Single Molecule Light Sheet Microscopy

Export of Native mRNA Molecules in Vivo

All genetic information is stored on DNA safely concealed in the cell nucleus. Here it is copied to messenger RNA molecules, which export the genetic information across the nuclear envelope into the cytoplasm, where it is translated into proteins. Light sheet fluorescence microscopy and minimally invasive labeling strategies make it now possible to observe the dynamics of this process at the single molecule level deep within living tissue.

Light Sheet Fluorescence Microscopy

More than 100 years ago, Siedentopf and Zsigmondy introduced the concept of ultramicroscopy for the observation of colloidal gold particles. Even though these particles were smaller than the wavelength of visible light, illumination by a thin light cone made it possible to observe light scattered from scarce particles [1]. Exactly 90 years later Voie et al. reintroduced the concept of ultramicroscopy for fluorescent samples [2]. The technique was then used to acquire time series of 3D image stacks of developing Medaka and Drosophila embryos at high, isotropic resolution [3], and the investigation of entire chemically cleared mouse brains [4], but also enabled the visualization of more dynamic biological processes deep inside live specimen [5]. In 2011, LaVision BioTec launched the first commercial instrument. Other groups have developed similar instruments for a number of applications under various acronyms.

However, light sheet fluorescence microscopy (LSFM) has become a widely accepted term for ultramicroscopy with laser illumination and fluorescent probes. Further developments pushed the limits to volumetric imaging of subcellular details in live cells [6] and nanometric single particle localization for super-resolved [7] or high-speed imaging [8]. Latest additions to the scope of LSFM include 3D localization methods [7] as well as further increased background rejection by synchronizing the readout of a confocal line detector with a scanned excitation beam [9].

Single Molecule Tracking with LSFM
In LSFM, a thin sheet of light within the sample illuminates the focal region of a detection objective to generate an optical sectioning effect.

In our setup (fig. 1), a cylindrical zoom unit [10] is used to transform the circular TEM$_{00}$ output of an optical single mode fiber into a collimated elliptical beam, which is subsequently focused into the specimen by an illumination objective. A large width of the incoming beam implicates a high effective illumination numerical aperture (NA), and thus a thin light sheet, but according to the laws of Gaussian optics also a large divergence (fig. 1b, c). Another cylindrical lens refracting the light along the axis perpendicular to the lenses in the zoom unit is used to adjust the lateral width of the light sheet. If its focal plane coincides with the back focal plane of the illumination objective, lateral divergence of the light sheet in the sample is minimized. Both, lateral sheet width and Rayleigh length along the illumination axis are usually matched to the camera field to render the illumination profile as homogeneous as possible. A fast-scanning mirror placed in the front focal plane of the same cylindrical lens can be used to laterally pivot the light sheet around the position of its axial focus (fig. 1a) during the camera exposure to diminish possible shadowing artifacts resulting from the sideways illumination [11]. In an alternative approach a circular beam is rapidly scanned across the image field to generate the light sheet [12].

In our experiments, specimen are kept in a specially designed glass cuvette with an optical grade sidewall through which the sample is illuminated, a coverslip bottom for use with an inverted microscope and an open top leaving the sample accessible for further manipulations, e.g. by a micro injection device. The cuvette is placed in a magnetic holder and attached to a motorized 3-axes-stage for convenient control of the sample position. Fluorescence is collected by a high NA detection objective from below and imaged onto an EMCCD or sCMOS camera. Here, we used a light sheet thickness of 2 µm (FWHM), corresponding to a Rayleigh length $X_0 = \pi \omega_0^2 / \lambda = 14 \, \mu m$ at $\lambda = 640\text{nm}$ (fig. 1c).
Contrast Enhancement by Selective Illumination

Using LSFM results in a number of advantages compared to other microscopy techniques. By sheet illumination an optical sectioning effect similar to a confocal microscope is achieved. This leads to greatly enhanced contrast and signal to noise ratio (SNR) due to reduced background fluorescence [8]. The higher SNR directly translates into improved localization precision of single particles. Furthermore, it enables single molecule detection at an unprecedented depth of well beyond 100 µm within semi-transparent live specimen. In LSFM, out-of-focus fluorescence does not need to be rejected by a confocal pinhole since it is not excited in the first place. On the one hand this reduces the photo-toxicity of fluorescence and saves on the overall photon budget since only fluorophores actually under observation are excited. On the other hand it allows for parallel image acquisition with high speed cameras rather than sequential point scanners. With the latest sCMOS cameras, single molecule imaging at hundreds to thousands of frames per second is possible. To further reduce photo-bleaching, illumination wavelength and intensity are controlled by an AOTF, which is triggered by the camera and transmits laser light only during the chip integration time.

Using LSFM to Study mRNA Export

Eukaryotic cells store their genetic information on DNA molecules in the nucleus. However, the machinery of protein translation is located in the cytoplasm and therefore the genetic information is exported in form of messenger RNA (mRNA) molecules. Export of nuclear mRNA is a key transport process in eukaryotic cells. To study the RNA translocation through the supramolecular nuclear pore complexes (NPCs) at the molecular level we used a classical biological system: the salivary glands of the dipteran *Chironomus tentans*. 15 years ago electron microscopy of gland cell nuclei allowed for the first time to visualize mRNA molecules during their NPC translocation. While enabling the direct observation of a completely unmodified endogenous mRNA, electron microscopy provided only a static view of this highly dynamic cellular process. LSFM finally enabled us to adopt the *C. tentans* system for light microscopy.

Kinetics of mRNA Export from Single Molecule LSFM Measurements

*In vivo* analysis of mRNA export requires fluorescence microscopy, which implies the generation of fluorescent mRNPs *in situ*. To this end we labeled the mRNA particles in living *C. tentans* salivary gland cells by microinjection of a fluorescent mRNA-binding protein: hrp36, the *C. tentans* hnRNP A1 homolog (fig. 2). During transcription the growing pre-mRNA associates with several mRNA-binding
proteins to form the so called ribonucleoprotein particles (mRNPs). Some proteins leave these particles during intranuclear trafficking, while others like hrp36 escort the mRNA through the NPC into the cytoplasm. Therefore hrp36 constitutes a perfect probe for in vivo labeling of native endogenous mRNA for export [13]. Very low amounts of fluorescence-labeled hrp36 molecules were injected into the cytoplasm, and after nuclear import and their integration into mRNPs we could follow single labelled mRNPs in the gland cell nuclei about 120 µm deep within the sample. To counterstain the NPCs we used fluorescent NTF2, a transport factor, which is unrelated to mRNA transport but is enriched in the central NPC channel. Co-injection of fluorescent NTF2 therefore resulted in distinct nuclear envelope visualization (fig. 3). NTF2- and hrp36-fluorescence signals were sequentially acquired using exactly the same imaging path to minimize alignment problems in the dual color measurements. Movies that were acquired with a frame integration time of 20 ms at 50 Hz frame rate allowed capturing single mRNP in the nucleoplasm approaching the nuclear envelope and during their export to the cytoplasm (fig. 3).

Since export events were only rarely seen due to the low label concentration used several thousand movies had to be recorded to collect enough data for quantification of the underlying kinetics. To efficiently screen for the rare export events we evaluated the movies by kymographs and an automated, computer-based evaluation protocol. The duration of several hundred mRNA export processes was measured and the time constants of mRNA export calculated (65±5ms and 350±25ms). The high SNR achieved by LSFM yielded a localization precision of as low as 10 nm for molecules bound to the nuclear envelope. This in turn allowed tracking the mRNA during their export and mapping the intra-NPC binding site distribution of mRNPs during translocation. Using the same imaging strategy for a further labeled protein involved, the RNA-helicase Dbp5, we could for the first time visualize the kinetics of this key enzyme of mRNA export at the single molecule level in vivo [13].

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References
For further literature please ask the authors.

Authors
Jan-Hendrik Spille (corresponding author)
Dr. Jan-Peter Siebrasse
Tim Kaminski
Prof. Dr. Ulrich Kubitscheck

Rheinische Friedrich-Wilhelms Universität
Institut für Physikalische und Theoretische Chemie
Bonn, Germany

Contact

Universität Bonn
Wegelerstr. 12
53115 Bonn
Germany