Superresolution Structured Illumination Microscopy (SR-SIM)

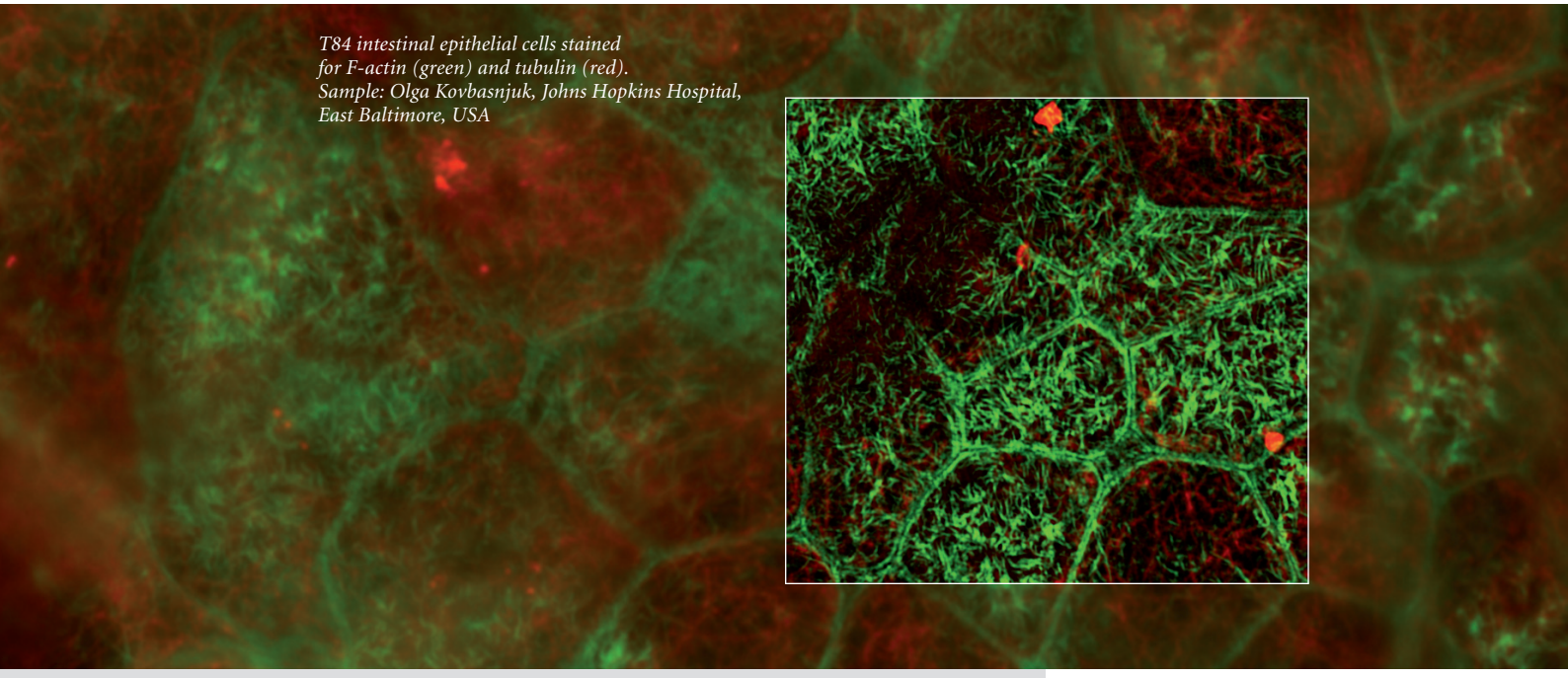
Superresolution Structured Illumination is one of the most flexible superresolution methods. Three dimensional resolution enhancement is achieved through an intricate combination of optics and image analysis.

The resolving power of a light microscope is limited to approximately 200nm in the lateral (XY) and 500nm in the axial (Z) direction. This so-called diffraction limit has been first described by Ernst Abbe in 1873 (reference 1) and still holds true today. In simple terms, it posits that objects cannot be resolved with a conventional light microscope if their distance is less than half the wavelength of the light used for observation (Figure 1).

In recent years, there have been several successful attempts to circumvent this problem and to achieve a resolution beyond the diffraction limit. In general, technologies with a resolving power exceeding the diffraction limit are nowadays collectively referred to as Superresolution Microscopy.

$$d = \frac{\lambda}{2n\sin\alpha}$$

Figure 1:
Formula describing the dependence of microscopic resolution on wavelength and opening angle of the objective lens.



T84 intestinal epithelial cells stained for F-actin (green) and tubulin (red). Sample: Olga Kovbasnjuk, Johns Hopkins Hospital, East Baltimore, USA

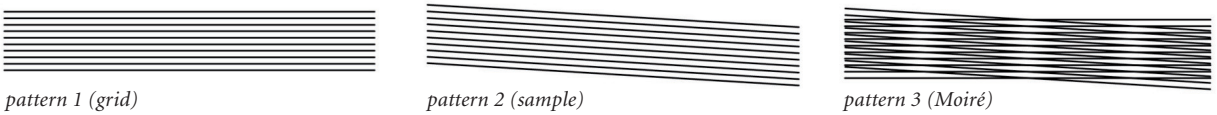
How Superresolution with ELYRA S.1 works

ZEISS

We make it visible.

Principle of SR-SIM imaging

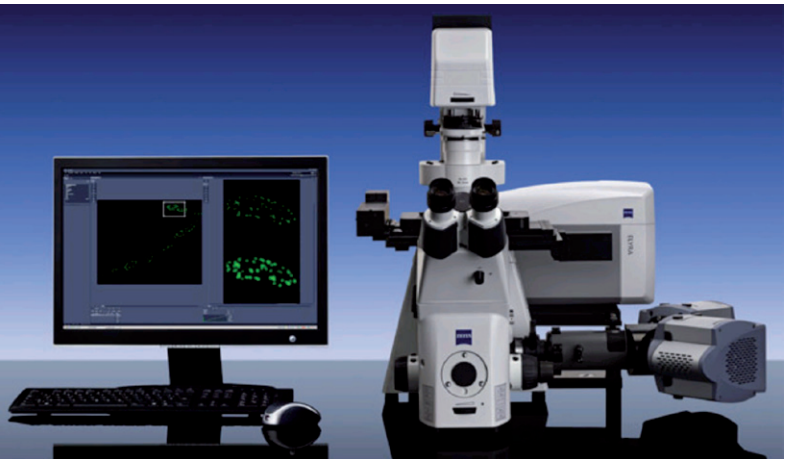
In SR-SIM, a fine sinusoidal pattern of light is used for exciting fluorescent samples.
In instruments like the Apotome or the Vivatome a similarly structured – yet much wider – pattern is used to produce optical slices.



pattern 1 (grid) *pattern 2 (sample)* *pattern 3 (Moiré)*

Figure 2: The Moiré patterns formed by superimposing regular patterns such as these grids are a familiar phenomenon.

Carl Zeiss combines the two most promising superresolution technologies (PAL-M and SR-SIM) in one new microscope system - the ELYRA PS.1.



PAL-M (Photo Activated Localization Microscopy) is a single molecule localisation method, achieving a lateral resolution down to 20 nm by imaging single molecules and subsequently determining their position by a Gaussian fit. It is compatible to all fluorescent dyes which can selectively be activated by all different methods of photo-transformation.

SR-SIM (Superresolution Structured Illumination Microscopy) is a technology which doubles both the lateral and the axial resolution of a light microscope. It is compatible to standard fluorescent dyes and staining protocols.

However, in order to achieve a true increase in 2D resolution, an additional effect must be utilized when illuminating the sample with patterned light: the Moiré fringes. Two superimposed patterns (in this case the illumination pattern and the structures in the sample) interfere with each other and produce a third, characteristic pattern: the Moiré fringes (Figure 2, pattern 3).

How does the superresolution information then pass the objective lens, if the structures in the sample are too small and the microscope optics are still diffraction limited? Essentially, the reason is that the Moiré fringes have a lower spatial frequency than the original structures within the sample. Therefore, the fringes can be transmitted by a normal objective lens. This fact is used as a central part in the reconstruction of the superresolution images: the interdependency of the three patterns is used to compute the unknown superresolution information of the sample. In other words, the superimposed pattern is transforming the inaccessible information into a wider pattern which can be transmitted by the lens. The concept to increase optical resolution by structured illumination was arguably introduced by Lukosz and Marchand in 1963 (reference 2).

The illumination pattern in SR-SIM is subject to the same restrictions as the light emitted from the sample. The finer the pattern is, the higher the gain in resolution. Therefore the pattern is chosen to be close to the diffraction limit. The frequency shift introduced by this light pattern can reach about a factor of two, thus the gain in light microscopic resolution by SR-SIM can be also up to the same factor of two. Technologically, to produce such a fine pattern with a high intensity contrast requires creating interference patterns with coherent laser illumination light. This differentiates SR-SIM from other structured illumination approaches like the ApoTome that require only incoherent illumination, for example from an arc lamp.

How is the gain in resolution along the Z-axis achieved technologically?

If the illumination pattern is additionally structured along the Z-axis, the same Moiré effects occur also in the 3rd dimension and can be used to collect superresolution information.
In order to create this illumination pattern in all 3 dimensions, a beam of coherent laser light is projected through a phase grating, diffracting the light into several orders. The 0th, +1st and -1st orders are focussed in the back focal plane of the objective. In the focal plane of the objective, the 3 beams interfere and form the 3-dimensional pattern. This effect is known as the Talbot effect. In Figure 3, a simulated 3-dimensional illumination pattern is illustrated as a 3D-rendering. Note that the pattern is slightly shifted along the Z-dimension.

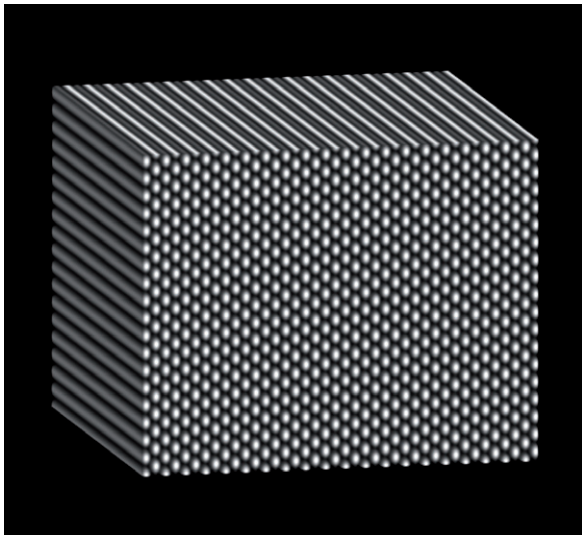


Figure 3: Rendering of the three-dimensional illumination pattern used in SR-SIM.

To completely cover the image plane, the sinusoidal pattern is phase-shifted 5 times and rotated in at least 3 steps. This results in a minimum of 15 raw data (widefield) images containing all necessary information, which are then used to compute one superresolution image of this plane.

What is the mathematical basis of SR-SIM?

In order to extract the superresolution information from the Moiré fringes, sophisticated computation steps are performed. To explain how this is done and how the resolution enhancement is then achieved, it is easiest to switch from real space or the spatial domain (as described above) into frequency space or the frequency domain.

By using Fourier transformations, complex information (i.e. brightness variations in an image) can be transformed into mathematically simpler parts – oscillatory functions. Those oscillations are the frequencies forming the complex information, very much like describing a chord of music with the individual notes used to play the chord.

A microscopy image in real space can be seen as information with varying fluorescence intensities. In the Fourier transformed image, the same information can be expressed by stating the strength and phase of a continuous set of frequencies.

Here, fine details in the image correspond to high frequencies and coarse ones to lower frequencies. An inverse Fourier transform can make the image information visible in real space again.

What is the scientific background of SR-SIM?

As stated above, light microscopes cannot resolve structures smaller than approximately 200nm in the lateral (XY) and 500nm in the axial (Z) direction. In Fourier space, an image produced by a light microscope contains only frequencies up to the so-called cut-off frequency. This means that there are no frequencies stemming from the sample present in the Fourier transformed image above this limit. The cut-off frequency corresponds to the smallest structures resolved by the light microscope in real space.

Figure 4 shows a Fourier transform of a microscopic image. The information is cut at a certain point (marked here by a red circle) representing the maximum frequencies the image contains in the XY plane. In order to increase the lateral resolution of an image, this red circle has to be transgressed and information has to be added beyond of it (see references 2 and 3 for details).

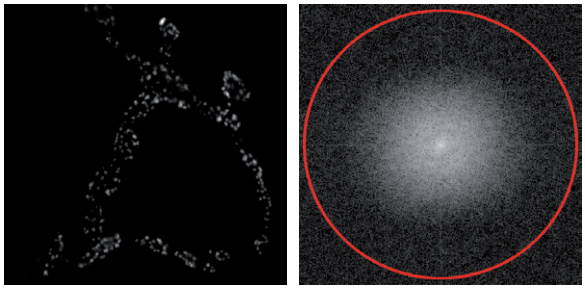


Figure 4: Micrograph acquired with a conventional microscope (left) and the graphic representation of the corresponding Fourier transform (right). The red circle marks the maximal frequency transmitted by the optical system.

By using the structured illumination approach, exactly this happens upon illumination of the sample with patterned light. Due to the Moiré effect, the high frequencies beyond the cut-off limit in the sample are shifted towards lower frequencies and therefore can pass the objective lens. In other words, information from outside the circle is shifted inside of it for the acquisition of the image. The image produced with patterned light now contains an overlap of shifted and non-shifted components which need separation. To make this information accessible for a human observer, the superresolution information has to be extracted and re-located to the correct location outside of the circle. In order to achieve isotropic resolution gain, this procedure has to be repeated for at least 3 angles and 5 phase-shifts of the illumination pattern. Figure 5 illustrates this schematically.

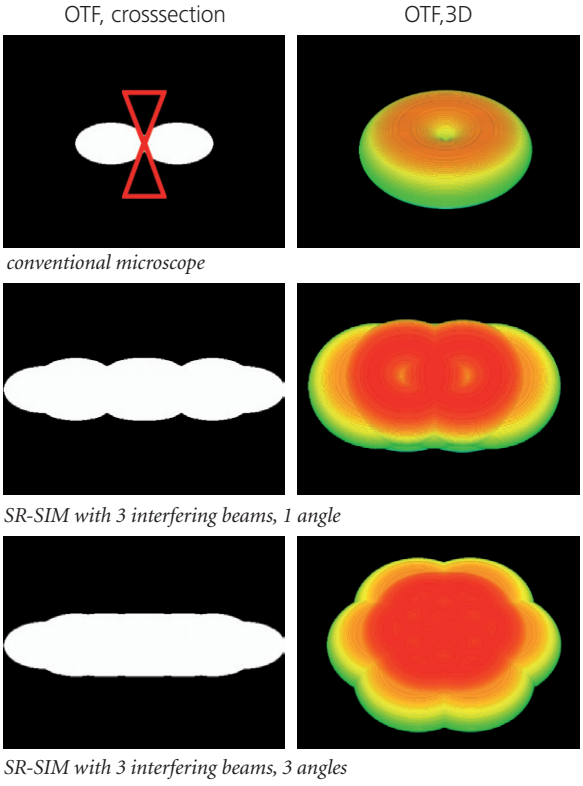


Figure 6: Schematic representations of the optical transfer functions (OTF) of different microscope systems. The boundaries of the OTFs are shown as xz crosssection (left row) and 3D rendered (right row). The "missing cone" in the OTF of a conventional microscope is indicated by red triangles.

Extended to the third dimension, the information inside the circle looks like a donut. (Figure 6, upper panel). This donut characterizes the optical transfer function (OTF) of the optical components, which is the Fourier transform of the so-called point spread function (PSF) which characterizes the image of an ideal point by the real optics of the microscopy system.

Adding more of those donuts around the central one increases the lateral resolution. The fact that the donuts have a dip along the Z-axis (the "missing cone") illustrates the problem to increase the resolution also in Z (Figure 6). No high frequency information is available in the missing cones.

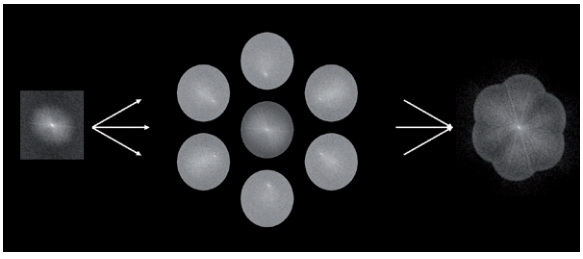


Figure 5: Separation and recombination of the different orders form the basis of superresolution information extraction in SR-SIM

How can the “missing cone” gap be filled?

Leading scientists in the field of superresolution have proposed that through the illumination of the sample with a light pattern which is extended in 3D, the “missing cone” can be filled and z resolution enhanced by a factor 2 (references 4-6). Transformed into Fourier space, a 3D light pattern “stacks” the OTF donuts above and relatively shifted to each other. The missing cones are thereby filled with information – thus higher frequencies are also detectable along the z-axis (see also Figure 6). An elegant technical solution was reported by Gustafsson et al. in 2008 (reference 7). In this approach, a three-dimensional illumination pattern is created through the interference of three laser beams. This method is covered by the US patent US RE38307E1 (reference 8) licensed to Carl Zeiss in October 2009. The license involves the right of commercializing the three-dimensional structured illumination method in a design using inverted microscope stands.

As stated above, the superresolution information has to be extracted from the raw data and separated from the lower frequency information. The high frequency information is subsequently shifted to the correct frequency positions and as a last step, the image is transformed back into real space. The algorithm used by Carl Zeiss is based on publications from Lukosz, Gustafsson and Heintzmann (references 2-6). Carl Zeiss has established a close collaboration with Rainer Heintzmann and his group regarding the development, optimization and testing of the algorithm. Experimental data acquired with ELYRA systems shows the expected resolution increase in X,Y, and Z (Figure 7).

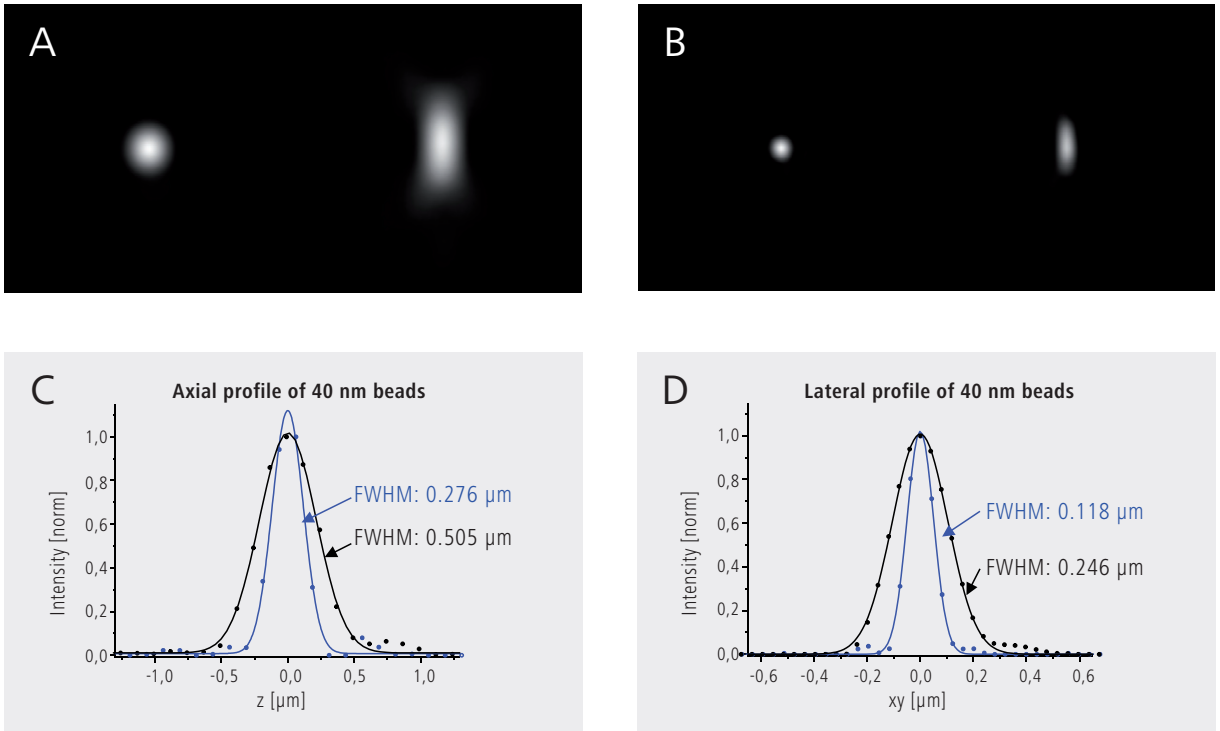


Figure 7: Subresolution fluorescent beads imaged with a conventional microscope (A) and the ELYRA S.1 system (B) . Full width half maximum measurements show the expected resolution increase in axial (C) and lateral (D directions).

How does the SR-SIM algorithm work?

As a first step, the acquired raw-images are Fourier transformed. 3 beams interfere in the focal plane and the resulting 3D illumination creates 5 components in Fourier space. All these components overlap within the lateral circle of the standard widefield transfer function, albeit two of them consist of two donuts each, displaced long the Z-frequency direction. Each of these components modulates differently with the lateral displacement of the grating. Thus taking 5 or more images at different grating positions allows the separation of the 5 individual components (this is a system of linear equations, which can easily be solved).

Now these separated components need to be placed at their correct positions, such that the object zero-frequency will coincide with the zero position of Fourier space.

For this operation precise knowledge of the grating constant is required. The separated components have areas of mutual overlap. From these overlap areas the information about the precise grating constant and phase positon is obtained by cross-correlation of the separated components, looking for a peak close to the design parameters (grating constant and orientation) of the setup. An insufficient contrast indicates experimental failure leading to an abortion of the reconstruction. This can have several reasons: When imaging deep in the sample, the excitation pattern can get distorted because of scattering. By determining the grating constant from the raw data, an indication for this effect is given to the user of the system. This is a way of keeping control of the experiment. The same holds true for the angle under which the pattern is projected into the sample. This is calculated from the raw data and eventual deviations from theoretical values are also taken into account when the image is reconstructed.
In a final step the various components are transformed back into real space, where the individual components are joined in a signal-to-noise optimized way. The information is weighted and stitched together to one superresolution image. A filtering parameter is used to further optimize the performance under noisy conditions. (references 3,5).

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