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# *In vivo* imaging of zebrafish embryogenesis

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ABSTRACT

The zebrafish *Danio rerio* has emerged as a powerful vertebrate model system that lends itself particularly well to quantitative investigations with live imaging approaches, owing to its exceptionally high optical clarity in embryonic and larval stages. Recent advances in light microscopy technology enable comprehensive analyses of cellular dynamics during zebrafish embryonic development, systematic mapping of gene expression dynamics, quantitative reconstruction of mutant phenotypes and the system-level biophysical study of morphogenesis.

Despite these technical breakthroughs, it remains challenging to design and implement experiments for *in vivo* long-term imaging at high spatio-temporal resolution. This article discusses the fundamental challenges in zebrafish long-term live imaging, provides experimental protocols and highlights key properties and capabilities of advanced fluorescence microscopes. The article focuses in particular on experimental assays based on light sheet-based fluorescence microscopy, an emerging imaging technology that achieves exceptionally high imaging speeds and excellent signal-to-noise ratios, while minimizing light-induced damage to the specimen. This unique combination of capabilities makes light sheet microscopy an indispensable tool for the *in vivo* long-term imaging of large developing organisms.



## 1. Introduction

The ability to acquire and quantitatively analyze light microscopy data of living biological organisms is crucial in many areas of the life sciences. However, live imaging of large multicellular specimens is fundamentally constrained by the limited optical penetration depth of light microscopy. In many model systems, light scattering and light absorption obscure the view of the inside of the specimen and preclude functional imaging or the live observation of developmental processes. Zebrafish embryos and larvae, which are highly transparent, are a remarkable exception. They offer the exciting opportunity to study development and function in a highly complex vertebrate model system by live imaging.

By using standard light microscopy techniques, detailed morphogenetic information can be obtained on the cellular and subcellular levels in a completely non-invasive manner [1]. By using advanced imaging approaches optimized for high-speed long-term imaging, it is even possible to study highly dynamic morphological processes over long periods of time and from the whole-embryo perspective, to obtain comprehensive gene expression atlases with high spatio-temporal resolution, to follow the formation of morphological defects in mutants, to reconstruct the dynamic building plans of entire organs and tissues, and to assess the level of morphogenetic variability between different embryos [2,3].

## 2. Challenges in long-term live imaging experiments

Several conflicting requirements need to be balanced in *in vivo* imaging applications. It is often desirable to achieve high imaging speed, low photo-bleaching and photo-toxicity, good three-dimensional resolution, high signal-to-noise ratio and excellent physical coverage at the same time. The latter aspect is particularly difficult to realize for large biological specimens, since limitations in optical penetration depth usually make it impossible to extract comprehensive information of the sample from a single view.

Some forms of light microscopy provide good overall performance, whereas others excel in one area by trading off performance in other areas. Conventional wide-field fluorescence microscopes provide high imaging speed and a good signal-to-noise ratio, but lack optical sectioning capability and are therefore unsuited for three-dimensional imaging. Point-scanning confocal fluorescence microscopes provide good spatial resolution and the intrinsic capability to eliminate scattered light [4], but perform poorly with respect to imaging speed, signal-to-noise ratio, photo-bleaching/toxicity and penetration depth. Point-scanning two-photon microscopes improve over confocal fluorescence microscopes with respect to penetration depth and usually also photo-bleaching/toxicity [5,6], but further reduce signal-to-noise ratio and imaging speed. Spinning disk confocal microscopes provide higher imaging speed than (single)

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point-scanning confocal fluorescence microscopes [7], but reduce spatial resolution.

To address the limitations arising from the intrinsic performance trade-offs encountered in conventional light microscopy techniques, new light sheet-based fluorescence microscopy techniques have been developed over the course of the past decade. Light sheet-based fluorescence microscopy, which will be discussed in Section 3, provides a particularly powerful approach to biological live imaging. Such microscopes combine intrinsic optical sectioning capability with high imaging speeds, good signal-to-noise ratio and low light exposure of the specimen [8–11].

A particular strength of this unique combination of capabilities is the possibility to study embryonic development on the system level while preserving the ability to follow cellular processes at the high spatio-temporal resolution required for global cell tracking [3]. For example, the reconstruction of zebrafish embryogenesis during the first 24 h of development requires the simultaneous tracking of several tens of thousands of cells, e.g. by fast long-term imaging of a nuclear fluorescent marker expressed in all cells [2]. High spatio-temporal resolution, low photo-bleaching rates, and an excellent signal-to-noise ratio are crucial. A volume of $\sim$1 mm$^3$ must be recorded approximately once per minute to keep track of cell identities, since cells move in average several micrometer per minute. In order to reliably detect all nuclei, good spatial resolution and axial sampling are necessary, which requires acquiring several hundred images at each time point to achieve full coverage of the embryo. Overall, the observation must thus be performed at a continuous imaging speed of at least 10 million volume elements (voxels) per second, which is approximately one order of magnitude faster than the typical acquisition speed of conventional point-scanning confocal or two-photon microscopes [2]. At the same time, a high dynamic range is required to address the varying expression levels of genetically encoded markers and signal heterogeneity due to signal degradation with increasing imaging depth into the embryo. The embryo's central yolk cell is opaque at visible wavelengths and imaging along multiple directions (providing a set of complementary views of the embryo) is needed to capture the development of the entire embryo. Finally, photobleaching and photo-toxicity [12] must be minimized to ensure the physiological development of the embryo [2].

Point-scanning confocal fluorescence microscopy is still the standard imaging technology used in most labs, and advanced light sheet fluorescence microscopy technology has become commercially available only very recently. However, the latter method provides a number of key advantages, which are of particular importance in long-term live imaging experiments. A comparison of both techniques is therefore provided in Section 4, which summarizes and expands on a previous discussion of these methods [34]. Two-photon fluorescence microscopy is not discussed as a separate method in this comparison, since two-photon excitation can effectively be implemented in both imaging techniques mentioned above [13–15].

## 3. Light sheet microscopy

### 3.1. Introduction to light sheet microscopy

Conventional and cofocal epi-fluorescence microscopes employ the same lens for fluorescence excitation and detection. In contrast, light sheet microscopes rely on the principle of sample illumination with a planar light sheet perpendicular to the axis of fluorescence detection (Fig. 1A) [16–18]. The light sheet is coplanar with the focal plane of the fluorescence detection system. This approach directly provides optical sectioning: Fluorophores are only excited in the illuminated plane and thus photo-bleaching and other types of photo-induced damage are avoided outside the thin volume of interest. This circumstance gives light sheet microscopes a decisive advantage in the fast imaging of sensitive biological specimens as well as in *in vivo* imaging over long periods of time [2,15,18–21].

Light sheet microscopes are furthermore particularly well suited for "multi-view imaging", which refers to the strategy of observing the same specimen along multiple different directions. Thereby, parts of the specimen become visible that would otherwise be hidden or obscured in the observation along a single direction [22]. The next two sections discuss the building blocks of a basic light sheet-based microscope [2], which is particularly cost-efficient yet very effective, as well as an advanced light sheet microscope for simultaneous multi-view imaging [15], which provides excellent physical coverage even of very large biological specimens.

### 3.2. Basic implementation of scanned light sheet microscopy

Different approaches have been developed to facilitate light sheet illumination, including the use of cylindrical optics to focus a Gaussian laser beam into a sheet of light [17,18] and fast laser-scanning of a laser beam that enters the specimen chamber from the side [2]. In scanned light sheet microscopy (DSLM), a "light sheet" is generated with a laser scanner that rapidly moves a micrometer-thin beam of laser light vertically through the specimen (Fig. 1A) [2]. The specimen is positioned in front of a second lens, the lens for fluorescence detection, and typically embedded in an aqueous gel [18] or in a transparent plastic compartment [19,23].

The basic DSLM implementation is shown in Fig. 1B. The DSLM illumination system comprises a multi-line argon-krypton laser (Melles Griot, 35 LTL 835–230), an acousto-optical tunable filter for laser wavelength selection and intensity control (AA Opto-Electronic, AA.AOTF.nC-400–650 nm-PV-TN), a two-axis high-speed scan head (GSI Lumonics, VM500+), an f-theta lens (Sill Optics, S4LFT0061/065*) and a low-NA illumination objective lens (Carl Zeiss, Plan-Apochromat 5×/0.16) operated with a regular tube lens. The illumination/excitation objective lens is mounted on a piezo nanofocus (Physik Instrumente, P-725.CLQ), which can move the lens by up to 400 μm along its optical axis [2].

The DSLM detection system can take advantage of several detection objective lenses (in particular Carl Zeiss C-Apochromat 10×/0.45 W and Plan-Apochromat 20×/1.0 W for zebrafish whole-embryo imaging) mounted on a second independently operated piezo nano-focus, a filter wheel (Ludl) with exchangeable long-pass filters (Semrock, RazorEdge RU 488 LP, RU 568 LP and RU 647 LP) and a detection tube, equipped with a tube lens and a CCD camera (PCO, pco.2000) [2].

### 3.3. Advanced implementation of light sheet microscopy for simultaneous multi-view imaging

The SiMView light sheet microscopy platform provides exceptionally high imaging speeds and allows simultaneous acquisition of four complementary views of the specimen for optimal physical coverage [15]. In order to realize simultaneous multi-view imaging, SiMView uses an orthogonal arrangement of four independently operated optical arms (Fig. 1C, Video 1). One pair of opposite arms is used for bi-directional light sheet illumination with two long working distance air objectives, similar to the illumination arrangement used in other light sheet microscopes [14,24,25]. The other pair, arranged at a right angle to the first, is used for bi-directional fluorescence detection with high numerical aperture water-dipping objectives and fast sCMOS cameras [15].

In addition to the illumination and detection arms, the SiMView microscope platform includes a laser light source, a four-view specimen chamber and a four-axis specimen positioning system (Fig. 1C, Video 1).