Supplementary Material to:
# STED Microscope with Spiral Phase Contrast

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Principle of Spiral Phase Contrast

Spiral Phase Contrast (SPC) is a particular implementation of phase contrast microscopy techniques developed to improve imaging of transparent objects. Briefly, a sample is illuminated with a coherent or incoherent source and imaged onto a detector by the combination of an objective and a tube lens. At the back focal plane of the microscope objective (Fourier plane), a phase mask adds a helical retardation to the wavefront of the beam. Phase retardation variations in the sample are converted into intensity variations in the image. Four possible implementations of SPC are schematized shown in Supplementary Fig. 1 and explained below.

In its original design, SPC imaging is obtained on a camera with collimated laser illumination of the sample[1] (Supplementary Fig. 1 a). After passing through the sample, the collimated beam is focused on the center of the helical phase mask (Supplementary Fig. 1 a, PM) at the back focal plane of the microscope objective (Obj.). The phase mask adds a helical retardation to the wavefront, leading to a toroidal intensity distribution (donut) with large diameter in the detector plane if the sample does not introduce further local phase gradients (flat sample). Light is redistributed into the donut rim, and when the camera is placed in the dark center, this lights falls outside the field of view. Diffraction by index inhomogeneities in the sample locally disturbs the destructive interference in the central region. Consequently these index variations appear bright on a dark background as phase gradients are converted into intensity variations.

A confocal scanning mode is achieved by focusing the illuminating laser beam in the sample plane and scanning the specimen (Supplementary Fig. 1 b). The beam is collimated by the objective lens before passing through the phase mask. At the image plane, the beam is focused into a small toroidal spot. A point detector can be placed at the center of this donut. Any phase gradient at the sample plane results in an asymmetrical illumination of the phase mask and distorts the donut at the detector. Thus, the detector receives light only in the presence of phase gradients. Alternatively to the central positioning, the donut center can be slightly shifted relative to the detector for providing a bas-relief effect with apparent side-illumination (see also Fig. 3 b).
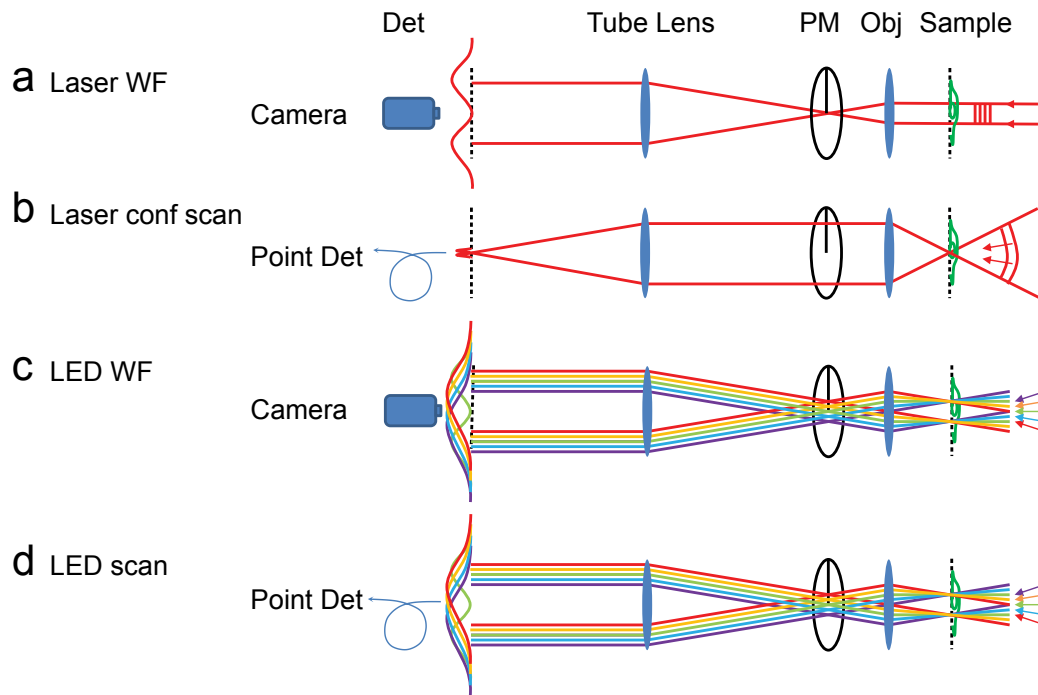
Supplementary Figure 1: Example of four possible implementations of spiral phase contrast (SPC); see text for detailed explanations. In all cases the illuminated sample is imaged by an objective lens (Obj.), the beam passes through a helical phase mask (PM) and an image is formed by a tube lens (TL) on a detector (Det.), which may be a camera or a point detector. (a) Widefield SPC with laser illumination. (b) Scanning confocal SPC with a fiber serving as a pinhole for the point detector. (c) Widefield SPC with an incoherent source. (d) Scanning SPC with an incoherent source.

SPC can also be obtained in widefield mode with incoherent illumination as provided by an LED (Supplementary Fig. 1 c). This illumination can be modeled as several incoherent collimated beams, represented with different colors in Supplementary Fig. 1. A single beam is centered on the phase mask and induces isotropic phase gradient detection as in the case of a collimated laser illumination. As the other beams are not centered on the singularity, each of them gives a phase contrast image against a bright background. Since the beams are mutually incoherent, their intensities superimpose in the detector plane, giving the final image.

SPC with LED illumination is also possible in scanning mode. For this configuration, the camera is replaced by a point detector and the sample is scanned (Supplementary Fig. 1 d). Despite point detection, this is not a confocal configuration, because a large area of the sample is illuminated.
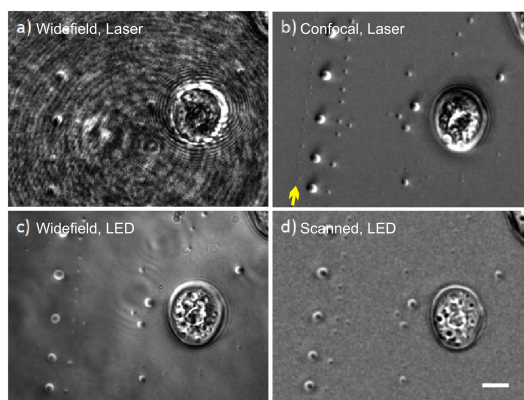
Supplementary Figure 2: Images of oil droplets in water, obtained in the four configurations presented in Supplementary Fig. 1 with an 100x, NA 1.40, oil immersion objective. (a) Widefield SPC on a camera with collimated laser illumination. (b) Scanning confocal SPC with a focused laser. Asymmetrical configuration which leads to a bas-relief effect is shown. (c) Widefield SPC with an LED as illumination source. (d) Scanning SPC with an LED. Scale bar 5 μm.

Examples

To compare the sensitivities of these configurations, we imaged oil droplets of various sizes in water (Supplementary Fig. 2).

In widefield mode with collimated laser illumination (Supplementary Fig. 2 a) a high background is present, steming from interference fringes and speckles, which arise from the coherent illumination. Large oil droplets are visualized, but small ones are hidden in the noise. To image weak objects such as cells[2], a more complicated configuration with a rotating diffuser can be used to average over different speckle patterns.

Confocal configuration, i. e. illumination with a focused laser and point detection, (Supplementary Fig. 2 b) gives a cleaner image. Focusing the laser into one spot avoids background all over the sample. Consequently, a row of tiny droplets (arrow) is clearly visible, while hardly revealed in the other modes. This configuration offers the best signal-to-noise ratio of the four methods shown here.

Widefield mode with LED illumination is also free of speckle background from the speckle background, since the source is incoherent (Supplementary Fig. 2 c). It reveals, therefore, almost all objects visible in confocal mode.

Scanning mode can also be implemented with LED illumination (Supplementary Fig. 2 d). A large area of the sample is illuminated, but a point detector is used, and the sample is scanned. Compared to widefield mode, this configuration removes illumination inhomogeneities and allows overlay with other scanned images. Compared to the confocal configuration, detection is performed against a

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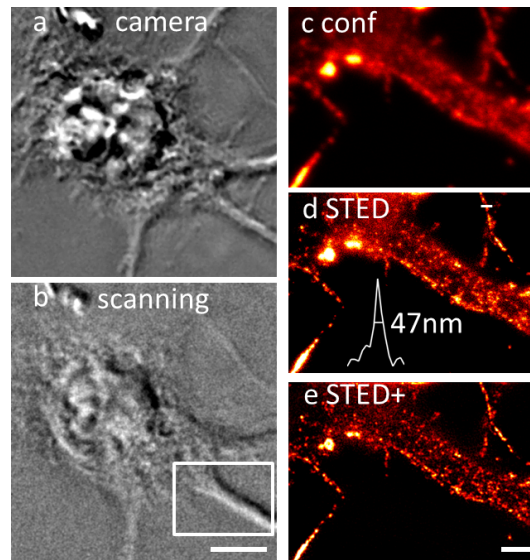
Supplementary Figure 3: SPC images obtained with incoherent LED illumination of a large area. Widefield (a) and scanning (b) SPC clearly visualize neurons including flat details such as neurites and allow for the selection of a region of interest for imaging the actin cytoskeleton, immunostained with Atto 532 in conventional confocal fluorescence microscopy (c) or in high-resolution STED microscopy (d). The inset trace shows an intensity profile according to the white bar. Noise can be reduced by Wiener filtering (e). Scale bars 5 μm (b) and 1 μm (e).

brighter background. The dynamic range of the detector (here an avalanche photo diode) becomes therefore a limiting factor.

This configuration is compared to widefield SPC for imaging of neurons in Supplementary Fig. 3. In widefield (Supplementary Fig. 3 a) and scanning (Supplementary Fig. 3 b) SPC with LED illumination, sub-cellular structures such as neurites are clearly visualized. The scanned image allowed precise selection of a region of interest for subsequent fluorescence imaging of the immunostained actin cytoskeleton in confocal (Supplementary Fig. 3 c) and STED (Supplementary Fig. 3 d) modes. Details of the actin structure smaller than 50 nm can be discerned in the STED image; an intensity profile is shown as inset in Supplementary Fig. 3 d. Linear deconvolution (Wiener filtering) reduces noise and sharpens the STED image (Supplementary Fig. 3 e).

References

1. Fürhapter, S., Jesacher, A., Bernet, S. & Ritsch-Marte, M. Spiral phase contrast imaging in microscopy. *Optics Express* **13**, 689–694 (2005).

2. Maurer, C., Jesacher, A., Bernet, S. & Ritsch-Marte, M. What spatial light modulators can do for optical microscopy. *Laser and Photonics Reviews* **5**, 81–101 (2011).

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