Diffusion measurements in early C. elegans embryos using SPIM-FCS

In the last decade Single-Plane Illumination Microscopy (SPIM) has emerged as a versatile technique for imaging fluorescently labeled samples in vivo. Going beyond mere imaging, we have used a combination of SPIM and fluorescence correlation spectroscopy (SPIM-FCS) to quantify protein diffusion in zygotes of the nematode Caenorhabditis elegans. By using our custom-built SPIM-setup [1] and one of the latest sCMOS cameras (Orca-Flash4.0 V2) we were able to derive spatially resolved diffusion maps of a peripheral membrane protein in the embryo's cytoplasm [2]. Our results compare favorably to previous reports on the diffusive behavior of this protein, thus showing the applicability of sCMOS sensors for SPIM-FCS as a promising new measurement technique.

1. Introduction

Diffusion is the basic means of molecular transport in living cells and hence acts as a trigger for vital biochemical processes. In order to understand biological processes it is therefore essential to quantify the diffusion behavior of proteins in the spatially inhomogeneous environment of a living specimen.

A well-established technique for local diffusion measurement is fluorescence correlation spectroscopy (FCS). By correlating the intensity fluctuations of the fluorescence (GFP) in a small focal spot it is possible to derive the diffusion behavior of labeled particles in the focal volume. However, in many cases one would like to carry out multiplexed data acquisition in order to obtain diffusion maps that assess diffusional transport throughout an inhomogeneous environment. Therefore, image based FCS-techniques have been developed.

Imaging dynamic processes in cells and multicellular systems requires fast and spatiotemporally resolved ultra-low light cameras to decrease photobleaching and -toxicity. SPIM combines rapid widefield detection with optical sectioning by detecting the fluorescence emission (GFP) of perpendicularly illuminated slices of a sample. Imaging only the illuminated slice results in reduced bleaching and allows for long-term, three-dimensional in-vivo imaging at a high spatiotemporal resolution [1, 3, 4, 9] with reduced background signals.

In SPIM-FCS [5, 6] each pixel of an acquired image represents a measurement point for the lateral diffusion behavior while the confined illumination by a thin sheet of light restricts the axial extension of the focal volume. The observed dynamics can be extremely fast. In order to resolve the decay of the autocorrelation in each pixel's intensity trace thousands of images have to be acquired at a very high frame rate (1000 to 25000 fps). New scientific complementary metal oxide semiconductor (sCMOS) cameras have the ability to dynamically image large field of views (2048 x 2048 pxls) very rapidly even at ultra-low light levels. The high quantum efficiency (peak QE = 72 % @ 580 nm) of the sensor makes it possible to resolve even the rapid diffusion of proteins in the cytoplasm of living cells without destroying the sample during measurement.

In this article, we have used SPIM-FCS on early embryos of the nematode C. elegans to obtain spatially resolved diffusion maps of the peripheral membrane protein PLC1δ1 in the cytoplasm.

2. Experiment

For SPIM-FCS, we have used a modified version of our previously published SPIM setup [1] as depicted in Fig. 1a. The widened beam of a DPSS-laser (491.5 nm) was focused in one dimension by a cylindrical lens on the back aperture of a water-dipping objective to obtain the illumination light sheet. To achieve the small observation volumes needed for FCS measurements, we overfilled the back aperture of the illumination objective to reduce the thickness of the light sheet to a waist FWHM of 1.2 ± 0.1 µm in a small rectangular region. Suitable eggs from transgenic worm lines expressing GFP-tagged PLC1δ1 were extracted in an early stage of development and then mounted on a custom-made metallic sample holder in a water-filled heating-chamber. Eggs were positioned in the waist of the light sheet with the long-axis of the ellipsoidal egg being oriented perpendicular to the propagation direction of the beam. By imaging the light sheet waist at the middle of the sCMOS camera (ORCA-Flash4.0, Hamamatsu Photonics, Japan) we reduced the number of horizontal lines to be read out (see Fig. 1d). In this way, frame rates of 1000 to 25000 fps were possible. In the rolling shutter mode of the sCMOS chip are two readout registers (one for each sensor half). After 9.7 µs two horizontal sensor lines with a width of 2048 pxls are read out. Reducing the number of horizontal pixels therefore increased the total acquisition speed. The emitted

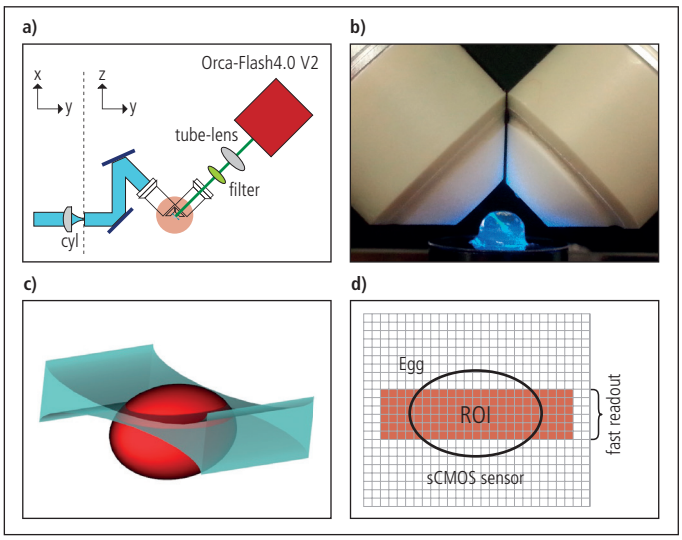

Figure 1: a) Sketch of our SPIM-FCS-Setup: The cylindrical lens focuses the beam in the x-dimension onto the excitation objective to form a lightsheet. Fluorescence (GFP) is collected perpendicular with a second objective and focused onto the sCMOS camera. b) Side-view of the objectives showing the lightsheet in the focus. c) The lightsheet was positioned in the upper half of the ellipsoidal embryo. d) Illustration of the small rectangular region of interest on the sCMOS sensor to achieve high framerates.

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fluorescence signal was filtered by a single-band filter and collected by a tube lens positioned perpendicular to the illumination light sheet (see Fig. 1b). The setup was controlled via a custom-made LabVIEW program using trigger signals to control the camera via the HoKaWo imaging software (Hamamatsu Photonics Deutschland GmbH). For measurements in the cytoplasm of the embryo up to 20,000 frames with exposure times in the range 152 – 1004 µs were taken. We imaged a layer in the upper half of the egg in order to reduce scattering and aberrations in the acquisition (Fig. 1c). Although the sCMOS sensor is very sensitive it was necessary to use fairly high excitation powers in the range of 0.8 – 20 mW (measured at the backaperture of the illumination-objective) which exceeded typical power-values used for gentle long-term imaging (~100 µW, 50 ms exposure-time) to maintain reasonable signal-to-noise ratios (SNR ~2.8 at light levels of ~210 photons/4 pixels [10]). The possibility of both on-chip (2x2 binning) and subsequent software-binning (3x3 binning) was used to further improve the SNR at the cost of spatial resolution. Because of the increased excitation power as compared to SPIM imaging, timetraces in each pixel had to be corrected for bleaching effects. The auto-correlation function (ACF) of the corrected time traces was calculated with an open-source data evaluation software (Quickfit 3.0 Beta, SVN: 3891 [7]). ACF-curves were then fitted using a model for three-dimensional diffusion to extract diffusion coefficients of each single pixel. Further details are available in Refs [1, 2]

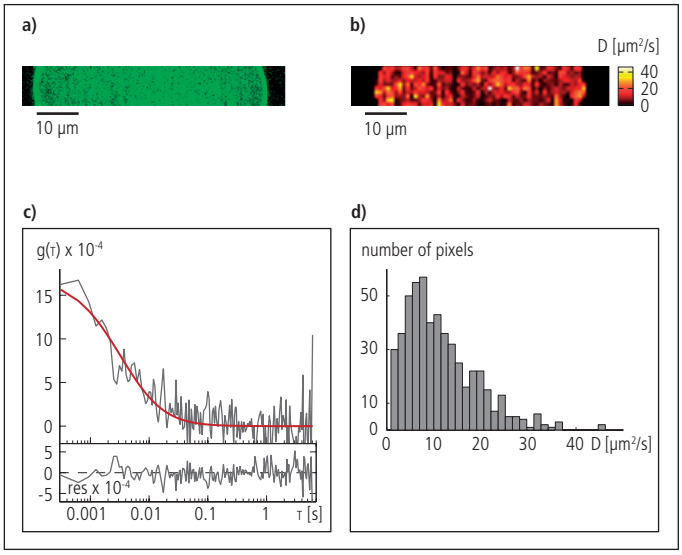

Figure 2: a) Fluorescence image of C. elegans embryo in one-cell stage expressing GFP-tagged PLC1δ1. b) The diffusion map of the embryo from (a) indicates the heterogeneous environment of the cytoplasm (additional 3x3 binning has been performed). c) Autocorrelation-function from the intensity time-trace of a single pixel. Fitting curve shown in red with residuals below. d) Distribution of all diffusion coefficient values from the measurement shown in (b).

3. Results

To test the performance of SPIM-FCS in a well-established model organism, we measured protein diffusion maps in the early embryo of C. elegans. The GFP-tagged protein PLC1δ1 is a peripheral membrane protein with a large cytoplasmic pool. Previous studies [8] determined the cytoplasmic diffusion to be in the range of 8.1 ± 2.0 µm²/s. The fast diffusion pushed the SPIM-FCS application to its limit. Fig. 2a illustrates the intensity image of an acquired layer in the early embryo in the one-cell stage. The lower intensity in the cytoplasm compared to the high signal on the membrane indicates a low amount of free proteins in contrast to elevated protein levels bound to PIP2 lipids on the plasma membrane. The autocorrelation function of a single pixel is shown in Fig. 2c. The acquisition speed of the camera is sufficient to catch even the rapid ACF decay due to cytoplasmic diffusion at a reasonable accuracy. The resulting diffusion map is shown in Fig. 2b. Pixels not including cytoplasmic sites, having poor SNR, or showing measurement artifacts were masked. The diffusion maps in the embryo reveal a heterogeneous distribution throughout the imaged layer, reflecting a considerable cytoplasmic heterogeneity. The distribution of diffusion coefficients is depicted in Fig. 2d. Pixel values of the shown measurement have a broad distribution around a median value of 9.7 µm²/s with a first and third quantile of 6.0 µm²/s and 15.5 µm²/s. This value is in a good agreement to previous reports [8]. The width of the distribution is the combination of the actual distribution of diffusive behavior throughout the embryo's cytoplasm and the fluctuating quality of the fitting-procedure for single pixels. A single measurement consisted of 528 individual pixels which correspond to hundreds of single point-FCS measurements. Therefore the multiplexed approach of a single SPIM-FCS measurement has the advantage of excellent statistics and reveals spatial differences in the diffusion behavior. To improve the data quality further a better SNR and shorter delay are crucial. Recently, Hamamatsu released an sCMOS camera which has a 10 % better quantum efficiency over the whole visible spectra. This improvement, in combination with fast readouts, allows for the measurement of even faster cell dynamics or more stable fitting results. In Summary, the results shown here demonstrate that the sCMOS camera enabled the quantification of fast diffusion processes in developing specimens.

4. Conclusion

In this report the performance of our custom-built SPIM-FCS setup and an sCMOS camera is demonstrated by measuring on the established model organism C. elegans.

By reading out only a small region parallel to the center-line of the camera sensor extremely high framerates were achieved. This enabled the autocorrelation of fast fluctuations in the fluorescence intensity signal caused by molecular diffusion. Due to the good SNR even at this short exposure times the resulting autocorrelation functions revealed diffusion maps of a model protein inside the cytoplasm of early C. elegans embryos. The presented data is in agreement with previous reports [8] on the same protein construct using scanning FCS measurements. The demonstrated SPIM-FCS method is well suited to uncover vital processes in developmental biology.

More detailed informations about our SPIM-FCS measurements, data evaluation, and results can be found in Ref [2].

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Key Words

C. elegans, Developmental Biology, Biophysics, Single-Plane Illumination Microscopy (SPIM), Fluorescence Correlation Spectroscopy (FCS), SPIM-FCS, sCMOS

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