

Video Article

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

Bernd Zobiak¹, Antonio Virgilio Failla¹

¹UKE Microscopy Imaging Facility, University Medical Center Hamburg-Eppendorf

Correspondence to: Antonio Virgilio Failla at a.failla@uke.de

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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 scale^{1,2,3,4,5,6}. 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

- 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.
- One day prior to the experiment, prepare the transfection reagents according to the manufacturer's recommendations or an empirically determined protocol, e.g.:
 - Dilute 1 μ g of RFP-Lifeact and 1 μ g of YFP-Vinculin in a total of 200 μ L reduced serum medium. Vortex the transfection reagent briefly, add 4 μ L to the DNA and vortex again. Incubate the transfection mix for 15-20 min at room temperature.
 - Add the transfection mix dropwise directly to the cells. Mix by shaking the plate and place it back into the incubator.
- On the day of the experiment, prepare the sample for live imaging:
 - Prepare a 10 μ g/ μ 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.
 - Dilute a 0.1 μ m 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.
 - 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.
 - Wash the cells with 2mL PBS, add 250 μ L 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.
 - 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

- 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.
- Fix all acquisition settings at the microscope before the cell suspension is applied:
 - 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").
 - 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.
 - Set the z-stack for the spinning-disk channels to 10 μ m with 0.4 μ m 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.
 - Activate the multi-point function "stage positions".
NOTE: Up to 3 positions can be recorded in a 30 s time interval.
- 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 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.
- Mix the cell suspension again by inverting the closed tube 2-3 times, and apply 1 mL of the cells to the imaging dish.
- 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.
- Start data acquisition by clicking on the "Sequence" button.

3. Image post-processing in ImageJ

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

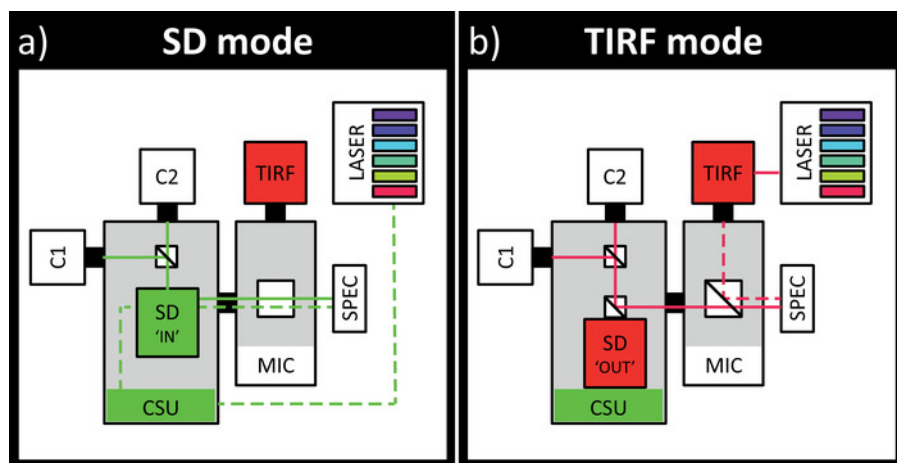


Figure 1: Schematic drawing of the SD-TIRF setup. A. SD imaging mode: the 6 different laser lines (405/445/488/515/561/640nm, green lines) are coupled into the confocal scanning unit (CSU), passing through the SD (position 'IN') and an empty filter cube in the microscope body (MIC). Fluorescence emission 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 [Please click here to view a larger version of this figure.](#)

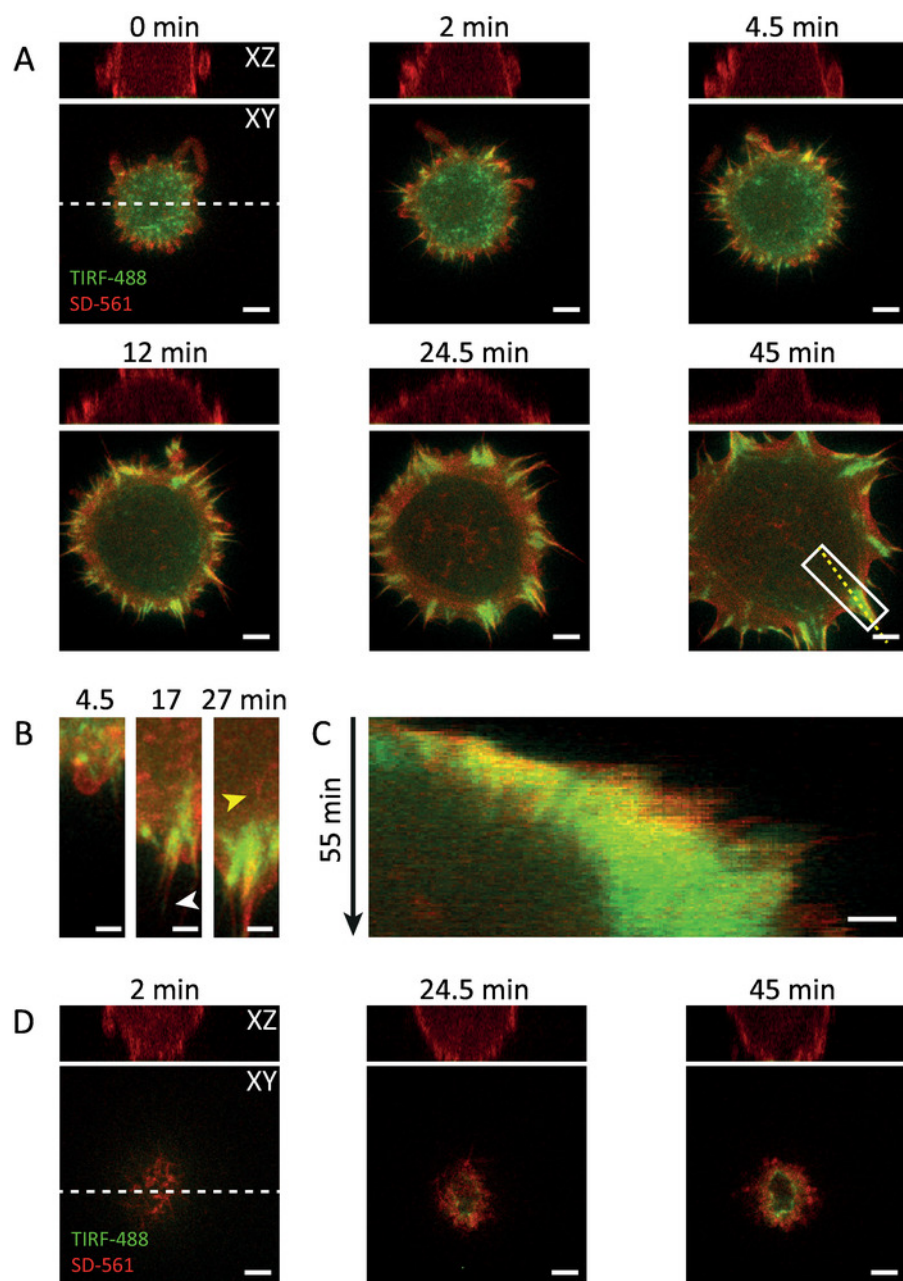
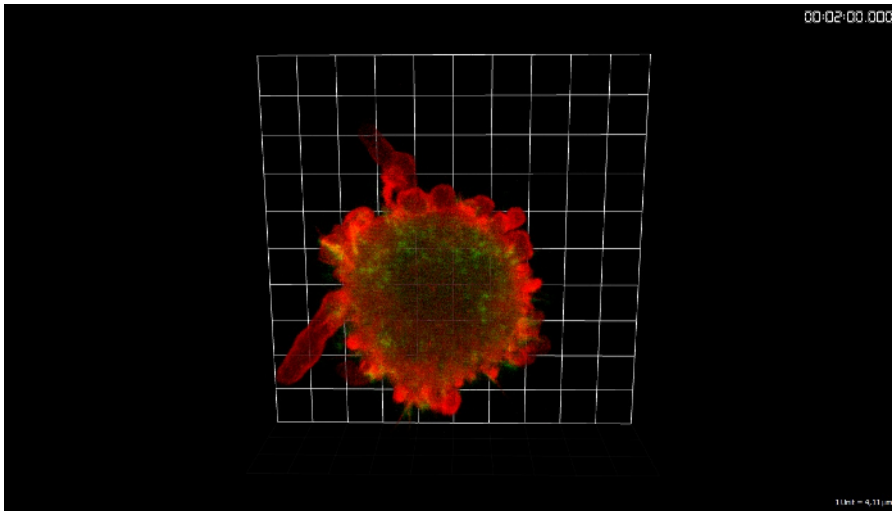
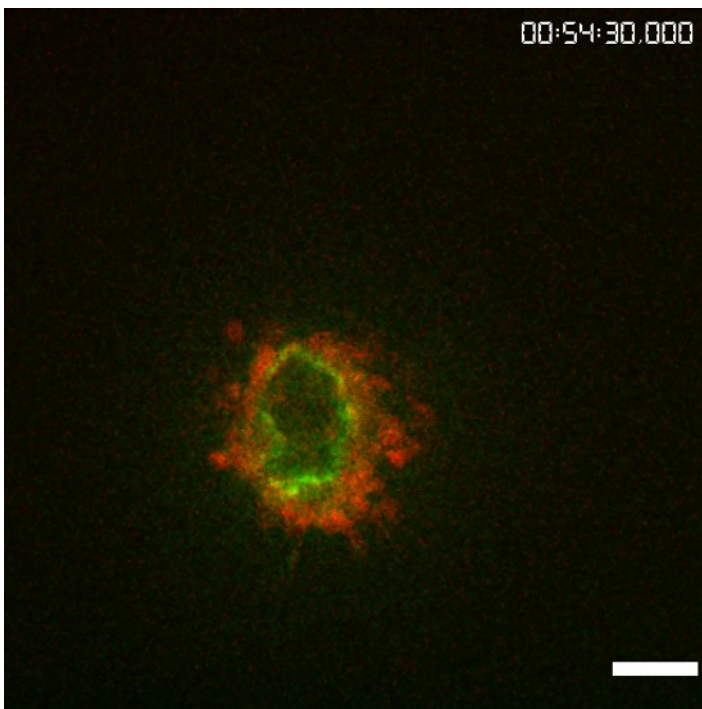


Figure 2: Representative results of cell spreading and adhesion formation using SD-TIRF microscopy. **A.** Double-transfected HeLa cell expressing RFP-Lifeact (red, SD-561 channel) and YFP-Vinculin (green, TIRF-488 channel) were trypsinized and re-seeded onto fibronectin-coated glass coverslips. Adhesion 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 μm (in A and D) and 2 μm (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. [Please click here to view a larger version of this figure.](#)



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. [Please click here to view this video.](#) (Right-click to download.)



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. [Please click here to view this video.](#) (Right-click to download.)

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 live 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.

Disclosures

The authors have nothing to disclose.

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