Correction: Micro-stepping extended focus reduces photobleaching and preserves structured illumination super-resolution features

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The authors wish to correct an error where Xue et al., 2015 was incorrectly cited instead of Jalal et al., 2019 in the final sentence of the ‘Biological sample preparation’ section of the Materials and Methods.

The correct text is as below:

Transfected fibroblasts were then seeded at a density of 5×10⁴ cells ml⁻¹ onto dishes containing adhesive fibronectin-coated circular islands of 1800 µm² in area that were fabricated by microcontact printing as described previously (Jalal et al., 2019).

Reference


The authors apologise to readers for this error, which does not impact the conclusions of the article. Both the online full text and PDF versions of the article have been corrected.
TOOLS AND RESOURCES

Micro-stepping extended focus reduces photobleaching and preserves structured illumination super-resolution features

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ABSTRACT

Despite progress made in confocal microscopy, even fast systems still have insufficient temporal resolution for detailed live-cell volume imaging, such as tracking rapid movement of membrane vesicles in three-dimensional space. Depending on the shortfall, this may result in undersampling and/or motion artifacts that ultimately limit the quality of the imaging data. By sacrificing detailed information in the Z-direction, we propose a new imaging modality that involves capturing fast ‘projections’ from the field of depth and shortens imaging time by approximately an order of magnitude as compared to standard volumetric confocal imaging. With faster imaging, radiation exposure to the sample is reduced, resulting in less fluorophore photobleaching and potential photodamage. The implementation minimally requires two synchronized control signals that drive a piezo stage and trigger the camera exposure. The device generating the signals has been tested on spinning disk confocal and instant structured-illumination-microscopy (iSIM) microscopes. Our calibration images show that the approach provides highly repeatable and stable imaging conditions that enable photometric measurements of the acquired data, in both standard live imaging and super-resolution modes.

This article has an associated First Person interview with the first author of the paper.

KEY WORDS: Microscopy, Extended focus, Super-resolution, Long-term live-cell imaging, Photobleaching, Rapid projections, Well-defined sectioning

INTRODUCTION

Imaging rapid cellular processes, such as following intracellular membrane trafficking, with sufficient time resolution needs to be weighed against the risk of inducing photobleaching and phototoxicity. Endosomes for example, often grow from resolution-limited spots that fuse to vesicles of a few microns in diameter while steadily undergoing fusion and fission events (Kerr and Teasdale, 2014).

A key event that marks endosome maturation from early to late endosomes, is the change of the Rab proteins coating the endosome, from Rab5 to Rab7 (Rink et al., 2005). This step in the endosome maturation process occurs within a few minutes and involves a dramatic kinetic change of membrane domains (Bergeland et al., 2008; Skjeldal et al., 2012). During maturation, the endosome, driven by intracellular motors along the cytoskeleton, also moves at various speeds up to a few microns per second in different directions inside the 3D cytoplasmic space (Kerr and Teasdale, 2014; Scott et al., 2014). The endosome of interest may then travel while the Z-stack is being captured, leaving visible motion artifacts making it difficult to conduct subsequent quantification analysis. Besides the motion artifacts, collecting such a 3D time lapse stack of 20–30 slices for each time point results in up to thousands of radiation exposures to each cell. This often causes heavy photobleaching of fluorophores and phototoxicity to the cell.

Commonly, to follow and study the dynamics of these vesicles in live cells, Z-stacks are recorded using a confocal microscope. However, even the more rapid spinning disk confocal with fast piezo Z drives take more than a second to record a stack of 20 slices and will hence fall short of the speed required for tracking the changing characteristics of endosomal vesicles. Thus, imaging and tracking the behavior of fluorescence-labeled endosome activity in live cells, is a challenging task despite the recent improvements in localization microscopy techniques (Dertinger et al., 2009; Gustafsson et al., 2016; Rosten et al., 2013; Sage et al., 2015, 2019; Zhao et al., 2018).

In order to capture rapid cellular events without disrupting processes due to photodamage and/or bleaching, cell biologists usually compromise on some imaging parameters, for instance, by not reaching Nyquist sampling of the Z-stacks or by lowering laser power, which worsen the signal-to-noise ratio. The endosomes in our project change dramatically in size, and we need subpixel localization accuracy and a temporal resolution in the order of seconds. Both photobleaching and motion artifacts worsen when aiming for a higher resolution, which makes it impossible for us to embrace the usual compromises.

To follow the membrane kinetics, we do not necessarily need a volume image of the endosome for our application, but merely a consistent projection to allow for accurate tracking, and after volume imaging we use the maximum or average projection feature to be able to follow the endosomes. Hence, we decided to sacrifice the Z-resolution to achieve higher temporal resolution and a reduced bleaching rate as described in this report.

To address this challenge, we introduced a new hardware adaptation, named ‘extended focus’ (EF) that greatly improves the imaging speed by generating a well-defined projection along the depth axis. EF allows any camera-based imaging system to synchronize the movement of the piezo stage and the camera in a precise manner. In addition, an optional signal shutters the lasers off, when the stage is moving, which provides perfectly horizontal sections. The EF Z-scan then combines multiple optical sections into a single exposure. The image generated by EF is equivalent to a sum or average projection virtually identical to summing up the
conventional 3D confocal images, but the EF results in a better than 10-fold increase in speed and more than a 10-fold decrease in radiation exposure and fluorophore bleaching.

Although the EF system in principle can be adapted to any camera-based imaging platform, it is best suited for spinning disk confocal systems. The confocal mechanism is far superior in confining the recorded volume compared to other competing approaches such as wide-field fluorescence (WF) microscopy. We have also tested the performance of EF on iSIM spinning disk systems. As the piezo Z-stage does not travel during light exposure, the EF projection preserves the iSIM super-resolution feature. In other words, the EF projection can be recorded in a super-resolved form, which is the most unique advantage over other extended depth of field methods, and greatly aids size and area measurements.

RESULTS
Designing the extended focus device
The principle of the EF feature is to use the piezo stage already installed in confocal microscopes, to scan in the Z-direction synchronized with the camera-based imaging system and collect the light from the entire scan. The way the stage moves, while the camera is exposed to light, has an influence on the image created; spinning disks scan an image in curved lines – commonly circles or spirals – so when the Z-drive is moving during the exposure, not all parts of an image slice come from the same depth. If the movement is slow, this will not distort the image noticeably. With speeds of less than half the image resolution per scan, the image looks ‘intact’ to the observer and gives the fastest artifact-free recording and only leads to very mild acceleration forces on the specimen. This provides speeds of up to 200 μm s⁻¹ on a CSU-X1 and 40 μm s⁻¹ on the CSU-W1 (two widespread spinning disk units manufactured by Yokogawa). Our initial design of EF used a signal generator (Fig. 1A) or an analog ramp circuit and this worked well for wide-field and confocal projections but disrupted the iSIM pixel-to-pixel connectivity. To improve this, we introduced micro-stepping along the Z-axis so that all sections are flat, that is, the stage is not moving during an exposure. Acceleration for the specimen is only around 0.3 g for millisecond steps, and very fast stepping is easily possible. This leaves the iSIM imaging pathway intact, allowing tracking with a far improved photons-per-frame budget than when recording full

Fig. 1. Development of the EF device. (A) Initial prototype of the EF device. (B) Precursor to current device. (C) Final EF product that can be easily integrated into most conventional microscopy setups. (D) Illustration of sector and turning time in EF imaging. The timing depends on the RPM of the CSUs, but also on how many times per revolution identical sectors repeat. Owing to a more sparse pinhole pattern, the CSU-W1 has a 5 times slower repeat than the CSU-X1. We measured and synchronized to the green pulses and then derived the sector timing internally on the controller. (E) Maximum speed timing of the two CSUs. After each step, the piezo stage was allowed to settle for 2 ms. The sector fly-by time plus the settling time, determine the fastest possible projection. The CSU-X1 has more than twice the projection speed of the CSU-W1 but 5 times less light exposure per image plane.
3D volumes of the same specimen. Visualizing long imaging sequences maintains the quality when integrating the whole projection into a single exposure.

The outline of the initial prototypes is shown in Fig. 1A–C, whereas Fig. 1D illustrates the sector and turning time in EF imaging. The timing depends on the rotation speed of the confocal scan unit (CSU) and also on how many times per revolution identical sectors are repeated. The CSU-W1 has a 5 times slower repeat frequency than the CSU-X1. We measured and synchronized to the turning time pulses (green in Fig. 1D) and then derived the sector timing internally on the controller (red pulses in Fig. 1D).

Maximum speeds in micro-stepping mode are more complex (Fig. 1E). After each step, the piezo stage was allowed to settle for 2 ms. The sector fly-by time plus the settling time, determine the fastest possible projection. The X1 beats the W1 by more than twice the projection speed, but has less than half the duty cycle and 5 times less light exposure per image plane compared to the W1. The X1 is superior in speed but one can predict that for low fluorescence intensities it will be necessary to increase the exposure time per slice to gain a higher signal.

We then tested the EF device on biological samples. We first tested the effect of different integration times on MDCK cells transfected with GFP–Rab5a (Fig. 2). When the same cell is imaged on a spinning disk confocal equipped with the EF device, different integration times (exposure times) result in different sectioning thicknesses (Fig. 2A); a longer integration time results in a thicker section thickness. At the integration time of 315 ms (Fig. 2A, EF, 6.0 μm), the EF image has better resolution than the wide-field image (Fig. 2B) and better penetration depth than the spinning disk image (Fig. 2C). The optical performance of EF with respect to biological samples is similar to that of projections of a Z-stack (Fig. 2D,E) but requires less total exposure time and file storage – the compression ratio is one divided by the number of slices.

**Validation of Z-depth of extended focus**

Despite the apparent success of achieving real time projection imaging of cells, the precision and the performance of the system needed to be validated by imaging well-defined objects. At a fixed stepping interval, the exposure time of the camera defines the (visible) travel range of the stage. In other words, theoretically the height of a Z-projection in EF should be proportional to the integration time (set as camera exposure time), as long as the exposure time is a multiple of the time-per-section and smaller than the maximum pre-set travel time.

To validate the precision control of projection depth of EF, we performed several experiments with the Argolight SIM test slides. The Argolight test slides series contains optical band gaps that mimic the behavior of fluorophores. Each test slide contains several known geometrical structures that are rendered very precisely. It is commonly used by optical microscopists to evaluate various performance parameters of the systems of interest, such as illumination homogeneity and the resolution limit. Patterns I and G of the Argolight SIM slide were chosen as quantitative tests for the depth resolution because they feature high-resolution structures in the Z-direction.

In the following test, we set the EF device to an amplitude of up to 1 V, driving the stage 20 μm up and down per cycle, each
Fig. 3. See next page for legend.
Fig. 3. Demonstration of different projection thicknesses from EF imaging. Pattern I (stair height of 0.5 μm) and pattern G on the ArgoSIM test slide were used to validate projection thickness of extended focus. (A) Simulation of pattern I from three different viewing angles. The small rings are flat (~20 nm depth) patterns and they descend by a constant step size from left to right and vice versa. In this example, the top left ring is 5 μm above the top right one and the lower left one is 5 μm below the lower right one. The locations of the four corner pillars are not accurate to accommodate all three perspectives in one image. (B) Images taken of pattern I as acquired by a spinning disk confocal, with a single Z-slice (upper panel) and the Z-projection (lower panel) shown. (C) Images of pattern I captured by EF with various travel times (corresponding to Z-travel ranges that are labeled on each panel), recorded at a speed of 0.2 μm per 5 ms. All images were taken with the same spinning disk confocal equipped with a 100×1.40 NA objective and a Prime95 sCMOS camera. (D) Single Z-slice images of pattern G taken by spinning disk confocal microscopy at different projection depths (labeled on the first three panels). A Z-projection image of pattern G taken by spinning disk microscopy is shown in the right-most panel. (E) Images of pattern G captured by EF with various travel times and the same speed.

forward travel taking 505 ms, with 100 micro-steps 200 nm apart. Pattern I — two crossing stairs (Fig. 3A) — contains two rows of 11 flat ring-like structures (stair elements) embedded at different depths, progressing at 0.5 μm stair-step height. As the confocal mechanism has a 0.6 μm to 0.8 μm sectioning depth, a single slice will either include two steps or one brighter step and two dimmer ones (Fig. 3B); 105 ms hence includes 21 5 ms slices and 20 0.2 μm travel steps in between. The depth captured is hence 0.8 μm+(20×0.2 μm)=4.8 μm. With the entire stairs being 5 μm tall, this explains the dim edge elements (Fig. 3C; EF 4.8 μm) — the movement and the stair design are very accurate. Likewise, for pattern G, 305 ms exposure time covers the whole hemisphere, that is, the 12 μm travel range plus the 0.8 μm sectioning thickness (Fig. 3D). Taken together, this data demonstrates a near-perfect agreement between the design sectioning volume and the actual recording of EF.

Extended focus preserves confocality

Many other approaches to achieve extended depth of view do so by sacrificing the confocality of the system, such as adjusting the pinhole size of a point scanning-based confocal system. To compare the performance of EF with other types of commonly used focus extension methods, we imaged the same ArgoSIM pattern I with several other imaging modalities, including spinning disk confocal, WF epifluorescence and point scanning confocal with various pinhole sizes. The microscope body and objectives of all the test systems are Nikon TiE with 100× NA 1.45 objectives. As illustrated in Fig. 4A, WF epifluorescence has the deepest penetration depth among all modalities, but the image of the stair-element that is further away from the focal plane appears dim and blurred. This is because the WF modality lacks the ability to reject out of focus light due to the lack of sectioning mechanisms. The two confocal modalities, spinning disk and point scanning confocal (pinhole sizes: 0.5 and 1 airy disk; Fig. 4B,C) have similar performance in terms of depth of penetration and image sharpness. However, when the pinhole setting on the point scanning confocal is set to the maximum pinhole size (Fig. 4D; 5.4 airy disks), the image obtained shows some features of the WF imaging modality. More stairs are
visible but the image of the stairs was no longer sharp; that is, the confocality was diminished while gaining penetration depth. This is not the case when using EF; at all depth distances the confocality is preserved (Fig. 3C). A semi-quantitative explanation can be seen in the simulated confocal point spread functions (PSFs) (Fig. S1). We computed the PSFs for the pinhole sizes used here and juxtaposed them to the EF mechanism. Note how well defined the EF PSF is in all directions – unlike the 5 airy unit PSF, it falls off steeply away from its core. Hence, the volume recorded with EF is well defined. Light from above or below is cut off and will not contribute to the background. In the XY plane the EF PSF has the same quality as the confocal PSF.

Extended focus preserves super-resolution capability of iSIM

Super-resolution microscopy has gained popularity among end users, yet none of the existing focus extension methods claims to coexist with any super-resolution method. We tested the EF device on the iSIM (also termed rescan spinning disk) system to demonstrate that EF does preserve the super-resolution capability of the iSIM system. Images of Argolight ARGO-SIM pattern E (gradually spaced lines; the biggest gap is 390 nm and smallest gap is 0 nm) were recorded by a W1 Gataca LiveSR scanner equipped with EF side-by-side with other imaging modalities (Fig. 5). The Gataca LiveSR (Müller et al., 2016) generates an optical SIM image that doubles the bandwidth of the instrument but realistically faces a contrast limited improvement of ∼1.7 over the raw spinning disk confocal image after post processing with a deconvolution and a Wien filter (Fig. 5C,D). Without post processing, the improvement appears to be ∼1.4 (Fig. 5F). When the EF is engaged, both integration time settings (100 ms, ∼4 μm and 500 ms, ∼20 μm travel) permit the resolution of the 150 nm gap. The performance is identical to that of the LiveSR without the EF device. With or without EF, LiveSR super-resolution mode provides an improved resolution over the spinning disk confocal mode (∼230 nm resolution for green emission and a NA 1.45 lens) and widefield mode (∼310 nm under the same conditions) on the same microscope system. Hence, we demonstrated that the EF not only preserves the sectioning of a spinning disk confocal system, but also the super-resolution capability of an iSIM spinning disk.

Extended focus reduces photobleaching

As EF generally shortens the minimum time required per data point compared to a confocal Z-stack, another expected benefit of the EF is reducing fluorochrome bleaching and phototoxicity during long-term imaging experiments. Photobleaching of fixed cells has been well studied (Axelrod et al., 1976; Kedziora et al., 2011; Pietrzyńska-Mach et al., 2014) and for a fixed input power to the sample the life expectancy (τ) of a fluorophore is constant and hence the exponential decay term is linear in time, given by e^−t/τ. The actual reduction of bleaching of an image sequence taken by spinning disk with EF compared with spinning disk without EF can be calculated as: (the exposure time per frame with EF)/(the exposure time per frame without EF×number of Z-slices per stack).

Fig. 5. EF preserves the super-resolution capability of iSIM on CSU. (A–F) Pattern E (45°) on ArgoSIM test slides were used to validate the resolution achieved by various imaging modalities with the same objective (100× NA 1.45). (A) Image taken with widefield modality. (B) Image taken with spinning disk modality. (C) Image taken with LiveSR modality with post processing. (D) Image taken with EF (105 ms exposure, Z-travel range of 4 μm) with LiveSR modality and post processing. (E) Image taken with EF (505 ms exposure, Z-travel range of 20 μm) with LiveSR modality and no post processing. (F) Image taken with EF (505 ms exposure, Z-travel range of 20 μm) with LiveSR modality and no post processing. Note the contrast (SNR) improvement as a consequence of the post processing step.
Live cells tend to slowly recover their intensity while being imaged. The speed of turnover can be affected by cell type, protein labeled, fluorophore properties, and duration and frequency of imaging. It is challenging to quantify the extent of bleaching in live-cell imaging. We conducted a qualitative live-cell imaging experiment to compare the photobleaching with and without EF. Long-term imaging experiments were conducted on the same microscope with the same sample with and without EF (Fig. 6); both experiments contain roughly the same number of frames (600 frames). Notice here that each frame in the EF experiment takes 315 ms (with the laser being on for 210 ms for each exposure, i.e. two-thirds of the time) and each frame in non-EF experiments takes a mere 100 ms. Trivially, EF exposes the specimen to a lower light dose per recorded stack – our original intention – but the considerably lower bleaching per amount of light pumped into the specimen is nonetheless remarkable. It is closely related to the very different bleaching curves between spinning disk confocal and point scanners. As the focal plane of EF scans through the volume, the EF image formation exposure is comparable to a weak WF exposure (as a large amount of pump light gets absorbed by the pinholes). Added to this is the fact that the beam interrupts every few milliseconds to allow for recovery and return to the ground state from the triple state. The mApple intensity curve illustrates this: we lose ∼30% of mApple intensity within 31.2 s cumulative exposure in confocal mode (Fig. 6B) and ∼15% within 63.2 s cumulative in EF (Fig. 6D).

If we calculate the time constants for the process $e^{-t/C_0}$, we obtain 931 s for EF and 198 s for confocal spinning disk. Note the considerable resting periods allowed by EF, which for a large part explain this strong difference. These are not physical decay constants but decay curves obtained under the above mentioned realistic imaging conditions.

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Fig. 6. EF imaging causes less photobleaching of live cell volumes than confocal sectioning. (A) Time series imaging of early endosomes labeled with GFP–Rab5 and mApple–Rab7. Two channels, 26 Z-slices, 12 time points (a total of 624 frames), 100 ms exposure and LiveSR (instant SIM) mode were used, resulting in a total exposure time of 31.2 s per channel in bursts of 2.6 s. Maximum intensity projects images of the entire image series are shown. Imaged with the CSU-X1 prototype in Curie Institute. (B) The intensity profile (average intensity of the whole image) of the images from A. (C) Bleaching profile of EF. Two channels, 301 time points, 210 ms (two-thirds of 315 ms) exposure (a total of 602 exposures), and LiveSR (instant SIM) mode (3 scans per slice) were used, resulting in a total exposure of 63.2 s per channel in much shorter 315 ms bursts which are again composed of 21 flashes of 10 ms. (D) The intensity profile of the images from C. Despite imposing double the photon dose, the short EF sequences seem to allow for a much better recovery in between exposures. A.U., arbitrary units.
Application of extended focus for imaging biological experiments

To further validate the applicability of EF on bioimaging experiments, we conducted two experiments with biological samples from ongoing imaging projects. The first experiment was the imaging experiment for tracking intracellular membrane vesicles, which was the driving force for developing the EF device. The aim of the experiment is to monitor the fluorescence intensity changes of Rab5 and Rab7 protein during the endosome maturation Rab switch process (Rink et al., 2005). As briefly discussed in the previous section, the main challenge for imaging in this type of experiment is the potential motion artifact while collecting a single Z-stack and the photobleaching.

In order to image and track the entire endosome maturation process with sub-pixel accuracy, at intervals of one volume per second, we ran into limitations of the stability of exposure and sectioning on our Yokogawa W1 spinning disk microscopes. The 50 μm pinhole disk, which scans one full sector in 5 ms, has a sectioning power of some 600–800 nm at 530 nm center wavelength, in an aqueous specimen. This is good enough to reject the cellular background but requires ~16–21 slices to be recorded to contain the height of the cell body, some 6 μm, which is the entire range an endosome could travel during the duration of maturation. If such a Z-stack is imaged every 10 s for 10 min, which is barely enough to cover our process of interest (endosome maturation), a total of 1500 frames of exposure are needed. This usually causes strong bleaching of the imaged sample.

The subsequent endosome tracking relies on a very uniform recording of the endosome. While it can adapt to slow changes of the fluorescent distribution, motion artifacts directly injure the localization accuracy. Our spinning disk (Yokogawa W1) with Metamorph (Molecular Devices) control software allocated ~4–5 s to record such a stack, leading to very visible elliptical distortions of the endosome. These can be somewhat amended by translating the frames of the recording against the conjectured direction of motion but this is a very heuristic process that introduces hard to control higher-order artifacts.

The result of the EF is illustrated in Fig. 7A (Movie 1); BJ-5ta human immortalized fibroblast cells transfected with Rab5a-mApple and Rab7a-GFP were imaged over time with a spinning disk equipped with Gataca LiveSR and EF (integration time 200 ms, Z-travel range of ~4 μm). This cell line usually has a height of around 5 μm above the coverslip. The cells of interest were imaged once (one single exposure of 200 ms) every 10 s for 20 min, which generates 121 time points. The resulting image series has the endosome of interest in-focus throughout 20 min, without noticeable photobleaching (Movie 1). As validated in previous sections, EF preserves the super-resolution capability of LiveSR, thus the resulting image series also has a high lateral resolution. All these make the subsequent tracking and quantification relatively straightforward (Fig. 7A–D).

We also applied EF imaging in a study of the self-organization of the actin cytoskeleton in fibroblasts. A typical experimental setup for this type of study involves confining cells to circular adhesive islands and then following the development of the actin cytoskeleton by spinning disk confocal sectioning through the volume of the cell (~10 μm in height) every few minutes for ~8 h. Images obtained from these experiments are normally presented as movies containing maximum intensity projections for each time point (Jalal et al., 2019; Tee et al., 2015), and motion artifacts that may arise during acquisition of a single time point are generally not a concern. The main challenges in these experiments are limiting photobleaching/phototoxicity during imaging and handling the large datasets that are generated. We observed actin cytoskeleton development in fibroblasts that were confined on fibronectin-coated islands at intervals of 1 min frame−1 for 10 min using iSIM without (Fig. 7E; Movie 3) and for 30 min with EF (Fig. 7F; Movie 4). A single time point from the normal confocal sectioning was captured in a TIFF file that is 291.8 MB in size while a single frame from imaging in EF mode was held in a TIFF file that was 100 times smaller at 2.9 MB in size. In the comparison experiment we conducted, the file of the same experiment collected without EF is 2GB (11 usable time points; Fig. 7E) while the file with extended focus is 85MB (31 usable time points; Fig. 7F). Hence EF reduces file size per usable data point with the actual reduction depending on the number of Z-slices per stack.

Consequently, we are confident that EF is compatible with many of the spinning disk-based bioimaging experiments, as long as Z-axis information is not required for the subsequent data analysis. EF offers faster projection collection over the whole volume, less photobleaching and phototoxicity and, for extensive use, an important added advantage is the great reduction in storage capacity and corresponding fast retrieval of small live imaging files.

DISCUSSION

The EF was designed to enable high lateral and temporal resolution tracking of endosomes in live cells by sacrificing detailed Z-resolution data. From an end-user point of view, EF greatly reduces light exposure per time point, reducing phototoxicity and favoring long-term live-cell imaging.

One potential issue regarding this type of application is focus drift during long-term imaging. At present this device cannot be used together with most of the differential reflective index-based focus stabilization or focus-searching devices, such as the Perfect Focus System (PFS) from Nikon. Normal Z-stack recordings face the same problem and it is usually countered purely mechanically by having a larger temperature chamber also enclosing the stage rather than installing an onstage incubator.

Although we primarily had fast kinetics of intracellular traffic in mind for developing the EF, it is clear that several other imaging applications can benefit from such a device, if they only require an accurate projection of the subject of interest. For instance, works from the Bershadsky group (Jalal et al., 2019; Tee et al., 2015) heavily relied on high-resolution projections from long-term imaging to describe organization of the actin cytoskeleton in a range of cell types. EF can reduce the overall photobleaching as we have demonstrated in the Results. Using EF also results in a reduced file size, thereby making the subsequent image processing and data storage less demanding for hardware infrastructures.

Optical extended field of depth phase masks have existed for some 15 years and are commercially available from Hamamatsu. The Hamamatsu EF2 and EF5 extended focus phase masks create elongated, high-NA PSFs. These are designed to provide extended depth of view in the camera light path of a widefield system but in a confocal spinning disk system they also extend the scanning focal spots, which is – in theory – useful. The remarkable loss of light we experienced and which worked against our design goal, is likely a result of poor alignment and them being used in the parallel light path. It would have still been our method of choice, if it did not also increase background visibility significantly. The background and its additional blur made this unsuitable for our tracking efforts. The masks are generally placed in the camera path near the relay’s magnification but can operate in the parallel beam path. The light loss, even in combination with a large pinhole disk was, however,
Fig. 7. See next page for legend.
Fig. 7. Application of EF to biological imaging experiments. (A) Final frame captured of BJ-5ta cell labeled via GFP–Rab7 and mApple–Rab5 from a time lapse series recorded every 10 s for 1200 s. The boxed area in ROI shows where endoskeleton tracking software was applied. See Movie 1. Scale bar: 10 μm. (B) A time lapse series of images from the ROI under the conditions imaged in A. Time in seconds after the start of imaging is shown in the lower right corner of frames. Scale bars: 1 μm. See Movie 2. (C) Outline of endosome tracked by photometric tracking algorithm of four representative frames. It seeds from the center of the endosome and extends in a star-shaped region. This way, it can maintain super-resolution accuracy and establish connectivity on a topological torus. It exploits frame-to-frame similarity in order to operate fully automatically. (D) The y-axis on the graph is the arbitrary intensity of Rab5 and Rab7; the x-axis is the number of frames (0.1 frame s$^{-1}$). The algorithm and the larger scale study are subject of a future publication. Total observable amount of each fluorophore over time. While the sum of both stays nearly constant, an evident exchange of the two components is observable. (E) Maximum intensity projections (14 slices taken in iSIM) of images from time lapse series of a fibroblast expressing Lifeact–GFP confined on a fibronectin-coated island imaged at 1 frame min$^{-1}$ for 10 min. Time in minutes after the start of imaging is shown in the upper left corner of frames. Scale bars: 10 μm. See Movie 3. (F) EF images from a time lapse series of a fibroblast expressing Lifeact–GFP confined on a fibronectin-coated island imaged at 1 frame min$^{-1}$ for 30 min with iSIM. Time in minutes after the start of imaging is shown in the upper left corner of frames. Scale bars: 10 μm. See Movie 4.

considerable and the homogeneity was not satisfactory. The fact that the mechanical approach supported structured illumination in the projection let us abandon the search for more-advanced and efficient projection methods.

Other previous works to achieve similar goals have been performed on fast focusing lenses (Liu and Hua, 2011). Here, thin liquid lens elements help refocusing optical systems over short focal ranges. They do not provide any real speed advantage over piezo-refocusing of the lens collar or the specimen holder in the low micrometer range; however, for long focus travel the speed is increased. All these efforts settle just below the kHz range and they introduce an inhomogeneous point spread function (Xue et al., 2015). The ready availability of piezo drives for our work, contributed to our choice in the end.

Similar considerations made us give up on using a simple single plane illumination scope (ASI diSPIM) for the projections. While sweep speeds, even with a scanning pencil beam, are far superior to those achievable with the spinning disk scan head – a plane can be scanned in as little as 0.5 ms and no laser shuttering is needed. At present there is no iSIM mechanism available for this, but one will likely emerge in the near future. SIM itself will also not supersede stochastic optical reconstruction microscopy (STORM) and direct (d)STORM imaging, but some tasks such as outlined in Bálint’s work (Bálint et al., 2013), could indeed be neatly implemented with a super resolving projection and it would improve temporal resolution.

The confocal sectioning also provides better homogeneity and repeatability – as in less illumination changes across the image plane and sharper confined slices – than current light-sheet microscopy. Tracking and photometry depend quantitatively on this repeatability but, on the downside, sequential sectioning greatly increases the risk of motion artifacts that manifest in distorted volumes or projections. Streaking fast through the observation volume, in the order of 100 ms, will eliminate this problem for the observation of most biological processes. The high repeatability and stability of the EF co-moving frames permit photometric measurements of dye concentrations, if Nyquist sampled, and in consequence, support localization microscopy.

Visible cell organelles can be localized with the same centroid approaches used for single-molecule localization (Fig. S3). Hence, the questions investigated by Jones et al. (2011) via 3D STORM, for the most part, can be settled with fast, high-resolution projections. As a larger object distributes its light over more camera pixels the localization is not as accurate as in single-molecule localization. A well-known application is the localization of elastic pillars for force measurements, which yields an accuracy better than 10 nm (Gupta et al., 2015). See Figs S2 and S3 for a brief overview of the performance of two-dimensional object localization. A ‘projection’ of an extended object becomes a two-dimensional object and hence can be localized as a whole. We also quantified how important image quality and sectioning (Sheppard, 1988) are in terms of accuracy.

The very confined optical sectioning of the confocal modality (Sheppard, 1988) is hence much preferable over other EF methods, such as PSF engineering (Abrahamsson et al., 2006) – whose commercial implementation we tested (Okita et al., 2018; data not shown) – Török et al.’s work in 2007 (Török and Kao, 2007) or assembling multiple sections from wide-field imaging. Also, using narrowband incoherent sources (Roche, 2013) does not overcome the restriction of poor sectioning. Zahreddine and Cogswell (Zahreddine and Cogswell, 2015) has an excellent outline of why engineered PSFs and alternative illumination systems such as axicons are suboptimal for use in a confocal setup.

A common concern raised by researchers who were shown the system in operation is the fast start-and-stop movement that micro-stepping requires in our examples as the specimen is moved up and stopped 60 to 200 times every second. The movements are, however, very small and the peak accelerations for a 0.2 μm jump in 1 ms are merely one-third of Earth’s gravitational pull and the mean acceleration is 5 to 20 times lower. The stress to the cells from these movements are thus negligible.

The Physik Instrumente 720 and 520 stages we used make no clear statement on how fast they can actually move, but they provide a position signal that can be compared to our input signal. Trivially, longer distances require more travel time, and movements below 1 μm were completed in <1 ms. In our setup, we obtained excellent image stability and acceptable brightness with 15 ms per section and <2 ms stage travel time. Then, EF with micro-stepping required 2 ms more per section but improved the image quality to the level of iSIM.

If the system has a fast acusto-optical tunable filter (AOTF), we recommend introducing the short interruptions of the laser as this reduces bleaching of the specimen. On the other hand, if the laser controller cannot accommodate shutter times <1 ms and you have a fast stage, it would be preferable not to interrupt the laser to avoid laser instability artifacts.

In summary, we have developed a simple and affordable ‘add on’ to extend the focus per image especially for the spinning disk confocal microscopes. The method sacrifices Z-resolution to achieve less bleaching and faster imaging speed, while maintaining the confocality and even the iSIM super-resolution capability. Any laboratory equipped with a spinning disk confocal system and a piezo stage can readily obtain this technology at very low parts cost. With the order list and the controller code provided in the Materials and Methods, the installation can be reproduced locally. Alternatively, a user-friendly commercial version could become available, as we have submitted a patent application of the method. As all parts are already present in a modern confocal microscope, we are aware that, in principle, this novel EF could be installed solely as a software feature by microscope manufacturers.
TOOLS AND RESOURCES

MATERIALS AND METHODS

Biological sample preparation

All three cell lines, MDCK cells stably transfected with CD74/Ii/Invariant Chain (Bergeland et al., 2008), BJ-5ta cells (ATCC) and HFF (ATCC catalog no. SCRC-1041), were maintained in standard DMEM (DMEM, Lonza, BioWHITTAKER) supplemented with 10% heat inactivated fetal bovine serum and 2 mM L-glutamine. MDCK and BJ-5ta cells were reseeded overnight in 35 mm IAWKI glass bottom dishes before they were transfected with 1μg of total DNA [0.5 μg Rab5–GFP or Rab5–mApple, and 0.5 μg Rab7–mApple or Rab7–GFP (Skjeldal et al., 2012)] by using either Lipofectamine 2000 (ThermoFisher) or JetOptimus (Polyplus) according to manufacturer’s instructions. The cells in the imaging dish were typically ~60% confluent at the time of transfection, and were imaged at least a day after transfection. Transient transfection of 1 μg of LifeAct–GFP (R. Wedlich-Soldner Group, Max Planck Institute of Biochemistry, Martinsried, Germany) into HFF cells was achieved by electroporation (Neon).

Circular islands of 1800 μm² in area that were fabricated by microfluidic circuitry. A1R microscope. The specifications of the base system are: Nikon Ti-E single turret microscope, Yokogawa Confocal Scanner Unit (CSU) W1 spinning disk engine [50 μm pinhole, 4 K revolutions per minute (RPM)] Photometrics Prime95B back illuminated sCMOS camera (11 μm pixels), Gataca Systems iLas2 6 line dual fiber laser launch, Physik Instrumente P524 piezo specimen stage and epi-fluorescence LED (ScopeLED). All images are collected with a Nikon CFI Plan Apochromat lambda 100×/1.45 oil immersion objective. In iSIM mode, the scan lens magnifies ~1.77 times, providing a projected pixel size of 62 nm on the 11 μm pixels of the sCMOS camera at 100× magnification. The point scanning confocal imaging experiment was carried out on a Nikon A1R microscope. The specifications of the base system are: Nikon Ti-E dual turret microscope and Nikon CFI Plan Apochromat lambda 100×/1.45 oil immersion objective.

Image processing

Simple image analysis is performed by FIJI (ImageJ). The ImageJ macro used to measure the mean average intensity in Fig. 2 (bleaching comparison) uses the ‘(Measure’) command for all slices.

Extended focus device

The spinning disk, wide field and iSIM imaging experiments were carried out on a Gataca LiveSR instant Structure Illumination Microscopy (iSIM) rescanning module microscope system. The specification of the base system are: Nikon Ti-E single turret microscope, Yokogawa Confocal Scanner Unit (CSU) W1 spinning disk engine [50 μm pinhole, 4 K revolutions per minute (RPM)] Photometrics Prime95B back illuminated sCMOS camera (11 μm pixels), Gataca Systems iLas2 6 line dual fiber laser launch, Physik Instrumente P524 piezo specimen stage and epi-fluorescence LED (ScopeLED). All images are collected with a Nikon CFI Plan Apochromat lambda 100×/1.45 oil immersion objective. In iSIM mode, the scan lens magnifies ~1.77 times, providing a projected pixel size of 62 nm on the 11 μm pixels of the sCMOS camera at 100× magnification. The point scanning confocal imaging experiment was carried out on a Nikon A1R microscope. The specifications of the base system are: Nikon Ti-E dual turret microscope and Nikon CFI Plan Apochromat lambda 100×/1.45 oil immersion objective.

Simulation of object tracking

Point-like emitters (e.g. single molecules) have been studied very extensively and in great detail. Early attempts to consider camera digitization, discretization, thermal noise and, today, predominantly photon noise, have led to the development of the idea of inverse square root dependency on the photon count, commonly cited today, and its most famous representation is the Thompson formula (Thompson et al., 2002), which elaborates on the mild detriment of pixelation.

As a visible object – somewhat imprecisely called a ‘large object’ – that always shows the same aspect ratio to the lens, is just a linear supposition of single emitters, the empty magnification rule of Ober et al. (2004) can be used with good accuracy, that the size of the projection introduces an estimation error of the centroid very similar to excessive empty magnification. The statement is only true for the centroid, not for imaging the actual shape. Absent of any camera readout noise, the localization is mildly deteriorated by the discretization (pixelation uncertainty) (Fig. S2).

When the typical electron read out noise of a current higher spec. sCMOS camera (~3 electrons) is added, the localization precision suffers a lot for very low light conditions but rapidly converges to the photon limited case for brighter objects (Fig. S3). The single point emitter statistics are again outlined in Stallinga and Rieger (2012) and we performed the statistics for these objects.

The graphs in Fig. S3 C,D powerfully demonstrates that background light ‘hurts’ object localization a lot more than it does single molecule tracking. The qualitative result is intuitive but the absolute cap in accuracy is a very serious limitation. The strong sectioning capability which rejects out of focus light, is hence ideal for obtaining localization accuracy.

Author contributions

The authors have filed a patent with Inven2 (DOFI 19087) and Dehns (ref. 12.147289) regarding the hardware implementation.

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Competing interests

The authors have declared no competing interests. 

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