Advanced spinning disk-TIRF microscopy for faster imaging of the cell interior and the plasma membrane

BERND ZOBIAK & ANTONIO VIRGILIO FAILLA
UKE Microscopy Imaging Facility, University Medical Center Hamburg-Eppendorf (UKE), Hamburg, Germany

Key words. Biology, fluorescence microscopy, illumination design, multiple imaging, three-dimensional microscopy, time imaging.

Summary
Understanding the cellular processes that occur between the cytosol and the plasma membrane is an important task for biological research. Till now, however, it was not possible to combine fast and high-resolution imaging of both the isolated plasma membrane and the surrounding intracellular volume. Here, we demonstrate the combination of fast high-resolution spinning disk (SD) and total internal reflection fluorescence (TIRF) microscopy for specific imaging of the plasma membrane. A customised SD-TIRF microscope was used with specific design of the light paths that allowed, for the first time, live SD-TIRF experiments at high acquisition rates. A series of experiments is shown to demonstrate the feasibility and performance of our setup.

Introduction
In the last 15 years, light microscopy underwent a profound revolution. Imaging with subwavelength-sized resolution of both fixed and living samples is now possible due to the flourish of different novel super resolution techniques, as are, for example, STED (stimulated emission depletion), PALM (photoactivation localisation microscopy), STORM (stochastic optical reconstruction microscopy) and SIM (structured illumination microscopy) (Hell & Wichmann, 1994; Gustafsson, 2000; Betzig et al., 2006; Hess et al., 2006; Rust et al., 2006; van de Linde et al., 2011). Moreover, high-resolution live imaging of macroscopic specimen became accessible after the implementation of novel approaches, that is SPIM (selective plane illumination microscopy) (Huisken et al., 2004; Keller et al. 2008). These imaging techniques benefit from technological developments that have strongly improved the sensitivity of detectors as well as the speed and capacity of data storage devices. All these progresses are extremely beneficial for the biological and biomedical field (Toomre & Bewersdorf, 2010; Eggeling et al., 2015; Sydor et al., 2015). Technological development, however, is still ongoing and will further permit relevant improvements to the speed, performance and resolution of imaging systems. For example, fast super resolution imaging of living animals with subcellular resolution is a target that might be achieved in the near future.

Light microscopy progresses result to be also beneficial for the research focused on studying membrane-mediated interaction between the cell and its environment. This is extremely important since a wide variety of processes are regulated via the plasma membrane, for example endo-/exocytosis, adhesion, migration or signalling. The understanding of those phenomena could provide relevant progress in many biological and biomedical branches such as neurobiology, developmental biology, tumour biology and microbiology (Axelrod, 2008; Mattheyses et al., 2010).

However, membrane-mediated processes are still not easily accessible by light microscopy. There are two prerequisites for imaging such events. The first: it is essential to isolate and localise the cell membrane. The second: it is necessary to track the fast movement of subcellular organelles in the volume enclosed by the cell membrane. Despite most of the available super resolution methods allow three-dimensional (3D) imaging, only TIRF microscopy, due to the exponential decay of its signal, can specifically localise the ventral plasma membrane of adherent cells, by comparing the time variation of the signal arising from subcellular structures, for example vesicles approaching or leaving it, with the exponential decay curves shown in Figures S1(C), (D). However, the localisation of the plasma membrane should be combined with the tracking of subcellular organelles, like vesicles, moving between the plasma membrane and the cytoplasm. In other words, TIRF microscopy needs to be combined with fast 3D imaging, a condition still not achievable by super resolution microscopy techniques.

At the present state of the art, fast highly resolved 3D stacks can be provided by three different approaches. The first is wide field microscopy supported by deconvolution algorithms. It is simple, fast and cheap, can be easily combined with TIRF microscopy but, as all the deconvolution-based imaging, is not fully reliable for processing low signal-to-noise ratio acquisitions. The second is 3D super-resolution microscopy
such as 3D SIM or lattice light sheet microscopy (Shao et al., 2011; Chen et al., 2014). Acquisition speed and resolution are high and thus might also be combined with TIRF in the future. This approach will prospectively provide the better signal-to-noise ratio without losing resolution. However, the implementation of this configuration appears to be complex. The third approach is spinning disk microscopy. This method provides high-speed, high-resolution and good signal-to-noise ratio, especially if compared with wide field microscopy.

The idea of integrating spinning disk and TIRF microscopy has already been published (Trache & Lim, 2009; Stehbens & Wittmann, 2014). However, none of these examples can be considered as a proof of principle for a setup thought to fulfill the demands illustrated in the previous paragraphs. In fact, in these microscopes, the spinning disk and the TIRF unit were placed onto two different detection paths, requiring long time for switching between them. The switching time is indeed the critical factor to prevent an efficient spinning SD-TIRF acquisition in a fast live imaging experiment.

In this work, we will show for the first time, according to our knowledge, a custom-designed microscope combining both dual channel spinning disk and TIRF microscopy. This imaging system is able to isolate the plasma membrane and, at the same time, to track events in the cell volume enclosing it. The key factor to achieve this goal was to use the same detectors for both SD and TIRF microscopy. This allowed us to perform fast sequential imaging switching between spinning disk and TIRF. As a proof of principle, we will show a series of examples of live cell movies, such as exocytosis, vesicle trafficking and cytoskeletal dynamics. Please note that the purpose of the experiments shown in this work is to demonstrate the capabilities of the setup without performing any quantitative analysis on the images. Thus, the description of imaging data is only qualitative.

Materials and methods

Microscope setup

In Figure 1, the custom setup of the integrated spinning disk-TIRF (SD-TIRF) microscope from Visitron systems (Puchheim, Germany) is illustrated. The setup is composed of an inverted Nikon Ti microscope (Tokyo, Japan) connected to the scan units via two independent ports. The left port is linked to a spinning disk unit (CSU, Yokogawa CSU W1, Tokyo, Japan) in two camera configuration. The back port is connected to an iLAS2 scanner unit (TIRF, Roper Scientific, Martinsried, Germany) designed for selective TIRF illumination and photo-bleaching/conversion experiments. As excitation sources, six laser lines, namely 405/445/488/515/561/640 nm, are coupled into the system via multiple beam-paths emerging from a laser combiner unit. For excitation and detection, a Nikon Plan-Apochromat TIRF 100×/NA 1.45 oil objective is used. In the detection path, the emitted light is split inside the CSU scan head by one of two switchable dichroics, that is 561 nm long-pass and 514 nm long-pass. After passing through emission filters (band-pass centred at 525 nm, width 50 nm, and band-pass centred at 609 nm, width 54 nm, for green and red fluorescence, respectively) placed in front of each detector, the light is finally directed to two EM-CCD cameras (C1, C2) can be used for simultaneous acquisition of, for example green/red or cyan/yellow fluorescence, the emission light (solid lines in A and B) is split by different dichroic mirrors inside the CSU (not shown).

![Fig. 1. Visitron SD-TIRF setup. The laser unit (LASER) hosts six different laser lines (405/445/488/515/561/640 nm, dashed lines in A and B, which are coupled via multiple fibres into either the confocal scanning unit (CSU) or the TIRF scanner (TIRF). Switching between SD (A, green light path) or TIRF (B, red light path) acquisition requires the change of the filter cube inside the microscope body (MIC; empty position for SD, quad cube for TIRF) and the movement of the SD in the CSU (‘IN’ for SD, ‘OUT’ for TIRF). Two EM-CCD cameras (C1, C2) can be used for simultaneous acquisition of, for example green/red or cyan/yellow fluorescence, the emission light (solid lines in A and B) is split by different dichroic mirrors inside the CSU (not shown).](image-url)

Images and movies in this manuscript were generated either by FIJI (Schindelin et al., 2012) (Figs. 2–4 and Movies S1–S3) in combination with the integrated plugins ‘Orthogonal Views’ and ‘3D Project . . . ’, or by Volocity (PerkinElmer, Rodgau, Germany) (Fig. 5 and Movies S4A–C). Vesicle tracking was performed with the ‘Track objects manually’ function in Volocity.

This microscope allows for fast simultaneous dual-channel spinning disk imaging followed by simultaneous dual-channel TIRF imaging. The fluorescence light emitted from the sample is either passing the dual spinning disks (SD mode, Fig. 1A) or bypassing them (TIRF mode, Fig. 1B). This configuration gives rise to three main advantages: first, the collected TIRF signal is maximised, since it is not reduced by the SD pinholes. Second, the system uses for the first time, according to our knowledge, the same detectors for SD and TIRF imaging, providing a perfect overlap of both TIRF and SD datasets. Thus, manual or
PKH26-labelled exosomes were incubated for 90 min with N2a cells. A total of 35 spinning disk (SD) image planes followed by 1 TIRF (TIRF) image plane were captured to generate a single volume. (A) The maximum intensity projection showed the distribution of exosomes in the cell at $t = 0\,\text{s}$. (B) Two vesicles whose axial positions were indistinguishable in the SD channel could be localised more precisely due to the presence (left) or absence (right) of a signal in the TIRF channel. (C) The $XZ$ projection showed the leverage of a single exosome (arrowhead) that was also visible in the TIRF channel for about 360 s and then detached from the plasma membrane at $t = 380\,\text{s}$. Scale bar = 5 $\mu\text{m}$ (in A and C) and 1 $\mu\text{m}$ (in B). Frame rate = 20 s frame$^{-1}$.

Sample preparation

All cells were cultured in a 37 $^\circ\text{C}$, 5% CO$_2$ and humidified incubator. The following growth media and transfection methods were used: HEK293 and HeLa cells were grown in DMEM/10% foetal calf serum/penicillin and transfected with Turbofect (ThermoFisher Scientific, Waltham, MA, USA). N2a cells were grown in DMEM with 10% exosome-depleted foetal calf serum (Atlas Biologicals, Fort Collins, CO, USA) and the exosomes were purified as described previously (Falker et al., 2016). Exosomes were PKH26-labelled (Sigma Aldrich, #MINI26-1KT, St. Louis, MO, USA) and measured to be 132 nm in diameter. Peripheral human macrophages were isolated from buffy coats, cultured as described previously (Linder et al., 1999) and transfected with the Neon® electroporator (Invitrogen, Carlsbad, CA, USA). Transient cell transfection was performed 1 day (N2a, HeLa, macrophages) or 3 days (HEK293), respectively, before the experiment. The following plasmids were transfected: GFP-Snx27, RFP-$\beta$1AR, GFP-CLN3, KIF9-GFP

Software-based image alignment issues and distortions, which could be experienced in another custom built setup similar to (Trache & Lim, 2009; Stehbens & Wittmann, 2014), are avoided. Third, the acquisition speed is enhanced, because only two components are moved for rapid switching between SD and TIRF mode: the fluorescence turret and the SD unit. In more detail, the switching time of the turret between the empty position for SD imaging and the adjacent position housing a quad-band filter cube for TIRF imaging is 150 ms (Nikon, 2016). To move the SD in or out requires about 500 ms. No additional time is consumed by rapidly changing the illumination ($<10$ ms) within the laser combiner. Altogether this results in a possible minimum cycle time for single plane, dual-channel SD-TIRF image datasets of 1286 ms with 100 ms exposure for SD and TIRF channels, respectively. In comparison, the same dataset acquired in single camera mode will take 1906 ms. Thus, the SD-TIRF switching time is faster than changing the microscope detection path as reported in other setups (Stehbens & Wittmann, 2014).
HEK293 cells were transfected with GFP-Snx27 and RFP-β1AR. After stimulation with isoproterenol, a 13-image planes dual channel SD stack (SD-488 and SD-561), followed by one dual channel TIRF image (TIRF-488 and TIRF-561) was captured. (A) A β1AR vesicle (arrowhead and insert) could be followed from the depth of the cell, accessing the plasma membrane and contacting a Snx27 vesicle. (B) The β1AR vesicle (SD/TIRF-561), after contacting a Snx27 vesicle (SD/TIRF-488), dissociated and returned to the perinuclear pool. All the images shown in this figure are displayed as they were acquired, exception made for the TIRF-488/TIRF-561 frames shown in B, where a median filter and linear contrast enhancement have been applied in order to improve visibility. Scale bar = 5 µm (in A) and 0.5 µm (in B). Frame rate = 3.5 s frame⁻¹.

Results

Single-colour spinning disk and TIRF imaging can be combined in a single dataset

In a first experiment, the interaction of fluorescently labelled exosomes with neuronal cells was investigated. As transmitting agents, exosomes seem to play a major role in the spreading of neurodegenerative diseases (Kalani et al., 2014). A day before the experiment, exosomes were purified from the neuronal cell line N2a and then marked with the red fluorescent dye PKH26. Before starting imaging, N2a cells were allowed to endocytose labelled exosomes for 90 min at 37°C at a ratio of circa 50 particles per cell. The dynamic redistribution in the cell was then recorded with SD-TIRF microscopy. A z-stack covering a 14 µm thick volume (35 z-planes with 0.4 µm spacing) was necessary to visualise the entire distribution of exosomes in the cell (Fig. 2A).

However, only by means of TIRF microscopy it was possible to see when and where there was a close contact of vesicles with the ventral plasma membrane. The time lapse sequence in Figure 2(C) (for a movie see Movie S1) clearly showed the contact of a single intracellular exosome with the plasma membrane (arrowhead, exosome was also visible in the cyan-coloured TIRF channel) for more than 360 s, when...
HeLa cells were transfected with GFP-CLN3 and stained for lysosomes with Lysotracker Red DND-99. A 13-image planes dual channel SD stack (SD-488 and SD-561), followed by one dual channel TIRF image (TIRF-488 and TIRF-561) was captured. (A). (B) A CLN3-positive lysosome (white arrowhead) was temporarily colocalising (possibly touching or fusing) with another lysosome (yellow arrowhead). The TIRF signal intensities varied, indicating a closer association with the plasma membrane and possible exocytosis. One vesicle reappeared at the plasma membrane at $t = 144\,\text{s}$ and finally lifted off at $t = 154\,\text{s}$. (C) XZ reconstruction of the lifting vesicle from $t = 149\,\text{s}$ and $t = 154\,\text{s}$ in B. Scale bar = 5 $\mu\text{m}$ (in A) and 1 $\mu\text{m}$ (in B). Frame rate = 4.6 s frame$^{-1}$.

Dual-colour SD-TIRF imaging reveals spatiotemporal interactions of vesicles at the plasma membrane

The mutual interactions between two fluorescently labelled structures and their correlation with the plasma membrane was studied taking advantage of dual-colour SD-TIRF imaging. For subsequent experiments, two SD and two TIRF acquisition channels were set. The first SD/TIRF channel was configured for detecting GFP (SD-488/TIRF-488), while the second SD/TIRF channel enabled the detection of red fluorophores such as RFP or Lysotracker Red DND-99, respectively (SD-561/TIRF-561). A z-stack of 5.2 $\mu\text{m}$ (13 z-planes with 0.4 $\mu\text{m}$ spacing) was a reasonable compromise to cover most of the fluorescent structures while maintaining a high acquisition speed.
Primary human macrophages were transfected with KIF9-GFP and alpha-tubulin-mCherry. A 11-image planes dual channel SD stack (SD-488 and SD-561), followed by one single channel TIRF image (TIRF-488) were captured. (A), (B) A KIF9 vesicle was followed over a course of 273 s, showing a close association with highly dynamic microtubules. Tracking the vesicle revealed its varying proximity with the plasma membrane, highlighted by intensity fluctuations of the TIRF signal present in most of the frames. Further lift-off abolished the TIRF signal completely (e.g. t = 149 and t = 154 s).

Scale bar = 5 μm (in A, left panel) and 1 μm (in A, right panel, and B). Frame rate = 6 s frame⁻¹.

In the first example, HEK293 cells were transfected with plasmids coding for GFP-Snx27, known to be involved in the recycling of cell surface receptors, and RFP-β1AR, the β1 adrenergic receptor (Nakagawa & Asahi, 2013). After stimulation with the β1AR-agonist isoproterenol, cells were investigated at the microscope. From a perinuclear pool of RFP-positive vesicles, a single vesicle could be followed while migrating to the cell periphery and touching the plasma membrane (Fig. 3A, arrowhead, SD-561 and TIRF-561 channel of magnified view in Fig. 3B, and Movie S2). The β1AR-vesicle was moving in proximity to the plasma membrane until it colocalised with a GFP-positive Snx27-vesicle (Fig. 3B), which was only visible in the SD-488 but not in the TIRF-488 channel. Upon temporal colocalisation, the β1AR-vesicle left the plasma membrane (no more signal in the TIRF-561 channel), but remained visible for about 20 s in the SD-561 channel until it also dissociated from the Snx27-vesicle and returned to the perinuclear pool.

In the second example, HeLa cells were transfected with GFP-CLN3, an endosomal/lysosomal glycoprotein, and stained for lysosomes with Lysotracker Red DND-99. From the majority of lysotracker-positive CLN3-vesicles, approximately...
20% were also detectable in the TIRF-561 channel (see Fig. 4A and Movie S3). Here, the dynamic interactions of CLN3-positive lysosomal vesicles have been studied, as depicted in the time lapse sequence in Figure 4(B).

In a zoomed area, two vesicles, marked with a white and a yellow arrowhead, were visible from time point \( t = 93 \) s on. One of the two vesicles (yellow arrowhead) was more distant from the plasma membrane, as indicated by the dimmer signal in the SD channels and the absence of the signal in the TIRF channels. We observed temporal colocalisation of several vesicles, when between \( t = 107 \) s and \( t = 116 \) s the signal increased in all channels and only a single vesicle was detectable. Further and more detailed studies might determine if this signal superposition is the result of a very close interaction, for example fusion. Upon separation or fission, two vesicles remained visible until \( t = 135 \) s in short distance to each other, but only one of them in proximity to the plasma membrane (white arrowhead, TIRF channels). Subsequently, both vesicles started to disappear from the plasma membrane. This was evident by watching at the white arrowhead marked vesicle in the XZ reconstruction of time points \( t = 149 \) s and \( t = 154 \) s in Figure 4(C).

**SD-TIRF imaging enhances the spatiotemporal resolution of the dynamic interaction of vesicles with the plasma membrane and the cytoskeleton**

It was demonstrated that GFP-KIF9 vesicles move along microtubules, regularly contacting podosomes (Cornfine et al., 2011), actin-rich structures that are involved in matrix-degradation and visible in the ventral site of adherent monocytic cells such as macrophages. To further elucidate the dynamic interplay of KIF9 with microtubules and the plasma membrane, SD-TIRF imaging was used. To do so, GFP-KIF9 was cotransfected with mCherry-alpha-tubulin in primary human macrophages. The GFP-signal was detected in the SD-488 and TIRF-488 channels, while the mCherry signal was displayed only in the SD-561 channel. A finer sampled 2.75-µm z-stack (11 z-planes with 0.25 µm spacing) was necessary and sufficient to resolve most of the peripheral microtubules and KIF9-vesicles albeit maintaining a high temporal resolution. The still image shown in Figure 5 and time lapse sequence (Movie S4A) clearly show temporal overlap between the signal arising from vesicles and microtubules that suggests the colocalisation of vesicles with microtubules. A single vesicle was tracked to reveal its position in all spatial directions over time (Fig. 5B and Movies S4B and S4C).

The XZ projection in Figure 5(B) clearly demonstrated that the vesicle was very dynamically moving in the axial and lateral directions. Intensity fluctuations visible in the TIRF-488 channel show repetitive contacts of the vesicle with the plasma membrane, possibly at podosomal sites. Please remember that the TIRF-signal of any isolated vesicle is directly correlated with its distance to the plasma membrane (Mattheyes et al., 2010). In details, the brighter is the fluorescence the closest the vesicle is to the plasma membrane (see also Fig. S1B), as confirmed by the axial position of the vesicle in the SD-488 channel in Figure 5(B).

**Discussion**

In this paper, we showed a greatly improved model of a SD-TIRF microscope and its application in a range of live imaging experiments. Although similar systems have been published before, there were two main disadvantages that might have hampered their application in biological experiments: first, image acquisition was too slow due to the light path design. Second, a perfect overlay between SD and TIRF images was not possible to be achieved, due to the separate detection paths for SD and TIRF modes that cause shifts and distortions. Hence, we decided to design the light path of our microscope to overcome those limitations. The Yokogawa CSU-W1 spinning disk unit has the possibility to move the dual-disk in and out of the detection path: this is a great benefit for the collection of the TIRF signal, which would otherwise be reduced by the pinholes. Additionally, the same cameras could be used for the detection of the SD and TIRF signal. The result was a high-precision overlap of the field of view of the wide field TIRF and laser scanned spinning disk images (checked by imaging fixed samples, see Fig. S2) as well as fast sequential acquisition of the two imaging modes. Running the Roper iLAS² scanner in so-called time-sharing mode allowed us to detect two TIRF channels simultaneously, speeding up the acquisition of complex, multichannel data. Indeed, the speed of the system is currently limited only by the movement of the dual-disk inside the scan head. This setup configuration can be easily improved by taking advantage of technological developments. A faster motor or the possibility to circumvent the disk movement, for example through bypassing the disk with galvanometer mirrors, would further reduce the switching time between SD and TIRF mode.

We demonstrated that, with our microscope, we were able to visualise the temporal overlapping of biological structures and to follow them in live imaging experiments (see Figs. 2–5). Please note that, in the previous mentioned experiments, we sacrificed imaging quality (high signal-to-noise ratio) to privilege noninvasive imaging conditions, i.e. negligible bleaching and reduced phototoxicity. Moreover, the signal-to-noise ratio can be improved easily by implementing a new generation camera with more sensitivity in the current setup. We were profiting from the fact that SD and TIRF microscopy are two imaging methods that are especially suited for live imaging, due to their low phototoxicity and high acquisition rates. Combining SD and TIRF datasets allowed 3D reconstructions of fast intracellular processes with high resolution of the bottom plane. This is of great importance, if interactions of subcellular organelles with the plasma membrane are investigated. SD imaging alone is too limited in the z-resolution and would not be able to reveal such contacts (see Fig. 2B). TIRF imaging...
allowed this differentiation. In fact, TIRF allows the specific localisation of a 100–200 nm excitatory zone above the coverslip from the residual volume of the cell (Axelrod, 2008) – a compartment known to comprise the plasma membrane. Furthermore, recent approaches with multigain TIRFM (Fu et al., 2016) have increased the axial resolution down to about 20 nm and could be implemented in our microscope.

Our setup is easy to build up and readily available to the scientific community. The combination of SD and TIRF in a single system designed, as shown in this paper, will encourage researchers to gain insight into cellular mechanisms that could not be unveiled before.

Acknowledgements

We greatly thank the scientific community of the University Medical Center Hamburg-Eppendorf for supporting us with samples for evaluation. Namely, we thank Alexander Hartmann for providing N2a cells and labeled exosomes, and Malte Klüssendorf, Johannes Brand and Andrea Mordhorst for providing transfected HEK293 cells, HeLa cells and macrophages, respectively.

References

Axelrod, D. (2008) Total internal reflection fluorescence microscopy. Methods in Cell Biology (ed. by J.J. Correia & H.W. Detrich). Chapter 7, 1st edn., Vol. 89, pp. 169–221. Elsevier B.V., Amsterdam, The Netherlands.

Bettaig, E., Patterson, G.H., Sougrat, R. et al. (2006) Imaging intracellular fluorescent proteins at nanometer resolution. Science (New York, N.Y.) 313(5793), 1642–1645.

Chen, B.-C., Legant, W.R., Wang, K. et al. (2014) Lattice light-sheet microscopy: imaging molecules to embryos at high spatiotemporal resolution. Science 346(6208), 1257998–1257998.

Cornfine, S., Himmel, M., Kopp, P., El Azzouzi, K., Wiesner, C., Krüssmann, T., Linder, S., Aldrich syndrome protein regulates podosomes in primary human macrophages. J. Cell Sci. 123(Pt 21), 3621–3628.

Nakagawa, T. & Asahi, M. (2013) beta1-adrenergic receptor recycling via a membrane organanelle, recycling endosome, by binding with sorting Nexin27. J. Membr. Biol. 246(7), 571–579.

Nikon. (2016) Eclipse Ti Brochure. Retrieved from https://www.nikoninstruments.com/content/download/14701/330516/file/2CE-MPFH-6.pdf. Accessed 20 August 2017.

Rust, M.J., Bates, M. & Zhuang, X. (2006) Sub-diffraction-limit imaging by stochastic optical reconstruction microscopy (STORM). Nat. Methods 3(10), 793–796.

Schindelin, J., Arganda-Carreras, I., Frise, E. et al. (2012) Fiji: an opensource platform for biological-image analysis. Nat. Methods 9(7), 676–682.

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

Stehbens, S.J. & Wittmann, T. (2014) Analysis of focal adhesion turnover. A quantitative live-cell imaging example. Methods in Cell Biology (ed by J.C. Waters, T. Wittman). 1st edn., Vol. 123, 335–346. Elsevier B.V., Amsterdam, The Netherlands.

Steyer, J.A. & Almers, W. (1999) Tracking single secretory granules in live chromaffin cells by evanescent-field fluorescence microscopy, 76(April), 2262–2271.

Sydor, A.M., Caymme, K.J., Puchner, E.M. & Mennella, V. (2015) Super-resolution microscopy: from single molecules to supramolecular assemblies. Trends Cell Biol. 25(12), 730–748.

Toomre, D. & Bewersdorf, J. (2010) A new wave of cellular imaging. Ann. Rev. Cell Dev. Biol. 26(1), 285–314.

Trache, A. & Lim, S.-M. (2009) Integrated microscopy for real-time imaging of mechanotransduction events in live cells. J. Biomed. Opt. 14(3), 34024.

van de Linde, S., Löschberger, A., Klein, T., Heidbreder, M., Wolter, S., Heilemann, M. & Sauer, M. (2011) Direct stochastic optical reconstruction microscopy with standard fluorescent probes. Nat. Prot. 6(7), 991–1009.

Supporting Information

Additional Supporting information may be found in the online version of this article at the publisher’s website:

Fig. S1. Point spread functions of the system were measured with multicolour, subresolution 100 nm beads. (A)–(D) Full width at half maxima (FWHM) in the lateral direction were determined to be 273 nm (A, SD-488), 274 nm (B, SD-561),
257 nm (C, TIRF-488) and 277 nm (D, TIRF-561), respectively. Axial FWHM were determined to be 759 nm (A, SD-488) and 791 nm (B, SD-561), respectively. (C), (D) The TIRF penetration depth was experimentally determined as reported elsewhere (Steyer & Almers, 1999) and overestimated to be 204 nm (C, TIRF-488) and 188 nm (D, TIRF-561), respectively. Scale bar = 1 μm.

**Fig. S2.** The image alignment of all four channels against each other is depicted using multicolour, fluorescent beads. Lateral line profiles of three selected beads (see overlay and zoomed images) are shown in the last column. (A) SD-561 channel versus TIRF-561 channel. (B) SD-488 versus TIRF-488 channel. (C) SD-488 versus SD-561 channel. (D) TIRF-488 versus TIRF-561 channel. Scale bar = 2 μm (full size images) and 0.5 μm (zoomed images), respectively.

**Movie S1:** 3D reconstruction movie of a time lapse sequence showing PKH26-labelled exosomes (SD-561 channel, red) on N2a cells (brightfield, bottom plane only). Time course is approximately 12 min at a rate of 20 s frame$^{-1}$.

**Movie S2:** 3D reconstruction movie of a time lapse sequence showing HEK293 cells transfected with GFP-Snx27 (SD-488 channel in green and TIRF-488 channel in magenta) and RFP-β1AR (SD-561 channel in red and TIRF-561 channel in cyan). Time course is approximately 11 min at a rate of 3.5 s frame$^{-1}$.

**Movie S3:** 3D reconstruction movie of a time lapse sequence showing HeLa cells transfected with GFP-CLN3 (SD-488 channel in green and TIRF-488 channel in magenta) and stained for lysosomes (SD-561 channel in red and TIRF-561 channel in cyan). Time course is approximately 4 min at a rate of 4.6 s frame$^{-1}$.

**Movie S4(A):** Orthogonal views movie of a time lapse sequence showing a GFP-KIF9 vesicle (SD-488 channel, green) randomly walking along mCherry-tubulin-labelled microtubules (SD-561 channel, red) in human macrophages. XZ and YZ reconstructions are flipped compared to the images in Figure 5(A). Time course is approximately 5 min at a rate of 6 s frame$^{-1}$. Time counter is hh:mm:ss.

**Movie S4(B):** Tracking of the same vesicle (SD-488 channel, green) as in Movie S4(A), but selecting the TIRF plane in the XY view (TIRF-488 channel, magenta) to demonstrate both plasma membrane contacts and axial movement in the XZ/YZ orthogonal views. XZ and YZ reconstructions are flipped compared to the images in Figure 5(B). Time counter is hh:mm:ss.

**Movie S4(C):** 3D reconstruction movie of the tracked vesicle shown in Movie S4(B).