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Combination of structured illumination and single molecule localization microscopy in one setup

Sabrina Rossberger1,2, Gerrit Best1,2, David Baddeley3,4, Rainer Heintzmann5,6,7, Udo Birk1,8, Stefan Dithmar2 and Christoph Cremer1,8,9,10

1 Kirchhoff Institute for Physics, University of Heidelberg, Heidelberg, Germany
2 Department of Ophthalmology, University of Heidelberg, Heidelberg, Germany
3 Department of Physiology, University of Auckland, Auckland, New Zealand
4 Department of Cell Biology, School of Medicine, Yale University, New Haven, CT, USA
5 Institute for Physical Chemistry, University of Jena, Jena, Germany
6 Institute of Photonic Technology, Jena, Germany
7 King’s College London, Randall Division, London, UK
8 Institute of Molecular Biology (IMB), Mainz, Germany
9 Institute of Pharmacy and Molecular Biotechnology, University of Heidelberg, Heidelberg, Germany

E-mail: sabrina.rossberger@kip.uni-heidelberg.de, cremer@kip.uni-heidelberg.de and c.cremer@imb-mainz.de

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Abstract

Understanding the positional and structural aspects of biological nanostructures simultaneously is as much a challenge as a desideratum. In recent years, highly accurate (20 nm) positional information of optically isolated targets down to the nanometer range has been obtained using single molecule localization microscopy (SMLM), while highly resolved (100 nm) spatial information has been achieved using structured illumination microscopy (SIM).

In this paper, we present a high-resolution fluorescence microscope setup which combines the advantages of SMLM with SIM in order to provide high-precision localization and structural information in a single setup. Furthermore, the combination of the wide-field SIM image with the SMLM data allows us to identify artifacts produced during the visualization process of SMLM data, and potentially also during the reconstruction process of SIM images.

We describe the SMLM–SIM combo and software, and apply the instrument in a first proof-of-principle to the same region of H3K293 cells to achieve SIM images with high structural resolution (in the 100 nm range) in overlay with the highly accurate position information of localized single fluorophores. Thus, with its robust control software, efficient switching between the SMLM and SIM mode, fully automated and user-friendly acquisition and evaluation software, the SMLM–SIM combo is superior over existing solutions.

Keywords: super-resolution microscopy, single molecule localization microscopy (SMLM), structured illumination microscopy (SIM), imaging, SPDM

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(Some figures may appear in colour only in the online journal)

1. Introduction

Different modes of super-resolution fluorescence microscopy have become a major tool in the analysis of biological nanostructures [1]. However, each method has its limitations and some biological questions may only be addressed by a particular microscopic technique. Therefore, a combination of different high-resolution imaging techniques in a single instrument is highly desirable.

The pointillistic imaging methods of single molecule localization microscopy (SMLM) methods (e.g. PALM/
FPALM [2–4], RPM [5, 6], SPDM [7], STORM) [8–11] currently provide the highest spatial resolution in wide-field fluorescence microscopy, which is currently within the 10 nm range. These approaches are based on the general principle of optical isolation of the diffraction images of point emitters due to differences in spectral signatures such as fluorescence lifetime [15], photo-switching behavior [12], differences in the absorption/emission spectrum [15], photobleaching [13], and blinking [14]. Localization data is basically a finite list of single molecule positions. In order to generate a structural image out of the position list, visualization methods are applied, which generate a visible structure by connecting the single position dots. A few numerical methods such as Gaussian blur or triangulation [16] provide visualization of SMLM data with regard to important parameters such as localization accuracy or local density. However, these methods become highly challenging (sometimes even impossible), when a very low yield of single molecule positions is detected (see figure 3). Even if the structure is well labeled, only a fraction of those single molecule signals is usually detected in SMLM. In some cases, the structure may then not be sufficiently visualized. A similar problem arises when a sparse target distribution is of interest, which inherently exhibits low labeling densities.

In both cases described, it often remains difficult to obtain structural spatial information from these images and thus to interpret images correctly. Moreover, visualization algorithms in SMLM often introduce artifacts in the generated images, which have to be considered. In many cases structural information can be provided by using a microscope technique with a lower resolution in a linear excitation mode, though a second co-localizing label is necessary in the case of sparse target distributions. Artifacts occurring during the visualization process can be identified and considered appropriately for data interpretation. A comparison with conventional fluorescence microscopy may be an option to solve this problem. However, the optical resolution (∼200 nm) may be inadequate to advantageously integrate the SMLM data.

Structured illumination microscopy (SIM) approaches are currently the most convenient choice in fluorescence microscopy in order to gain highly resolved structural information because this method yields a resolution twice as good (∼120 nm) compared to standard fluorescence microscopy [17–19]; it is also superior to the resolving power of confocal [20] or multi-photon microscopy [21]. Theoretically, SIM provides unlimited resolution when nonlinear fluorescence response is utilized [22–24]. However, for practical applications this is quite complicated to realize. Special fluorophores with a long life time, high laser-intensities, or complicated microscope setups are generally necessary in order to achieve a high degree of nonlinearity. Therefore, the applicability of nonlinear SIM has been limited up to now. For practical applications the two-point resolution [15] of SIM is generally about one order of magnitude lower than that of SMLM.

Nonetheless, an essential advantage of SIM is the opportunity to obtain images with an enhanced resolution already at low laser-intensities with conventional fluorophores. This enables imaging, including repeated imaging of the same target up to several hours or ideally even a few days. Furthermore, the imaging process is much faster, which leads to a much shorter exposure time resulting in substantially less phototoxic damage of the biological object [22, 24]. In order to transfer fluorophores into a spectrally different state (e.g. dark), SMLM often requires either high illumination intensities when using conventional single fluorophores combined with standard preparation conditions [7], or very special fluorophores and complex illumination schemes (e.g. PALM/FPALM). At present, time lapse live-cell or other in vivo applications have been extremely difficult to realize. Toxic illumination intensities and switching buffers or molecule mobility pose a major problem [28–34]. Other present limitations of SMLM relate to the fluorophores or buffer media employed [8, 35, 36].

The contrast mechanism in photo-switching based SMLM is considerably different from conventional fluorescence transitions. Conventional fluorescence is typically based on transitions between the electronic singlet states $S_1$–$S_0$, whereas in the SMLM method, photo-switching transitions between different long-lived spectral states are involved. Therefore, both methods (SIM and SMLM) give complementary information about the object. In principle, even a completely different set of fluorophores can be used for each method. Despite its more stringent restrictions in terms of specimen labeling, SMLM also exhibits benefits over SIM. (a) The setup of SMLM is generally much simpler to realize and also the alignment is easier. While more complex and time-consuming to align, a SIM setup is still less complex than focused super-resolution microscopes such as STED [37] or 4Pi-CLSM [38]). (b) The imaging process of SIM is faster as it does not require a data stack of several thousand images as in SMLM. (c) As reported, SMLM is capable of 3D imaging with a highly enhanced resolution compared to SIM, but it is necessary to introduce additional optical elements and algorithms for SMLM imaging in order to gather 3D information [3, 9, 39].

So far, imaging of thicker 3D structures (beyond the ∼10 µm range) has been impractical. A possibility to overcome this problem is the use of a perpendicular illumination e.g. light sheet microscopy [40–42].

Moreover, both methods, SMLM [43–47] and SIM [48–50] allow imaging of multi-color labeled targets, though SIM benefits from a simple sample preparation as no special fluorophores and embedding media are needed.

We have previously described the usefulness of SIM in order to access and resolve autofluorescent (AF) structures within the retina at a high-resolution level [48, 49, 51, 52]. It was stated that SIM allows a differentiated analysis of AF tissue. In comparison, it is usually quite challenging to image AF structures with SMLM, as bleaching and switching of fluorophores contributing to the AF signal is difficult to control [6].

To combine the advantages of both approaches, we developed a custom-built microscope setup, providing SIM with SMLM acquisition modes. As a result, single molecule
position information can be correlated directly with the super-resolved structural information of the specimen. This also significantly facilitates imaging of the same region of interest (ROI) in 2D/3D with two different super-resolution microscopy methods. Here, we present a detailed description of each component of our custom setup including several additional fully automated and software-controlled features, which have been developed in order to speed up and simplify the imaging process. Further, a user-friendly interface of all software-controlled features will be outlined.

2. Methods

2.1. SIM

SIM is a fluorescence microscopy method. Illuminating through the detection objective lens yields a lateral resolution typically twice as high as in commonly used wide-field fluorescence microscopes, in which resolution is limited to about half the wavelength of excitation light [18, 19].

SIM is based on a spatial frequency shift of information in the object plane. Thus information of high, but usually non-resolvable frequencies is shifted into the pass-band of the detection objective lens [17, 19, 48, 53]. The sinusoidal illumination pattern is modified regarding its phase and orientation. Typically nine images for each reconstructed high-resolved 2D image need to be acquired, which are composed of three phase-shifted images for three different orientations of the grating. For reconstruction, custom software was used as described previously [19, 42, 48]. Commercially available fluorophores for labeling are sufficient in order to perform SIM imaging by applying relatively low laser intensities (focal plane intensity: ~100 W cm\(^{-2}\)).

2.2. SMLM

Many variants of SMLM methods exist. Measurements presented here were acquired by using the SMLM approach SPDM\textsuperscript{pyramid} [1, 5, 7, 44], which is based on photo-switching using conventional fluorophores and standard sample preparation conditions. High laser intensities (focal plane intensity of about 10 kW cm\(^{-2}\)) are applied to the specimen, which transfer (presently) most of the fluorophores into a ‘dark’ state (i.e. non-fluorescent at the emission wavelengths registered) from which they recover stochastically towards their fluorescent state. No second laser was necessary in order to photo-switch fluorophores. As main prerequisite for SMLM, only a sparse number of fluorophores of the labeled target are in a ‘bright’ state within each image frame. Thus the diffraction limit can be circumvented as the detected signals of the single fluorophores ideally do not overlap on the detector, i.e. they are optically isolated. Hence, each of these diffraction-limited signals essentially represents a measured point spread function (PSF) of the microscope, and the position of the molecules can be approximated very accurately by the center/maximum of the respective measured PSF. The accuracy depends on signal, background and noise levels. Theoretically the position of each fluorophore can be determined with ‘unlimited accuracy’ if the photon count is high enough. This procedure is repeated for several thousand frames. In each frame different optically isolated fluorophores are detected in a bright state, which afterwards add up to a whole image accounting for the accurate position information. Thus, in theory an infinite resolution can be achieved [4, 10, 11]. In practice, a resolution in the 10 nm range has been proven to be realistic. Commercially available standard fluorophores used in these experiments were able to recover several times before they were irreversibly photobleached, which increases the localization precision.

2.3. Experimental setup

2.3.1. Hardware. The setup is a custom built wide-field microscope of extraordinary mechanical stability, which combines the two high-resolution microscopy methods SIM and SMLM (figure 1).

Five different monochromatic laser wavelengths from the UV-range to the IR-range are currently available for illumination (405 nm: DPSS, 150 mW, BFi Optilas, Dietzenbach, Germany; 488 and 568 nm: Coherent sapphire 488/568 HP, 200 mW, Coherent, Dieburg, Germany; 671 nm: DPSS, VA-1-300-671, 300 mW, Beijing Viasho Technology Co. Ltd, Beijing, PRC; 808 nm: diode, 800 mW, Roithner Lasertechnik, Vienna, Austria).

The laser lines are coupled into the optical path using several dichromatic mirrors. Custom-built shutters enable automated, either sequential or simultaneous, switching between the different laser lines. The laser intensity can be controlled with a custom-built neutral-density filter wheel (figure 1, orange asterisk), which contains 12 neutral gray filters of different densities from 100% to 0% transmission. An attached stepper motor enables an automated rotation of the neutral-density-filter wheel. Calibration of the wheel is done by a photosensor consisting of a photodiode and a light emitting diode (OPB980, OPTEK Electronics, Carrollton, Texas, USA).

The illumination pattern is generated by a Twyman Green interferometer as previously described [48]. A 50% beam splitting mirror transmits one half of the beam and reflects the other half onto a reflective mirror positioned directly next to the cube in order to minimize beam displacement. Both beams are then redirected back into the cube by mirrors, which are controlled by piezoactuators and appropriate piezoamplifiers (both PI, Karlsruhe, Germany). The interference pattern is generated in the plane of the beam splitting cube, which is mounted on a rotatable stage, including an appropriate linear actuator and its control element (M-RS 65 and CMA-12CCL, controller: Esp300, all Newport Spectra-Physics GmbH, Darmstadt, Germany). Thus the cube can be automatically rotated around its axis perpendicular to the optic table. The incident excitation beam is reflected by an angle of $\Theta$ instead of 90°. This results in two beams, which leave the cube at an angle of $\Theta$ and $-\Theta$ respectively ($z$-axis). Each beam is 1/4 of the intensity of the original excitation beam and passes through the focusing lens.
Figure 1. Schematic of the ‘combo’ setup: the present combo setup can be used with up to five laser excitation frequencies, which can be selectively switched on and off by using custom-built shutters. A neutral-density filter wheel (orange asterisk) regulates the laser intensity. An automated mirror system switches the setup between two modes depending on whether the mirrors are moved in or out of the beam path. In the case of mirror A being placed into the beam path the laser beam is reflected into the direction of the custom-built interferometer in order to perform SIM imaging (dark blue). For this purpose the second automated mirror B needs to be removed from the excitation path. In reverse mode, SMLM imaging (green) is performed. A fully automated dichromatic splitter filter wheel (yellow asterisk) as well as an automated blocking filter wheel (blue asterisk) were introduced. The tube lens focuses the fluorescence light onto a CCD chip of the high quantum efficiency camera. The setup can be supplemented with a second CCD camera in order to split fluorescence light emitted by different fluorophores, but excited by the same wavelength. In addition to the SPDM/Phymod/SIM mode used here, the setup is designed in such a way that PALM/FPALM, or STORM/dSTORM modes can also be applied.

As the splitting cube is at the focal point of the focusing lens, the generated sinusoidal illumination pattern is imaged into the object plane.

The illumination pattern can be shifted by the piezoactuators attached to the mirrors. A minimum of three different positions of the patterns is required, which results in sequential shifts of the mirror by $\lambda/(2 \times 3)$ (2 due to the doubling of the optical path). In order to achieve an isotropic resolution improvement, a minimum of three orientations of the illumination pattern are generally generated. For this purpose the splitting cube is tilted by a second attached linear actuator (CMA-12CCCL, Newport Spectra-Physics GmbH, Darmstadt, Germany), which results in a rotation of the two beams around a horizontal axis ($x$- and $y$-axis). In order to achieve a high modulation of the excitation pattern in SIM, it is important that the polarization of the interfering light beams is perpendicular to the plane spanned by the two beams. Therefore, a half-wave plate mounted on a motorized rotational stage (Newport PR50CC) is used in order to rotate the polarization according to the orientation of the pattern. All optical parts in the beam path after the wave plate are unpolarizing.

The focusing lens, an achromat (VIS ARB2, $d = 31.5, f = 300$ mm) mounted on a $x$–$z$-translation system (both QiOptiq Photonics, Goettingen, Germany) for easier adjustment, focuses the laser light into the high numerical objective (HCX PL APO 100×/1.4 oil CS, Leica, Wetzlar, Germany). The objective lens then refocuses the generated sinusoidal illumination pattern into a plane of the sample. The objective lens is mounted on a fine-adjustment micrometer (G061061000, QiOptiq Photonics, Goettingen, Germany), which allows a coarse adjustment of the focus into the sample. In order to scan the sample in the $z$-direction for fine adjustment of the focus position, a further focusing element (P-725.1CD, 100 µm, pfoc, Physik Instrumente (PI), Karlsruhe, Germany) is introduced.

To obtain a visual control of the grating generated and projected into the object plane, a small part of the beams is split and redirected by an additional beamsplitter cube within the optical path onto the CCD chip of a web camera (MANHATTAN Webcam 500) before passing through the focusing lens. This visual control is necessary for the adjustment of the interferometer, which can be controlled by several piezoactuators as described above.

As the splitting cube is at the focal point of the focusing lens, the generated sinusoidal illumination pattern is imaged into the object plane.
The rest of the excitation light is reflected on appropriate dichromatic edge filters (405/488/568: Di02-R405/Doi02-R488/Doi02-R568 and 680: Q680LP, Semrock, Rochester, USA; 810: Q810LP, Chroma, Olching, Germany) mounted on a commercially available manual dichromatic filter wheel (CDFW5/M, Thorlabs, Munich, Germany, figure 1, yellow asterisk). In order to enable a fully automated image acquisition, the dichromatic filter wheel was equipped with a stepper motor (P430 258 005 01, Portescap, La Chaux-de-Fonds, Switzerland). A stepper control element (RN-Stepp297, Robotikhardware, Sontra, Germany) allows automated control of the stepper motor utilizing custom software.

The reflected excitation light illuminates the aperture of the objective lens and is focused into the sample. Microscope slides or a live-cell chamber are mounted on a Nikon Eclipse microscope table (for Nikon Eclipse TI and TE 2000, 00-24-437-0000, Maerzhaeuser, Wetzlar, Germany); the table itself is mounted on a custom-built stage made of Invar. This material benefits from its unusually low coefficient of expansion and thus minimizes thermal and mechanical drifts. Control of the table is performed by a commercial controller (mc2000, also Maerzhaeuser) which is additionally equipped with a joystick.

For easier adjustment, a periscope (not shown in figure 1, silver mirrors, 21010, Chroma, Olching, Germany) built of custom illumination optics is positioned in front of the dichromatic filter wheel. This is used to direct the laser beam into the objective and to perform imaging at different illumination angles. TIRF (total internal reflection fluorescence) microscopy can be conducted as well.

The fluorescence signal emitted by the sample passes the high numerical objective lens and is transmitted by the same appropriate dichromatic edge filter, which separates the excitation light from the fluorescent emission light (figure 1, yellow asterisk). To remove any remains of the excitation light, a stepper motor-driven filter wheel (FW102C, Thorlabs, Munich, Germany, figure 1, blue asterisk) is placed in front of the tube lens prior to a high quantum efficiency CCD camera (Sensiscam QE, PCO, Kelheim, Germany). This filter wheel houses up to 6 emission filters—one for each excitation wavelength (405/488/561/808: BLP01-405/488/561/808, Semrock, Rochester, USA; 690: LP XF 3104, Omega Optical, Olching, Germany)—and is fully automated. The diameter of the illuminated field of view is ∼65 μm, which corresponds to a full use of the CCD chip of the camera. The setup can be further extended by implementing a second dichromatic beam-splitter and a second CCD camera. Thus the light emitted by different fluorophores excited with the same (or different) wavelengths can be split on two CCD chips, similar to the setup described by Baddeley et al [6]. As a consequence, the range of fluorophores can be further extended.

A software-controlled, fully automated mirror-based mechanism allows fast switching between the two microscope modes (figure 1, large arrows). In order to perform SIM measurements, the first movable mirror A (figure 1) needs to be placed within the beam path in order for the laser light to travel through the Twyman green interferometer. In this case the second movable mirror B (figure 1) has to be removed from the optical path. When imaging in SMLM mode, the interferometer needs to be bypassed. For this purpose the first mirror needs to be removed from the beam path, whereas the second mirror is inserted in order to deflect the excitation beam onto the focusing lens. Apart from the beam expander, the rest of the beam path is the same for both microscope modes. If high-intensity illumination is used for SMLM (e.g. for SPDM), a different beam expansion compared to SIM is implemented into the optical path by utilizing lenses of different focal points (SIM: first lens f = 10 mm and second lens f = 250 mm; SMLM: first lens f = 40 mm and second lens f = 100 mm).

Additional equipment for the microscope includes a halogen lamp (TQ/FOI-1, 150 W, Techni-Quip, Pleasanton, California, USA) for studying samples using transmitted light and for wide-field fluorescence microscopy. Furthermore, a heated incubator (UNO Top Stage Incubator, H501, Okolab, Ottaviano, Italy) is available in order to perform live-cell experiments. An exchangeable 35 mm Petri-dish plate adapter (H501-EC-35PA, Okolab, Ottaviano, Italy) is implemented into the incubator. CO2 and humidity control during experiments is ensured by an additional air pump (OKO-AP, Okolab, Ottaviano, Italy).

2.3.2. Software. The acquisition software is based on the programming language Python. The basic microscope software ‘PYME’ for SMLM is available at http://code.google.com/p/python-microscopy/. Our custom-modified acquisition software (see also supplementary information available at stacks.iop.org/JOpt/15/094003/mmedia) features a graphical user interface (GUI) in which all hardware controls are displayed including piezocontrols for the interferometer/grating assembly and for the focusing unit, all filter wheel controls and a shutter control for the different excitation laser lines. Further controls include the joystick, a fast switching option, which allows changing between both microscope modes, and camera controls.

An acquisition script allows a fully automated imaging process for both microscope modes, which only needs to be set up once prior to imaging. Parameters necessary for SMLM and SIM are kept in separate parts of the control window. The microscope stage positions for each acquisition are automatically saved, which allows repeated relocation and imaging of the same region.

2.4. Sample preparation, imaging and visualization

HEK293 cells were stably transfected with Claudin3-YFP as described previously by Piontek et al [54], mounted on microscopy slides and embedded with Immu-Mount (Thermo Scientific, Pittsburg, PA, USA). Imaging was performed with the microscope setup described, which is capable of the two microscope modes SIM and SMLM. Conventional wide-field fluorescence microscopy is also possible. Excitation illumination for imaging was performed with a 568 nm laser line (Coherent Sapphire 568 HP, 200 mW,
Figure 2. Comparison of wide-field fluorescence microscopy, SIM, SMLM and illustration of visualization methods for SMLM (simulation details: see section 3.5): (I) simulated structure within a target object; (II) simulated standard wide-field fluorescence image; (III) simulated SIM image; (IV) simulated single molecule localization data (SMLM, position data set); (V) visualization of the localization data in IV using a Gaussian based algorithm [16]; (VI) visualization of the localization data in IV using a triangulation algorithm [16]; (VII) simulated sparse target distribution of localization signals assuming the same structure within the target object; (VIII) visualization of the localization data in (VII) using the Gaussian algorithm and (IX) visualization of localization data in (VII) using the triangulation algorithm. Scale-bar is 2 µm.

Coherent, Dieburg, Germany) with an output intensity of 100 mW.

For a high-resolved SIM image a total of nine images with standard resolution were recorded with SIM mode. A fine sinusoidal grating (350 nm period) was projected onto the object plane and then shifted by \( \frac{1}{3} \times 350 \, \text{nm} \) along the direction of modulation (three phase positions). Afterwards, the orientation of the grating was changed in order to achieve an isotropic resolution improvement (0º, 60º and −60º; three orientations). For each image frame, the fluorescence emission was integrated over 50 ms. Intensity in the focal plane for imaging was estimated to be 46.8 ± 2.7 W cm⁻². SIM Images were reconstructed using custom software, which is described in detail elsewhere [17, 19].

After registration of the SIM images, the microscope mode was changed to SMLM by filter switching, without moving the specimen. The SMLM image sequences consisted of 1000 frames with an integration time of 50 ms/frame and a focal plane intensity of 3.84 ± 0.1 kW cm⁻². Position determination of the optically isolated individual molecule signals and visualization was performed using custom software. The mean localization accuracy was \( \Delta x = 10.6 \, \text{nm} \). Visualization was conducted using a triangulation algorithm with a 10 nm pixel-size and 100 random jittering in order to smooth edges. Further details concerning image acquisition and reconstruction algorithms are described elsewhere [6, 16, 39].

2.5. Simulation

Whereas both SIM and SMLM simulations have been individually published previously [19, 44], in this report we present a direct comparison between SIM, SMLM, and wide-field microscopy, using one microscope setup with fast switching options between the different microscopy modes. In particular, we include the results of various visualization methods. The simulations shown in figure 2 were performed using Matlab 2009a (The MathWorks Inc.,...
Figure 3. Simulation of different event densities for SMLM: (I) wide-field image; (II) SIM image; (III) highly bleached SMLM data (low event densities); (IV) medium densely distributed single molecule events visualized with a triangulation algorithm; (V) very densely distributed single molecule event; (A)–(C) show the corresponding magnifications of the crossing regions for the different event densities. (A) Low event densities (III) are not able to resolve the crossing of the two lines properly while (B) medium densely packed (IV) single molecule events clearly reveal the crossing section. However, gaps within the lines occur although the structure is continuous. (C) Multiple fluorophores (V) are localized within the same frame and within a diffraction-limited area. As a result, the localization algorithm used eliminates events with insufficient optical isolation from the image; while the crossing is still clearly resolved, this produces various artifacts in the apparent signal density (white arrows).

Natick, MA, USA). Four sinusoidal patterns with different offsets respective to each other were generated. Afterwards, images were overlaid in order to generate a double sinusoidal structure. Image size was 1000 pixels × 1000 pixels with a pixel-size of 10 nm. The simulation for the SMLM image was performed by assuming ~20,000 single molecule events randomly distributed within the structure. Single molecule positions were jittered by the corresponding randomly generated localization error (mean = 10 nm, standard deviation = ±10). The background was simulated by using ~10,000 random single molecule events per 1000 pixels × 1000 pixels, with identical parameters for all simulated SMLM images. For simulating a sparse target distribution of a SMLM image ~3000 single molecule
events were generated. In order to generate a wide-field image, simulated single molecule events were blurred assuming a PSF of 200 nm. The SIM image was generated accordingly, assuming a PSF of 100 nm. As usually more fluorophores contribute to a SIM and wide-field-image compared to a SMLM image, five times more fluorophores were assumed for simulation. Gaussian-based visualization was performed by blurring single molecule positions with the localization error (pixel-size: 10 nm). Visualization based on a triangulation algorithm was performed by inversely translating next-neighbor distances into intensity (jittering: 100, pixel-size: 10 nm). In order to smooth edges, single molecule positions were randomly jittered as described in detail by Baddeley et al 2010 [16].

The simulations shown in figure 3 were performed using PYME. The pixel size for SIM was 70 nm, the pixel size for SMLM images was 5 nm. Visualization was performed using a triangulation algorithm. The simulations for single molecule events also consider multiple events within one frame in near vicinity in the case of very high labeling densities.

3. Results

Figures 2 and 3 compare simulated wide-field, structured illumination and single molecule localization microscopy images. For this purpose, in figure 2 (SMLM/SPDM\textsubscript{Phymod}) and SIM have been applied to a simulated sinusoidal pattern (figure 2(I)). Figure 2(II) shows the simulated wide-field fluorescence image, as expected when using a standard fluorescence microscope. At many sites, the structure is not resolved (arrow). Figure 2(III) displays the corresponding SIM image (resolution enhanced by a factor of two). The SIM image is not only able to resolve the two sinusoidal lines within the peaks of the generated structure but also within the area of high gradients, whereas the standard wide-field method (figure 2(II)) is not capable of this. The SMLM image is able to reveal this detail even better, due to the higher resolution provided (figures 2(IV)–(VI)). Applying a Gaussian algorithm for the localization of SMLM signals is the most common procedure in order to generate structural information (figure 2(V)). However, this visualization method usually results in a resolution loss compared to the actual data, as positions are often blurred with the standard deviation of the corresponding localization accuracy. A triangulation algorithm [16] is usually superior to a Gaussian visualization in terms of conserving resolution provided by the raw data (figure 2(VI)). In this case, the two lines of the sinusoidal pattern are clearly resolved. A limitation arises when the triangulation algorithm is applied to a sparse target distribution (figures 2(VII)–(IX)), which does not produce sufficient structural information [16] (figure 2IX)). In this case an overlay of the SMLM image with a wide-field fluorescence image is desirable.

The standard wide-field fluorescence image is not able to resolve the inner structure of the simulated pattern (arrow in figure 2(II)), and structural information can be assigned to the single molecule events of the SMLM image in a limited manner only. The highly resolved SIM image can be used in order to provide sufficient structural information. As a result, in most cases single molecule events can be located within the corresponding structure of the target and thus provide actual information about location and distribution, making it finally possible to gain nanostructural insights that have previously been inaccessible.

Figure 3 presents another simulation example, the crossing of two lines. In biology, such lines might be represented e.g. by two actin fibers crossing each other. Simulations were performed in order to compare structures for which only a few, an average number, or many fluorescent bursts were detected. The extremes correspond to highly bleached (few detected signals) or densely labeled (many detected signals) structures. In terms of the desired resolution, these simulations indicate that the results depend strongly on the label densities assumed.

Figure 4 shows first experimental results. As biological model we used HEK293 cells, which were stably transfected with Claudin3-YFP. Claudins (Cld) form the backbone of tight junction (TJ) structures regulating the paracellular permeability of tissue barriers. Pore-forming Claudins determine the permeability for ions, whereas that for solutes and macromolecules is assumed to be crucially restricted by the strand morphology (i.e., density, branching and continuity). Previously, we have shown [54] by SMLM/SPDM\textsubscript{Phymod} that the Claudin3-YFP labeled strands form extended TJ networks. Two populations of elliptic meshes (with mean diameters of 100 nm and 300–600 nm, respectively) were revealed. In addition, SPDM\textsubscript{Phymod} images suggested that the Claudin3-YFP distribution along the membrane in protrusions of such strands was highly heterogeneous. The distribution of membrane-bound Claudin3-YFP in such a protrusion can be approximated by a cylinder the diameter of which is defined by the diameter of the protrusion, and the wall thickness is defined by the distribution/spatial arrangement of the Claudin3-bound YFP. A cross section through such a protrusion should therefore exhibit two peaks more or less at the position of the membrane on either side. The curvature of the membrane (cylinder) leads to the width of each of the peaks. Using computer calculations with the aforementioned localization accuracy, the peak width for a 500–600 nm wide protrusion as shown in figure 4 was estimated to be 50–60 nm for an ideally dense label. Therefore, such specimens may constitute a good test object to demonstrate the resolution obtainable with the various modes of the combo setup constructed. Ideally, fluorescent nanospheres (beads) should be suitable test object with a well-defined size. However, due to the labeling density in these beads, SPDM\textsubscript{Phymod} could not be performed on beads because reversible photobleaching of a sufficient amount of the fluorophores in order to obtain optical isolation of a single remaining fluorophore per recorded image frame is not possible.

Figure 4 shows a comparison (overview SMLM/SPDM\textsubscript{Phymod} image figure 4(IV)) between conventional wide-field (figures 4(I), (A), structured illumination/SIM ((II), (B)), and localization microscopy/SMLM (III), (C)). While the wide-field image (II), (A) does not reveal any substructure of the protrusion, the SIM image ((III), (B))...
clearly indicates a substantial heterogeneity in the distribution of the Claudin-YFP signals. On the single molecule level ((III), (C)) this heterogeneity in the signal distribution becomes still more prominent. For a quantitative analysis, figure 4(V) shows intensity plots for a cross section of a protrusion as indicated in figures 4(A)–(C). Whereas in the conventional wide-field mode, the apparent width of the line plot was around 1 μm, the SIM and SPDM Phymod line plots indicated a considerably smaller width of the protrusion walls (120 ± 10 nm for SMLM, 150 ± 20 nm for SIM). In contrast to the conventional wide-field image, the SIM line plot reveals a substantial heterogeneity of the Claudin-YFP signal distribution. This heterogeneity becomes much more prominent in the SPDM Phymod line plot. In this case, two clearly separated signal distributions across the protrusion are observed, each of them with an apparent half-width below the conventional resolution limit. Another important feature is the substantial gain in image contrast obtained by the suppression of background noise by the algorithms used.

The localization data of the image shown in figure 4(III) is exemplarily visualized in figure 4(IV) using a triangulation method published elsewhere [16]. The resolution of the triangulation image is comparable to the resolution of SIM, though differences can be seen. The SMLM image (figure 4(III)) as well as the visualized SMLM image using a triangulation algorithm (figure 4(IV), white arrows) shows...
interspersions within the protrusions of the cells. When comparing this observation to the SIM image, significant differences can be seen. In order to explain this, we need to look at the z-thickness of optical sections generated by the two methods. The 2-beam SIM reconstruction software used here provides an optical sectioning of 500–600 nm along the optical axis, whereas the SMLM algorithm was able to increase the optical sectioning to values smaller than 300 nm. In this case, detected signals exceeding a certain width were eliminated from the positions taken into account: signals with a large FWHM are usually arising from out-of-focus objects. Consequently, in the SMLM image, more parts of the protrusions of the cell are potentially out-of-focus than in the SIM mode. Another reason may be a varying micro-environment within the sample or a different suppression capability for out-of-focus signals. Photo-switching of fluorophores is highly dependent on the micro-environment. Within the area of the interferences of the protrusions, the micro-environment might potentially not have favored the blinking behavior of the single molecules and thus did not contribute to the image acquisition, although labeling was present (figure 4(II)). In comparison, the fluorescence signal can be detected in the SIM image due to the lower optical sectioning from other focal planes, where labeling was present. The opposite is possible as well: high labeling densities could often result in several fluorophores in the near vicinity to each other being in a ‘bright’ state within the same frame. Thus their PSFs might overlap, and these events are therefore discarded in the post-processing of the single molecule data using present algorithms (‘collision’ artifacts, figures 4(IV) and (C)). As a result, an information loss could potentially occur though the labeling efficiency was very good. A combination with the SIM image could reduce such effects (figure 4(II)). This can for example be observed in figure 4 in the upper-right quadrant of figures 4(I)–(III). The two bright spots appear dimmer within the SMLM image (figure 4(III), white arrows) compared to the wide-field and SIM image (figure 4(II), white arrows). In case of high event densities, visualization algorithms may also tend to fill gaps between closely spaced structures (figures 4(IV) and (C), white arrows) although the SIM image would potentially reveal two separate structures (figure 4(II)). In addition, background issues may play a key role in single molecule detection as out-of-focus single molecule events contribute to locally evaluated background levels (‘shading’-artifacts). As a result, several single molecule events cannot be separated from the high background; thus, locally reduced total numbers of detected signals are evaluated. Moreover, background levels do not tend to be homogeneous throughout the whole specimen. Usually inhomogeneities are present, resulting in a variable detection efficiency. Drifts occurring during the relatively long data acquisition may also distort single molecule positions. A SIM image can help to identify drift-related displacements and would allow mathematical corrections of SMLM data.

4. Discussion

In this paper we have presented a unique ‘combo’-microscope setup, which combines the two high-resolution methods SIM and SPDMPhymod, in one custom setup. In contrast to presently available commercial microscope systems, this setup is able to use up to five excitation channels, and can easily be utilized with multiple cameras simultaneously. First proof-of-principle images demonstrate that this instrument is a promising tool for future studies combining SIM and SMLM. A combination of both methods in one setup allows biological questions to be addressed on a highly advanced resolution level.

In the following we will discuss two particular applications which might benefit most from this combined approach: imaging of sparsely labeled structures and imaging in the presence of autofluorescence. Furthermore, details of the particular advantages for routine application of our combined microscope setup will be discussed.

The mathematical generation of structural information of SMLM targets is highly challenging or even impossible if the yield of single molecule events is very low. Here, two possible scenarios can be distinguished. In the first case the structure within the specimens is well labeled (as given by the spatial distribution of fluorophore molecules). However, due to various effects such as bleaching and blinking efficiency, the single molecule statistics in the SMLM is too low in order to allow visualization of the data set. In this case a highly resolved SIM image of the same structure may deliver this information, due to its different contrast mechanisms. In the second scenario the target structure itself is quite sparsely labeled and therefore SMLM visualization algorithms are not easily applicable; furthermore SIM will also not be able to resolve the structural content. Applying a second label in order to resolve structural information would solve this problem [16]. In our combined setup, it is possible to use a highly resolved image of the specimen acquired with SIM in order to gain structural information, which may be correlated with the sparse target distribution gained with SMLM. Furthermore, combinations of ‘switchable’ and ‘non-switchable’ fluorophores will extend the total number of channels available for high-resolution fluorescence imaging.

The other great advantage of combining both methods is the detection of visualization artifacts in SMLM images, which do not occur in SIM images, and vice versa. A direct comparison easily reveals those details, and thus a combination of both methods helps to interpret single molecule data correctly. With the combination of both methods in one setup and by utilizing multi-labeled targets a relatively easy and sufficient solution for this frequently encountered problem is now available.

Another potential application will affect biological questions dealing with AF structures. As already demonstrated, SIM is a useful tool to study AF structures generally found in the aging human eye [48, 49]. However, if additional fluorescence markers to target specific structures are used, the influence of the AF signal on the signal of the targeted fluorescent markers during the imaging process with SIM and
SMLM needs to be studied. When utilizing SIM, the ratio of the AF signal intensity versus the intensity emitted by additional fluorescent markers will be of main interest. SMLM will be more challenging as the AF signal might outshine the single molecule signal, which is presumably much weaker. In future measurements, it needs to be tested whether it is possible to separate the single-molecule signal from the AF signal in order to reconstruct an image. As the AF is generally strongest in the shorter-wavelength band (e.g. AF of lipofuscin in retinal pigment epithelium cells [48, 49] and AF due to fixation such as PFA [55]), additional markers within the red or near-infrared range might be most promising in order to separate signals from each other [6].

Finally, the AF signal given by the SIM image and its structural information can be merged into one image with the signals of other labeled targets on SMLM resolution level. As a result, the relative orientation to each other is revealed and can be assigned to separate compartments within the biological object.

In other applications it might be impossible to distinguish between AF signal and labeling. Here SMLM would reveal the AF signal as it is usually not ‘blinking’. Thus the AF signal itself could be imaged with SIM and correlated with the SMLM data.

As the ‘combo’ microscope presented here provides a unique, user-friendly, fast and fully automated imaging device, it is possible to conduct live-cell experiments, for which a fast imaging process of low phototoxicity is absolutely necessary. This requires imaging of the same region of interest over long periods of time as well as an automated relocation of a target, which is now possible with the newly developed combo setup using the SIM approach.

First attempts show promising results e.g. performing live-cell imaging using SIM over long periods of time applied on RPE cells (data not shown), where we exploited the AF signal of the cells with no additional labeling necessary. SIM already delivers a sufficient resolution at low laser intensities and is thus a much less invasive imaging tool compared to SMLM methods based on ultraviolet and/or high illumination intensities.

The application of SMLM methods on living cells has already been demonstrated, although no information about the influence on the cells have been given [33]. Labeling methods e.g. via SNAP-tag fusion proteins are advantageous for live-cells experiments [31] with combined SIM and SMLM imaging. However, as SMLM in the SPDM$_{Phymod}$ mode presented here is highly invasive due to high laser intensities (other current approaches use ultraviolet light and/or toxic switching buffers), the influence of these methods on living cells needs to be tested thoroughly. While the combo setup described could potentially be used in conjunction with low illumination intensity SMLM methods, such as PALM or FPALM, the problem of image construction in spite of extended molecule movements in the thousands of image frames remains. Whether it is possible to repeatedly image living specimens with SMLM is highly dependent on the necessary intensity (in the presence of appropriate non-toxic switching buffers), the absorption of light by the cell and the resistance of the cell to phototoxic damage.

In summary, both methods benefit from each other as each method compensates the weaknesses of the other. SMLM/SPDM$_{Phymod}$ images deliver a very high single molecule localization accuracy resolution but without further information about the structural context the highly resolved position information might even be useless. Assigning the SMLM position information to the structural information of a standard wide-field fluorescence image would not necessarily increase information as in this case the wide-field resolution may be too low to sufficiently reveal single structural details of the object. However, SIM images provide a sufficient resolution gain in order to visualize structures of biological objects in much more detail than conventional fluorescence microscopy. Thus position information can directly be assigned to improved structural features and increase the amount of information gained of the specimen. The newly constructed custom-built microscope presented here is a tool superior over existing separate SIM and SMLM methods for future nanostructural studies dealing with biological/medical questions.

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Author contributions

All authors have made substantive contributions to this study. S Rossberger: concept and design, software development, analysis and interpretation of data, drafting the paper, final approval of the version to be published. G Best: concept and design, software development, analysis and interpretation of data, drafting the paper, final approval of the version to be published. D Baddeley: concept and design, software development, drafting the paper, final approval of the version to be published. R Heintzmann: substantial contributions to concept, SIM reconstruction software and critically revising the paper, final approval of the version to be published. U Birk: substantial contributions to the interpretation, writing and critically revising the paper, final approval of the version to be published. S Dithmar: critically revising the paper, final approval of the version to be published. C Cremer: substantial contributions to concept, writing and critically revising the paper, final approval of the version to be published.
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