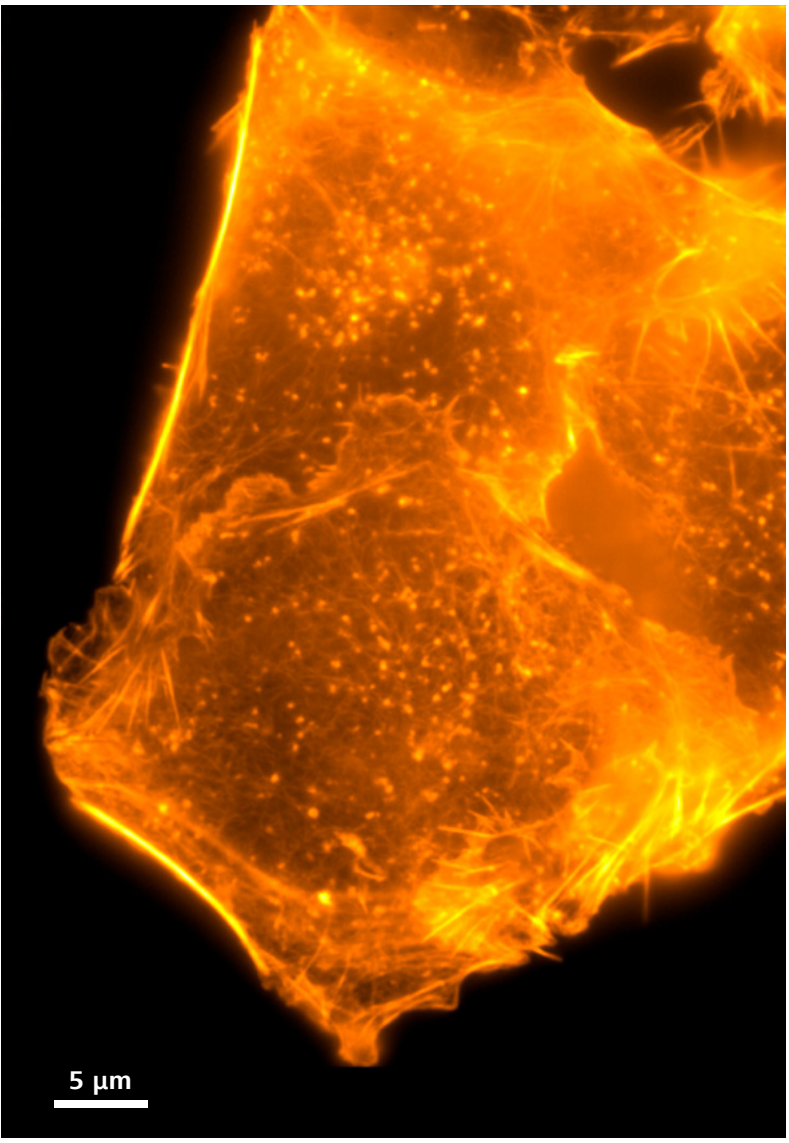

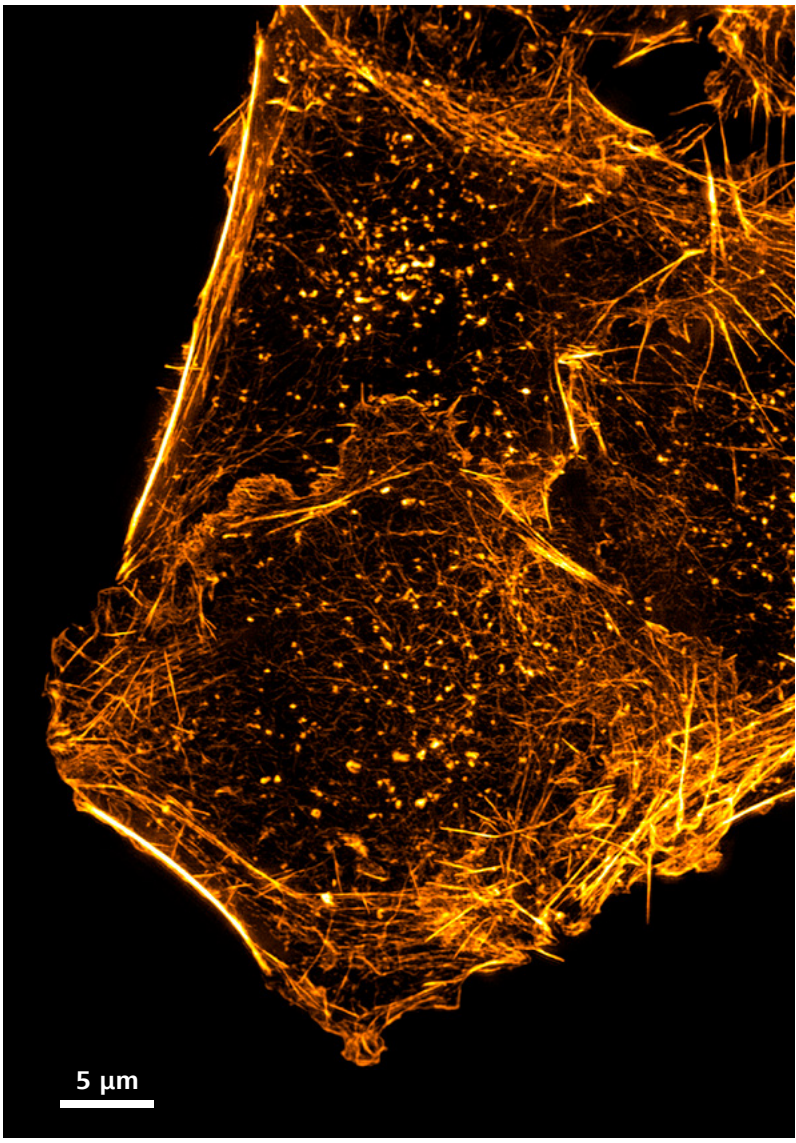

Introducing Lattice SIM for ZEISS Elyra 7

Structured Illumination Microscopy with a 3D Lattice for Live Cell Imaging

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Structured Illumination Microscopy with a 3D Lattice for Live Cell Imaging

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Introduction

Structured Illumination Microscopy (SIM) is one of the most powerful and versatile methods in fluorescence microscopy. Today, microscopists use it for optical sectioning and to achieve superresolution in all three spatial dimensions. In recent years, it developed into a routine method for the study of biological structures and biochemical processes (Ströhl and Kaminski 2016). The basic principle behind SIM is the reconstruction of a final image from multiple different illuminations of the sample, thereby revealing information that is otherwise lost in widefield microscopy.

Compared to other superresolution (SR) techniques, SIM has many advantages for live cell imaging. First of all, it can be used with standard sample preparation and virtually all fluorescent dyes and proteins. Secondly, SIM is very light efficient: it outperforms other far-field fluorescence SR techniques by minimizing photo damage (Li et al, Wäldchen et al.). However, live cell imaging of dynamic processes in living samples is still challenging, because of the time it takes to acquire the multiple raw images it takes to reconstruct a single SR frame. That's why researchers often see themselves restricted to acquire time-lapse images only in 2D. An increase in temporal resolution - either to resolve fast processes or to be able to get from 2D acquisition to volume imaging – is therefore highly desirable. At the same time, fast live cell imaging not only requires to minimize the photon dosage per frame but also robustness against background noise from out-of-focus fluorescence, limiting raw image quality. In addition, imaging with low photon numbers influences the achievable resolution in SIM.

With Elyra 7, ZEISS introduces Lattice SIM. This new superresolution technology is extremely light efficient and fast – and additionally offers superfast optical sectioning. The inherent live cell compatibility of Lattice SIM is further increased by new possibilities to digitally increase the achievable frame rate, while minimizing the number of images required for volume imaging. Microscopists profit from less photodamage and highest imaging speed.

The Lattice SIM technology

Generating the optical lattice for ZEISS Elyra 7

The classic SIM approach for superresolution fluorescence imaging was pioneered by Gustafsson [3]. A line grid pattern with intensity modulation in one single lateral direction is imaged onto the sample to produce a structured illumination pattern. This line-by-line structuring allows to obtain higher resolution in one direction (perpendicular to the lines). The pattern needs to be rotated in order to improve the resolution in other lateral directions. Classic SIM is conducted with at least three orientations of the line grid. For each rotation angle, the grid is swept laterally in five steps, creating the so-called phase images. These 15 raw images are then combined into the final superresolution image. Up to now all commercial implementations of SIM rely on this rotation of the illumination pattern. Unfortunately, this rotation approach is quite time consuming, which limits the achievable maximum frame rate of this promising superresolution technology.
Three-dimensional point patterns (here referred to as Lattice SIM) for SIM have already been described by Betzig [1] and Heintzmann [7]. In Lattice SIM, the spot pattern is only shifted laterally, without rotation steps. This allows live cell imaging of dynamic processes with much higher frame rates.

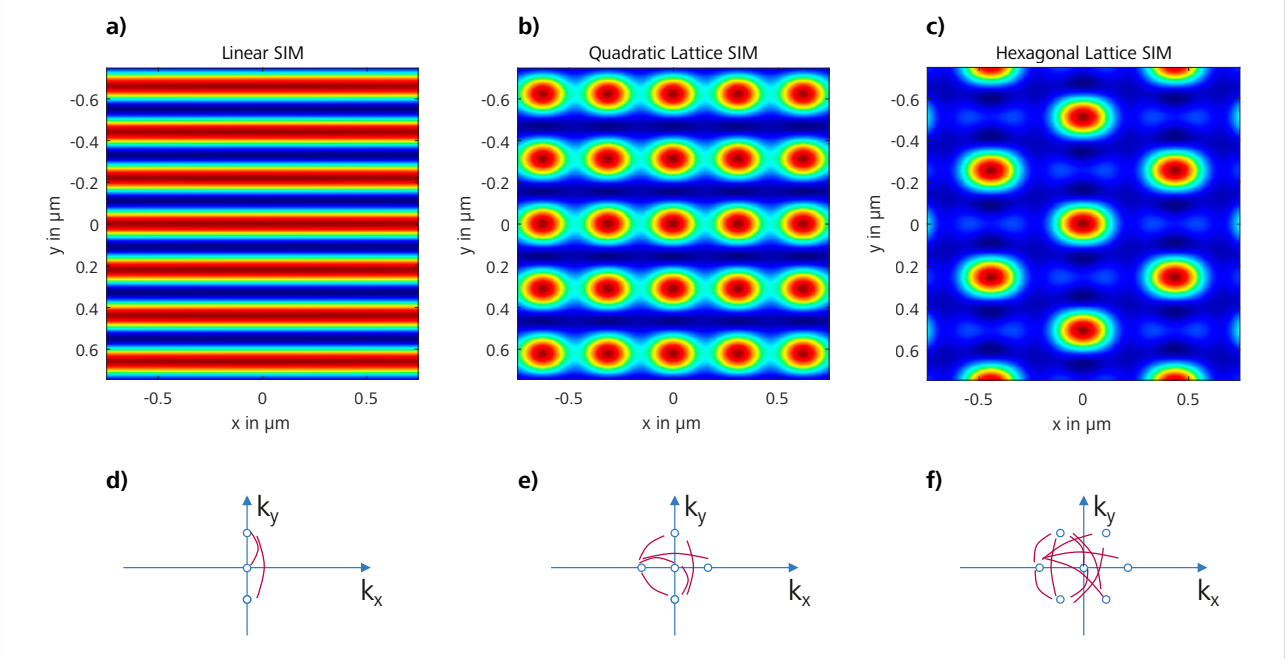

Figure 1 *Top row: lateral illumination pattern at a wavelength of 488 nm and a numerical aperture (NA) of 1.4. Bottom row: illumination distribution in the objective lens back focal plane. The linear polarization is parallel to the x-axis. d – f) excited frequency components indicated as red lines in the pupil patterns of structured illumination*

Here, we compare Lattice SIM with the performance of classic SIM using three relevant criteria for live cell imaging: (1) optical resolution – given by frequency support, (2) the number of required raw images which influences imaging speed, susceptibility to motion artefacts and phototoxicity and (3) signal to background ratio (SNB). The last point determines how thick and how densely labeled the sample can be. And it also influences the required photon dosage for good image quality.

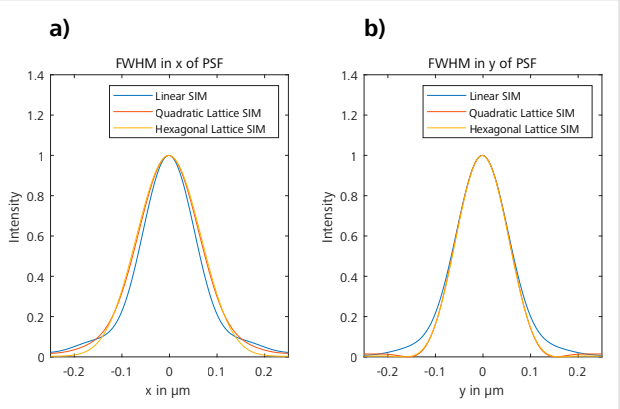

Figure 2 *PSF represented as line plots along the lateral PSF in x (Fig. 2a) and y (Fig. 2b) direction. The FWHM x/y are 134nm/134nm for the 1D linear SIM, 154nm/136nm for the 2D quadratic and 160nm/138nm for the 2D hexagonal SIM.*

Figure 1 shows simulations of (b) quadratic and (c) hexagonal lattice patterns in comparison to a stripe pattern (a). For all three cases, we assumed a structured illumination at 80 % of the cut-off frequency of the objective lens. The simulations are based on a vectoral beam propagation in order to take polarization effects into account.

The optical resolution of classic SIM and Lattice SIM are de facto identical, as can be seen in the in-focus PSF line profiles along the x and y direction (figures 2a and 2b). The data show that the frequency support of Lattice SIM is similar to classic SIM. Small differences stem from the linear polarization of the illumination beam, which for classic SIM rotates with the direction of the pattern. Overall, the differences are minor, because of their low amplitudes in the optical transfer function.

A critical factor determining the sensitivity of SIM is the modulation depth of the structured illumination pattern as detected by a camera in a single raw image. Figure 3 shows simulations of the modulation depth when imaging a planar and homogeneously labeled fluorescent sample of thickness D comparing the three different structured illumination patterns.

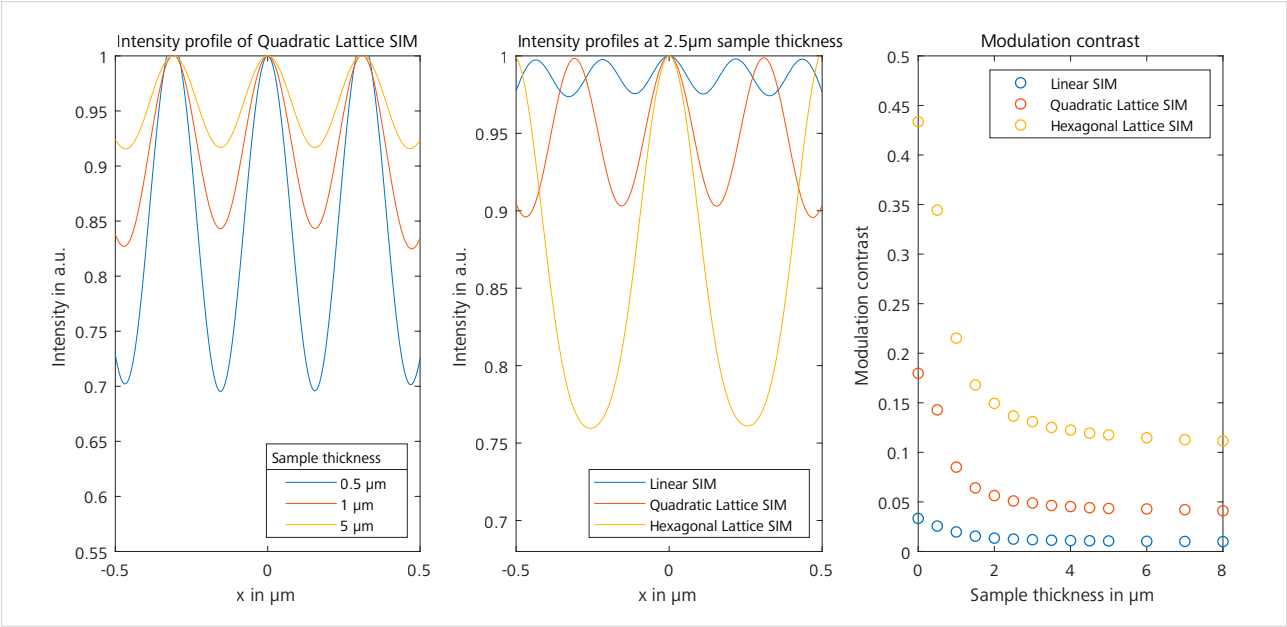

Figure 3 *Modulation depth as detected by the camera in a homogenously labeled sample of varying thickness for 1D linear, 2D quadratic and 2D hexagonal SIM modes. a) Intensity profiles of captured images for three different sample thicknesses illuminated with 2D quadratic SIM pattern. b) Intensity profiles at a sample thickness of 2.5 µm for the three SIM modes. c) Modulation Contrast vs. sample thickness.*

For a given structured illumination pattern, the modulation depth decreases with increasing thickness of the samples (Fig. 3a), because of out-of-focus blur. This in general limits detected signal and hence impacts the penetration depth of SIM. Fig. 3b shows the modulation for a fixed thickness of the sample when applying various grid patterns. Fig. 3c summarizes the modulation contrast defined as

$$MC = \frac{I_{max} - I_{min}}{I_{max} + I_{min}}$$

as a function sample thickness. The data indicate that Lattice SIM patterns provide greater modulation depth than grid patterns. Consequently, Lattice SIM allows to image deeper in the sample. Lattice SIM also outperforms classic SIM when imaging spatially extended or densely labeled structures. This is due to the high modulation contrast of Lattice SIM and results directly in a better Signal-to-Noise-Ratio (SNR). Consequently, the deconvolution step during SIM processing becomes more robust. In real-life applications – when imaging spatially extended structures – the slightly larger frequency support of classic SIM cannot be exploited to its full extent, because of noise. This means that the lateral resolution of Lattice SIM will be comparable to classic SIM. Setting resolution specs aside, the most important factor for live cell imaging is that Lattice SIM requires lower illumination intensity and fewer emitted photons than classic SIM.

The number of raw images (phases and rotation) that need to be acquired for each of the SIM methods depends on the number of excited frequency components (in Fig. 1d–f) indicated as red lines in the pupil patterns of structured illumination) [1]. Hexagonal lattice patterns need almost twice the number of raw images compared to quadratic lattice patterns or grid patterns, because they generate more frequency components f_N. Imaging fast processes in living cells demands a low number of required raw images. That's why ZEISS Elyra 7 uses a quadratic lattice pattern for structured illumination microscopy.

A simplified schematic of the beam path is shown in Figure 4. Illumination light is delivered by a fiber and passes a 2D grating. The grating is then imaged onto the sample via the tube lens TL and the objective lens. A phase shifter moves the 2D pattern across the sample. The phase shifter consists of 2 glass plates that are mounted on galvanometric scanners. The scanners can rotate the glass plates at very high speed, allowing rapid scanning of the 2D pattern across the sample. Emission light is collected by the objective lens and delivered to up to two sCMOS cameras for multicolor detection. One camera port is fitted with an adjustable lens to compensate for any chromatic length aberrations (CHL). This enables multicolor imaging of the sample with best precision.

Figure 5 shows an image of 120 nm DNA-origami (GATTA-SIM Nanoruler 120B, Gattaquant Germany) imaged at a wavelength of 488 nm with a ZEISS Plan-APOCHROMAT 63× 1.4 oil lens on ZEISS Elyra 7. Molecules at different lateral orientations have been measured and angles are shown. 120 nm resolution in all major lateral directions using the quadratic Lattice SIM can clearly be shown. The resolution is in good agreement to what one would expect in classic SIM at NA 1.4.

Reconstructing multiple planes with the new Leap mode

With ZEISS Elyra 7, a new acquisition and processing mode – called Leap mode – is introduced (see Fig. 6). Leap mode allows to reconstruct z-planes within the depth of focus around the sampled position ("digital sectioning"). This new mode reduces the number of images required to generate a given 3D image stack by a factor of three. In other words, Leap mode allows to image three times faster or with only a third of the photon dosage.

Each camera image is a projection of light along the z-axis encoded by the point spread function. The illumination pattern along z also modulates the intensity detected in the projection when the sample is shifted laterally. Thus, the phase images from a single position in z also contain information from adjacent planes, especially within the depth of focus – in an order of 500 nm. Although 3D-SIM processing takes this 3D information into account, the sampling in z and

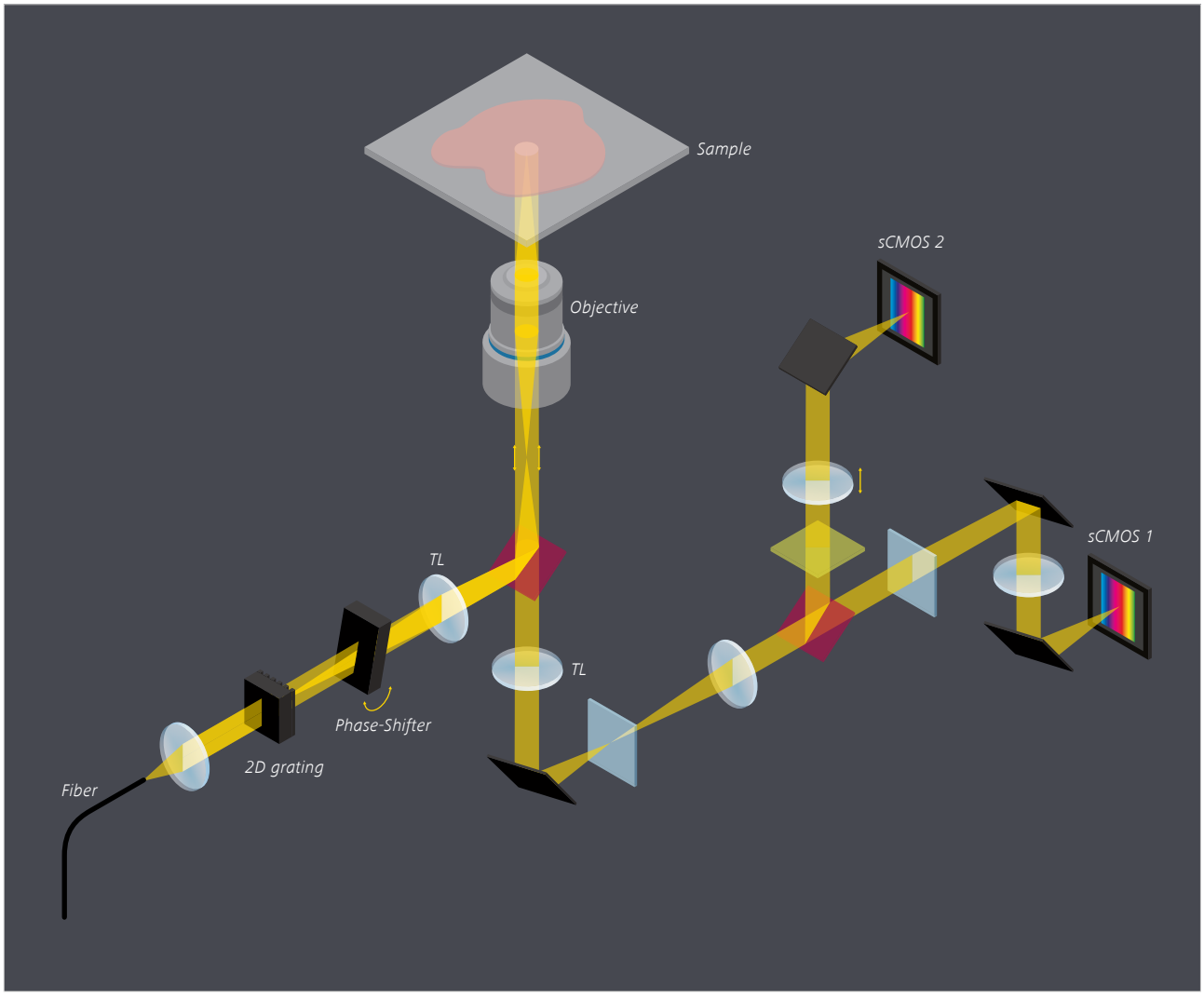

Figure 4 *Schematic beam path of ZEISS Elyra 7 with a 2D quadratic pattern for Lattice SIM*

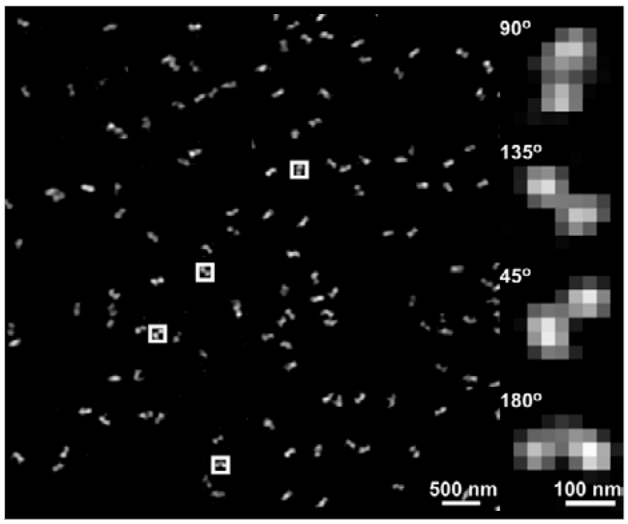

Figure 5 *Lattice SIM image of 120 nm DNA-origami. Imaged with ZEISS Elyra 7, orientations are indicated within the image*

number of reconstructed planes must match. As recently shown by Jost and collaborators [11] – and successfully applied in the 2D Superresolution Mode for ZEISS Airyscan [12] – it is possible to reconstruct 3D data from a series of 2D raw images, all acquired in the same focus position. Both publications focus on the excellent suppression of out-of-focus information by these algorithms.

However, for the Leap mode, this algorithm was developed further: it now allows to fully reconstruct additional z-planes by unmixing the various contributions of adjacent z-planes. The solution space for unmixing requires an overlap within the depth of focus of the sample planes as illustrated in Fig 6.

Studying dynamic processes in superresolution with the Burst mode

Sliding processing is a useful method to increase the temporal resolution when studying dynamic processes in living samples. This method was introduced with the Burst mode for ZEISS Apotome.2 [10]. Another prominent example of the usage of sliding processing was employed in single molecule localization microscopy [8]. Here, this type of processing allowed a totally new class of experiments, e.g. investigation of molecular interactions in living cells. Recently, sliding processing has also been applied to classic SIM [9]. Figure 7 shows the principle behind the Burst mode. Two singular events (indicated in red and green) occurring in single raw frames are classically not resolved in time as long as they fall into one block of 15 images (indicated in blue), as they are processed into a single superresolution image. The Burst mode uses interleaved sets of 15 images to process one superresolution image. Now, these two events can clearly be separated.

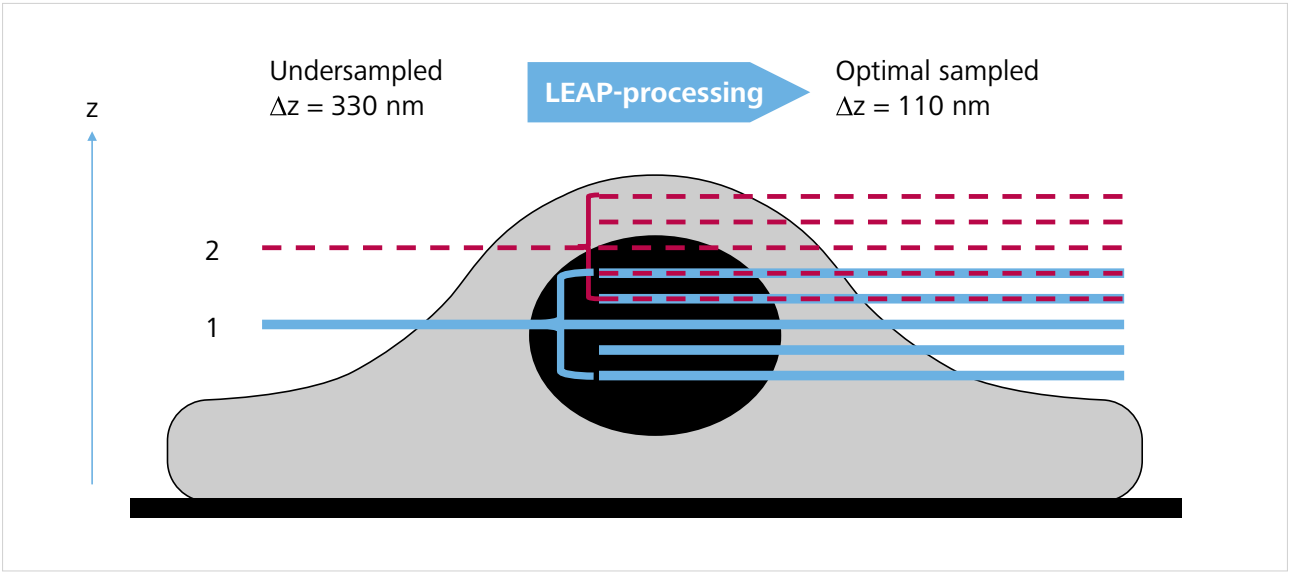

Figure 6 *Schematic representation of LEAP-mode of multiplane reconstruction using typical values for 63× 1.4 Oil Immersion objective. The left side shows the measured z-planes and the right hand side the reconstructed planes with optimal z-sampling.*

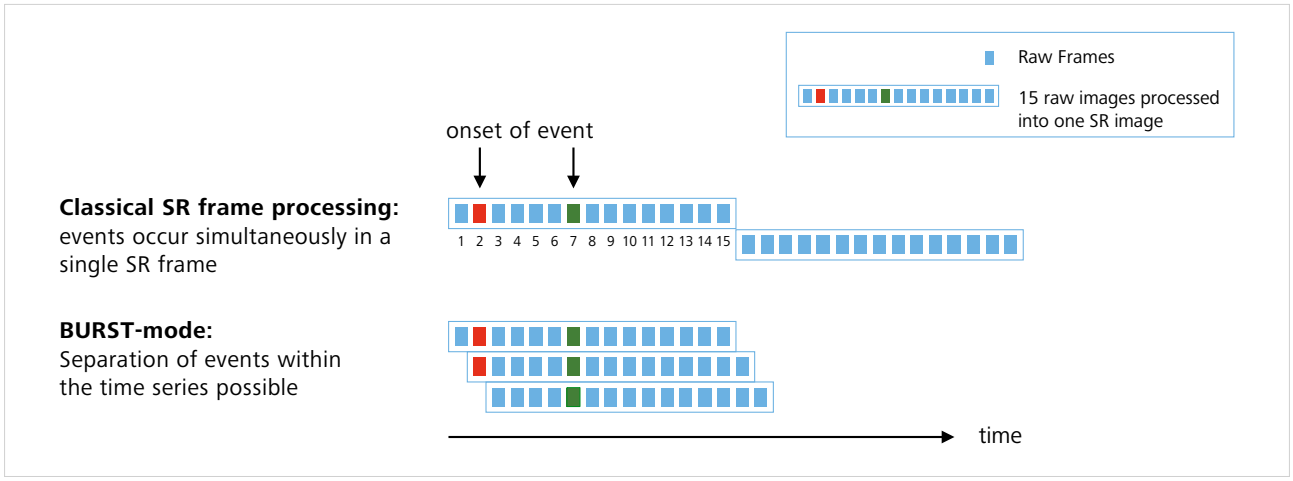

Figure 7 *Schematic of SIM BURST-mode processing. In BURST-mode superresolved images are processed using raw frames 1-15, 2-16, 3-17 and so on.*

Summary

Lattice SIM for ZEISS Elyra 7 allows imaging with a lateral optical resolution of 120 nm in all directions. Additionally, the modulation contrast of Lattice SIM is greatly improved compared to classic SIM. This is highly beneficial when imaging sparsely labeled living samples – and it allows fast live cell imaging. The new Leap mode increases frame rate by a factor of three and reduces photodamage, as the number of required images for acquisition of a 3D stack is reduced. The Burst mode for Lattice SIM allows to image dynamic processes with a frame rate of up to 255 fps. ZEISS Elyra 7 with Lattice SIM enables live cell imaging with fast and gentle 3D superresolution.

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