Stray light rejection by structured illumination

P. K. Fink, D. Hillmann, G. L. Franke, D. Ramm, P. Koch and G. Hüttmann

***Abstract*—Structured illumination microscopy (SIM) combines confocal microscopy and wide field microscopy. By illuminating the specimen with a grid which is shifted in phase, it is possible to discriminate out of focus information without lateral scanning. A problem of structured illumination is that the visibility of the illumination pattern drops significantly for high numerical apertures (NA). The reason for low visibilities is the modulation transfer function (MTF) of the objectives which reveals the ability of transferring a modulation frequency of an object to an image. The MTF for incoherent illumination is not as good as for coherent light. For high NA imaging, a fiber based method to create the illumination grid by interference of two plane waves was developed. The illumination grid is projected on the specimen and not as in classical structured illumination microscopy imaged onto it. The visibilities of the grid are by far higher with this technique.**

I. Introduction

Wide field reflection microscopy suffers from the problem that reflexes from optical elements in the setup and from cover glass contribute to the image by adding a high level of noise. Confocal microscopy solves this problem of discriminating out of focus light by adding pinholes in front of the light source and in front of the detector [1] (Fig. 1).

A drawback of this technique is, that the specimen has to be scanned laterally in order to achieve an image. In structured illumination microscopy out of focus information is discriminated by imaging a grid into the probe [2]. The grid is shifted laterally in phase three times with a respectively shift of 120°. This method has been feasible for low numerical apertures but the visibility of the grid degrades at higher numerical apertures. For fast high resolution reflection imaging with a high depth of field and no parasitic reflexes we developed an illumination technique where the grid was created by interference of two plane waves. With this technique the lines of the grid are seen in every axial position and not just in one plane (Fig. 2). Furthermore the achievable visibility should be better because of the coherent illumination.

II. Theory

In structured illumination microscopy a grid is projected on the specimen (Fig. 4) and modulated in phase to get at least three pictures from which one picture without grid can be calculated as following [3][4][5]:

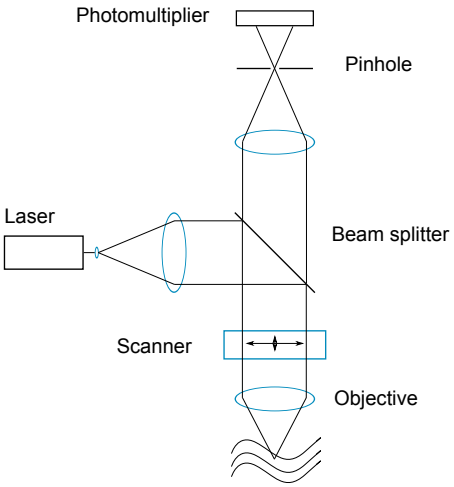

Fig. 1. Schematic setup of a confocal microscope

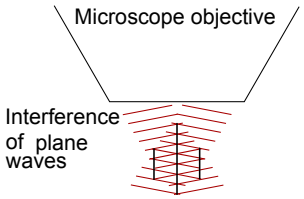

Fig. 2. Schematic illustration of the interference of two plane waves; black points represent lines of constructive interference

$$I_A = \sqrt{(I_1 - I_2)^2 + (I_1 - I_3)^2 + (I_2 - I_3)^2} \qquad (1)$$

I_A is the calculated image, $I_{1,2,3}$ are the images on which the modulation grid is projected. In I_1 the phase of the grid is 0° in I_2 and I_3, 120° are added respectively. To retrieve the original image the modulated images have to be added.

$$I_0 = I_1 + I_2 + I_3 \qquad (2)$$

The intensity of the calculated image I_A depends on the visibility of the grid: the higher the visibility of the grid, the better will be the contrast in the calculated image. That is because the constant component in the modulated images is discriminated by equasion 1. An example is shown in Fig. 3.

In conventional SIM the axial resolution is dependent on the frequency of the illumination grid. The higher the grid frequency, the faster the visibility decreases when it is defocused and the better the axial resolution gets.

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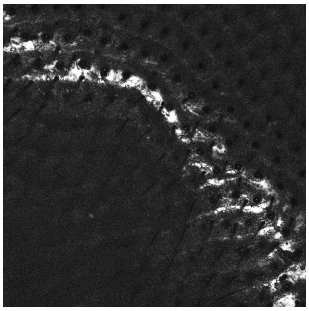

Fig. 3. Image of the wing of a fly with a conventional SIM setup

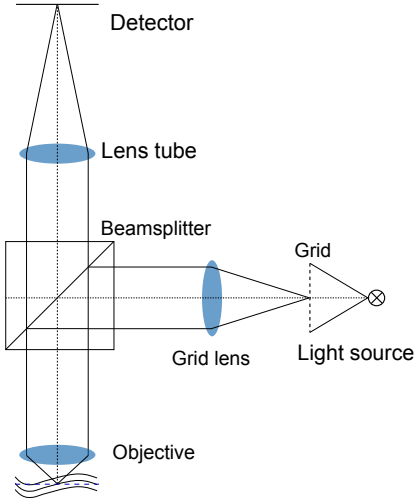

Fig. 4. Conventional SIM-setup

The modulation transfer function (MTF) is a property of an imaging system which reveals the ability of transferring a modulation of the object to an image. So it is the ability of adjacent pixels to change from black to white in response to patterns of varying spatial frequency. This means it is the actual capability to show fine details with good contrast. If an image appears blurred in areas with high spacial frequencies, the MTF of the imaging system is too low at high frequencies to images these objects. The illumination grid of SIM is very close to the limitation of the axial resolution, it is important that the MTF of the system is good enough to image the grid in a good contrast. Otherwise the intensity and the contrast of the calculated image I_A drops significantly.

Interference is a phenomenon that arises when two light waves are superpositioned. If the conditions of spacial and temporal coherence are met, the combined illumination I_C is calculated as following:

$$I_C = I_1 + I_2 + 2 \cdot \sqrt{I_1 \cdot I_2} \cos(\delta) \tag{3}$$

δ is the phaseshift between both waves

$$\delta = \frac{2\pi}{\lambda}(l_{opt1} - l_{opt2}) \tag{4}$$

l_{opt1} and l_{opt2} is the optic path the light has travelled after splitting.

III. Material and Methods

In classical SIM an amplitude grid is imaged onto the sample. The axial adjustment of the position of the grid is very important to achieve acceptable visibilities of the grid. For implementing our fiber based method of two interfering plane waves for structured illumination into a common SIM setup a light is splitted into two arms with a fiber based 50-50 beam splitter. The two fibers are coupled onto an array of eight fibers of identical length which are positioned next to each other with a distance of $500\,\mu\mathrm{m}$. By switching between fibers, the distance between them changes and different interference frequencies can be chosen. A lens collimates both exiting divergent beams. Because of the limited coherent length the optical path difference between the fibers has to be very small to create interference. To achieve the identical length of both fibers and to modulate the phase of the grating, two fiber stretchers were built into the arms (Fig. 5). The fiber stretchers are made of two metal cylinders which can be moved towards and away from each other either with a fine thread or with a piezo translator. The fibers are wrapped around the cylinders several times. The fibers are not stetched more than 1%. This is important because the fibers are very sensitive. If the fibers are stretched more than 1%, the fiber could break. With the stretchers the optical path length can be adjusted by approximately $3\,\mathrm{cm}$. This seems much, but that is needed to adjust the over 8 meter long fiber. To create the required phase shift of the interference pattern, the second piezo controlled fiber stretcher was used. With this fiber stretcher it is possible to add optical path lengths to the sample arm which result in a phase shift of the interference pattern. The possible adjustment of optical path length is between a few hundred nm and a few $\mu\mathrm{m}$. The piezo controlled fiber stretcher is based on a P-753 LISA Linear-Aktor from Physik Instrumente (PI).

The used light source was a Superlum Broadsweeper 840 with a wavelength of $840\,\mathrm{nm}$. The detection was realized with a Mikrotron Eosens MC3010 highspeed camera. To redirect the light to the camera a Thorlabs 50/50 beam splitter Cube with NIR (near infrared) coating was used. The collimation was realized with an NIR coated achromat with a focal length of $25\,\mathrm{mm}$. The used objectives were a $40\mathrm{x}$ objective with an NA of 0.75 (WN-Achroplan, Zeiss) and a $5\mathrm{x}$ objective with an NA of 0.14 (M Plan Apo NIR, Mitutoyo).

IV. Results and Discussion

To characterize the quality of the grid, it was projected onto a silver mirror. Measurements were performed with the high NA objective (0.75 NA WN-Achroplan, Zeiss). The visibility V of the grid was calculated with the Michelson contrast:

$$V = \frac{I_{max} - I_{min}}{I_{max} + I_{min}} \tag{5}$$

Where I_{min} is the lowest intensity that was detected in the analyzed region and I_{max} is the highest intensity that was detected in the analyzed region. The measured maximal visibility V of the grid shown in Fig.6 was 70%.

That is by far higher than the realistic acquirable visibilities that are achieved with classical structured illumination. In the

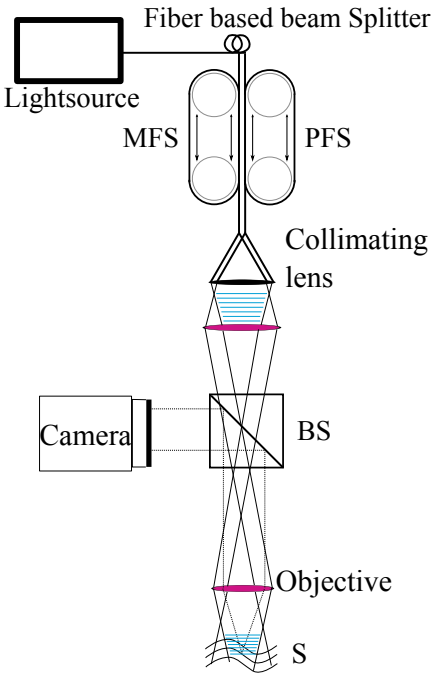

Fig. 5. Schematic setup with fiber based structured illumination and piezo fiber stretcher (PFS), manual fiber stretcher (MFS), a Beamsplitter (BS) is used to guide the reflected light to the camera

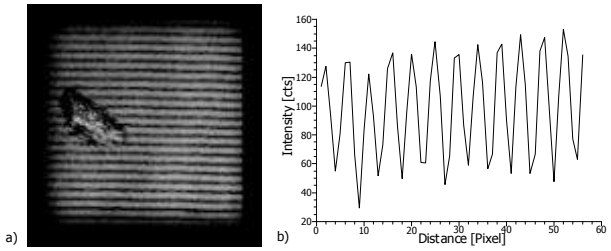

Fig. 6. a) Focused silver mirror with Zeiss $40\mathrm{x}/0,75/\infty/0$ WN-Achroplan objective, size: $145x145\,\mu\mathrm{m}$; b) plot of intensity profile

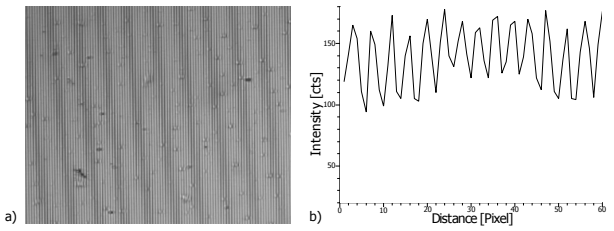

Fig. 7. a) With conventional SIM focused silver mirror with Mitutoyo $20\mathrm{x}/0.4$ M Plan Apo NIR objective; b) plot of intensity profile

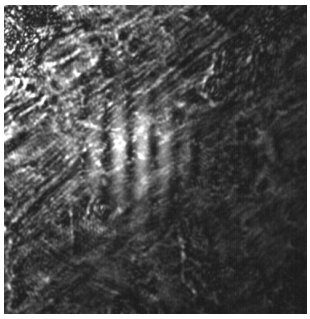

Fig. 8. Microscopic image of a coin

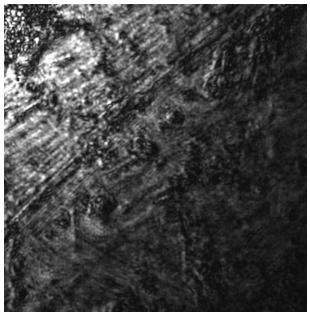

Fig. 9. Reconstructed SIM image of a coin

classical setup visibilities of 50% are considered very good [3]. That is because the MTF of incoherent illumination used in structured illumination microscopy drops contineously with the frequency. Additionally the visibility of the interference pattern does not change in the image. In an experimental classical SIM setup (Fig. 4) with a NA of 0.4 the best achieved visibilities were 30% (Fig. 7). But that was not achievable on the entire field of view. The visibility dropped on the edges of the image to 0.15. With higher NA (0.8) the visibility of the grid decreased to a maximum of 0.1.

Also in the fiber based setup the theoretical visibility of 100% was not achieved. The visibility was better when the objective with the NA of 0.14 is used.

To demonstrate stray light rejection, a coin was imaged with the setup of Fig. 5. Three images were taken with a respectively phase shift of $120°$. The final image was calculated with equation 1. The reconstructed image is seen in Fig. 9. In the wide field microscopic picture there is a reflex of the beam splitter in the center, which was successfully discriminated (Fig. 8).

The phase of the grating in the three images was adjusted with an accuracy of 1.3% which is the reason that there are nearly no vertical lines seen in the calculated image. To further reduce this error, the fiber stretcher has to get a better housing so that the thermal influence of the environment is minimized. Over a few seconds the phase of the grid shifts over more than one radiant (Fig. 10).

V. Conclusions

It was shown that it is possible to supress parasitic reflections by projecting the interference pattern of two coherent beams onto a sample. The contrast of the grid is much higher than in classical SIM and the interference frequency can be chosen without altering the setup or an imaged grid. Accuracy of the phase of the grating is limited by drifts caused by thermal expansion of the fiber stretchers. A new thermally stabilized housing will reduces this effect. It should be possible to do single-shot topography with our setup if the object is illuminated and viewed at different angles (Fig. 11).

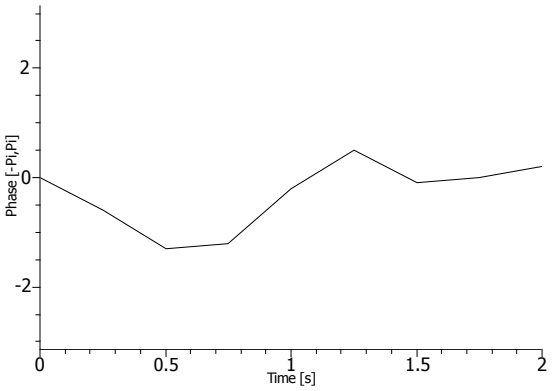

Fig. 10. Change of the phase of the interference pattern over time

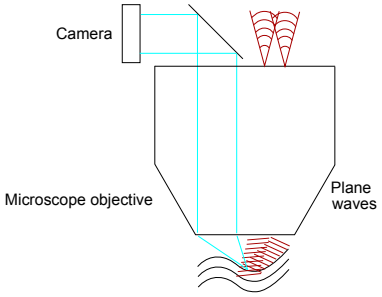

Fig. 11. Setup for single-shot topographic measurements

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