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Structured illumination generation with an integrated optical chip

GIANMARIA CALISESI(*)
Politecnico di Milano, Department of Physics - Milano, Italy

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Summary. — Structured illumination is a widely used technique in fluorescence microscopy that allows for super-resolution imaging. In this paper, a brief introduction to the topic will be presented, together with a compact and integrated optical device that can generate and spatially translate a structured light pattern suitable for SIM microscopy. The device uses optical waveguides and directional couplers made with femtosecond laser micromachining. The beams are directed through an optical coupler to create the illumination pattern after interfering. The phase of the beams in the waveguides can be adjusted using thermal phase-shifters so that it is possible to shift the illumination pattern over the field of view of the microscope, enabling the acquisition of multiple phase images for SIM reconstruction without the need for additional optical elements. The effectiveness of the device is demonstrated by showing its use in a commercially available inverted microscope for superresolution imaging of Bovine Pulmonary Artery Endothelial Cells (BPAE Line) deposited on a commercial glass slide.

1. – Structured illumination microscopy

Structured Illumination Microscopy (SIM) is a well established optical technique that allows for high-resolution imaging beyond Abbe's limit for epifluorescence microscopes. Initially used for obtaining optical sectioning, SIM is now commonly used for super-resolution microscopy [1]. The basic principle of SIM is to project a pattern of light onto the sample, and then to image the sample with a conventional microscope while the pattern is shifted or rotated. By analyzing the fluorescence emitted by the sample excited with a modulated pattern, it is possible to extract information about the sample that is not visible with a conventional microscope. The technique relies on the fact that the resolution of a microscope is limited by the wavelength of light used to illuminate the sample, which is typically on the order of a few hundred nanometers. By illuminating the sample with a pattern that has a much smaller period than the wavelength of light,

(*) E-mail: gianmaria.calisesi@polimi.it

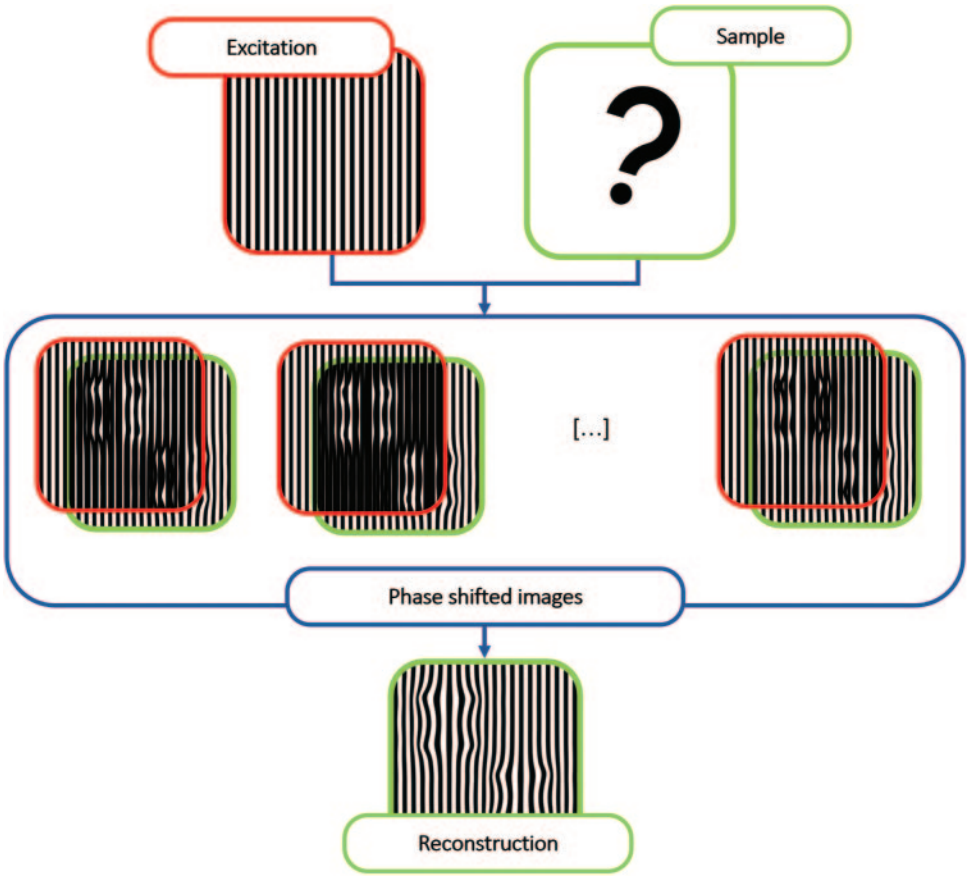

Fig. 1. – Working principle of Structure Illumination Microscopy (SIM). An unknown sample is illuminated with a structured pattern of light. By shifting (and in some cases also rotating the illumination pattern) and capturing multiple images, it is possible to extract more information about the sample and create a super-resolution image with a resolution beyond the diffraction limit of standard epifluorescence microscopes.

it is possible to extract information about the sample at much higher resolution, as schematically shown in fig. 1.

When implemented with linear excitation, SIM can double the resolution of a standard diffraction-limited epifluorescence microscope by making fluorescence emission proportional to the excitation intensity. When operated in a nonlinear regime, it can further increase resolution down to the tens of nanometers scale. Recently, structured illumination has opened up new possibilities in three-dimensional localization microscopy, light sheet microscopy, and far-field optical nanoscopy [2, 3].

The generation of a coherent SIM pattern is typically achieved through the interference of two or more laser beams on the sample. For example, in an epifluorescence microscope, a sinusoidal pattern can be generated by focusing two coherent laser beams into the microscope's pupil. By changing the relative phase of the beams, the pattern moves (spatially shifts) through the field of view. In its original implementation, a diffraction grating was used to create the two coherent beams, and a mechanical translator (and

rotator) was used to change the pattern's spatial phase (and angle). More recent SIM applications use a spatial light modulator to shape the beam [4]. This approach has the advantage of allowing the creation of complex modulation patterns with high phase precision, as well as the ability to measure microscope aberrations. However, even in modular implementations, a bulk optical setup for SIM pattern generation can be cumbersome and require daily adjustments.

The potential for increased spatial resolution of SIM can be fully realized by bringing mutually coherent beams to interference, which, as said, generates the highest modulation frequency and contrast for the laser excitation pattern. However, this requires certain design considerations for the optical excitation paths. The decision between 2D or 3D mode of operation must be made, and early adopters typically chose two-beam total internal reflection fluorescence (TIRF) excitation. This is achieved by generating a standing wave pattern above the glass surface using two laser beams from a single source that interfere at angles beyond the angle of total internal reflection. Recent implementations use TIRF through microscope objective lenses with a numerical aperture exceeding 1.4. To achieve mostly isotropic resolution in the lateral direction, three angles with three phase steps per angle are used, resulting in nine raw images that are assembled into a single reconstructed SIM image.

To achieve the highest modulation depth in SIM, the polarization of the interfering laser beams must be parallel to the stripe direction. This can be easily met using the standing wave approach, but for other methods, such as two-beam or three-beam illumination, this requires dynamic adjustments of the polarization. This can be done using fast electrooptical devices, or by using segmented wave plates. In the case of three-beam SIM, circularly polarized light is used for the central beam to ensure similar contrast ratios along the vertical direction. To generate the different interfering laser beams and angles, methods such as diffraction gratings, spatial light modulators, acoustooptic deflectors, and digital mirror devices have been used. Recent extensions to the SIM approach have been used in light-sheet illumination, and can also improve the spatial resolution of two-photon microscopy [5, 6].

2. – Application: HexSIM

As stated in the introduction of this paper, SIM patterned illumination can be generated in a variety of ways. One of which is explained in depth in [7] and will be the subject of this paragraph. The proposed approach makes it easier to exploit SIM, since it comes with a stable and pre-aligned illumination scheme thanks to integrated optics. It is in fact possible to use an integrated device for patterned illumination in SIM microscopy. The device consists of a miniaturized glass chip that incorporates optical waveguides, beam splitters, and thermal phase shifters. The light coupled into the input waveguide of the chip is split into three waveguides at its output. The relative phases of the three waveguides are controlled by a voltage applied to the thermal shifters. Three optical fibers are connected to the waveguides at the output of the chip and placed on a glass ferrule at the vertices of an equilateral triangle, with each fiber core 120 degrees apart from the others similarly as described in [3]. These three point sources, azimuthally polarized, interfere at the sample, producing a hexagonal intensity pattern suitable for SIM microscopy, specifically for its hex-SIM implementation as shown in fig. 2. Hence, the hex-SIM approach involves illuminating the sample with a hexagonal pattern created by aligning three coherent laser beams on the objective pupil. The pattern is then shifted laterally along a straight line, and only seven images are required to deduce an enhanced

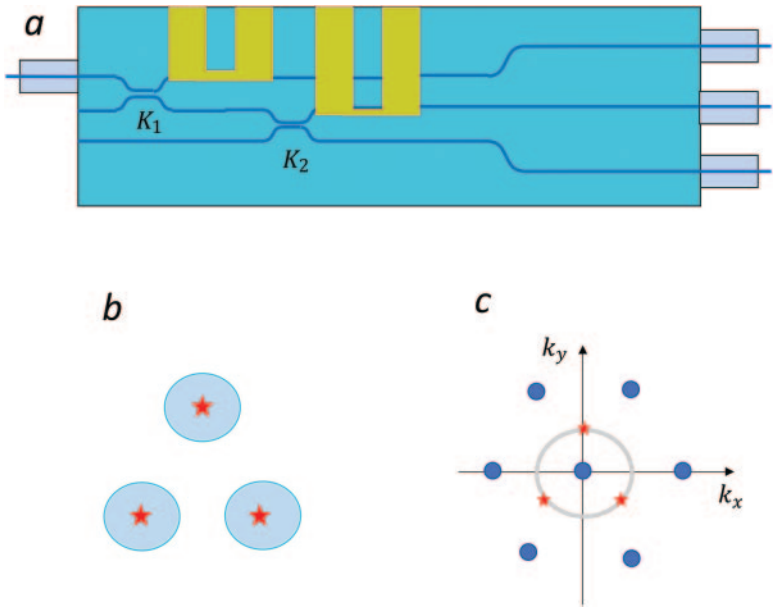

Fig. 2. – (a) Schematic drawing of the integrated optical circuit with two metal phase shifters (in yellow) for phase control. The thermal shifters are placed on top of the waveguides, which are coupled together in K_1 and K_2, by a factor of 33% and 50%, respectively. (b) Fiber core positioning (represented by red stars) in real space at the back focal plane of the objective. (c) The relative positions of the three illumination beams and the seven spatial frequencies from the illumination pattern (represented by blue spots) in relation to the illumination pupil (represented by a grey circle).

resolution image without the need for pattern rotation, which is typical of conventional SIM [8]. By controlling the relative phase of the three waveguides, the patterns are thus shifted on the sample, acquiring the seven images required for hex-SIM reconstruction.

The positioning of the three light dots at the pupil plane determines the spatial shape of the obtained interference pattern and is therefore a crucial parameter. For this reason the so-called filling factor is defined. The filling factor is evaluated as the ratio between the radius of the circle containing the three points in the back focal plane and the pupil radius.

The device which has the three light dots as output is constructed as a glass-based optical circuit that splits the laser light coupled to the chip into three output beams as shown in fig. 2. Femtosecond laser micromachining on glass is one of the possible techniques for manufacturing such photonics devices in the visible range and beyond. Femtosecond laser micromachining (FLM) is a well-established microfabrication technique that uses fs-pulsed laser irradiation on transparent or absorptive materials to selectively change their structural and optical properties. The material modifications depend on the irradiation parameters, and among the possible changes, a localized increase in the refractive index can be induced with respect to the pristine material. This allows the creation of optical waveguides with controlled birefringence, basically reduced to 0, so that there is no major modification to the input polarisation state. Another common technology involves the use of optical circuits based on silicon nitride, which allows for

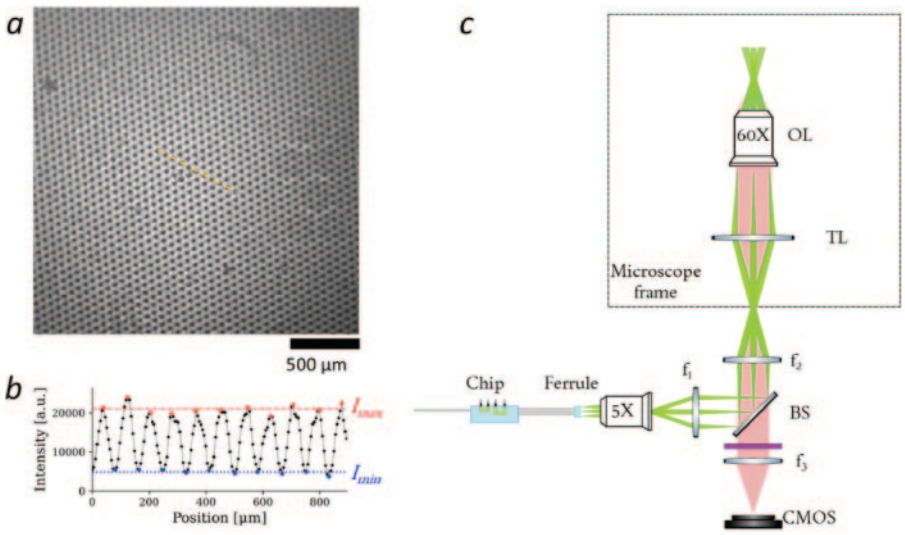

Fig. 3. – Analysis of the pattern formed, directly imaged onto the camera (b) Contrast analysis obtained by line plotting along the yellow line (c) Structured illumination microscope. The three green dots are imaged onto the back focal plane of the objective thanks to a 5× objective and a relay system (f_1 and f_2), which also determines the filling factor. Reprinted with permission from [7] © The Optical Society.

the creation of waveguides with a high refractive index. Silicon nitride technology is ideal for total internal reflection microscopy, but due to high coupling losses, it is less suitable for far-field illumination [9]. In contrast, femtosecond laser micromachining on glass allows for the manufacturing of waveguides with small refractive index variations that can be easily coupled with standard optical fibers and has been widely used for many applications, including microscopy on chip [10].

In SIM, the phase shift of the pattern is critical. That is because it controls the position of the interference fringes that will interact with the sample. In other words, the shift of the pattern controls the position of the dots in the Fourier domain, in figs. 2 and 3. Hence, the phase shift is used to control the relative positions of the fringes, which in turn controls the amount of information that can be extracted about the sample. If the phase shift is not carefully controlled, the fringes may not be in the correct position, and the information that can be extracted about the sample will be limited. By using metal deposition and exploiting thermo-optic effects, phase shifters can be built on the optical circuits obtained with FLM, with switching responses as low as 200 microseconds, potentially allowing for switching rates greater than kHz [11]. It is worth noting that, although this approach is ten times slower than fiber-based modulation, in typical SIM measurements, acquisition time is limited by the fluorescence signal reaching the camera, and kHz switching rates are beyond what is typically required with scientific CMOS image acquisitions.

Finally, the developed optical circuit consists of two integrated couplers, which act as beam splitters and distribute laser light among the three output waveguides according to their coupling ratio. Laser light is coupled into one waveguide through a polarization-maintaining fiber. The first splitter is designed with a coupling ratio of 33%, so one-third

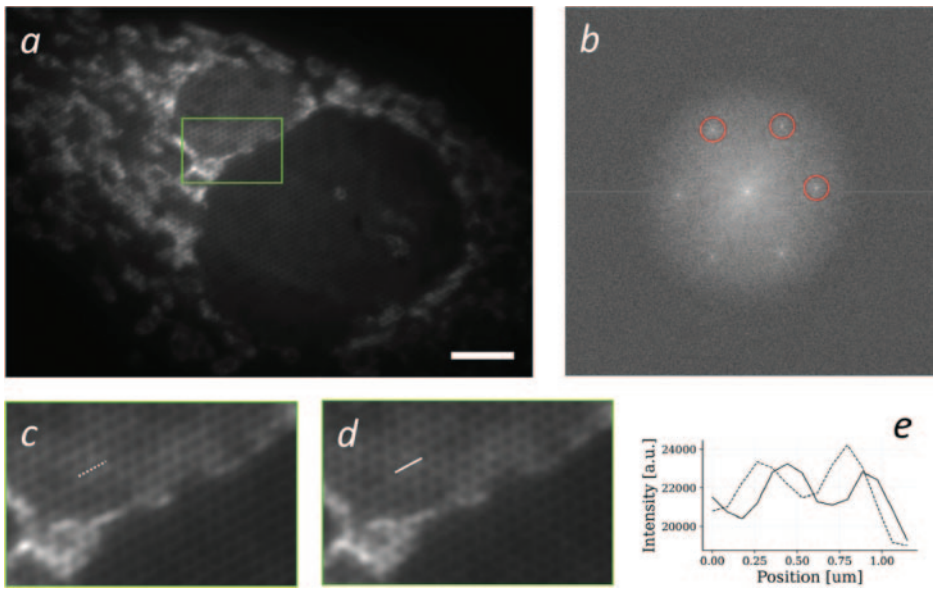

Fig. 4. – (a) A pattern is created on a fluorescent sample, with a period of approximately 450 nm. Scale bar is 5 μm. (b) A power spectrum (on a logarithmic scale) of the image from panel (a) displays 7 peaks due to the presence of the hexagonal pattern, with the carrier frequencies highlighted in red circles. Panels (c) and (d) are zoomed-in regions from panel (a) captured with different voltages applied to the thermal shifters. (e) A line plot from the white lines of panel (c) (dotted) and (d) (solid) illustrates the effective translation of the illumination pattern. Reprinted with permission from [7] © The Optical Society.

of the initial power continues traveling in the first arm, while the remaining power is guided to the second splitter. The latter is designed as a 3 dB coupler with a coupling ratio of 50%. With such power splitting, the initial power is ideally equally distributed among the three output ports. In our device, we found that the power ratio at the three outputs was 33%, 36%, and 30%. It is worth noting that the optical waveguides fabricated with femtosecond laser micromaching are slightly birefringent, with the main axis parallel to the writing beam direction. This is due to the ellipticity of the waveguide cross-section and the mechanical stress induced in the modified region. As a result, the polarized light from the PM input fiber is maintained throughout the chip.

The device has been validated and shown to be effective as an add-on system for a standard fluorescence microscope, allowing for the implementation of structured illumination microscopy. The system has been tested at a relatively low filling factor of 0.57, which can be a limitation for some high-resolution SIM applications. However, filling factors of this level are commonly used in super-resolution applications, and the technology used in this device is also useful for applications where space constraints are present, such as in multimodal microscopes and miniaturized devices. Future work will focus on improving the filling factor and numerical aperture, optimizing the contrast and polarization, and increasing the speed of the chip. In addition, the use of broadband directional couplers, which can be optimized for layout and interaction length, distance between waveguides, and curvature radius, can enable the creation of integrated multi-color SIM pattern generators.

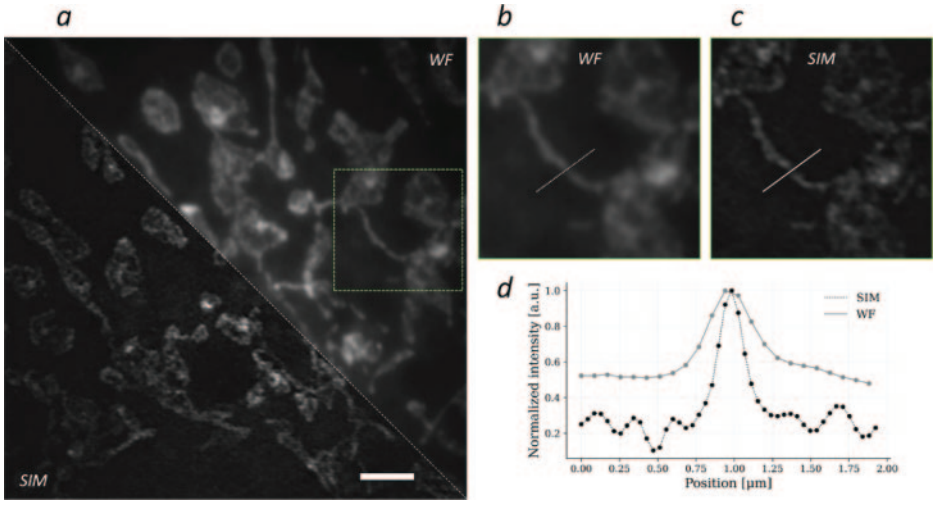

Fig. 5. – Imaging results. (a) Widefield (top right) and SIM reconstruction (bottom left) of a commercial fluorescent slide. Panels (b) and (c): zoom of the regions in the dotted green box for both techniques. (d) Resolution comparison on a line for the two images, obtained with a line profile. Scale bar is 3 μm. Reprinted with permission from [7] © The Optical Society.

The whole chip (phase shift and optical guides) was mounted in order to have the three dots at the back focal plane of an Olympus 60×/1.4NA oil immersion objective and was used to image a cell slide (FluoCells Prepared Slides #1) stained with MitoTracker Red CMXRos in the mitochondria. The generated pattern measurement is shown in fig. 3, together with the experimental sketch. The characterisation of the pattern, measured when overlapped to the prepared slide, is shown in fig. 4 as well as its Fourier transform. The structured illumination pattern used had a period of 380 nm and a filling factor of 0.57. After calibration, 7 images were acquired with the correct phases and processed using the reconstruction method described in [7]. The results of the reconstruction procedure are reported in fig. 5. In comparison to a standard widefield image, the SIM reconstruction showed an improvement in contrast due to a reduction in background noise and an enhanced resolution when imaging the mitochondrial membranes. This was exemplified by a line profile of a filament, which appeared narrower in the SIM reconstruction. To quantify this resolution improvement, we applied a decorrelation analysis and found an improvement of 1.5 times, which was consistent with the filling factor used.

3. – Conclusions

The presented optical device generates phase-controlled point sources arranged in a circle with azimuthal polarization. The chip, which can be installed on the illumination port of a widefield microscope, can be used as the light source for structured illumination, upgrading a conventional widefield system to a SIM microscope. By dynamically controlling the phase of the waveguides, the illumination pattern can be shifted over the field of view of the microscope. Using this device, the resolution of imaging biological samples is improved by a factor of 1.5. This chip opens new possibilities for creating multiple point-sources with controlled phase. It has been demonstrated in the context of hexago-

nal SIM, but similar devices can be made to produce other SIM patterns. Additionally, the chip design can be easily modified to increase the number of waveguides, allowing for greater flexibility and even higher resolution in 2D and 3D structured illumination microscopy.

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