Optical sections by means of "structured illumination": background and application in fluorescence microscopy

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Fluorescence is a standard technique in modern biomedical research that is used for the specific detection of cell and tissue structures. A problem arises when "thick" specimens are used whose thickness in the direction of the optical axis is greater than the objective's depth of field. In this instance, the sharp image information from the focal plane is overlaid with blurred image information from out-of-focus planes. As a result contrast and resolution in the z-direction are reduced and 3D reconstruction of the sample is inhibited. An "optical section" through the sample, which only extracts the information from the sample that is in focus, can prevent or even reverse this problem. One method for generating optical sections is to use "structured illumination", as has been implemented in the ApoTome from Carl Zeiss.

In conventional fluorescence microscopy, the image on the retina or in the camera plane always consists of signal contributions from the focused object plane and from the object parts or structures situated above and below it. These structures are either out of focus and, consequently, perceived as faded, or – if they clearly lie outside of the focal plane – are perceived as visible background and therefore as a reduction in contrast. This background arises as a result of the fact that, during fluorescence excitation in the microscope, the excitation light from the illumination source not only hits the focal plane but radiates through the whole sample. This means that the fluorescence emission of object structures located above and below focal plane can also enter the imaging beam path through the objective.

In order to analyse the optical imaging properties of a fluorescence microscope, fluorescent beads that have a diameter below the optical resolution limit (smaller than 0.2 µm) are used. A sequence of image planes (z-stack) is acquired of individual beads in order to examine how their light is imaged in the three-dimensional space. By visualising the acquired data, it is possible to analyse the point spread func-

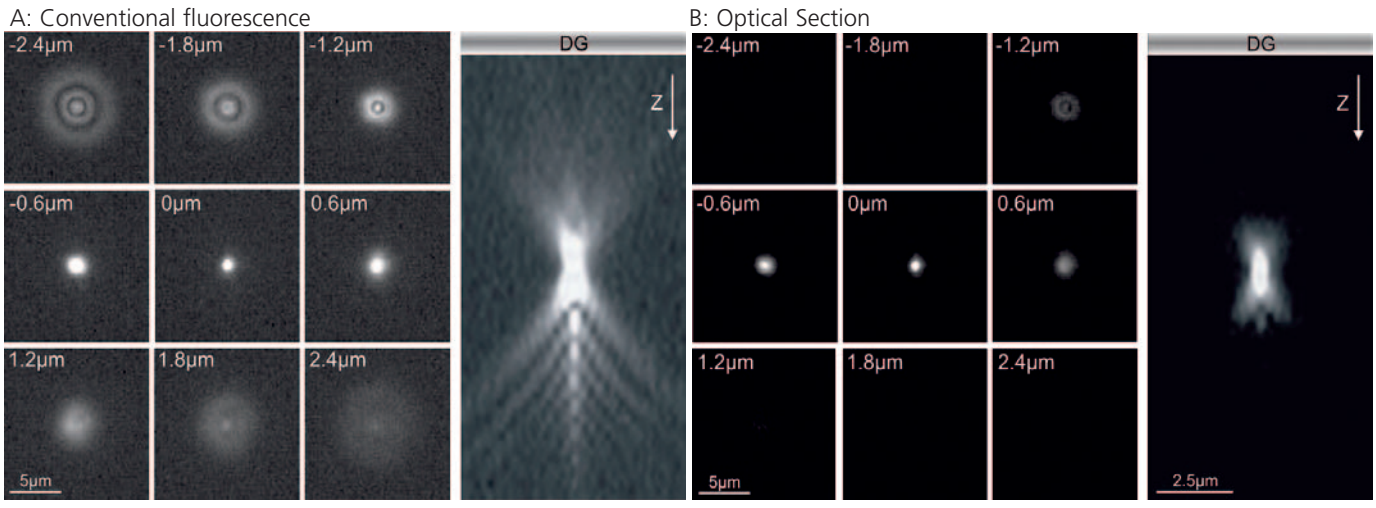

***Figure 1**: Point spread function (PSF) of a conventional fluorescence microscope (A) and of the ApoTome with structured illumination (B). For both A and B, shown on the left are the different focal planes of the acquired z-stack (the relative position to the central focus plane of the bead is given in µm), and on the right is shown a section along the optical axis (z-direction) through the centre of the PSF (DG: cover glass; objective: Plan-Apochromat 63x/1.4; bead Ø 0.175 µm)*

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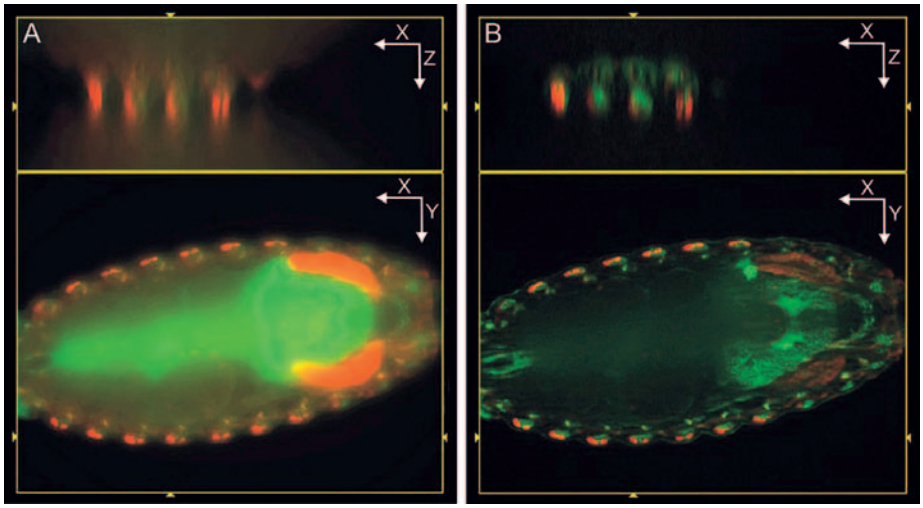

***Figure 2**: Drosophila melanogaster (common fruit fly) embryo. In conventional fluorescence (A) it is possible to observe the reduction in image contrast due to light from planes above and below the set focal plane. Image acquisition using the ApoTome (B) clearly improves the contrast (objective: Plan-Apochromat 20x/0.75; 2-channel fluorescence, specimen from Christian Klämbt, University of Münster)*

tion (PSF) and thus draw conclusions on the resolution and imaging quality of the optical system.

Figure 1 shows different views of the PSF for a conventional fluorescence microscope. The light from a truly round structure is imaged at planes far above and below the object focus. The magnitude of this problem is particularly well illustrated by the section along the z axis. An additional aspect is that the light from the bead above and below the central plane is imaged differently: above it appears diffuse and cloudy, while below the light shows a ring structure (known as Airy disc). This difference can be traced back to the fact that the image stack is acquired by moving the object and the PSF is thus distorted by the (unavoidable) spherical aberrations that occur. Ideally, the acquisition of the PSF should be made by generating the z-stack in the image space, for example by moving the camera along the optical axis.

1 Optical sections

For thick specimens, light from parts of the object above and below the chosen focus plane reduces the contrast and resolution along the optical axis (z resolution). In terms of microscopy, specimens are deemed "thick" when their dimension in the z-direction is greater than the objective's depth of filed. This depth of field (*Tv*) is dependent on the numerical aperture (*NA*) of the objective being used, the refractive index of the immersion medium (*n*) and the wavelength of the light (λ). The following definition is generally recognized:

$$Tv = \frac{n \cdot \lambda}{NA^2}$$

If a combination of specimen and objective is used in which the specimen is thicker than the objective's depth of field, the image is disrupted by light from planes above and below the focal plane. A sample thickness several times greater than the depth of field can actually prevent detailed image acquistion entirely (**figure 2**).

Various techniques have been developed for the generation of optical sections, including different systems for biological research such as confocal laser scanning microscopy and 3D deconvolution, that have now achieved universal acceptance. A relatively new method is "structured illumination" or "grid projection" on the basis of a conventional fluorescence microscope.

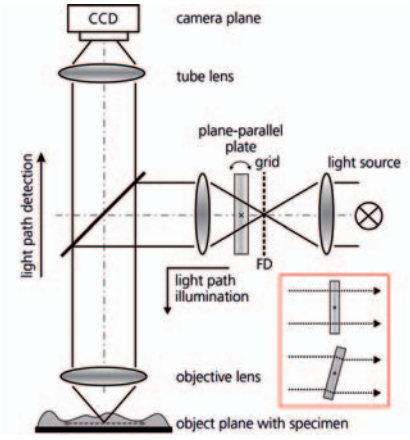

***Figure 3**: Beam path for structured illumination in a conventional fluorescence microscope (FD = field diaphragm plane). The inset shows the parallel movement of the projected grid resulting from tilting a plane-parallel glass plate*

2 Structured illumination: principle

Figure 3 illustrates the principle of the set-up used for structured illumination. A grid structure is inserted at the field diaphragm plane (FD) in the illumination path of a fluorescence microscope and projected onto the specimen (**figure 4**). At least three (raw) images are acquired with the

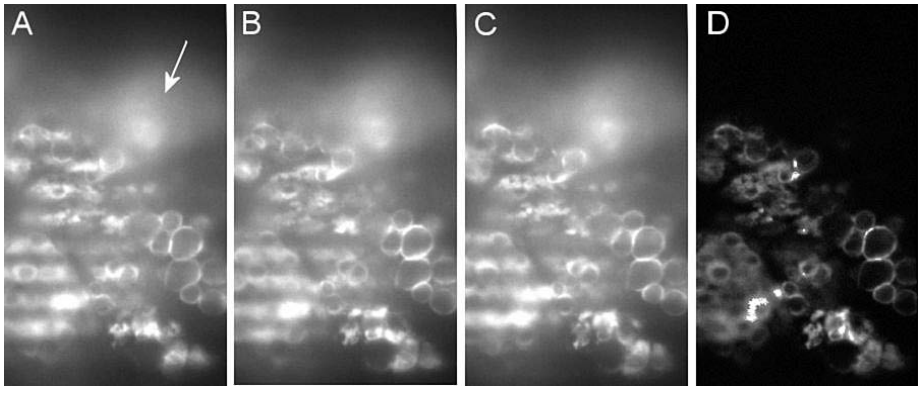

***Figure 4**: For structured illumination, three raw images (at least) are acquired, each with a different grid position (A-C). An image-analysis algorithm calculates an optical section (D) from the raw images online*

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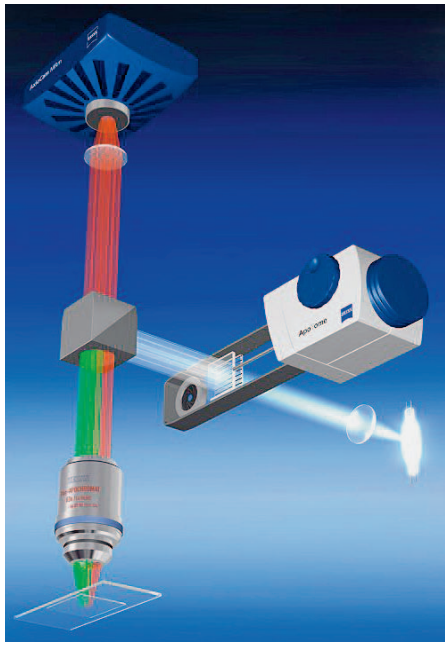

***Figure 5**: Structured illumination with the ApoTome. The slider contains a glass plate with a vapor-deposited grid structure and a plane-parallel glass plate which can be tilted by means of a galvo scanner mechanism*

grid structure in different positions, these images then being combined in real-time to form an optical section and displayed online. This imaging principle has been implemented in the ApoTome from Carl Zeiss as an insert into the fluorescence illumination path of the Axio Imager and Axiovert 200 research microscopes (**figure 5**).
Lateral movement of the grid projection within the focus plane at the specimen is achieved by simply tilting a plane-parallel glass plate located directly behind the grid in the beam path.
How does this approach lead to an optical section through the sample? First, the projected grid becomes visible in the focal plane as a result of the object structures fluorescing after they have been excited by the structured light. At the points where no light has hit the object (dark grid lines) no fluorescence is triggered. The projected grid becomes most clear in the focal plane, on out-of-focus specimen structures, it is imaged with reduced contrast (figure 4A, arrow).
In extreme cases, when regions of the specimen lie well outside the focal plane and are only perceived as a blurred background, the image of the grid is no longer visible. It is thus possible to establish through image analysis, which areas of the camera images show regions of the specimen that are in the focal plane of the objective, and which areas contain signals from those outside of the focal plane. This is realised by simply determining the grid contrast as a function of location (xy) pixel by pixel over the field of view in each image. Removing the out-of-focus image information and collating the 3 images then results in an optical section through the specimen.

3 Applications

Structured illumination is particularly suitable for fluorescence applications in cytology, neurobiology and developmental biology. In these research fields, specimens of complete embryos of drosophila, zebra fish, etc. are often specifically stained with fluorescent dyes. Due to the thickness of these "whole mounts", optical sectioning techniques are essential for imaging biological structures at adequately high contrast and resolution in the axial direction. The ApoTome permits the very simple and inexpensive use of conventional fluorescence imaging systems for exactly this type of application.
The generation of an optical section is a crucial prerequisite for visualising microscopic z-stacks in 3D. For this application too, structured illumination is capable of producing high-quality results which serve as the basis for reconstructing 3D visualisations using software algorithms (**figure 6**).

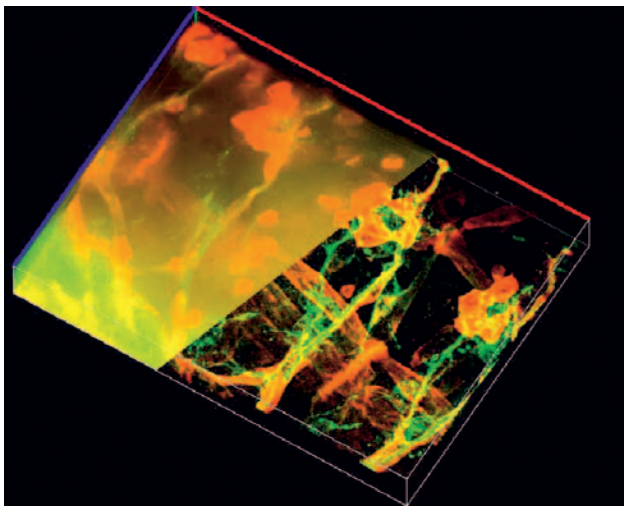

***Figure 6**: 3D visualization of nerves and glial cells in a D. melanogaster embryo; objective: Plan-Neofluar 40x/0.75. When images are acquired with a conventional fluorescence microscope, the cell structures and interconnections are overlaid by out-of-focus light (top left). Optical sections allow object details to be displayed with high contrast and improved resolution along the optical axis*

4 Summary

Use of the "structured illumination" method enables conventional fluorescence microscopy to create optical sections through specimens and thereby improve the contrast and resolution along the optical axis. Optical sections facilitate the 3D reconstruction of specimens, enabling structures to be displayed in their proper spatial context.

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