Scanned LSFM with Confocal slit Detection

THE QUESTION: Improvement of SNR and contrast in Light sheet fluorescence microscopy by using a sCMOS camera for detection

Light sheet fluorescence microscopy (LSFM) is a more and more popular technique for 3D imaging of living specimen. In contrast to epi-illumination microscopy the sample is illuminated with a thin light sheet orthogonally to the detection path. Most often the sheet is formed by rapidly scanning a laser beam across the sample. This illumination yields intrinsic optical sectioning and reduction of photobleaching and phototoxicity since excitation is limited to fluorophores inside the observation plane. This is particularly beneficial for long term imaging in developmental biology and reduces image background.

THE BARRIER

By using a CCD or sCMOS camera as detectors images can be acquired with high frame rates and sensitivity. The readout of a classical CCD occurs in the so-called „global shutter" mode, i.e. the pixel exposure is initiated and terminated for all pixels simultaneously and all light is detected during global exposure time. However, not all detected photons convey information, which is useful to create a sharp image, since they are also elastically scattered on their way through the specimen. Scattering occurs for both the excitation and the emission light. Scattered photons are likely to hit a random detector pixel and contribute to a nonspecific image background reducing contrast, signal to noise ratio and the effective resolution.

THE SOLUTION: Using a Scientific CMOS Camera in Rolling Shutter Mode for Confocal Slit Detection

In order to improve image quality, we developed a setup that exploits a scientific CMOS (Orca FLASH2.8 or FLASH4, Hamamatsu Photonics K.K.) camera as a confocal slit detector, explains Eugen Baumgart, Ph.D student in Professor Kubitschecks group. This can be accomplished in a very elegant manner by taking advantage of a special readout mode featured by sCMOS cameras, the "rolling shutter". Each pixel of a sCMOS chip has its own readout and amplification unit, which allows this sequential readout pattern. In the rolling shutter mode the exposure is initiated at the topmost pixel line and advances to the next row until it reaches the bottom of the chip.
The time between activation of subsequent rows is referred to as the line activation time, and the exposure time is an integer multiple thereof.
The shortest possible exposure time for a pixel is equal to the line activation time. Selecting it would result in only one single active row at a time moving from top to bottom of the chip. Increasing the exposure time means to increase the number of simultaneously exposed rows thus forming a slit shaped region of active pixels with adjustable width, which moves along the detector and truly forms a confocal slit mask. By synchronizing a scanned Gaussian laser beam for sample illumination with the rolling shutter of an Orca Flash4 camera, a confocal arrangement of spatially confined illumination and detection volumes can be realized. Reduction of the rolling shutter width allows for preventing a larger fraction of scattered photons to hit the active detector lines and from contributing to image formation, while the ballistic photons directly hit the active pixels in the rolling shutter region.

This technique amalgamates the better of two strong techniques: the superior contrast and resolution capabilities of confocal imaging with the elegant illumination scheme, high detection efficiency and high frame rates of LSFM. It does not require any modifications of the optical detection path and no image post-processing.

The setup of our scanned light sheet fluorescence microscope, in which illumination beam and detection slit as realized by the rolling shutter of a sCMOS were synchronized, is described in Figure 1.

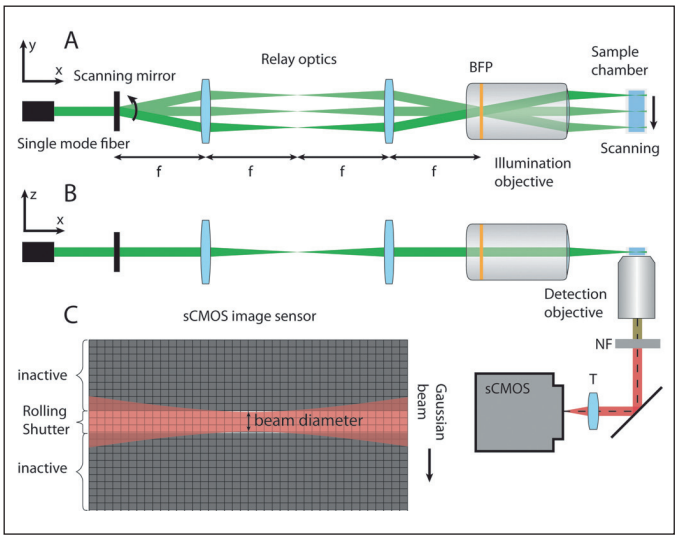

Figure 1:
(A) The beam exits a single mode fiber and is scanned by a galvo scanner. The relay optics, consisting of two lens systems with overlapping focal lengths, is used to image the scanning point into the back focal plane (BFP) of the illumination objective, generating a light sheet by scanning a focused Gaussian beam telecentrically.

(B) Side view of the setup schematics. Fluorescence light is collected by a detection objective and passes a notch filter (NF). The final image is formed by a tube lens (TL) on the camera in such a manner, that the position of the scanned beam is always conjugated to the rolling shutter.

(C) Since only a narrow region of the camera detection array is active, a significant amount of scattered light is rejected, which reduces image background. The smaller the rolling shutter, the more scattered photons are dismissed from detection.

Figure 2A shows an image of the illumination beam entering from the left into a sample containing a non-scattering fluorescence solution (Alexa Fluor 647 in buffer). Figure 2B reveals the deterioration of the beam and the fluorescence background in a scattering sample corresponding to immobilized red-fluorescent beads (∅ 200 nm) and additional Alexa Fluor 647 dye molecules. Scattering of the excitation laser beam and the excited fluorescence created a substantial fluorescence background outside of the excitation volume.

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This grossly diminished contrast and sectioning quality. Next, we acquired images of the latter sample with a scanned beam, and compared the image contrast for various rolling shutter widths. Image contrast acquired with a rolling shutter with an extension equal to the beam waist diameter was almost doubled compared to the global shutter data (fig. 2C). The signal-to-noise ratio, measured as the ratio of the intensity amplitude divided by the background noise was more than tripled in this confocal detection mode.

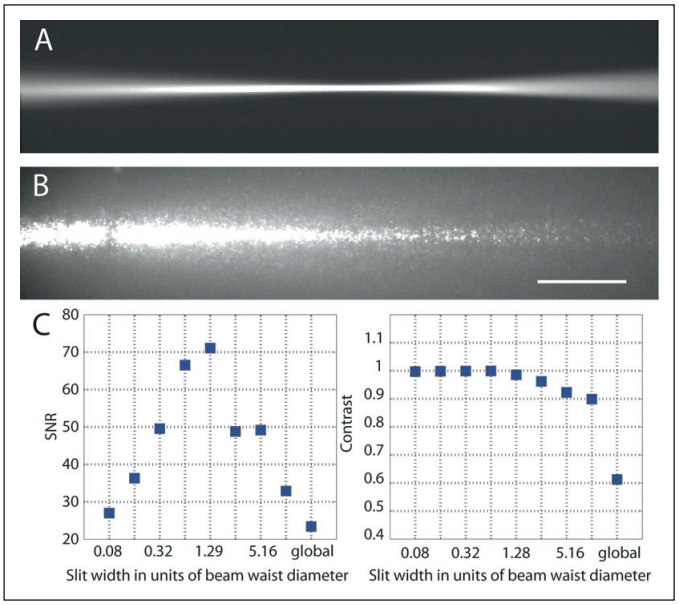

Figure 2: Deterioration of imaging quality in a scattering sample.

(A) Illumination beam in a non-scattering dye solution.

(B) Sample with fluorescent beads. The illumination beam is scattered and broadened on its way through the sample. Also, scattered fluorescence sums up to a homogenous image background.

(C) Improvement of contrast and signal-to-noise ratio by using rolling shutter detection. Illumination was from left. Scale bar, 100 µm.

THE FUTURE

Certainly, it is more relevant to examine the usefulness of the confocal line scanning mode using biological specimen. We did that when imaging scattering specimens like Chironomus tentans salivary gland cells. Light Sheet Fluorescence Microscopy improved the sample illumination path compared to wide field microscopy. By this development we took the next step by optimizing also the detection path. Currently we apply this instrument to the study of zebra fish embryos.

For more information see:

Baumgart E. and Kubitscheck U., Opt. Expr.20, 21805–21814 (2012)
Baumgart E. and Kaminski T, Imaging & Microscopy 1, 2-4 (2013)
http://www.chemie.uni-bonn.de/pctc/kubitscheck

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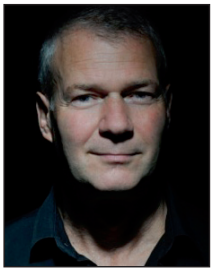

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