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Structured illumination microscopy of autofluorescent aggregations in human tissue

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ABSTRACT

Sections from human eye tissue were analyzed with Structured Illumination Microscopy (SIM) using a specially designed microscope setup. In this microscope the structured illumination was generated with a Twyman-Green Interferometer. This SIM technique allowed us to acquire light-optical images of autofluorophore distributions in the tissue with previously unmatched optical resolution. In this work the unique setup of the microscope made possible the application of SIM with three different excitation wavelengths (488, 568 and 647 nm), thus enabling us to gather spectral information about the autofluorescence signal.

1. Introduction

Compared to other microscopy methods used in biomedical research, fluorescence microscopy has several advantages in terms of sample preparation as well as the variety of possibilities to extract biologically significant information. A major problem, however, of any standard fluorescence microscopy method compared to non-light-optical microscopy methods remains the intrinsically limited conventional resolution of about 200 nm (Abbe, 1873; Rayleigh, 1896). Over the last years different techniques referred to as super resolution fluorescence microscopy have been established to compensate for this deficiency. These techniques (i.e. 4Pi (Cremer and Cremer, 1978; Hell and Stelzer, 1992), STED (Hell and Wichmann, 1994), SIM/PEM (Gustafsson, 2000; Heintzmann and Cremer, 1999) and localization methods (Cremer et al., 2010)), are based on conditions not considered in the original contributions of Abbe and Rayleigh. In combination with novel optoelectronical and mathematical tools, these different approaches have allowed the microscope to surpass the conventional resolution limit substantially, both in the object plane and in the direction along the optical axis of the microscope system.

In this report the method of Structured Illumination Microscopy (SIM), also referred to as patterned excitation microscopy (PEM), was applied to the investigation of the retinal pigment epithelium (RPE) in human eye tissue samples. SIM utilizes, in contrast to standard widefield (non-scanning) fluorescence microscopy, a periodically modulated excitation intensity in the object plane.

The RPE, which supports the functioning of the retina, is a cell monolayer between the rod outer segments and the Bruch's membrane. Age-related macular degeneration is primarily caused by a degeneration of the RPE. This degeneration is accompanied by excessive aggregations of lipofuscin. Lipofuscin consists of a variety of fluorescent and non-fluorescent proteins (Eldred and Katz, 1988) and lipids (Ng et al., 2008), and is mostly contained in the lysosomal residual bodies forming lipofuscin granules.

Lipofuscin exhibits autofluorescence across the visual spectrum (Sparrow et al., 2000), making fluorescence microscopy an attractive method to investigate the deposits of these pigments. Structured illumination imaging at three different wavelengths (488, 568, 647 nm) has allowed us to study the distribution of lipofuscin at a level of detail inaccessible to conventional fluorescence microscopy and to attain additional spectral information about the aggregations.

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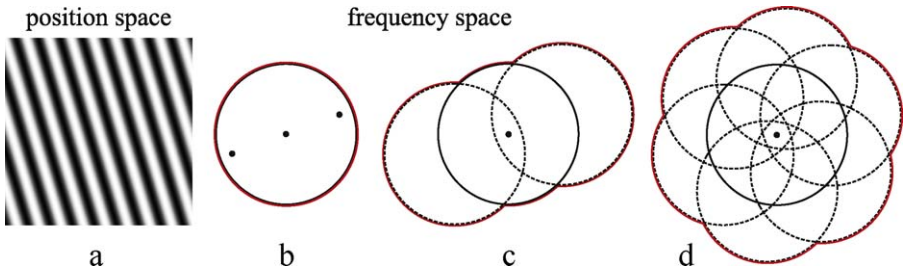

Fig. 1. Extension of the accessible frequency region by SIM. The sinusoidal illumination pattern (a) contains three delta-peaks in frequency space as illustrated in the Fourier transformed raw image (b). The red outline demonstrates the frequency limit. The raw data (b) consists of superposed original image information positioned at three different origins. After separation, the information can be shifted back to its respective position resulting in an expanded accessible frequency area (c). To expand the resolution in the object plane isotropically, the illumination is rotated (d).

2. Methods

2.1. Structured illumination microscopy

2.1.1. Principle of SIM

In conventional microscopy, the optical resolution is constrained by the wavelength of the detected light to approximately 200 nm.

Although this limit is a universal principle that cannot be broken directly, SIM is able to code high resolution information into the low resolution supported region of the microscope and thus circumvent the limit. The required conditions are generated in SIM by illuminating the object with a periodic pattern (e.g. a sine squared as described by Heintzmann and Cremer (1999) and Gustafsson (2000)).

The effect of this process in the image plane is visualized in Fig. 1: The object is illuminated with a sine square intensity pattern (a). By this operation in position space (multiplication of the object distribution with a sine square), in Fourier Space (b) the object's spatial frequencies are shifted by the spatial frequencies $\nu_x = 1/P_x$, $\nu_y = 1/P_y$ with the period of the sine square in x and y direction P_x and P_y. In total thereby three copies of the original Fourier-transformed object are generated. Two copies are originated at the negative and the positive grating frequency, while a central copy stays originated at the zero spatial frequency. The three copies exist jointly in the Fourier-transformed image (b). As the high spatial frequencies are cut off by the objective (the cut-off frequency is illustrated by the red circle in (b)) object information of high frequency that usually would lie outside the supported region ($\nu > \nu_{max}$) is coded in the transmittable frequency region.

An acquisition of a series of images with the grating at different positions makes it possible to identify the shifted information afterwards and transfer it back to its proper frequency position (Fig. 1c). This procedure results in a broadened support region in frequency space and thereby in an improvement of optical resolution in the direction of the modulation of the illumination pattern. To achieve an isotropic resolution in the lateral (object) plane, the direction of modulation of the excitation intensity has to be changed (i.e. the illumination pattern has to be rotated). Here the illumination pattern is rotated by 60° subsequently to three different acquisition orientations, which results in an accessible frequency domain of a shape as indicated in Fig. 1d and a sufficiently isotropic resolution improvement.

Since the illumination pattern is projected into the object plane through the objective lens, the spatial frequencies of the pattern have to be smaller than the cut-off frequency of the objective lens for the excitation wavelength. As the excitation wavelength is usually close to the fluorescence emission wavelength, the excitation cut-off frequency is close to the accordant frequency for the emission light. Because of these facts, the maximal resolution improvement that can be achieved with linear SIM is roughly by a factor of two.

The theory behind linear structured illumination resolution improvement has been described in detail by Heintzmann and Cremer (1999).

However, the benefit of SIM is not only the improved lateral resolution but also the improved axial optical sectioning resulting especially in an improved contrast compared to conventional widefield microscopy. A theoretical analysis of the optical sectioning ability of SIM has been done by Karadaglić and Wilson (2008).

2.1.2. The microscope

Most commonly used SIM microscopes use either a physical grating (Heintzmann and Cremer, 1999; Gustafsson, 2000), or a synthetic grating generated by a spatial light modulator (Hirvonen et al., 2008; Kner et al., 2009) in an intermediate image plane to create the modulated pattern in the object plane.

In this work, a different approach applying an interferometer configuration was used. This will be denoted as SIM_{HD}.

The setup, built from custom-made elements, is based on an inversely applied specially designed widefield (non-scanning) fluorescence microscope. The structured illumination in the object plane is generated by a Twyman-Green interferometer. A schematic of the setup is displayed in Fig. 2. The excitation laser beam (λ_{exc} = 404, 488, 568, 647 nm) is directed to a 50% beam splitting cube (Fig. 2, cube A), positioned in a focal point of the focusing lens. Half of the beam is reflected by 90° as the other half passes through the cube without change of direction. The resulting two beams are then reflected with mirrors by 180° back into the cube. After the light passes the beam splitter again, two beams, each at ¼ intensity are generated, that leave the cube heading towards the focusing lens. As the two beams travel the same distance, they are coherent and generate an interference pattern. If the beam splitting cube is rotated around the axis perpendicular to the ground by an angle θ, one beam is deflected by 2θ, as the other beam is deflected by -2θ. Thus the interference pattern can be adjusted by rotating the beam splitter. The beams pass the focusing lens, and are then deflected by a dichromatic beam splitter (AHF Z488/568/647) by 90° towards the high numerical objective (Leica HCX PL APO 100x/1.4-0.7 OIL CS). After passing through the focusing lens and the objective, the interference of the two beams in the object plane generates a sinusoidal pattern with a modulation parallel to the object plane. The orientation of the modulation can be changed by rotating the beam splitter around an axis parallel to the ground and perpendicular to the previous rotation axis. It is therefore possible to generate sinusoidal interference patterns in the intermediate image plane with arbitrary period and direction, which makes it possible to adjust the period of the pattern in accordance with the particular task and wavelength. To control the generated pattern, a part of the exci-