



Research article

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High-speed multiplane structured illumination microscopy of living cells using an image-splitting prism

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Abstract: Super-resolution structured illumination microscopy (SR-SIM) can be conducted at video-rate acquisition speeds when combined with high-speed spatial light modulators and sCMOS cameras, rendering it particularly suitable for live-cell imaging. If, however, three-dimensional (3D) information is desired, the sequential acquisition of vertical image stacks employed by current setups significantly slows down the acquisition process. In this work, we present a multiplane approach to SR-SIM that overcomes this slowdown via the simultaneous acquisition of multiple object planes, employing a recently introduced multiplane image splitting prism combined with high-speed SIM illumination. This strategy requires only the introduction of a single optical element and the addition

of a second camera to acquire a laterally highly resolved 3D image stack. We demonstrate the performance of multiplane SIM by applying this instrument to imaging the dynamics of mitochondria in living COS-7 cells.

Keywords: super-resolution optical microscopy; PACS10: 42.30.Wb; multiplane image acquisition; structured illumination microscopy.

1 Introduction

Conventional fluorescence microscopy is inherently limited in its spatial resolution due to diffraction. Optical super-resolution imaging techniques allow us to overcome this limitation. For super-resolution structured illumination microscopy (SR-SIM), this is achieved by using high spatial illumination frequencies that down-modulate spatial frequencies beyond the cutoff into the passband of the microscope [1–4].

Linear implementations of SR-SIM create a sinusoidal interference pattern in the sample plane, leading to a spatial resolution improvement of at best twofold over wide-field microscopy and additional contrast enhancement for high spatial frequencies and strong suppression of out-of-focus light by filling the missing cone of the instrument's optical transfer function [3, 5, 6]. In return, only a small number of raw images with defined illumination patterns [nine in the standard two-dimensional (2D) implementation, less when using advanced algorithms [7]] are needed for the image reconstruction process. The excitation powers are comparable to conventional wide-field imaging, so photo-damage can be minimized, and no special dyes (e.g. dyes that are photo-switchable or inherently blinking) are required for the approximately twofold resolution enhancement. The combination of these features makes SR-SIM a very fast super-resolution imaging technique, with current instruments [8, 9] providing 2D

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imaging with approximately 100-nm spatial resolution in the lateral direction at video-rate speed and even faster.

Live-cell imaging is the primary domain for SR-SIM. Here, its high temporal resolution excels, especially when observing highly dynamic processes on subsecond time scales. If, however, three-dimensional (3D) volumetric imaging is required, current state-of-the-art SR-SIM systems slow down the acquisition rate significantly, because of the need to physically move the object through the focal plane, e.g. by piezo-translation stages. The slowdown arises not just from the repeated image acquisitions, but also from the intermediate sample movement that needs to be accounted for. Also, advanced control electronics are required if stage movement, SR-SIM illumination, and camera exposure are to be synchronized precisely.

Such delays can be avoided if a full 3D volume of the sample could be acquired at once. Two different approaches to multiplane image detection currently exist: a diffractive element, based on a phase-shifting spatial light modulator or a lithographically-produced optical element [10, 11], can be introduced into a conjugated image plane and create almost arbitrary multiplane detection schemes. Chromatic aberrations can be corrected using additional elements [12]. Recently, this approach was combined with a commercial SR-SIM microscope [13]

and provided the first multiplane SR-SIM images in a prototype system. Although this proved to be a great demonstration of the future of SR-SIM imaging, these tests ended up being limited by the slow speed of the commercial SR-SIM platform, which was not originally conceived for video-rate super-resolution imaging. Here, we present a multiplane imaging approach to SR-SIM that circumvents these issues and achieves high-speed imaging rates previously available only to 2D SR-SIM.

2 Multiplane SIM

Our work is based upon an imaging strategy for multiplane detection that divides the detected fluorescence light into multiple image planes by introducing discrete optical path length differences to the image path [14–16]. This path length difference guarantees perfect object-image conjugation for eight object planes equally displaced along the axial direction. As seen in Figure 1, the classical 8f setup of the detection path allows us to easily realize a telecentric instrument ensuring identical scaling of all eight images obtained from different axially displaced object planes.

While image splitting via subsequent changes in detection path length is a robust concept, its typical

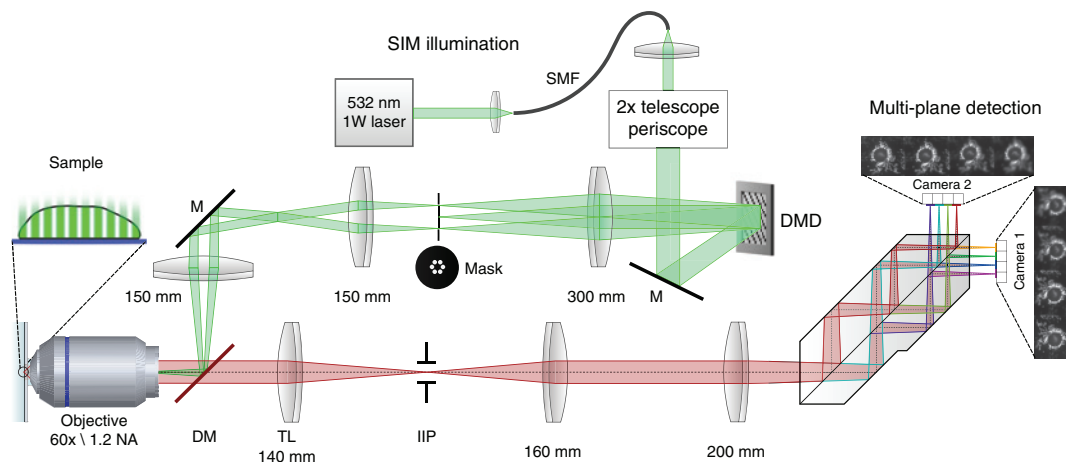


Figure 1: Optomechanical schematics of the high-speed multiplane SR-SIM imaging system.

The SR-SIM pattern is created by a 532-nm 1-W laser (Roithner) coupled into a single-mode fibre for mode cleaning. The light at the distal end of the fibre is then collimated, expanded by a factor of 2 and directed onto a digital micromirror device (DMD), which is used as a fast, electronically controlled optical grating. A segmented aperture mask removes spurious diffraction orders, arising due to the binary nature of the DMD. A relay lens system focuses the light into the back focal plane of the objective lens (Olympus UPLSAPO 60 \times /1.2 NA water immersion). Excitation and emission light is wavelength-separated by a dichroic mirror (DM). In the detection path, the tube lens (TL) forms an intermediate image (*image plane*), where a field stop (IIP) sets the field-of-view size and prevents overlapping signals in the multiplane detection system. A magnification of $\times 58.5$ is achieved, which corresponds to a projected pixel size of 111 nm for the sCMOS cameras (ORCA Flash 4.0, Hamamatsu). Lenses form a relay telescope into the main component, the precision-made multiplane prism [16]. It provides 2×4 copies of the image onto two scientific sCMOS cameras, where each copy has a defined path-length difference and thus defined axially offset focal plane.

implementation with discrete optical elements is sensitive to thermal drifts and vibration and rather difficult to align. A recent development [16] solved this problem by integrating this approach into a single, stable, precision-made optical element: a compound image-splitting prism. This device allows for the simultaneous detection of up to eight diffraction limited images obtained at equally spaced vertical planes with only minimal alignment of the overall optical setup. Compared to diffraction-based multiplane microscopy [13], our approach has the advantage that the prism consists of a single, compound refractive element, instead of two separate gratings and a refractive correction prism. This greatly improves the stability and simplifies the alignment of the system. While the diffractive solution can in principle be tailored to a specific imaging application, the method presented here is very versatile and, through a careful selection of tube lenses, can be used at any wavelength, allowing for example 3D correlative microscopy [16].

We combined our prism-based detection path with a coherent SIM excitation path based on a high-speed digital mirror device (DMD-DLP7000, Vialux) and a high-power laser (532 nm, 1 W, Roithner). By utilizing two synchronized sCMOS cameras (Hamamatsu Orcaflash V4.0), we achieved high-speed volumetric imaging with 50-ms exposure time. Taking camera readout and device synchronization into account, this results in about 1.3 reconstructed SIM data sets per second, to image a full volume of about $40 \times 40 \times 2.45 \mu\text{m}^3$. Both the DMD-based spatial light modulator and the cameras could, in principle, be tuned to provide even higher imaging speeds [8], if required by the application and if sufficiently bright fluorophores are used to compensate for the lower signal-to-noise ratio.

In its current configuration, the SIM is operated in coherent two-beam mode. Here, the ± 1 st diffraction orders generated by the DMD grating pattern can interfere in the sample plane to generate a lateral modulation of the excitation pattern. The 0th order is blocked, so no axial modulation of the SIM pattern is introduced. This approach sacrifices the axial resolution improvement possible in full three-beam SIM, but comes with a set of advantages. First, including SIM modulation along the axial direction would entail aligning each z -plane with the axial periodicity of the SIM pattern, which would take away some of the systems robustness and simplicity. Also, the acquisition of 15 instead of nine raw images is required for a direct SIM reconstruction of three-beam SIM data, which impacts overall imaging speed, as well as sample photobleaching and phototoxicity. Most importantly, the optical sectioning intrinsically provided by three-beam SIM can also be obtained in a two-beam approach (albeit at lower axial resolution) by trading

in some of the lateral resolution enhancement for optical sectioning capability. In brief, using a slightly coarser SIM pattern (frequency of $1.8 \mu\text{m}^{-1}$, or 555-nm periodicity, here) leads to an overlap of the shifted copies of the optical transfer function obtained by SIM, which, in turn, allows to fill their missing cone and thus provides axial sectioning [5, 6]. Additionally, using a coarser SIM pattern simplifies the aspect of polarization control. For optimal pattern contrast, the polarization of the interfering beams would have to be rotated to match the three orientations of the SIM pattern. However, when using a coarser pattern, and thus steeper interference angles, the influence of polarization becomes less pronounced [17]. This allows us to use a fixed linear polarization while maintaining reasonable pattern modulation contrast for all pattern orientations, which both simplifies the optical setup and avoids light loss in the filter elements that would otherwise occur. To summarize, we tuned the SIM system to a compromise of both optical sectioning and lateral resolution improvement, while operating at the minimum of nine raw SIM frames to achieve high imaging speeds.

3 Results

We applied our multiplane SIM system to the fast imaging of mitochondrial dynamics [18, 19]. Here, SIM is an enabling imaging method, as its increase in spatial resolution and inherent background suppression allow us to visualize the mitochondria and their 3D morphology and dynamic changes thereof, which cannot be observed with conventional wide-field resolution [20]. Furthermore, the rapid movement of mitochondria requires high imaging speed, while their 3D nature greatly benefits from acquiring signals from multiple z -planes. However, the structures are still rather “thin” compared to their length, and a coverage of $2.45 \mu\text{m}$ (eight planes with an axial sampling of 350 nm) as provided by our system is typically sufficient to capture the entire 3D mitochondrial structure in a single-shot multiplane exposure. In this case, no axial movement of the sample is needed at all, which greatly simplifies data acquisition and decreases the acquisition time. The data processing and results are presented in Figure 2, where our imaging was performed with 50-ms exposure time per raw data frame. As mentioned earlier, three angles and three phases are required for the 2D SIM image reconstruction process, thus reaching 770-ms acquisition time for the full imaged volume.

Figure 2A summarizes the flow of data. A key challenge is that the eight planes are all slightly shifted with

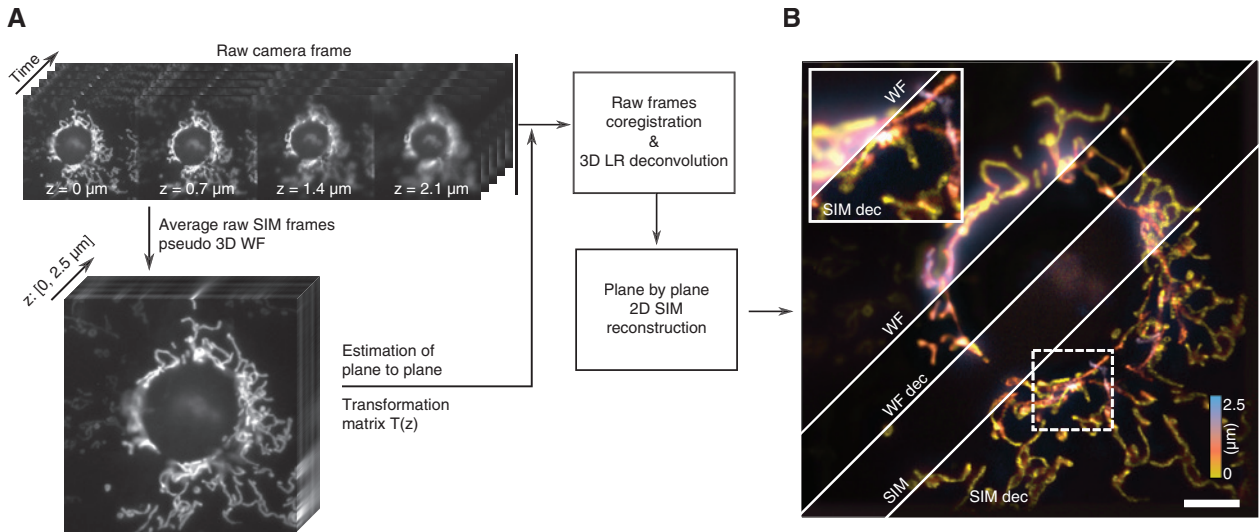


Figure 2: Data processing and SIM image reconstruction procedure.

(A) The raw images are first averaged to produce a pseudo wide-field image. This image is then used to estimate the interplane transformation matrix $T(z)$. This matrix is used to coregister the raw SIM images and reorder the data in x, y, z, t space. Then, the data are either averaged (WF), deconvolved in 3D (WF dec), reconstructed (SIM), or deconvolved and reconstructed (SIM dec). (B) Colour-coded maximum intensity z -projection of 3D images for all four modalities on a fixed COS-7 cells labelled with MitoTracker Orange. Inset shows a zoomed in comparison between WF and deconvolved SIM. Scale bar $5 \mu\text{m}$.

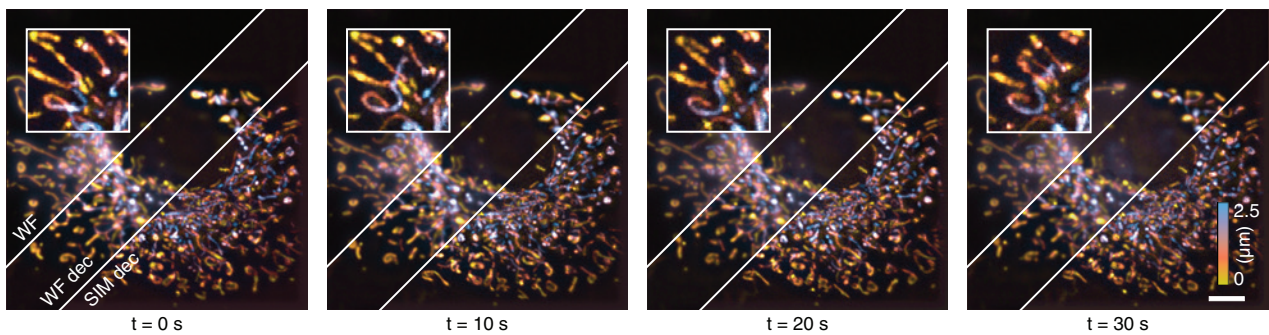


Figure 3: Time series of SR-SIM imaging data following mitochondrial dynamics (stained with MitoTracker Orange) in COS-7 cells, colour-coded maximum intensity z -projection of the deconvolved and SIM reconstructed eight planes (Figure 2). The full series contains 74 time points, each acquired with 50-millisecond SR-SIM acquisition time to allow for motion artefact-free imaging (see Supplementary Material: Visualization 1). The series has been renormalized in brightness to compensate for photo bleaching, but no further processing beyond a standard SIM reconstruction had to be performed. Insets (field of view $7 \times 7 \mu\text{m}^2$) show deconvolved SIM exhibiting high spatial motility of the mitochondria during the imaging. Scale bar $5 \mu\text{m}$.

respect to each other, due to unavoidable limitations in the prism manufacturing. By cross-correlating two consecutive planes, we are able to recover the prism transformation matrix $T(z)$ and perform a subpixel coregistration for all the raw SIM frames. We then average all the frames to form a pseudo-wide-field image (WF), deconvolve the 3D WF using 10 iterations of Lucy–Richardson (LR) deconvolution (deconvlucy, MATLAB 2017b), reconstruct the SIM image plane by plane (SIM), or deconvolve in 3D the raw SIM frames and reconstruct the SIM images plane by

plane (SIM dec). Results are shown in Figure 2B, where we encoded the depth information using a colour-coded maximum z -projection. Using the recently introduced image resolution estimation method [21], we measure a resolution of 460 nm for WF and 434 nm for WF dec. This relatively high value is due to the presence of out-of-focus light, which is partially removed by the 3D deconvolution. For SIM, we measure 266 nm (263 nm for SIM dec), which corresponds to a resolution gain of about 1.7 compared to WF. We note that for SIM the deconvolution

mostly improves the image contrast and does not change the resolution. We used a custom 2D SIM reconstruction algorithm implemented in MATLAB, following closely the work from Müller et al. [22]. For the 3D LR deconvolution, we used an experimentally acquired 3D PSF of the setup, obtained by imaging, localizing, and averaging the 3D image of 15 sparsely distributed subdiffraction (100-nm diameter) fluorescent beads.

In Figure 3, the time series of a second data set is shown. With an exposure time of 50 milliseconds, we achieved about 1.3-fps volumetric SIM imaging speed. We estimate for the resolution 520 nm for WF, 460 nm for WF dec, 375 nm for SIM, and 265 nm for SIM dec. The small degradation of resolution for SIM and WF is due to unavoidable sample movement. No further degradation of resolution over time is observed. However, we find (see Supplementary Material: Visualization 1) that this speed allows us to capture the 3D dynamics of the mitochondrial network. While the reconstructed sequence had to be bleach-corrected by normalizing the average of each frame, 74 time points (about 60 seconds) could easily be acquired, without the need to resort to any advanced image reconstruction algorithms.

4 Conclusion

The results presented here demonstrate the first proof of concept of high-speed two-beam multiplane SIM imaging of living cells using an image-splitting prism. They show that the combination of multiplane image detection with fast two-beam SIM illumination indeed yields the desired high-speed volumetric imaging. These results also indicate that this system is well suited to image mitochondrial dynamics, as both the necessary temporal and spatial resolution is reached.

Our work also provides several insights into current limitations and challenges. For example, the current DMD implementation encounters significant losses of the excitation light due to spurious diffraction, with a maximum of about 3 mW reaching the sample (single-mode fibre coupling: 25%, DMD transmission: 6%, SIM mask: 30%, dichroic and objective lens: 70%). Newer liquid crystal on silicon-based designs will allow for a 10× improvement in power management albeit at the cost of more complex timing requirements. By utilizing polarization control and higher pattern frequencies, the lateral spatial resolution could also be pushed towards ~130 nm. In combination with more advanced algorithms taking full advantage of the 3D information

for low signal-to-noise image reconstruction, we envision that 5- to 10-millisecond exposure times, and thus volumetric imaging within 50 to 100 milliseconds, will be possible. Very recent development into advanced denoising SR-SIM reconstruction algorithms [23] points to solutions that will allow for the use of significantly lower signal levels in the image reconstructions and thus might allow us to push for even faster imaging speed. Advanced fluorescent dyes will allow for even more extended observation times. Novel, smart data-driven feedback loops should also be able to dynamically adapt the imaging speed depending on the observed dynamics. These approaches will all complement multiplane video-rate SR-SIM imaging quite well.

In its current state, image-splitting multiplane SR-SIM technology provides an early demonstration of what this technology will be able to achieve in more improved configurations. As the approaches and their implementations evolve, we believe they will provide an important tool for future high-speed super-resolution 3D imaging of living cells and organisms.

5 Sample preparation and staining

No. 1.5 cover glass coverslips were cleaned with a piranha solution and coated with fibronectin (0.5 $\mu\text{M}/\text{ml}$). Cells were grown in Dulbecco modified eagle medium without phenol red medium, containing 10% of foetal bovine serum. Mitochondria were stained with 100 nM MitoTracker Orange (Thermo Fisher) according to manufacturer-provided staining protocol for 30 min. Then cells were either fixed with 4% paraformaldehyde or used for live-cell imaging. For live-cell imaging, cells were washed twice with grown medium and imaged in phosphate-buffered saline, pH 7.4, which proved to reduce background fluorescence, and short-term imaging was performed in a custom-built incubator at 37°C.

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Conflicts of interest: The authors declare that they have no conflicts of interest related to this article.

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