Light Sheet Fluorescence Microscopy (SPIM) and laser excitation in orange for imaging of live organisms

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The observation of biological processes deep inside tissues is in no way an easy task and can readily be compromised by many competing processes, especially light scattering and autofluorescence. By using appropriate fluorescent proteins these sample-specific influences can, however, be reduced. For example bright red fluorophores have been developed [1,2] especially for in-toto imaging of self-developing organisms which help minimize the impact of such processes. These bright red fluorophores are typically excited by sources in the orange-red spectral region. This article discusses these very advantages and shows the improvement in results when using the newer longer wavelength excitation sources for the three-dimensional in-vivo observation of the early cleavage divisions in the fruit fly embryo.

1 Lasers for fluorescence microscopy

In fluorescence microscopy, the most widely used "longer" wavelengths available from modern lasers are typically 561 nm (yellow) or 640 nm (red). The 640 nm diode lasers are in general too long in wavelength to efficiently excite the bright red fluorophores (such as mCherry) and hence the benefit of using the longer wavelength lower energy light to achieve deeper penetration is lost. Likewise the 561 nm light is too short in wavelength. The ideal excitation wavelength is around 590 nm (orange), and this wavelength in combination with the bright red fluorophores gives the optimum conditions resulting in substantially less scattering and absorption and thus less autofluorescence occurs within the sample, thereby leading to an overall higher imaging quality.

Lasers operating in the orange part of the spectrum (580–600 nm) are, however, not new. The helium-neon (HeNe-) laser, a gas laser that apart from its main line at 632.8 nm can also emit at 594.1 nm, has been available for many years. However, its relatively low output power of maximum 3 mW (polarized) is not enough for many applications. Fibre lasers, with emission at 592 nm, offer on the one hand power levels of several hundred mW, but suffer from high intensity noise, often severely reducing the signal-to-noise ratio and thus the image recording quality.

In the following sections we exemplarily describe SPIM applied to the investigation of embryos from Drosophila using an orange laser, as well as discussing the design and characteristics of this innovative DPSS-laser emitting at 594 nm.

2 SPIM – technical concept

The visualisation and quantification of biological processes in living organisms require microscopy methods that can give 3-dimensional fluorescence data with high spatial and temporal resolution but at the same time minimize the phototoxic effects. The light sheet based fluorescence microscopy (LSFM), or SPIM (from Selective Plane Illumination Microscopy), has, especially for developmental biology studies [3], in the last years proven to be a strong alternative to widespread confocal and multiphoton microscopy techniques.

Just as in ultramicroscopy [4–6], SPIM is a combination of conventional wide-field microscopy and optical sectioning of different focal planes in the object, based on a separation of the illumination and the detection axis. The main features of light sheet microscopy are shown in **figure 1 and 2**. Contrary to conventional microscopy methods, the illumination of the sample is perpendicular to the optical detection axis. As a result, only the fluorophores in the direct vicinity to the focal plane of the detection lens are excited. The bleaching of fluorophores outside the focal plane can therefore be reduced compared to confocal microscopy, for thick samples like fish embryos perhaps as many as several hundred times reduced, and at the same time higher contrast images can be obtained. The emitted photons are collected by the detection lens and imaged on a camera chip with the help of a tube lens. Due to the high quantum efficiency of modern CCD and CMOS cameras, high frame rates with low noise components and high bit depths are possible.

3 Application example – fruit fly

The fruit fly, Drosophila melanogaster, is a highly studied model organism. The first 13 nuclear divisions occur in the absence of cytokinesis yielding roughly 6000 nuclei in the common cytoplasm (syncytium). The short time interval between divisions and

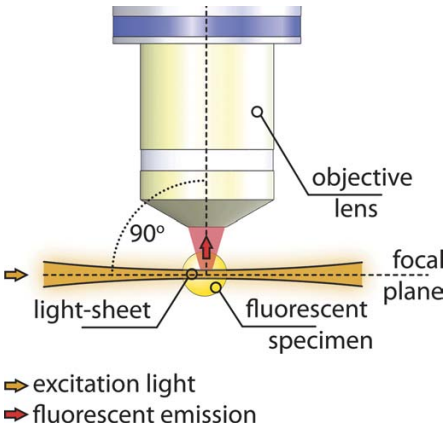

Figure 1: Functional principle of a light sheet fluorescence microscope. Fluorophores in the embryo are illuminated from the side with a just a few µm thick light sheet. The emitted photons are collected by the detection lens perpendicular to the illumination plane. The focal plane of this lens coincides with the illumination plane

the intrinsic autofluorescence of the yolk (vitellus) are challenges for live images. The vitelline membrane and the yolk are the main causes for autofluorescence in the fruit fly embryo [7]. When using blue to yellow fluorescent proteins (e.g. ECFP, EGFP and EYFP), the required excitation wavelengths in the blue or blue-green spectral region (<520 nm) generate a broad autofluorescence emission spectrum (up to 570 nm), which often has a considerable negative impact on the imaging quality. In the red spectral region, on the other hand, the contribution from autofluorescence is very small.

By choosing longer wavelength fluorophores (e.g. mCherry, mRFP – red fluorescent protein [1,2]) in combination with a suitable excitation laser wavelength, both the scattered light and the autofluorescence can be reduced. **Figure 3** shows the chronological sequence of the cleavage divisions in an early embryonic stage of the fruit fly.

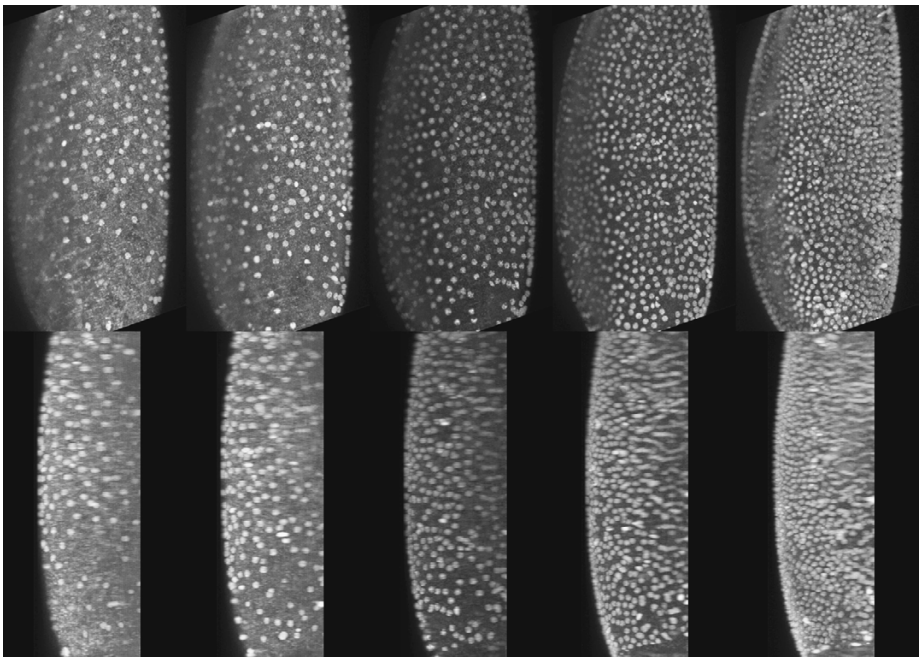

Figure 3: Fruit fly embryo in mitotic cleavage cycles 10 to 14 (from left to right). Histone proteins enclosed by the cell nuclei are marked with red fluorescent protein (H2Av-mCherry). The excitation wavelength is 594 nm, the emission is filtered by a 641/75 nm bandpass filter. The density of the nucleus is increasing with each cell division. Above the view along the apical-basal axis. Shown is the local background-corrected maximum intensity projection of the 3D data at each point in time. The fluorescent proteins in the embryo are dorsally excited (in the picture from right). Below the dorsoventral view at the respective points in time. The resolution is a factor 6 lower than above, and the image has been accordingly interpolated. (Images with EMCCD camera Andor Ixon 885, detection objective 20X NA=0.5, light sheet thickness 4 µm, fly strain Bloomington No. 23651)

4 Orange laser light source

Diode-pumped solid-state lasers (DPSSLs) are a proven and mature technology for accessing the visible part of the spectrum so often required to excite targeted fluorophores typically used in cell biology. Moreover, when designed to operate single longitudinal mode (SLM) they can provide exceptionally low noise and stable performance. The near infrared (NIR) fundamental laser wavelength is converted to the visible in a periodically poled non-linear frequency conversion crystal in a process commonly known as either second harmonic generation (SHG) or sum frequency generation (SFG). SHG is the least complex of the two processes but can not be used to access all wavelengths, e.g. in orange, in which case SFG can then be applied.

By selecting a non-linear frequency conversion crystal with a large non-linearity, in this case a periodically poled potassium titanyl phosphate (PPKTP) crystal (**figure 4**), and in combination with the high intra-cavity beam intensity, the resulting inceased conversion efficiency is leading to higher output powers compared with other crystals. Inherent also in the design, such lasers have very low-noise (<0.3% rms) and high beam profile quality (TEM_{00}, M^2<1.1). Their long-term power stability is also extremely good (**figure 5**, intensity variations <2% over 8 hours) and is considered an important condition for the day- or weeklong observation of cell development in living organisms.

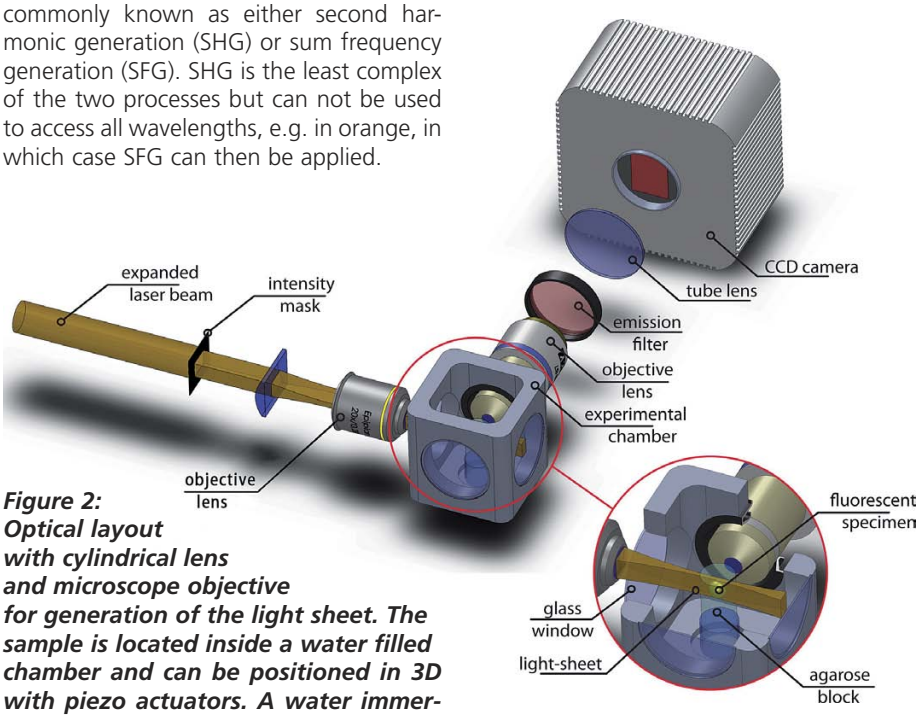

Figure 2: Optical layout with cylindrical lens and microscope objective for generation of the light sheet. The sample is located inside a water filled chamber and can be positioned in 3D with piezo actuators. A water immersion objective with tube lens and emission filter generates the camera image

5 Conclusion

A method such as light sheet fluorescence microscopy has important strengths for the study of biological processes in living organisms. However, in practice the method is only as good as the laser used to excite the fluorophore. In the meantime, not only microscopy techniques such as 3D live cell imaging, but also other analytical methods like flow cytometry, benefit from the development of 594 nm DPSS lasers with adequately high output power.

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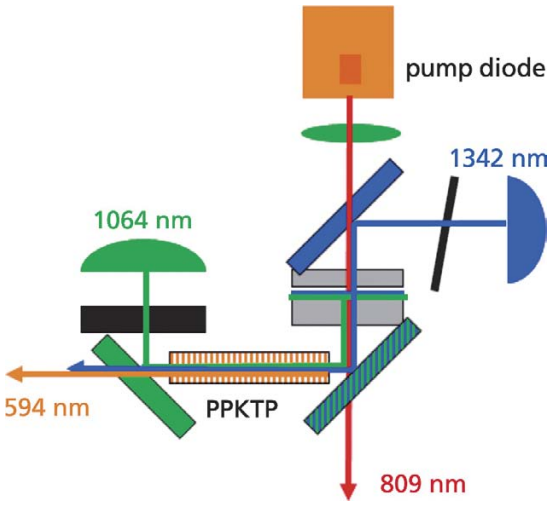

Figure 4: Functional principle of a DPSS laser with sum-frequency generation of 594 nm in a PPKTP crystal

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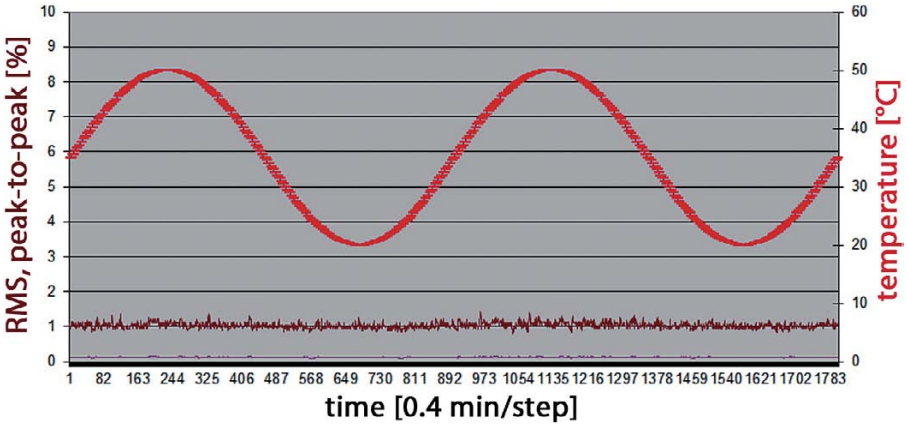

Figure 5: Typical long-term intensity noise of a 594 nm DPSS-laser as a function of temperature at thermal cycles from 20 to 50°C in 12 hour periods

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