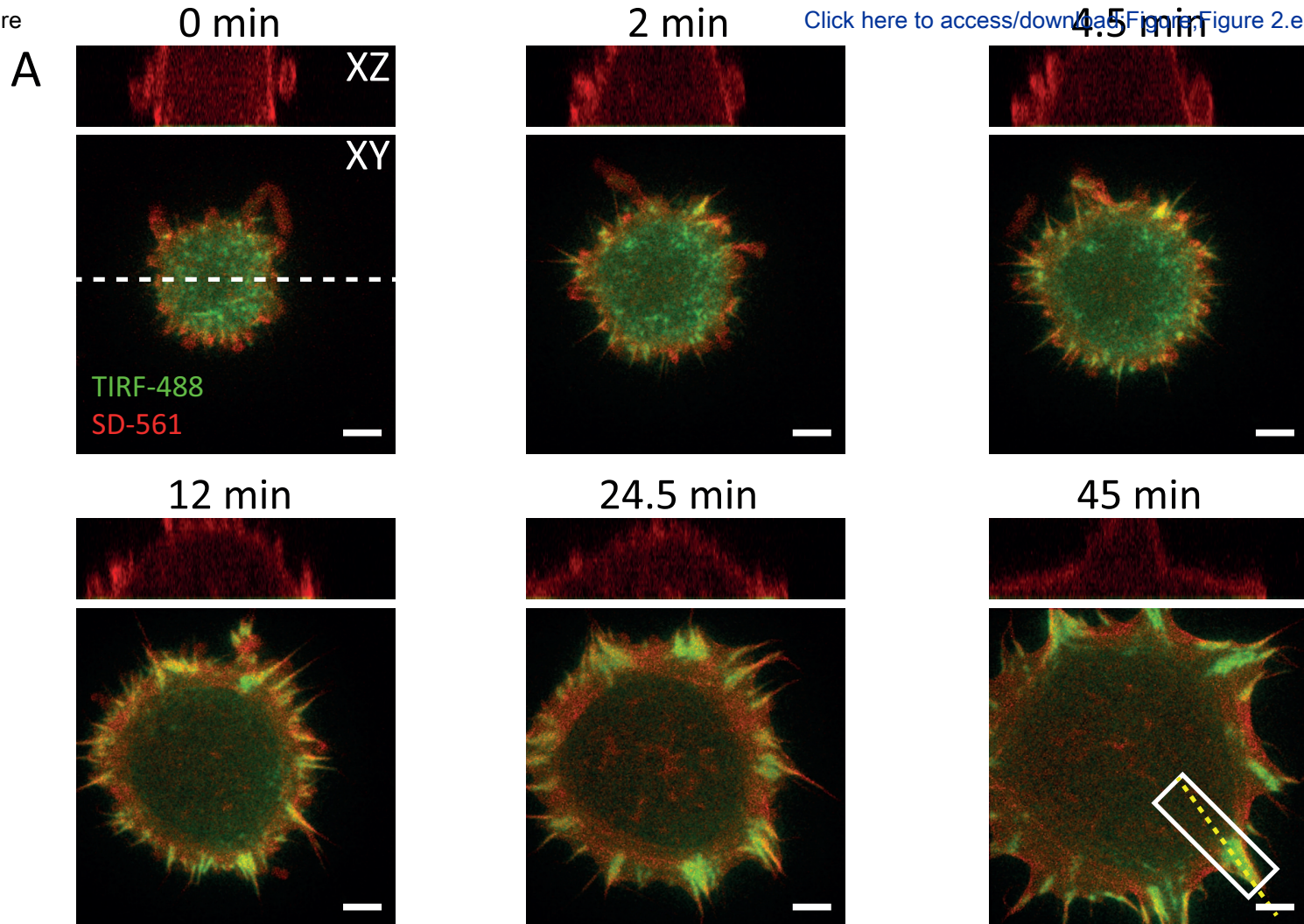
444 22. Shao, L., Kner, P., Rego, E.H., Gustafsson, M.G.L. Super-resolution 3D microscopy of live
445 whole cells using structured illumination. *Nat. Methods*. **8** (12), 1044–1046 (2011).

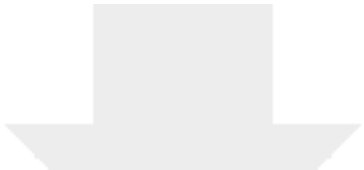
446 23. Chen, B.-C. *et al.* Lattice light-sheet microscopy: Imaging molecules to embryos at high
447 spatiotemporal resolution. *Science*. **346** (6208), 1257998–1257998 (2014).

448 24. Fu, Y., Winter, P.W., Rojas, R., Wang, V., McAuliffe, M., Patterson, G.H. Axial
449 superresolution via multiangle TIRF microscopy with sequential imaging and
450 photobleaching. *Proceedings of the National Academy of Sciences*. **113** (16), 4368–4373
451 (2016).

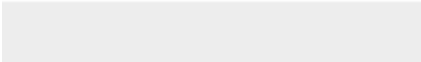

452 25. Boulanger, J. *et al.* Fast high-resolution 3D total internal reflection fluorescence
453 microscopy by incidence angle scanning and azimuthal averaging. *Proceedings of the*
454 *National Academy of Sciences*. **111** (48), 17164–17169 (2014).

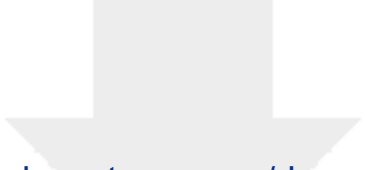




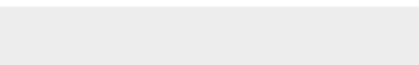



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Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Microscope and accessories			
SD-TIRF microscope	Visitron Systems		
Ti with perfect focus system	Nikon		Inverted microscope stand
CSU-W1 T2	Yokogawa		Spinning disk unit in dual-camera configuration
iLAS2	Roper Scientific		TIRF/FRAP scanner
Evolve	Photometrix		EM-CCD cameras
PiezoZ stage	Ludl Electronic Products		Motorized Z stage
Bioprecision2 XY stage	Ludl Electronic Products		Motorized XY stage
Stage top incubation chamber	Okolab	Bold Line	Temperature, CO ₂ and humidity supply
Cell culture			
HeLa cervical cancer cells	DSMZ	ACC-57	
NIH3T3 fibroblasts	DSMZ	ACC-59	
Dulbecco's phosphate buffered saline (PBS)	Gibco	14190144	
Trypsin-EDTA 0.05%	Gibco	25300054	
Dulbecco's Modified Eagle Medium + GlutaMAX-I (DMEM)	Gibco	31966-021	
OptiMEM	Gibco	31985070	Reduced serum medium
Fetal calf serum (FCS)	Gibco	10500064	
Penicillin/Streptomycin (PenStrep)	Gibco	15140148	
Full growth medium (DMEM supplemented with 10% FCS and 1% PenStrep)			
TurboFect	ThermoFisher Scientific	R0531	Transfection reagent
Ascorbic acid (AA)	Sigma	A544-25G	
6-well cell culture plate	Sarstedt	83.392	
Glass bottom dishes	MatTek	P35G-1.5-10-C	35mm, 0.17mm glass coverslip

Fibronectin, bovine plasma	ThermoFisher Scientific	33010018
Neubauer improved chamber	VWR	631-0696
TetraSpeck beads	ThermoFisher Scientific	T7279

Plasmids

RFP-Lifeact
YFP-Vinculin

Maren Rudolph, Institute of Medical Microbiology, Univ
Andrea Mordhorst, Institute of Medical Microbiology, U

Software and plugins

VisiView	Visitron Systems
ImageJ	
Turboreg plugin	
Volocity	PerkinElmer

Version 3
Version 1.52c
<http://bigwww.epfl.ch/thevenaz/turboreg/>
Version 6.2.2

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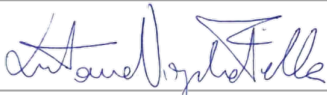
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Dr. Antonio Virgilio Faiilla

Manuscript Revision

Date 31.08.2018

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. Zobiak¹ (b.zobiak@uke.de), Antonio Virgilio Faiilla¹ (a.faiilla@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.



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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 past¹³. 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 demonstrated⁷.” (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 imaging¹⁴. 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 illumination⁸." (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 FIJI¹⁰, 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 FIJI¹⁵, 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 plugin¹¹ 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 plugin¹⁶ 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 200nm⁷) 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 report¹⁷, 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.

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- 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 illumination⁸.” (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 % CO₂ 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

A handwritten signature in blue ink, appearing to read 'Antonio Virgilio Failla', with a stylized flourish at the end.

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Manuscript number: JMI-2017-0043.R1

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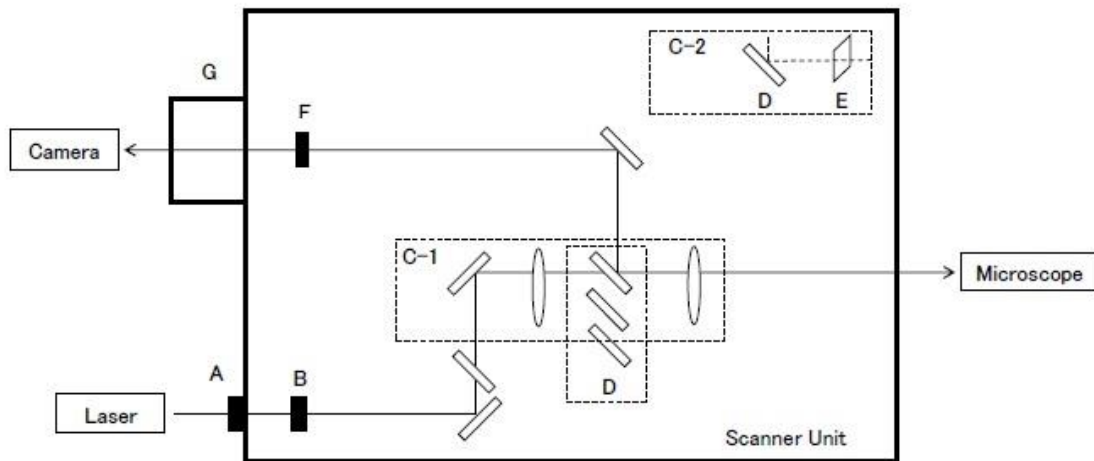
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Date:

T1 Model



A: VIS Port

B: Shutter

C-1: Confocal path

C-2: BF path

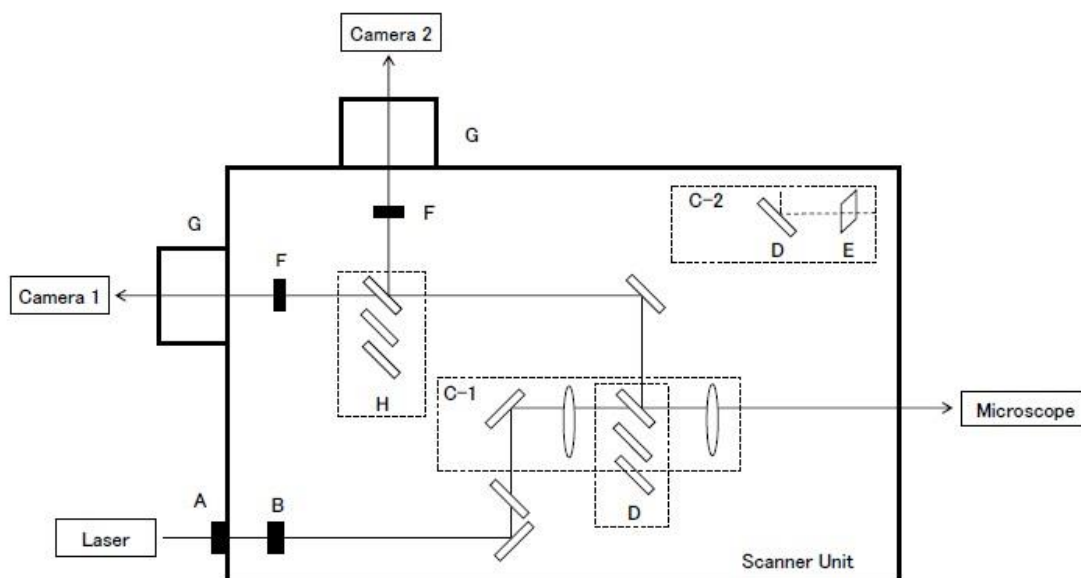
D: Dichroic mirror (Refer to the specification sheet)

F: EM Filter (Refer to the specification sheet)

G: Camera port

Selected dichroic mirror and EM filters are installed at factory

T2 Model



A: VIS port

B: Shutter

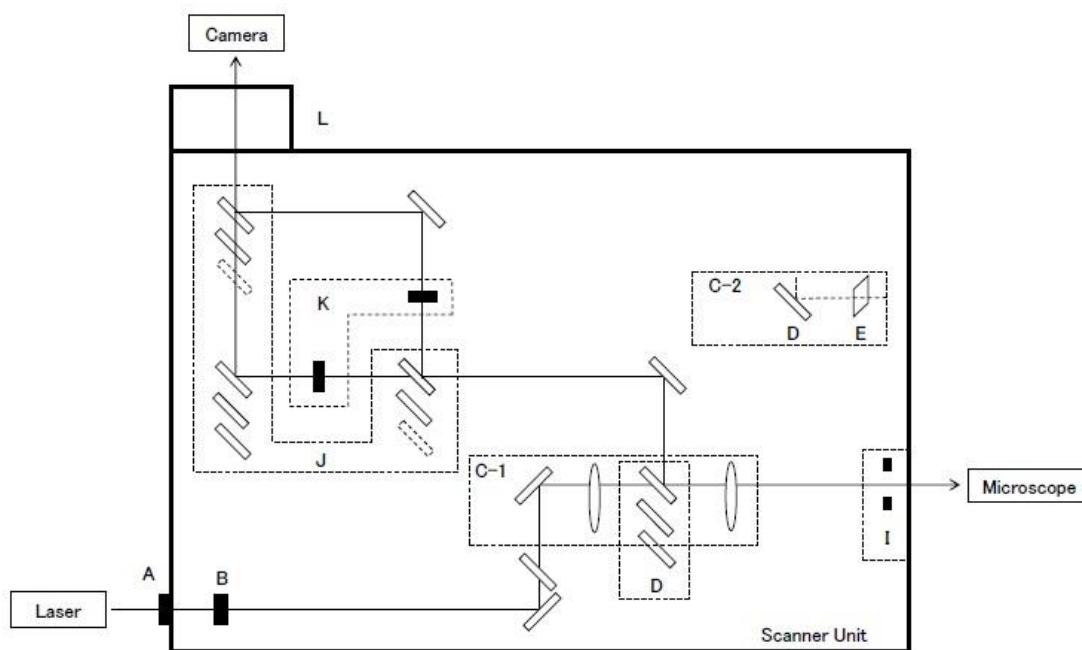
C-1: Confocal path

C-2: BF path

- D: Dichroic mirror (Refer to the specification sheet)
 F: EM Filter (Refer to the specification sheet)
 G: Camera port
 H: Light path exchange unit (Either a dichroic mirror or a 100% mirror)

Selected dichroic mirror and EM filters are installed at factory

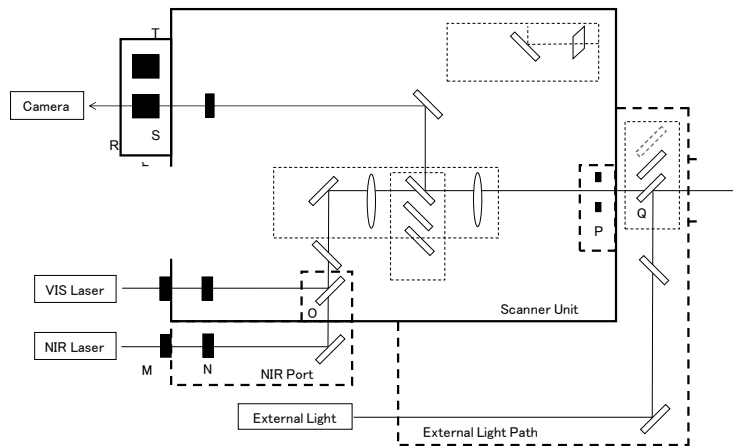
T3 Model



- A: VIS port
 B: Shutter
 C-1: Confocal path
 C-2: BF path
 D: Dichroic mirror (Refer to the specification sheet)
 I: Variable aperture
 J: Light path exchange unit (Dichroic mirror)
 K: EM filter (Refer to the specification sheet)
 L: Camera port

Selected dichroic mirror and EM filters are installed at factory

Options (Example: T1 Model with full options)



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M: NIR Port

N: Shutter for NIR Port

O: Dichroic mirror for NIR Port (Refer to specification sheet)

P: Variable aperture

Q: Dichroic mirror for External Light Path (Refer to specification sheet)

R: Lens Switcher

S: Lens1

T: Lens2

Selected dichroic mirrors are installed at factory