

¹ A new angle on light-sheet microscopy
² and real-time image processing

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¹ Abstract

² Light-sheet fluorescence microscopy, also called single plane illumination microscopy, has
³ numerously proven its usefulness for long term imaging of embryonic development. This
⁴ thesis tackles two challenges of light-sheet microscopy: high resolution isotropic imaging
⁵ of delicate, light-sensitive samples, and real-time image processing and compression of
⁶ light-sheet microscopy images.

⁷ A symmetric light-sheet microscope is presented, featuring two high numerical aperture
⁸ objectives arranged in 120°. Both objectives are capable of illuminating the sample
⁹ with a tilted light-sheet and detecting the fluorescence signal. This configuration allows
¹⁰ for multi-view, isotropic imaging of delicate samples where rotation is not possible. The
¹¹ optical properties of the microscope are characterized, and its imaging capabilities are
¹² demonstrated on *Drosophila melanogaster* embryos and mouse zygotes.

¹³ To address the big data problem of light-sheet microscopy, a real-time, GPU-based
¹⁴ image processing pipeline is presented. Alongside its capability of performing commonly
¹⁵ required preprocessing tasks, such as image fusion of opposing views immediately during
¹⁶ image acquisition, it also contains a novel, high-speed image compression method. This
¹⁷ algorithm is suitable for both lossless and noise-dependent lossy image compression, the
¹⁸ latter allowing for a significantly increased compression ratio, without affecting the results
¹⁹ of any further analysis. A detailed performance analysis is presented of the different
²⁰ compression modes for various biological samples and imaging modalities.

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¹ List of Abbreviations

- ² **2D** two-dimensional.
- ³ **3D** three-dimensional.
- ⁴ **BFP** back focal plane.
- ⁵ **CCD** charge coupled device.
- ⁶ **CLSM** confocal laser scanning microscopy.
- ⁷ **CMOS** complementary metal oxide semiconductor.
- ⁸ **CPU** central processing unit.
- ⁹ **DCT** discrete cosine transform.
- ¹⁰ **DFT** discrete fourier transform.
- ¹¹ **DPCM** differential pulse code modulation.
- ¹² **DSLM** digitally scanned light-sheet microscopy.
- ¹³ **dSTORM** direct stochastic optical reconstruction microscopy.
- ¹⁴ **DWT** discrete wavelet transform.
- ¹⁵ **eCSD** electronic confocal slit detection.
- ¹⁶ **EM-CCD** electron multiplying CCD.
- ¹⁷ **FEP** fluorinated ethylene propylene.
- ¹⁸ **FN** field number.
- ¹⁹ **FOV** field of view.
- ²⁰ **FPGA** field programmable gate array.
- ²¹ **fps** frames per second.
- ²² **FWHM** full width at half maximum.
- ²³ **GFP** green fluorescent protein.
- ²⁴ **GPU** graphics processing unit.
- ²⁵ **HEVC** high efficiency video codec.
- ²⁶ **ICM** inner cell mass.

- ¹ **JPEG** joint photographic experts group.
- ² **LSFM** light-sheet fluorescence microscopy.
- ³ **MIAM** multi-imaging axis microscopy.
- ⁴ **MuVi-SPIM** Multiview SPIM.
- ⁵ **NA** numerical aperture.
- ⁶ **OPFOS** orthogonal plane fluorescent optical sectioning.
- ⁷ **OTF** optical transfer function.
- ⁸ **PEEK** polyether ether ketone.
- ⁹ **PSF** point spread function.
- ¹⁰ **RESOLFT** reversible saturable optical fluorescence transitions.
- ¹¹ **ROI** region of interest.
- ¹² **sCMOS** scientific CMOS.
- ¹³ **SMLM** single molecule localization microscopy.
- ¹⁴ **SNR** signal-to-noise ratio.
- ¹⁵ **SPIM** single plane illumination microscopy.
- ¹⁶ **STED** Stimulated Emission Depletion.
- ¹⁷ **TE** trophoectoderm.
- ¹⁸ **TLSM** thin light-sheet microscopy.
- ¹⁹ **WNL** within noise level.
- ²⁰ **YFP** yellow fluorescent protein.

¹ Introduction

2 Imaging techniques are one of the most extensively used tools in medical and biological
3 research. The reason for this is simple: visualizing something invisible to the naked eye is
4 an extremely powerful way to gain insight into its inner workings. Our brain has evolved
5 to receive and process a multitude of signals from various sensors, and arguably the most
6 powerful of these is vision.

7 As a branch of optics, microscopy (from ancient Greek *mikros*, “small” and *skopein*,
8 “to see”) is based on observing the interactions of light with an object of interest, such
9 as a cell. To be able to see these interactions, the optics of the microscope magnifies the
10 image of the sample, which can be recorded on a suitable device. For the first microscopes
11 in the 17th century, this was just an eye at the end of the ocular, and the recording was
12 a drawing of the observed image [1].

13 Microscopy is a truly multidisciplinary field: even in its simplest form, just using
14 a single lens, the principles of physics are applied to gain a deeper understanding of
15 biology and nature. Today, microscopy encompasses most of natural sciences and builds
16 on various technological advancements. While physics and biology are still in the main
17 focus, chemistry (fluorescent molecules), engineering (automation) and computer science
18 (image analysis) are all integrated in a modern microscopy environment.

19 Light-sheet fluorescence microscopy (LSFM), also called single plane illumination
20 microscopy (SPIM), is a relatively new addition to the arsenal of tools that comprise light
21 microscopy methods, and is especially suitable for live imaging of embryonic samples over
22 extended periods of time [2–5]. It is also easily adapted to the sample, allowing to image
23 a large variety of specimens, from entire organs [6], to the subcellular processes occurring
24 inside cultured cells [7].

25 This work tackles two challenges in light-sheet microscopy: high-resolution live imaging
26 of delicate samples, such as mouse embryos, and real-time image processing and
27 compression of large light-sheet datasets. Before discussing the work in detail, Chapter 1
28 and Chapter 2 will give a short introduction to the concepts this thesis builds on. The
29 basics of fluorescence microscopy, light-sheet microscopy, information theory and image
30 compression will be covered.

31 Chapter 3 is devoted to presenting a new light-sheet microscope, the Dual Mouse-

1 SPIM, designed for isotropic imaging of light-sensitive specimens. This microscope is
2 based on two identical objectives positioned in 120° as opposed to the conventional 90°
3 orientation used in light-sheet microscopy, and it offers dual-view detection through both
4 lenses. We discuss the benefits of this arrangement and the design principles and optical
5 layout of the microscope. After characterizing the optical properties of the microscope,
6 we demonstrate its imaging capabilities on various samples.

7 In Chapter 4 we present a GPU-based real-time image processing pipeline designed
8 to efficiently handle large amounts of microscopy data. As 3D imaging is gaining more
9 and more traction, image datasets are generated at a faster pace than ever. We present
10 a real-time preprocessing solution for multiview light-sheet microscopy, and a new im-
11 age compression algorithm to significantly reduce data size by taking image noise into
12 account. The theory behind these methods will be discussed before demonstrating their
13 capabilities and evaluating their performance on multiple biological samples.

14 Finally, Chapter 5 will give a summary of the new results presented in this thesis,
15 and discuss the potential future applications.

¹ Chapter 1

² Live imaging in three dimensions

³ Live imaging is indispensable to understand the processes during embryonic development.
⁴ In an ideal setting, the ultimate microscope would be able to record a continuous, three
⁵ dimensional (3D), multicolor dataset of any biological process of interest with the highest
⁶ possible resolution. Due to several limitations in physics and biology this is not possible.
⁷ Therefore, a compromise is necessary. The diffractive nature of light, the lifetime of
⁸ fluorescent probes and the photosensitivity of biological specimens all require microscopy
⁹ to be adapted so that the question at hand may be answered. In order to acquire useful
¹⁰ data one has to choose a tradeoff between spatial and temporal resolution and signal
¹¹ contrast, while making sure the biology is not affected by the imaging process itself
¹² (Figure 1.1) [8].

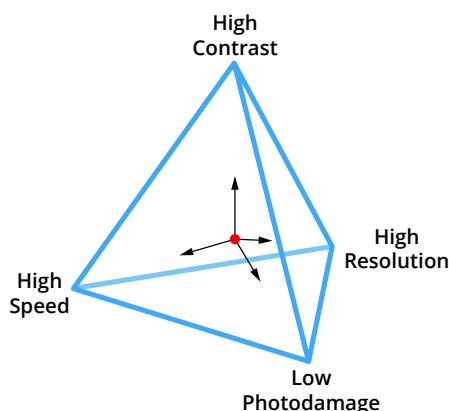


Figure 1.1: Tradeoffs in fluorescence microscopy for live imaging. Also called the “pyramid of frustration”. When optimizing the imaging conditions (red dot), a tradeoff has to be made between resolution, contrast, and imaging speed, while avoiding photodamage. One can only be improved at the expense of the others due to the limited photon budget of the fluorescent molecules. Adapted from [8].

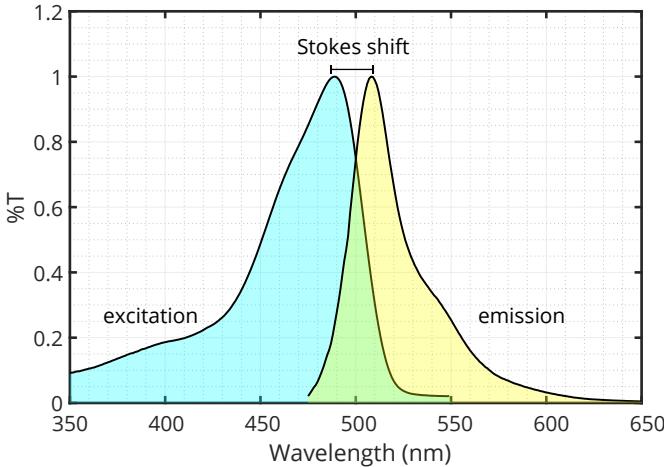


Figure 1.2: Excitation and emission spectrum of enhanced green fluorescent protein (EGFP). Excitation spectrum in blue, emission spectrum in yellow. The separation between the two spectra is due to the Stokes shift, which is 19 nm for EGFP. Emission and excitation light can be separated by a long-pass filter at 500 nm. Data from [12].

¹ 1.1 Wide-field fluorescence microscopy

Fluorescence microscopy [9, 10], as a subset of light microscopy, is one of the few methods that allow subcellular imaging of live specimens with specific labeling of structures of interest. The first use of the term fluorescence is credited to George Gabriel Stokes [11], and it refers to the phenomenon of light emission following the absorption of light or other electromagnetic radiation. As the name of the technique suggests, this method collects fluorescent light from the specimens. Since biological tissues are usually not fluorescent, except for some autofluorescence mostly at shorter wavelengths, fluorescent dyes or proteins have to be introduced to the system in order to be able to collect the necessary information. The advantage of this is that the signal of the labeled structures will be of very high ratio compared to the background.

A fluorescent molecule is capable of absorbing photons in a given wavelength range (excitation spectrum) and temporarily store its energy by having an electron in a higher energy state, *i.e.*, in an excited state. This excited state, however, is not stable, and the electron quickly returns to the ground state while emitting a photon. The energy of the absorbed and emitted photons are not the same, as energy loss occurs due to internal relaxation events, and the emitted photon has lower energy than the absorbed photon. This phenomenon is called the Stokes shift, or red shift, and can be exploited in microscopy to drastically increase the signal-to-noise ratio by filtering out the illumination light (Figure 1.2).

1 1.1.1 Fluorescent proteins

2 Traditionally, synthetic fluorescent dyes were used to label certain structures in the
3 specimens. Some of these directly bind to their target, and others can be used when
4 conjugated to an antibody specific to the structure or protein of interest. A requirement
5 for these methods is that the fluorescent label has to be added to the sample from an
6 external source, and, in many cases, this also necessitates sample preparation techniques
7 incompatible with live imaging, such as fixation [13].

8 The discovery of fluorescent proteins has revolutionized fluorescence microscopy.
9 Since these molecules are proteins, they can be produced directly by the organism if
10 the proper genetic modifications are performed. Even though this was a hurdle at the
11 time of discovering the green fluorescent protein (GFP) [14], genetic engineering tech-
12 niques evolved since then [15], and not only has its gene been successfully integrated
13 in the genome of a multitude of organisms [16–18], but many variants have been also
14 engineered by introducing mutations to increase fluorescence intensity, and to change the
15 fluorescence spectrum to allow multicolor imaging [18–21]. The usefulness and impact of
16 these proteins are so profound, that in 2008 the Nobel Prize in chemistry was awarded to
17 Osamu Shimomura, Martin Chalfie, and Roger Tsien “for the discovery and development
18 of the green fluorescent protein, GFP” [22].

19 1.1.2 Wide-field image formation

20 By imaging fluorescently labelled specimens, a wide-field fluorescence microscope has the
21 capability of discriminating illumination light from emitted fluorescent light due to the
22 Stokes shift described in the previous section. The microscope’s operating principle is
23 depicted in Figure 1.3.

24 Light from a source, typically a mercury lamp is focused on the back focal plane of
25 the objective to create even illumination of the sample. Before entering the objective,
26 the light is filtered, so only the wavelengths that correspond to the excitation properties
27 of the observed fluorophores are transmitted. Since the same objective is used for both
28 illumination and detection, a dichroic mirror is utilized to decouple the illumination
29 and detection paths. The emitted light is filtered again to make sure any reflected and
30 scattered light from the illumination source is blocked to increase signal-to-noise ratio.
31 Finally, light is focused by a tube lens to create a magnified image on the camera sensor.

32 This type of arrangement is called infinity-corrected optics, since the back focal point
33 of the objective is in “infinity”, meaning that the light from a point source exiting the back
34 aperture is parallel. This is achieved by placing the sample exactly at the focal point of
35 the objective. Infinity-corrected optics have the advantage that they allow placing various
36 additional optical elements in the infinity space, (*i.e.*, the space between the objective
37 and the tube lens) without affecting the image quality. In this example such elements

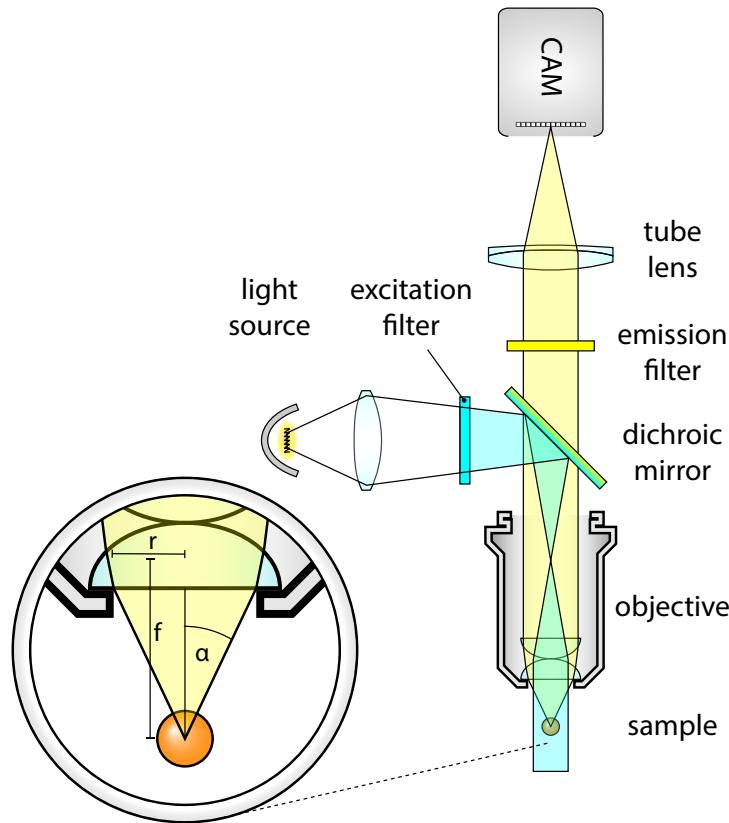


Figure 1.3: Wide-field fluorescence microscope. The light source is focused on the back focal plane of the objective to provide an even illumination to the sample. Emitted photons are collected by the objective, and are separated from the illumination light by a dichroic mirror. Inset: Light collection of an objective lens. α : light collection half-angle; f : focal length; r : radius of aperture.

- 1 are the dichroic mirror and the emission filter.
- 2 The combination of the objective and tube lens together will determine the optical
- 3 magnification of the system, which will be the ratio of the focal lengths of these lenses:

$$M = \frac{f_{TL}}{f_{OBJ}}. \quad (1.1)$$

- 4 The final field of view (FOV) of the microscope will depend on the magnification, and
- 5 also on the size of the imaging sensor (D), and the objective field number (FN , specified
- 6 by the manufacturer, the diameter of the view field in the image plane):

$$FOV = \frac{\min(D, FN)}{M}. \quad (1.2)$$

- 7 Apart from the magnification, the most important property of the objective is the
- 8 half-angle of the light acceptance cone, α (Figure 1.3, inset). This not only determines
- 9 the amount of collected light, but also the achievable resolution of the system (see Sec-
- 10 tion 1.1.3). This angle depends on the size of the lens relative to its focal length. In other

¹ words it depends on the aperture of the lens, which is why the expression *numerical*
² *aperture* (NA) is more commonly used to express this property of the objective:

$$\text{NA} = n \cdot \sin \alpha. \quad (1.3)$$

³ where n is the refractive index of the medium, and describes the light propagation speed
⁴ in the medium relative to the speed of light in vacuum. For air vacuum and air, $n = 1$,
⁵ for water $n_{\text{H}_2\text{O}} = 1.33$ and for commonly used optical glass $n_{\text{BK7}} = 1.52$.

⁶ For small α angles, the following approximation holds true: $\sin \alpha \approx \tan \alpha \approx \alpha$. Thus,
⁷ the numerical aperture can also be expressed as a ratio of the radius of the lens and the
⁸ focal length:

$$\text{NA} \approx n \frac{r}{f}, \quad \text{when } \alpha \ll 1. \quad (1.4)$$

⁹ 1.1.3 Resolution of a wide-field microscope

¹⁰ The resolution of an optical system depends on the size of the smallest distinguishable
¹¹ feature on the image. A practical way of quantifying this is by measuring the smallest
¹² resolved distance, *i.e.*, the minimum distance between two point-like objects so that the
¹³ two objects can still be distinguished. This mainly depends on two factors: the NA of
¹⁴ the objective, and the pixel size of the imaging sensor.

¹⁵ Even if the imaging sensor would have infinitely fine resolution, it is not possible to
¹⁶ reach arbitrarily high resolutions due to the wave nature of light and diffraction effects
¹⁷ that occur at the aperture of the objective. This means that depending on the wavelength
¹⁸ of the light, any point source will have a finite size on the image, it will be spread out,
¹⁹ limiting the resolution. The shape of this image is called the *point spread function*, or
²⁰ PSF (Figure 1.4), as this function describes the behavior of the optical system when
²¹ imaging a point-like source. This property of lenses was already discovered by Abbe in
²² 1873 [23], when he constructed his famous formula:

$$\delta = \frac{\lambda}{2 \cdot \text{NA}}. \quad (1.5)$$

²³ where δ is the smallest distance between two distinguishable features.

²⁴ Another representation of the optical performance, is the *optical transfer function*,
²⁵ or OTF (Figure 1.4), which is the Fourier transform of the PSF:

$$\text{OTF} = \mathcal{F}(\text{PSF}). \quad (1.6)$$

²⁶ As this function operates in the frequency space, it describes how the different frequencies
²⁷ are affected by the system. The resolution can also be defined as the support of the OTF,
²⁸ since this describes the highest frequency that is still transmitted by the optical system.

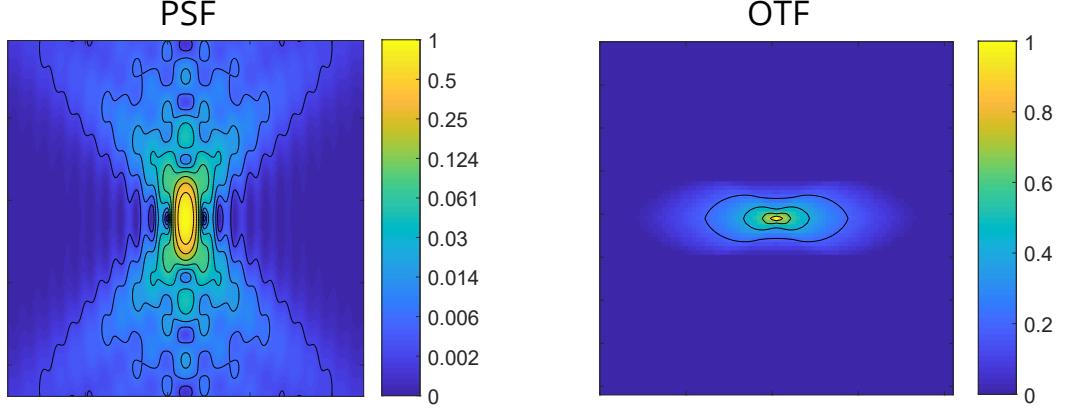


Figure 1.4: Axial cross section of the PSF and OTF of a wide-field microscope. Simulated PSF (left) and OTF (right) for a wide-field microscope with a water immersion objective ($n = 1.33$). $NA = 1.1$, $\lambda = 510 \text{ nm}$

1 Any pattern with higher frequency will be lost, thus lies beyond the resolution limit. For
 2 circularly symmetric PSFs, the OTF will have real values. However, if this is not the
 3 case, the Fourier transform also introduces complex components.

4 Abbe's formula can be derived from the scalar theory of diffraction using a paraxial
 5 approximation (Fraunhofer diffraction, [24]). It is useful to define the following optical
 6 coordinates instead of the commonly used Cartesian coordinates x , y and z :

$$v = \frac{2\pi nr}{\lambda_0} \sin \alpha, \quad u = \frac{8\pi nz}{\lambda_0} \sin^2 \frac{\alpha}{2} \quad (1.7)$$

7 where $r = \sqrt{x^2 + y^2}$ is the distance from the optical axis, and α is the light collection
 8 angle as shown on Figure 1.3. In this system the intensity of the electric field in the focus
 9 of a lens is [25]:

$$H(u, v) = C_0 \left| \int_0^1 J_0(vr) e^{-i\frac{1}{2} \cdot ur^2} r dr \right|^2, \quad (1.8)$$

10 where C_0 is a normalization constant, and J_0 is the zeroth order Bessel function of the
 11 first kind.

12 To determine the lateral resolution of the system, let's substitute $u = 0$ as the axial
 13 optical coordinate, and evaluate Equation 1.8 which will give the intensity distribution
 14 in the focal plane:

$$H(0, v) = C_0 \left| \int_0^1 J_0(vr) r dr \right|^2 = \left(2 \frac{J_1(v)}{v} \right)^2, \quad (1.9)$$

15

16 where J_1 is the first order Bessel function of the first kind. This equation describes the

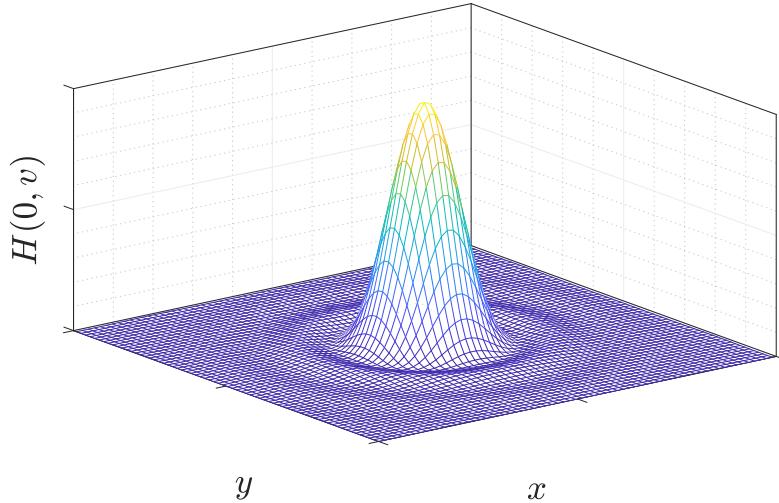


Figure 1.5: Airy pattern. Airy pattern calculated in Matlab based on Equation 1.9

1 famous Airy pattern (Figure 1.5) which will be the shape of the PSF in the focal plane.
 2 The width of this pattern is the resolution, and although there are multiple definitions
 3 for this, the most commonly accepted is the Rayleigh criterion [24, 26] which defines
 4 the resolution as the distance between the central peak and the first local minimum.
 5 As this lies at $v = 3.38$, the resolution can be expressed by substituting this value into
 6 Equation 1.7 and solving it for r :

$$\delta_{xy} = r(v = 0.338) = \frac{3.83}{2\pi} \frac{\lambda_0}{n \cdot \sin \alpha} \approx 0.61 \frac{\lambda_0}{NA}, \quad (1.10)$$

7 which is equivalent to Abbe's original formula (Equation 1.5). The only difference is the
 8 scaling factor which is due to the slightly different interpretations of width of the Airy
 9 disk as mentioned earlier.

10 Similarly, to calculate the intensity distribution along the axial direction, let's sub-
 11 stitute $v = 0$ to Equation 1.8:

$$H(u, 0) = C_0 \left(\frac{\sin \frac{u}{4}}{\frac{u}{4}} \right)^2. \quad (1.11)$$

12 For this expression the first minimum lies at $u = 4\pi$. Converting back to Cartesian
 13 coordinates, the axial resolution can be expressed as:

$$\delta_z = \frac{2n\lambda_0}{NA^2}. \quad (1.12)$$

14 So far we only considered a single, point-like emitter. As the intensity function de-
 15 scribes how an optical system “spreads out” the image of a point, it is also called the
 16 Point Spread Function (PSF, Figure 1.4). In a more realistic scenario, however the emit-

ters are neither point-like, nor single. Effectively, however, for every emitter the PSF would be imaged on the sensor, and this creates the final image. In mathematical terms, this can be expressed as a convolution operation between the underlying fluorophore distribution of the object (O) and the PSF (H):

$$I(u, v) = O(u, v) * H(u, v). \quad (1.13)$$

The effective result of this kind of diffraction-limited image formation is a blurred image with a finite resolution of δ_{xy} in the lateral direction, and δ_z in the axial direction.

The PSF is further affected by the illumination pattern as well. Since the number of emitted fluorescent photons are roughly proportional to the illumination intensity, the illumination pattern will have an effect on the overall PSF of the system, which can be expressed as:

$$H_{sys} = H_{ill} \cdot H_{det}, \quad (1.14)$$

where H_{ill} is the point spread function of the illumination, and H_{det} is the point spread function of the detection.

1.2 Point scanning methods

In most cases, a wide-field microscope is used to image a layer of cultured cells, or a sectioned sample, thus axial resolution is not a concern. Imaging live specimens, however is not so straightforward, as these samples are usually much thicker than a typical section. For these samples 3-dimensional (3D) imaging is highly beneficial, which necessitates the use of optical sectioning instead of physical sectioning to be able to discriminate the features at different depths.

Due to the design of the wide-field microscope, any photons emitted from outside the focal plane will also be detected by the sensor, however as these are not originating from the focus, only a blur will be visible. This blur potentially degrades image quality and signal-to-noise ratio to such an extent that makes imaging thick samples very difficult if not impossible in a wide-field microscope.

Evaluating Equations 1.10 and 1.12 for a range of possible numerical apertures reveals the significant differences in lateral and axial resolution for any objective (Figure 1.6). Especially for low NAs, this can be significant, a factor of ~ 20 difference. For higher (>0.8) NAs the axial resolution increases faster than the lateral, however they will only be equal when $\alpha = 180^\circ$. This means that isotropic resolution with a single lens is only possible if the lens is collecting all light emitting from the sample, which seems hardly possible, and would be highly impractical. For commonly used high NA objectives the lateral to axial ratio will still be around 3–6.

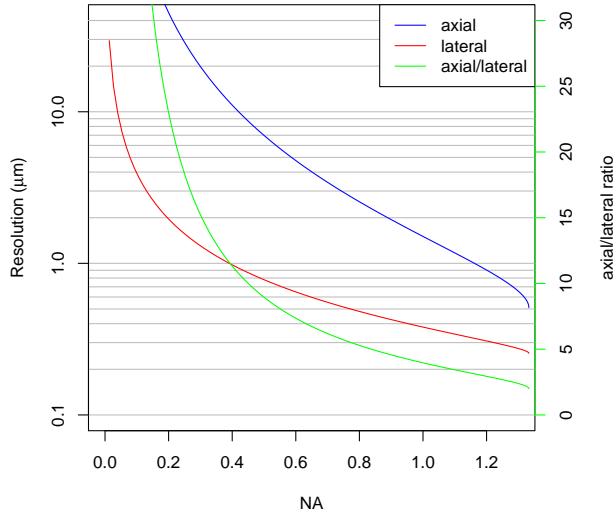


Figure 1.6: Resolution of a wide-field microscope. Axial (blue) and lateral (red) resolutions of a wide-field microscope are shown with respect to the numerical aperture (NA). Resolutions are calculated with $\lambda = 510\text{nm}$, the emission maximum of GFP and $n = 1.33$, the refractive index of water, for water dipping objectives.

Instead of using a single lens to achieve isotropic resolution, it is more practical to image the sample from multiple directions to complement the missing information from different views. When rotating the sample by 90° for example, the lateral direction of the second view will correspond to the axial direction of the first view. If rotation is not possible, using multiple objectives can also achieve similar results, such as in the case of Multi-Imaging Axis Microscopy (MIAM) [27, 28]. This microscope consisted of 4 identical objectives arranged in a tetrahedral fashion to collect as much light as possible from multiple directions, and provide isotropic 3D resolution, albeit at the expense of extremely difficult sample handling, since the sample was surrounded by objectives from all directions.

1.2.1 Confocal laser scanning microscopy

Confocal laser scanning microscopy (CLSM) [29, 30] addresses most of the problems of wide-field microscopy we mentioned in the previous section. It is capable of optical sectioning by rejecting out-of-focus light, which makes it a true 3D imaging technique. Furthermore, the light rejection also massively reduces out-of-focus background, and increases contrast.

This is achieved by two significant modifications compared to the wide-field optical path. To be able to reject the out-of-focus light, an adjustable pinhole is placed at the focus of the tube lens. Light rays originating from the focal point will meet here, and

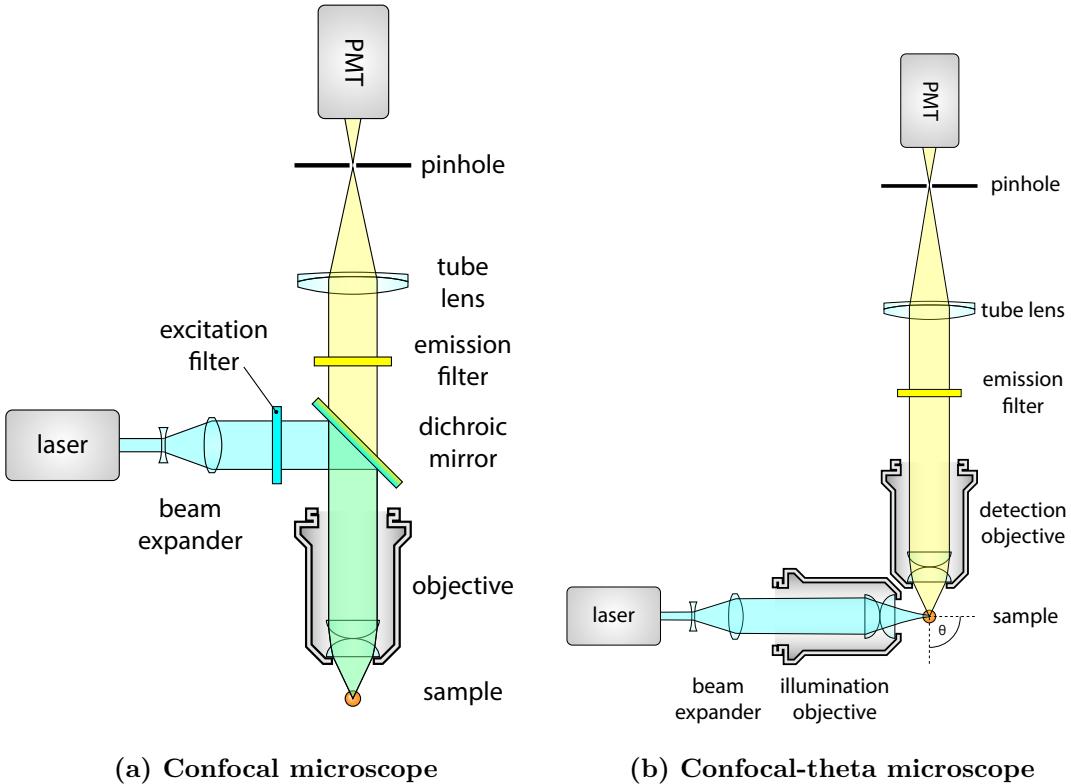


Figure 1.7: Basic optical components of a confocal laser scanning and confocal-theta microscope. Both types of microscopes use confocal image detection, which means that a pinhole is used to exclude light coming from out-of-focus points. Light intensity is measured by a photomultiplier for every point in the region of interest. The final image is generated on a computer using the positions and recorded intensity values. A regular confocal microscope (a) uses the same objective for illumination and detection, while a confocal-theta microscope (b) uses a second objective that is rotated by θ around the focus. In this case, $\theta = 90^\circ$.

1 are able to pass through the pinhole. However, out-of-focus light will converge either
 2 before or after the aperture, and thus the aperture blocks these rays. To maximize the
 3 fluorescence readout efficiency for the single focal point, a photomultiplier tube is used
 4 instead of an area sensor (Figure 1.7a).

5 To maximize the signal from the focal point, the illumination light is also focused
 6 here by coupling an expanded laser beam through the back aperture of the objective.
 7 This not only increases illumination efficiency (since other, not detected points are not
 8 illuminated), but has the added benefit of increasing the resolution as well. This is due
 9 to the combined effect of illumination and detection PSFs as described in Equation 1.14
 10 (Figure 1.8). For Gaussian-like PSFs, the final resolution (along a single direction) can
 11 be calculated in the following way:

$$\frac{1}{\delta_{sys}^2} = \frac{1}{\delta_{ill}^2} + \frac{1}{\delta_{det}^2}, \quad (1.15)$$

12 where δ_{ill} and δ_{det} are the resolutions for the illumination and detection, respectively.

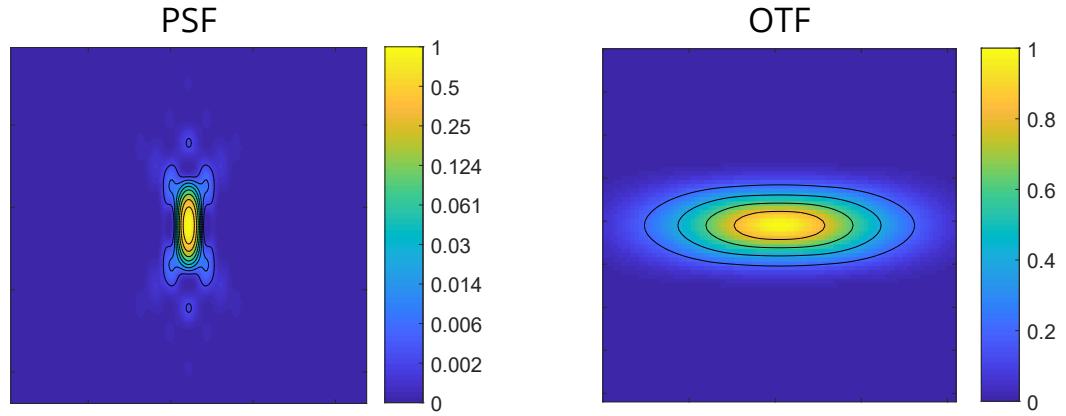


Figure 1.8: Axial cross section of the PSF and OTF of a confocal laser scanning microscope.
Simulated PSF and OTF for a laser scanning confocal microscope with a water immersion objective ($n = 1.33$). $NA = 1.1$, $\lambda = 510 \text{ nm}$

- ¹ Since the same objective is used for both illumination and detection, and the difference
- ² in wavelength is almost negligible, $\delta_{ill} = \delta_{det} = \delta$, the final system resolution will be:

$$\delta_{sys} = \frac{1}{\sqrt{2}}\delta. \quad (1.16)$$

- ³ This means that the distinguishable features in a confocal microscope are ~ 0.7 times
- ⁴ smaller than in a wide-field microscope using the same objective.

⁵ Because of the different detection method in a confocal microscope, direct image
⁶ formation on an area sensor is not possible, since at any given time, only a single point is
⁷ interrogated in the sample. Instead, it is necessary to move the illumination and detection
⁸ point in synchrony (or in a simpler, albeit slower solution, to move the sample) to scan
⁹ the entire field of view. The image can be later computationally reconstructed by a
¹⁰ computer program that records the fluorescence intensity of every point of the field of
¹¹ view, and displays these values as a raster image.

¹² 1.2.2 Confocal-theta microscopy

¹³ Although confocal microscopy already has 3D capabilities, its axial resolution is still
¹⁴ limited compared to the lateral, since it uses only one objective. An alternative realiza-
¹⁵ tion of the confocal microscope, the confocal theta microscope [31] introduces a second
¹⁶ objective to the system, that is used to illuminate the sample (Figure 1.7b). Since this
¹⁷ decouples the illumination and detection, using a dichroic mirror is no longer necessary.
¹⁸ The second objective is rotated by θ around the focus, this is where the name of this
¹⁹ setup originates from.

²⁰ As in the case of standard confocal microscopy, the system PSF is improved by the

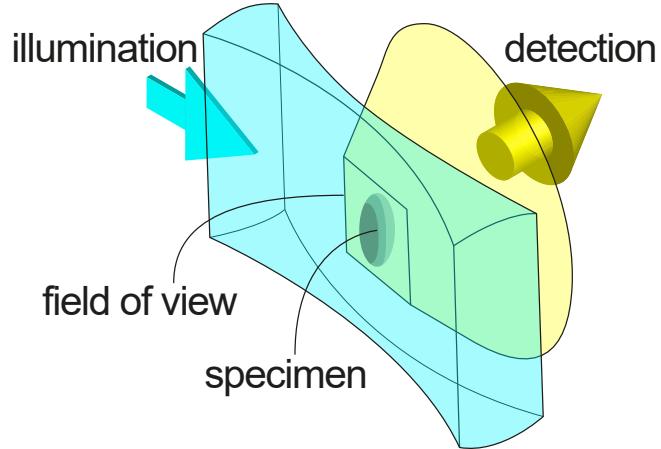


Figure 1.9: Basic concept of single-plane illumination microscopy. The sample is illuminated from the side by laser light shaped to a light-sheet (blue). This illuminates the focal plane of the detection lens, that collects light in a wide-field mode (yellow). The image is recorded, and the sample is translated through the light-sheet to acquire an entire 3D stack.

1 illumination pattern. Here, however, the axial direction of the detection coincides with
 2 the lateral direction of the illumination, which results in a dramatic improvement of
 3 axial resolution compared to standard confocal microscopy. Lateral resolution will also
 4 be increased, but by a smaller extent, resulting in an almost isotropic PSF, and equal axial
 5 and lateral resolutions. Although this is a big improvement to confocal microscopy in
 6 terms of resolution, this technique did not reach a widespread adoption as it complicates
 7 sample handling, while still suffering from two drawbacks of confocal microscopy that
 8 limit its live imaging capabilities.

9 Imaging live specimens for an extended period of time with confocal microscopy
 10 although possible [32, 33], is not ideal. For each voxel imaged, a large portion of the
 11 specimen has to be illuminated, which results in a very high dose of radiation on the
 12 sample. This can be as much as 30–100 times larger, than the dose used for the actual
 13 imaging [34]. Illumination with high power of laser for an extended time frame can
 14 result in bleaching the fluorophores, which in turn will lower the signal at later times.
 15 Furthermore, any absorbed photon has the possibility to disrupt the chemical bonds
 16 inside the specimen, which can lead to phototoxic effects. Moreover, the usage of the
 17 pinhole although increases resolution, also decreases the detectable signal intensity, thus
 18 has a negative impact on image contrast [35].

19 1.3 Light-sheet microscopy

20 A selective-plane illumination microscope (SPIM) uses a light-sheet to illuminate only a
 21 thin section of the sample (Figure 1.9). This illumination plane is perpendicular to the
 22 imaging axis of the detection objective and coincides with the focal plane. This way, only

1.3 Light-sheet microscopy

1 the section in focus will be illuminated, thus providing much better signal-to-noise ratio.
2 In case of conventional wide-field fluorescence microscopy, where the whole specimen
3 is illuminated, out-of-focus light contributes to a significant background noise. With
4 selective-plane illumination, this problem is intrinsically solved, and it also provides a
5 true optical sectioning capability. This makes SPIM especially suitable for 3D imaging.

6 The main principle behind single-plane illumination microscopy, that is illuminating
7 the sample from the side by a thin light-sheet, dates back to the early 20th century, when
8 Siedentopf and Zsigmondy first described the ultramicroscope [36]. This microscope used
9 sunlight as an illumination source, that was guided through a precision slit to generate
10 a thin light-sheet. This allowed Zsigmondy to visualize gold nanoparticles floating in
11 and out of the light-sheet by detecting the scattered light from the particles. Since these
12 particles are much smaller than the wavelength of the light, the device was called an
13 ultramicroscope. His studies with colloids, and the development of the ultramicroscope
14 led Zsigmondy to win the Nobel Prize in 1925.

15 After Zsigmondy, this method was forgotten until rediscovered in the 1990s, when
16 Voie *et al.* constructed their Orthogonal-plane Fluorescent Optical Sectioning (OPFOS)
17 microscope [37]. They used it to image a fixed, optically cleared and fluorescently labelled
18 guinea pig cochlea. In order to acquire a 3D dataset, the sample was illuminated from the
19 side with a light-sheet generated by a cylindrical lens, then rotated around the center axis
20 to obtain multiple views. Although they only reached a lateral resolution around 10 µm
21 and axial resolution of 26 µm, this method allowed them to generate a 3D reconstruction
22 of the guinea pig cochlea [38].

23 Later, in 2002, Fuchs *et al.* developed Thin Light-Sheet Microscopy (TLSM) [39]
24 and used this technique to investigate the microbial life in seawater samples without
25 disturbing their natural environment (by, *e.g.*, placing them on a coverslip). Their light-
26 sheet was similar to the one utilized in OPFOS, being 23 µm thin, and providing a
27 1 mm × 1 mm field of view.

28 Despite these early efforts, the method did not gain larger momentum. The real
29 breakthrough in light-sheet imaging happened at the European Molecular Biology Labo-
30 ratory (EMBL) in 2004, where Huisken *et al.* [40] combined the advantages of endogenous
31 fluorescent proteins and the optical sectioning capability of light-sheet illumination to
32 image Medaka fish embryos, and the complete embryonic development of a *Drosophila*
33 *melanogaster* embryo. They called this Selective-Plane Illumination Microscopy (SPIM),
34 and it quickly became popular to investigate developmental biological questions.

35 Since then, light-sheet-based imaging has gained more and more popularity, as it can
36 be adapted and applied to a wide variety of problems. Although sample mounting can
37 be challenging because of the objective arrangement, this can also be an advantage, since
38 new microscopes can be designed with the sample in mind [3, J3] (Figure 1.10). This

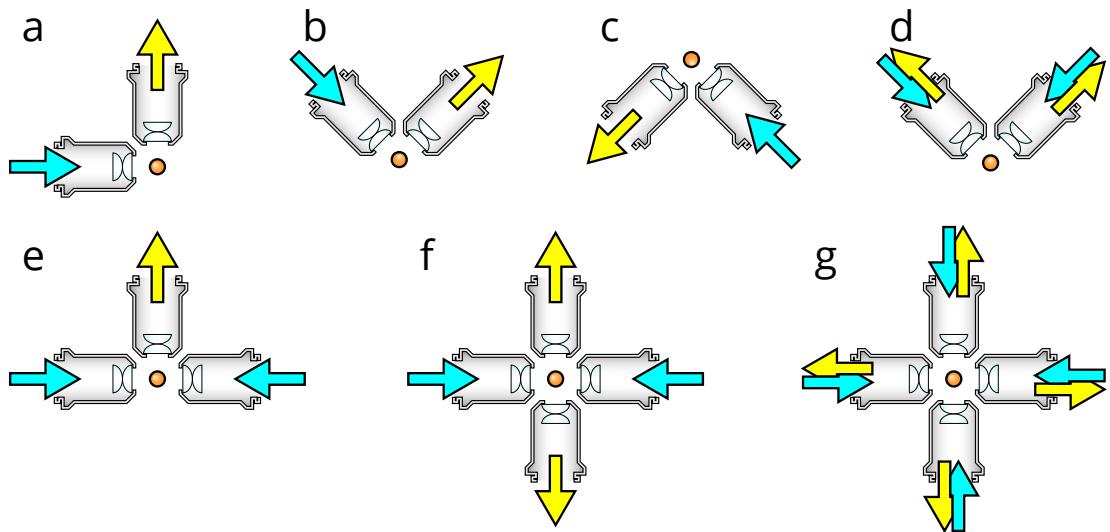


Figure 1.10: Different optical arrangements for light-sheet microscopy. (a) Original SPIM design with a single lens for detection and illumination. [40] (b) Upright SPIM to allow for easier sample mounting such as using a petri dish (iSPIM, [41, 42, J1]). (c) Inverted SPIM, where the objectives are below the sample, which is held by a thin foil [J2]. (d) Dual-view version of the upright configuration, where both objective can be used for illumination and detection (diSPIM, [43]). (e) Multidirectional-SPIM (mSPIM) for even illumination of the sample with two objectives for illumination [44]. (f) Multi-view SPIM with two illumination and detection objectives for *in toto* imaging of whole embryos (MuVi-SPIM [45], SimView [46], Four-lens SPIM [47]). (g) A combination of (d) and (f), using four identical objectives, where both can illuminate and detect in a sequential manner, to achieve isotropic resolution without sample rotation (IsoView [48]).

made it possible to adapt the technique for numerous other specimens, such as zebrafish larvae [49], *C. elegans* embryos [42], mouse brain [6], and even mouse embryos [J2, 50, 51].

As many of these specimens require very different conditions and mounting techniques, these microscopes have been adapted to best accommodate them. An upright objective arrangement for example (Figure 1.10b) allows imaging samples on a coverslip, while its inverted version is well suited for mouse embryos, where a foil is separating them from the immersion medium (Figure 1.10c). A modified version of the upright arrangement allows for multi-view imaging using both objectives for illumination and detection in a sequential manner (Figure 1.10d) [52].

To achieve a more even illumination in larger samples, two objectives can be used from opposing directions to generate two light-sheets (Figure 1.10e) [44]. This arrangement can further be complemented by a second detection objective, to achieve parallelized multi-view imaging (Figure 1.10f) [45–47]. For ultimate speed, 4 identical objectives can be used to achieve almost instantaneous views from 4 different directions by using all objectives for illumination and detection (Figure 1.10g) [48].

Furthermore, because of the wide-field detection scheme it is possible to combine SPIM with many superresolution techniques, such as single molecule localization [53],

- ¹ STED [54], RESOLFT [J1], or structured illumination [7, 55, 56].

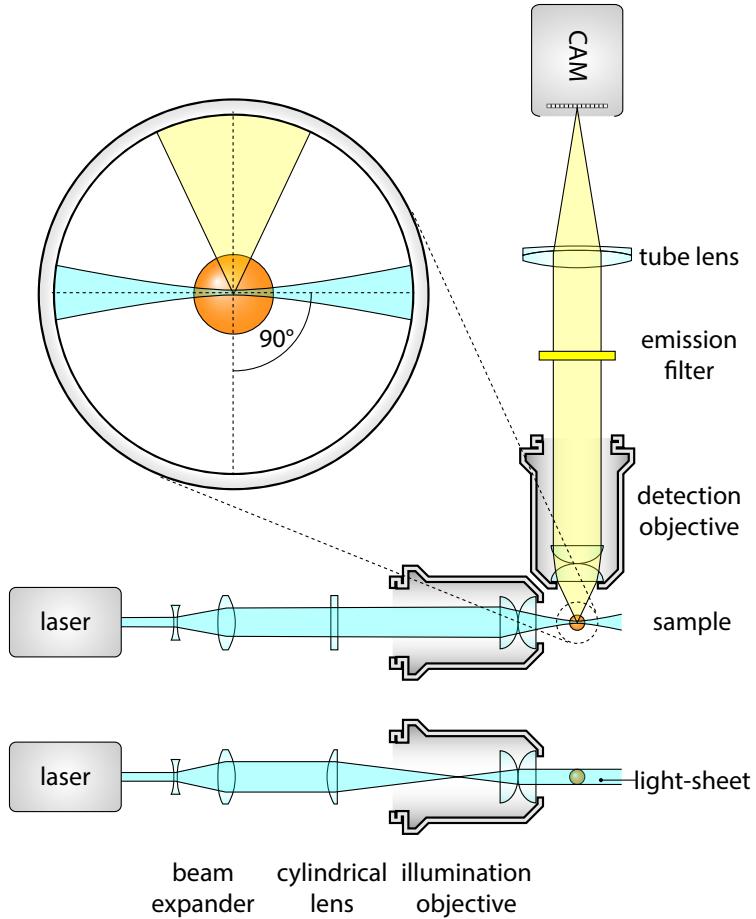


Figure 1.11: Basic optical components of a SPIM. A dedicated illumination objective is used to generate the light-sheet, which is an astigmatic Gaussian beam, focused along one direction. Astigmatism is introduced by placing a cylindrical lens focusing on the back focal plane of the objective. Detection is performed at a right angle, with a second, detection objective. Scattered laser light is filtered out, and a tube lens forms the image on an area sensor, such as an sCMOS camera.

- ² Since illumination and detection for light-sheet microscopy are decoupled, two independent optical paths are implemented.

⁴ The detection unit of a SPIM is basically equivalent to a detection unit of a wide-field microscope without the dichroic mirror (Figure 1.11). The most important components are the objective together with the tube lens, filter wheel, and a sensor, typically a charge coupled device (CCD) or scientific complementary metal–oxide–semiconductor (sCMOS) camera.

⁹ The resolution of a light-sheet microscope mainly depends on the detection objective. ¹⁰ Since imaging biological specimens usually requires a water-based solution, the objectives ¹¹ also need to be directly submerged in the medium to minimize spherical aberrations. As

the refraction index of water ($n = 1.33$) is greater than the refraction index of air, these objectives tend to have a higher NA, resulting in higher resolution. As the illumination is decoupled in this system, the light-sheet thickness also has an influence on the axial resolution. Finally, the resolution also depends on the pixel size of the sensor which determines the spatial sampling rate of the image.

Although image quality and resolution greatly depend on the detection optics, the real strength of light-sheet microscopy is the inherent optical sectioning which is due to the specially aligned illumination pattern, that confines light to the vicinity of the detection focal plane.

There are two most commonly used options to generate a light-sheet: either by using a cylindrical lens, to illuminate the whole field of view with a static light-sheet, as in the original SPIM concept [40]; or by quickly scanning a thin laser beam through the focal plane, thus resulting in a virtual light-sheet. This method is called Digitally Scanned Light-sheet Microscopy (DSLM) [49].

1.3.1 Static light-sheet illumination

For a static light-sheet, the normally circular Gaussian laser beam needs to be shaped in an astigmatic manner, *i.e.*, either expanded or squeezed along one direction, to shape it into a sheet instead of a beam. This effect can be achieved by using a cylindrical lens, which as the name suggests has a curvature in one direction, but is flat in the other, thus focusing a circular beam to a sheet (Figure 1.11).

However, to achieve light-sheets that are sufficiently thin for optical sectioning, one would need to use cylindrical lens with a very short focal length, and these are hardly accessible in well corrected formats. For this reason, it is more common to use a longer focal length cylindrical lens in conjunction with a microscope objective, which is well corrected for chromatic and spherical aberrations [57]. This way, the light-sheet length, thickness and width can be adjusted for the specific imaging tasks.

Light-sheet dimensions

The shape of the illumination light determines the optical sectioning capability and the field of view of the microscope, so it is important to be able to quantify these measures. The most commonly used illumination source is a laser beam coupled to a single mode fiber, thus its properties can be described by Gaussian beam optics.

For paraxial waves, *i.e.*, waves with nearly parallel wave front normals, a general wave equation can be approximated with the paraxial Helmholtz equation [58]

$$\nabla_T^2 U + i2k \frac{\partial U}{\partial z} = 0 \quad (1.17)$$

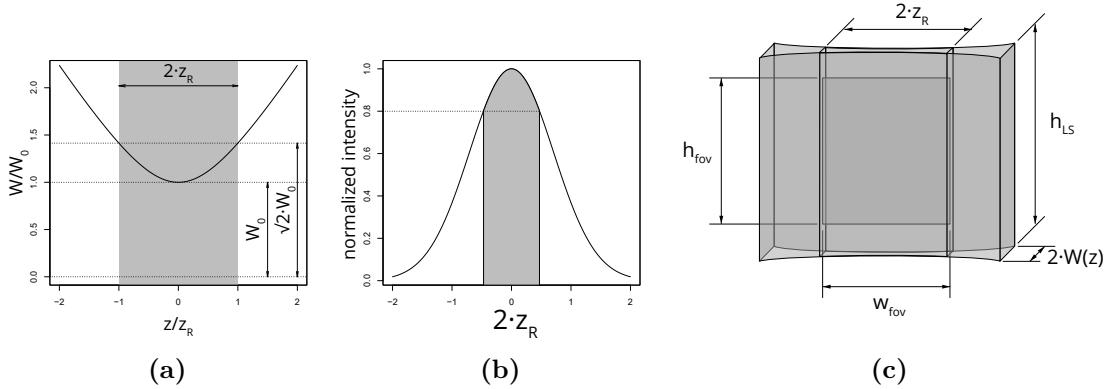


Figure 1.12: Light-sheet dimensions. (a) The width and thickness of the field of view depends on the Rayleigh length of the beam ($z_{R,y}$) and the beam waist (W_0). (b) The height of the field of view is determined by the Gaussian profile of the astigmatic beam. (c) shows a light sheet, with the field of view indicated. Since the light-sheet intensity is uneven, the field of view has to be confined to a smaller region.

1 where $\nabla_T^2 = \frac{\partial^2}{\partial x^2} + \frac{\partial^2}{\partial y^2}$, $U(\vec{r})$ is the wave function, $k = \frac{2\pi}{\lambda}$ is the wavenumber and z is in
 2 the direction of the light propagation.

3 A simple solution to this differential equation is the Gaussian beam:

$$U(r, z) = A_0 \cdot \frac{W_0}{W(z)} \cdot e^{-\frac{r^2}{W^2(z)}} \cdot e^{-i \cdot \phi(r, z)} \quad (1.18)$$

4 where A_0 is the amplitude of the wave, W_0 is the radius of the beam waist (the thinnest
 5 location on the beam), $r = \sqrt{x^2 + y^2}$ is the distance from the center of the beam, $W(z)$
 6 is the radius of the beam at distance z from the waist, and $\phi(r, z)$ is the combined phase
 7 part of the wave-function (Figure 1.12a). Furthermore:

$$W(z) = W_0 \sqrt{1 + \left(\frac{z}{z_R}\right)^2} \quad (1.19)$$

8 where the parameter z_R is called the Rayleigh-range, and is defined the following way:

$$z_R = \frac{\pi W_0^2}{\lambda}. \quad (1.20)$$

9 Apart from the circular Gaussian beam, the elliptical Gaussian beam is also an eigen-
 10 function of Helmholtz equation (Equation 1.17) which describes the beam shape after a
 11 cylindrical lens:

$$U(x, y, z) = A_0 \cdot \sqrt{\frac{W_{0,x}}{W_x(z - z_{0,x})}} \sqrt{\frac{W_{0,y}}{W_y(z - z_{0,y})}} \cdot e^{-\frac{x^2}{W_x^2(z - z_{0,x})}} \cdot e^{-\frac{y^2}{W_y^2(z - z_{0,y})}} \cdot e^{-i \cdot \phi(x, y, z)} \quad (1.21)$$

This beam still has a Gaussian profile along the x and y axes, but the radii ($W_{0,x}$ and $W_{0,y}$), and the beam waist positions ($z_{0,x}$ and $z_{0,y}$) are uncoupled, which results in an

elliptical and astigmatic beam. The beam width can now be described by two independent equations for the two orthogonal directions:

$$W_x(z) = W_{0,x} \sqrt{1 + \left(\frac{z}{z_{R,x}}\right)^2} \quad \text{and} \quad W_y(z) = W_{0,y} \sqrt{1 + \left(\frac{z}{z_{R,y}}\right)^2}. \quad (1.22)$$

Since the beam waist is different along the two axes, the Rayleigh range is also different:

$$z_{R,x} = \frac{\pi W_{x,0}^2}{\lambda}, \quad z_{R,y} = \frac{\pi W_{y,0}^2}{\lambda}. \quad (1.23)$$

Based on these equations, the light-sheet dimensions and usable field of view can be specified (Figure 1.12c). The light-sheet thickness will depend on the beam waist, $W_{0,y}$ (if we assume the cylindrical lens is focusing along y), and the length of the light-sheet can be defined as twice the Rayleigh range, $2 \cdot z_{R,y}$. As these are coupled (see Equation 1.23), having a thin light-sheet for better sectioning also means that its length will be relatively short. Fortunately, because of the quadratic relation, to increase the field of view by a factor of two, the light-sheet thickness only needs to increase by a factor of $\sqrt{2}$.

Light-sheet height is determined by the intensity profile of the beam along the vertical axis (Figure 1.12b). Since this is a Gaussian function (see Equation 1.18), only a small part in the middle can be used for imaging, because towards the sides the intensity dramatically drops. To allow a maximum 20% drop-off in intensity at the edges, the light-sheet height becomes $h_{fov} = 2 \cdot 0.472 \cdot W_{x,0} = 0.944 \cdot W_{x,0}$.

1.3.2 Digitally scanned light-sheet illumination

Although generating a static light-sheet is relatively straightforward, with the simple addition of a cylindrical lens to the light path, it has some drawbacks. As already mentioned in the previous section, the light intensity distribution along the field of view is not constant, as the light-sheet is shaped from a Gaussian beam. Furthermore, along the lateral direction of the light-sheet the illumination NA is extremely low, resulting in effectively collimated light. Because of this, shadowing artifacts can deteriorate the image quality [44].

A more flexible way of creating a light-sheet is by scanning a focused beam in the focal plane to generate a virtual light-sheet (digital scanned light-sheet microscopy, DSLM [49]). Although this method might require higher peak intensities, it solves both drawbacks of the cylindrical lens illumination. By scanning the beam, the light-sheet height can be freely chosen, and a homogenous illumination will be provided. Focusing the beam in all directions evenly introduces more angles in the lateral direction as well, which shortens the length of the shadows.

The basic optical layout of a DSLM is shown on Figure 1.13. A galvanometer con-

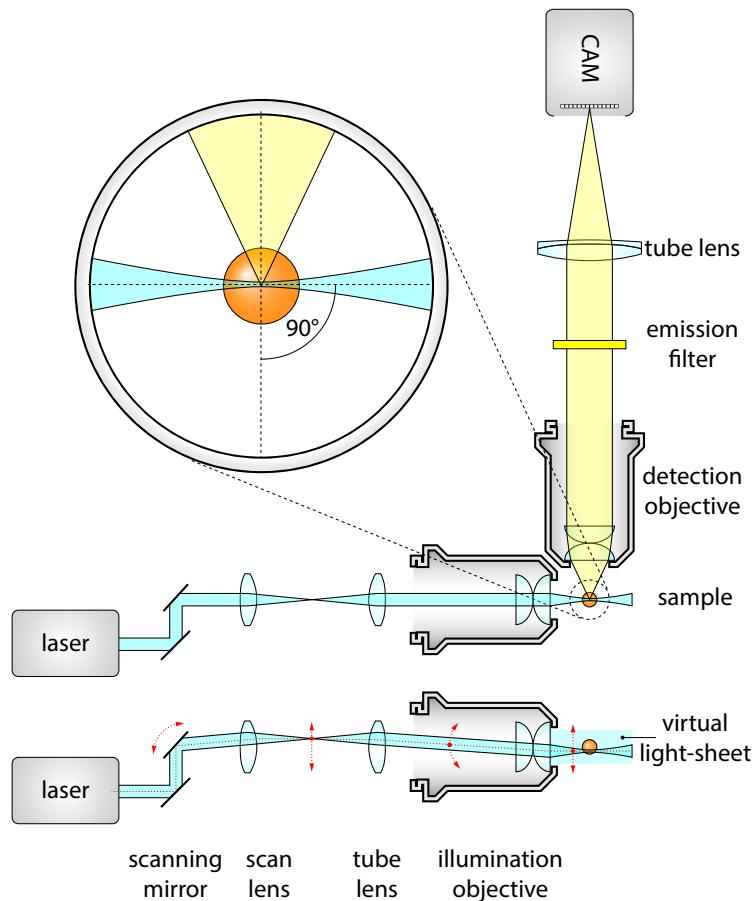


Figure 1.13: DSLM illumination. DSLM illuminates a specimen by a circularly-symmetric beam that is scanned over the field of view. This creates a virtual light-sheet, which illuminates a section of a specimen just like the SPIM. The light-sheet in DSLM is uniform over the whole field of view and its height can be dynamically altered by changing the beam scan range.

1 trolled mirror is used to alter the beam path that can quickly turn around its axis which
 2 will result in an angular sweep of the laser beam. To change the angular movement to
 3 translation, a scan lens is used to generate an intermediate scanning plane. This plane is
 4 then imaged to the specimen by the tube lens and the illumination objective, resulting
 5 in a scanned focused beam at the detection focal plane. The detection unit is identical
 6 to the wide-field detection scheme, similarly to the static light-sheet illumination. By
 7 scanning the beam at a high frequency, a virtual light-sheet is generated, and the flu-
 8 orescence signal is captured by a single exposure on the camera, resulting in an evenly
 9 illuminated field of view.

1 1.4 Light-sheet microscopy for imaging mmalian development

3 Because of its optical sectioning capabilities, combined with the high specificity of flu-
4 orescent labels, confocal microscopy had an immense influence on biological research,
5 and has been the go-to technique for decades for many discoveries [59–61]. Live imaging
6 capabilities of confocal microscopy are, however, limited. This is mostly due to the illu-
7 mination scheme: to collect information from a single point, almost the whole embryo
8 has to be illuminated. This unwanted illumination can easily lead to bleaching of the
9 fluorophores, and also to phototoxic effects that can prevent further embryonic develop-
10 ment. The point scanning nature of image acquisition also implies an inherent constraint
11 to the maximum imaging speed, as it can be only increased at the expense of reducing
12 signal intensity (Figure 1.1).

13 One improvement to address both of these drawbacks is the use of a spinning disk with
14 a specific pattern (also called Nipkow disk) of holes to generate multiple confocal spots
15 at the same time [60]. If these spots are far enough from each other, confocal rejection
16 of out-of-focus light can still occur. As the disk is spinning, the hole pattern will sweep
17 the entire field of view, eventually covering all points [62]. The image is recorded by an
18 area detector, such as a CCD or EM-CCD, which speeds up image acquisition [63].

19 Although out-of-focus illumination is still an issue, spinning disk confocal microscopy
20 was successfully used to investigate the mechanisms of symmetry breaking in early mouse
21 embryonic development [64], and the mechanisms leading to the first cell fate specification
22 that will differentiate extraembryonic tissue from the embryo proper [33, 65, 66]. Even
23 though these studies were carried out with live embryos, only a few hours of time-lapse
24 imaging is possible before the accumulation of phototoxic effects arrests development
25 [67]. Compared to this, just the pre-implantation phase of mouse embryonic development
26 spans 3 days, and the full development is 18 days [68].

27 1.4.1 Imaging mammalian pre-implantation development

28 Pre-implantation is the first phase of mouse embryonic development, that starts right
29 after fertilization. The embryo in this phase is still in the oviduct, travelling towards
30 the uterus, where it will implant into the uterine wall. The developmental stage between
31 fertilization and the implantation is called the pre-implantation stage. Here the embryo
32 divides, and already the first cell fate specifications start when forming the trophoecto-
33 derm (TE) and the inner cell mass (ICM) at the blastocyst stage. ICM cells will form
34 the embryo proper, while TE cell will contribute to the formation of the extraembryonic
35 tissues.

36 During this process the embryo is still self-sufficient, which makes it possible to image

1.4 Light-sheet microscopy for imaging mmalian development

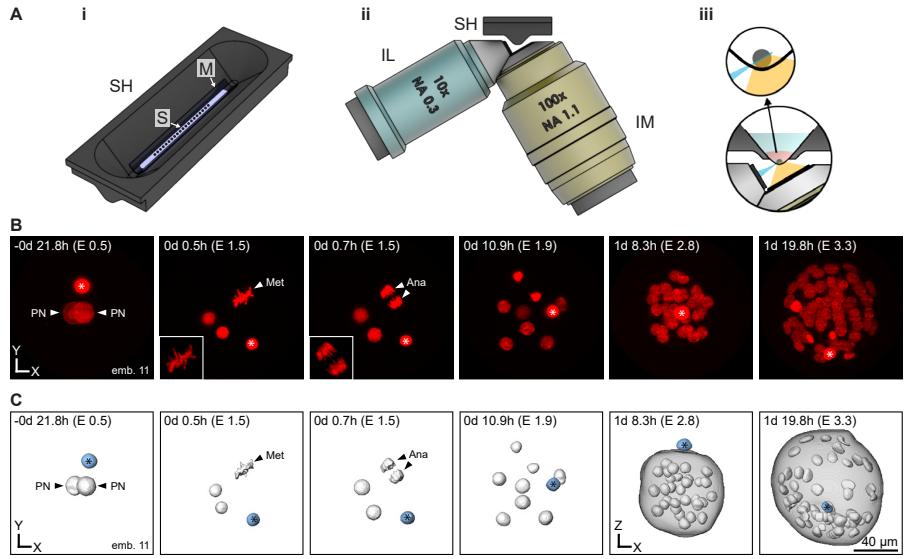


Figure 1.14: Inverted light-sheet microscope for multiple early mouse embryo imaging.. (i) A sample holder (SH), containing a transparent FEP membrane (M) allows multiple embryo samples (S) to be placed in line for multisample imaging. (ii) Inverted objective orientation with side view of the sample holder. One possible configuration is to use a 10 \times 0.3 NA illumination objective (IL) and another 100 \times 1.1 NA detection objective placed at a right angle to the illumination. (iii) Close up on side view of sample on FEP membrane with both objectives. Since the FEP membrane is transparent on water, it provides no hindrance to the illumination beam in penetrating the sample or for the emitted fluorescence on reaching the detection objective. (B) Still images of one particular timelapse experiment, and (C) corresponding segmented nuclei. The star depicts the polar body. Adapted from Strnad *et al.* [J2]

1 this stage in an *ex vivo* embryo culture by providing the proper conditions [69]. Long
2 term imaging, however, is extremely challenging due to the very high light sensitivity of
3 the specimens. Imaging these embryos in a confocal microscope will lead to incomplete
4 development, even if the imaging frequency is minimized to every 15 mins [J2].

5 Imaging for just a few hours is already enough to investigate important processes, such
6 as cell fate patterning [70]. Other approaches aim to lower the phototoxicity by either
7 using 2-photon illumination which operates at longer wavelengths [71–73], or by lowering
8 imaging frequency as a compromise [74]. These approaches, however either require highly
9 specialized equipment, such as an ultra-short pulsed laser, or is compromising on the time
10 resolution.

11 Light-sheet microscopy, on the other hand, drastically lowers phototoxic effect by
12 using a much more efficient illumination scheme (see Section 1.3), and thus makes a
13 better use of the photon budget. Using this technique, it is possible to image the full pre-
14 implantation development at high spatial and temporal resolution without any negative
15 impact on the developmental process. Such a microscope was developed by Strnad *et al.*
16 at EMBL [J2], who used it to understand when exactly the first cell fate specification is
17 decided in the embryonic cells.

18 As a mouse embryo culture is not compatible with the standard agarose-based sample
19 mounting techniques, a completely new approach was taken, which resulted in a micro-

1.4 Light-sheet microscopy for imaging mmalian development

1 scope designed around the sample. The sample holder forming a V-shape was built with
2 a bottom window, and it is lined with a thin FEP (fluorinated ethylene propylene) foil
3 that supports the embryos (Figure 1.14A, i). This arrangement allows the utilization of
4 the standard microdrop embryo culture, while providing proper viewing access for the
5 objectives. As the embryos are relatively small (100 µm) and transparent, a single illu-
6 mination and single detection objective arrangement is enough for high quality imaging.
7 A low resolution (NA=0.3) objective is used to generate the scanned light-sheet, and
8 a high resolution (NA=1.1) objective is detecting the fluorescence at 50×magnification
9 (Figure 1.14A, ii). As the foil is curved, it allows unrestricted access to the embryo, while
10 separating the imaging medium from the immersion liquid (Figure 1.14A, iii). Further-
11 more, its refractive index is matching the refractive index of water, so optical aberrations
12 are minimized.

13 Using this setup, Strnad *et al.* were able to pinpoint the exact timing of the first cell
14 fate decision that leads either to ICM or TE cells. More than 100 embryos expressing
15 nuclear (H2B-mCherry) and membrane (mG) markers were imaged for the entire 3 days
16 of pre-implantation development (Figure 1.14B). The image quality was sufficient to
17 segment all nuclei in the embryos (Figure 1.14C), and track them from 1 to 64 cell stage,
18 building the complete lineage tree. Based on the lineage trees, and the final cell fate
19 assignments, it was determined that at the 16 cell stage the final specification is already
20 decided, while earlier than this it is still random.

21 1.4.2 Imaging mammalian post-implantation development

22 After the initial 3 days of pre-implantation, the embryo undergoes the implantation
23 process, during which it is inaccessible to microscopical investigations. Although a new
24 method was recently developed that allows the *in vitro* culturing of the embryos em-
25 bedded in a 3D gel [76], this has not reached wider adoption yet. Hence, developmental
26 processes during implantation have only been investigated in fixed embryos.

27 Following the implantation process, at the post-implantation phase, *ex vivo* embryo
28 culturing becomes possible again [77, 78], and these embryos can be kept alive for several
29 days in an artificial environment. During this process especially interesting stages are the
30 late blastocyst (~E4.5), gastrulation (~E6.5), and somite formation (~E8.5). Before live
31 imaging techniques became available, these stages were mostly investigated using *in situ*
32 visualization techniques to shed light on several developmental processes [79]. Many
33 pathways playing important roles have been identified this way, however live imaging is
34 still necessary to validate these results, and ensure continuity in the same specimen [80].

35 Light-sheet microscopy is a good choice for imaging these stages, just like in the case
36 of pre-implantation embryos. These embryos, however, present new challenges for sample
37 handling and culturing. Owing to their extreme sensitivity, dissection can be difficult,

1.4 Light-sheet microscopy for imaging mammalian development

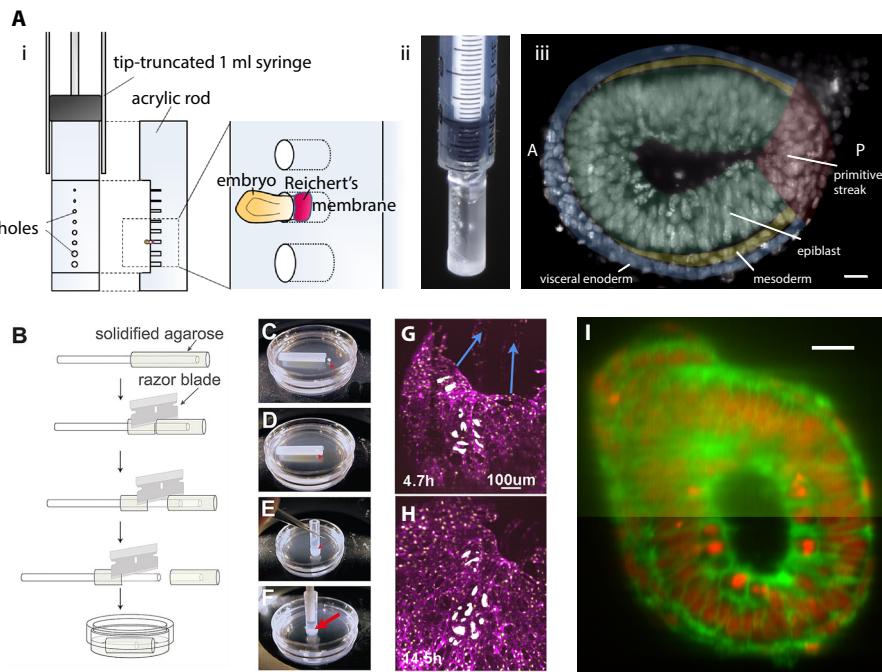


Figure 1.15: Imaging mouse post-implantation development. (A) (i, ii) Mounting technique for E5.5 to E6.5 embryos. A tip-truncated 1 mL syringe holds an acrylic rod, cut and drilled with holes of different size in order to best fit the mouse embryo by its Reichert's membrane, leaving the embryo free inside the medium. (iii) Maximum intensity projection of a 13 μm thick slice at 78 μm from distal end of an E6.5 mouse embryo. The different tissues corresponding to the rudimentary body plan are annotated. Scale bar: 20 μm . (B) For stages ranging between E6.5 and E8.5, mounting using a hollow agarose cylinder has also successfully been proposed. Optimal sizes for the corresponding embryonic stage to be imaged can be produced, so that the embryo can grow with least hindrance. (C–F) Steps for mounting the mouse embryo inside the agarose cylinder. The inner volume of the cylinder can be filled with optimal medium, allowing the much larger chamber volume to have less expensive medium. (G–H) Example images of a 9.8 h timelapse with the mounting shown in (B) where the expansion of the yolk sac can be observed in direction of the blue arrows. (I) In order to aid multiview light-sheet setups in overcoming the higher scattering properties of embryos at this stage, and to allow faster and easier data recording, electronic confocal slit detection allows better quality images to be taken at shorter acquisition times. Scale bar: 20 μm . Adapted from Ichikawa *et al.* [50], Udan *et al.* [51] and de Medeiros and Norlin *et al.* [75].

especially for earlier stages (E4.5). Furthermore, since the embryo is also growing during development, gel embedding is not an option, as this might constrain proper development. Thus, special handling and mounting techniques had to be developed in order to allow live 3D imaging of these specimens.

Ichikawa *et al.* [50] designed a custom mounting apparatus manufactured from acrylic in the shape of a rod that fits in a standard 1 mL syringe with its tip truncated (Figure 1.15A, i). In the rod several holes were drilled with different sizes that can accommodate different sized embryos, which are held by an extraembryonic tissue, the Reichert's membrane (Figure 1.15A, ii). Mounting this way doesn't disturb the embryo itself, and it can freely develop in the culturing medium, while it is also stationary for the purpose of imaging. Using this technique, Ichikawa *et al.* were able to image through several stages of development, including interkinetic nuclear migration at stages E5.5–6.5 (Figure 1.15A, iii).

1.4 Light-sheet microscopy for imaging mammalian development

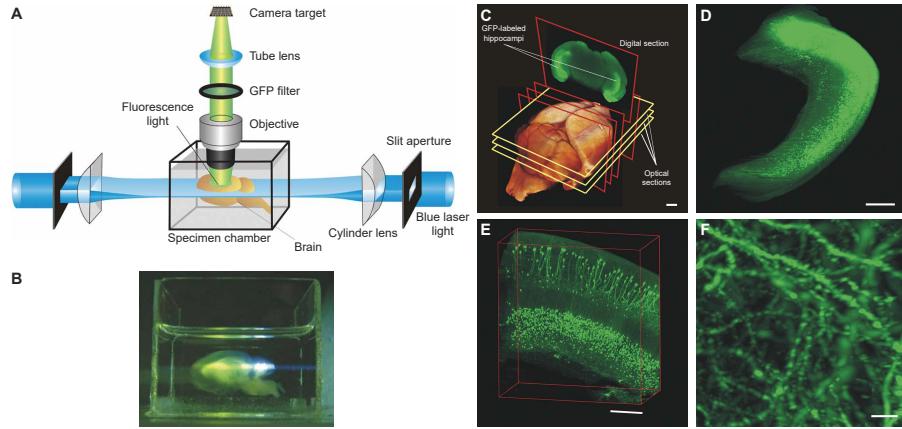


Figure 1.16: Imaging adult mouse brain with light-sheet microscopy.. (A) Schematics of the ultramicroscope for brain imaging. The specimen is embedded in clearing medium to ensure necessary imaging depth. Illumination is applied from two sides to achieve even illumination for the whole field of view. Light-sheet is generated by a slit aperture followed by a cylindrical lens. The specimen is imaged from the top using wide-field detection method. (B) Photograph of the imaging chamber with a mounted cleared specimen and light-sheet illumination. (C) Surface rendering of a whole mouse brain, reconstructed from 550 optical sections. GFP and autofluorescence signal was recorded. Hippocampal pyramidal and granule cell layers are visible in the digital section. Scale bar: 1 mm. Objective: Planapochromat 0.5×. (D) Reconstruction of an excised hippocampus from 410 sections. Note that single cell bodies are visible. Scale bar: 500 µm. Objective: Fluar 2.5×. (E) 3D reconstruction of a smaller region of an excised hippocampus from 132 sections. Scale bar: 200 µm. Objective: Fluar 5×. (F) 3D reconstruction of CA1 pyramidal cells imaged with a higher resolution objective (LD-Plan Neofluar 20× NA 0.4) in a whole hippocampus (430 sections). Dendritic spines are also visible, even though usually a higher NA objective (>1.2) is required to visualize these. Scale bar: 5 µm. Adapted from Dodt *et al.* [6].

1 A second method of sample mounting for light-sheet imaging was developed by
 2 Udan *et al.* who were able to record a full 24 h time-lapse of living embryos focusing
 3 on the gastrulation and yolk sac formation processes (Figure 1.15G–I). Their mounting
 4 technique comprised of producing a hollow agarose container shaped like a cylinder that
 5 could support the embryo from below without constraining its growth (Figure 1.15B–F).

6 Another consideration to keep in mind, is the growing size of the embryo. As it
 7 gets bigger, illumination is less efficient, and scattering can dominate at larger depths.
 8 As mentioned in earlier (Figure 1.3) this can be alleviated by multi-view imaging: illu-
 9 minating and detecting from multiple directions. Electronic confocal slit detection can
 10 further improve the signal-to-noise ratio by rejecting unwanted scattered light, which
 11 allows deeper imaging in large specimens, even up to E7.5 (Figure 1.15I) [75].

12 1.4.3 Imaging adult mice

13 Imaging adult mice is especially interesting for answering neurobiological questions. Since
 14 development is over at this stage, the use of an environmental chamber is no longer neces-
 15 sary. The biggest challenge for imaging these samples is their size, as they are centimeters
 16 in size instead of less than a millimeter as in the embryonic stage. Furthermore, the tis-
 17 sues of adult mice are much more opaque which severely limits imaging depth. Light-sheet
 18 microscopy can already deal with large specimens, however to achieve (sub)cellular res-

1.4 Light-sheet microscopy for imaging mmalian development

1 olution for an entire brain for example, multiple recordings have to be stitched together
2 after acquisition [81].

3 Light scattering and absorption depend on the tissue composition and imaging depth.
4 Especially the brain with a high concentration of lipids in the myelinated fibers pose a
5 real challenge for imaging. Live imaging is usually performed with 2-photon microscopy
6 which can penetrate the tissue up to 800 µm deep [82]. Using fixed samples, however, the
7 scattering problem can be eliminated by the use of tissue clearing methods.

8 Tissue clearing is a process that removes and/or substitutes scattering and absorbing
9 molecules by a chemical process while keeping the tissue structure intact and preserving
10 fluorescence. The most dominant contributors to these effects are the proteins and lipids.
11 Proteins in the cells locally change the refractive index of the tissue which leads to
12 scattering, while lipids predominantly absorb the light. Clearing methods tackle these
13 problems by chemically removing and substituting lipids by certain types of gel, and
14 immersing the whole sample in a medium with higher refractive index to match the
15 optical properties of proteins. Numerous methods have been developed for tissue clearing,
16 such as ScaleA2 [83], 3DISCO [84, 85], SeeDB [86], CLARITY [87], CUBIC [88] and
17 iDISCO [89].

18 The first combination of optical clearing and light-sheet microscopy for whole brain
19 imaging was performed by Dodt *et al.* using a custom ultramicroscope consisting of
20 two opposing illumination arms and a single detection with an objective from above
21 (Figure 1.16A). The light-sheets were positioned horizontally, and the cleared samples
22 could be placed in a transparent imaging chamber filled with the clearing medium (Figure
23 1.16B). Imaging was performed from both top and bottom after rotating the sample
24 180°. By changing the detection lens, it is possible to adapt the system to different sam-
25 ples: low magnification is capable of imaging the whole brain (Figure 1.16C), while for
26 smaller, dissected parts, such as the hippocampus, higher magnification with higher res-
27 olution is more appropriate (Figure 1.16D). With this configuration individual cell-cell
28 contacts can be recognized (Figure 1.16E), and even dendritic spines can be visualized
29 (Figure 1.16F).

30 Although light-sheet microscopy is highly suitable for imaging cleared specimens, even
31 entire mice [90], brain imaging in live animals is more challenging due to the standard
32 two-objective setup of a conventional SPIM microscope. Two light-sheet-based methods,
33 however offer a solution for this, axial plane optical microscopy (APOM) [91] and swept
34 confocally-aligned planar excitation (SCAPE) [92] both use only a single objective to
35 generate a light-sheet and detect the fluorescence as well. This is done by rotating the
36 detection plane at an intermediate image (APOM), or by rotating both the light-sheet
37 and detection plane simultaneously (SCAPE).

¹ Chapter 2

² Image compression

³ Light-sheet microscopy is capable of generating an immense amount of data in a short
⁴ amount of time. For this reason not only fast data processing, but data compression also
⁵ plays an important role when evaluating, sharing and storing the images. In this chapter
⁶ we will briefly introduce the core concepts of image compression and show some examples
⁷ as a background for Chapter 4. For a more comprehensive review on data compression
⁸ fundamentals, the interested reader is referred to the works of Sayood [93], and Salomon
⁹ and Motta [94].

¹⁰ There are two distinct ways of data compression: lossless and lossy. This is an impor-
¹¹ tant difference, and the choice between the two greatly depends on the application itself.
¹² For some types of data no loss is acceptable, for example computer programs or text.
¹³ Here, even a small change could drastically influence the meaning of the original data.
¹⁴ For other applications, such as audio and video compression, some loss can be tolerated
¹⁵ as long as there is no perceived change or distortion. For scientific applications mostly
¹⁶ lossless compression is used. Even for image data, where lossy compression can signifi-
¹⁷ cantly increase the compression ratio (original size / compressed size), lossy compression
¹⁸ is usually not recommended, as it can alter the measurement results in unpredictable
¹⁹ ways [95].

²⁰ 2.1 Basics of information theory

²¹ As compression is the art of reducing the size of a message while keeping the same
²² amount of information, it is necessary to briefly discuss the theory behind information,
²³ and how to quantify it. These concepts were first introduced by Shannon, in his highly
²⁴ influential papers [96–98]. As a rigorous mathematical explanation is outside the scope
²⁵ of this work, we refer the interested reader to the aforementioned works.

²⁶ Informally, information can be quantified as the amount of surprise. Any statement,
²⁷ if it has a high probability carries a low amount of information, while if it has a small

1 probability, there is more information. Consider the following example: “It is July”. If it is
 2 indeed July, this statement doesn’t provide too much information. Continuing with “It is
 3 snowing outside”, carries more information, as it is unexpected under the circumstances
 4 (provided the conversation takes place on the Northern Hemisphere).

5 The self-information of event A can be defined the following way:

$$I(A) = \log_b \frac{1}{P(A)} = -\log_b P(A), \quad (2.1)$$

6 where $P(A)$ is the probability of event A occurring. The base of the logarithm can
 7 be chosen freely, but usually the preferred base is $b = 2$. With this choice, the unit
 8 of information is 1 bit, and $I(A)$ represents how many bits are required to store the
 9 information contained in A .

10 The average self-information, also called entropy, for a random variable X can be
 11 calculated based on the self-information of the outcomes A_i :

$$H(X) = \sum_i P(A_i)I(A_i) = -\sum_i P(A_i) \log_b P(A_i) \quad (2.2)$$

12 If we consider a data stream S , where each symbol records the outcome of independent,
 13 identical random experiments X , then the average information in each symbol will be
 14 $H(X)$. As a consequence, as stated by Shannon’s source coding theorem [96], the shortest
 15 representation of S will require at least $N \cdot H(X)$ bits, where N is the number of symbols
 16 in the stream. This defines the theoretical lower bound that any naïve compression
 17 algorithm, also called entropy coding, can achieve without losing any information.

18 The requirement, however, is that the symbols are independent of each other, which
 19 is usually not true for most of the data types we (humans) are interested in. In order to
 20 achieve ideal compression, the input needs to be modeled, or transformed, to represent it
 21 as a sequence of independent variables. This pattern of modeling and coding [99] is the
 22 core of all modern compression algorithms. The following sections will briefly introduce
 23 these concepts by two examples: Huffman coding, and pixel prediction.

24 2.2 Entropy coding

25 Entropy coding algorithms aim to reduce data size and reach the limit of entropy for
 26 any kind of input, based on the probability distribution of the possible symbols. All of
 27 these algorithms work in a similar way, as their aim is to represent the symbols of the
 28 input data by codewords in a way to minimize the overall length of the data. Variable
 29 length codes for example replace the more common symbols with short codewords, while
 30 for less frequent symbols they use longer codewords. The ideal length of a codeword for
 31 symbol A is actually equal to $I(A)$, as this was the definition of self-information.

Table 2.1: Examples of a random binary code (#1) and a prefix-free binary code (#2). Code #2 is uniquely decodable, while for code #1 it is necessary to introduce boundaries between codewords to be able to distinguish them.

Letter	Code #1	Code #2
a_1	0	10
a_2	11	11
a_3	00	00
a_4	10	010
a_5	111	011

1 Prefix-free codes

2 Fixed-length codes have the convenience that there is no need to indicate the boundaries
3 between them, as they are all the same length (*e.g.*, 8 bits for ASCII codes). For variable
4 length codes, as the name implies this is not possible, which necessitated the development
5 of various strategies to demarcate these codewords.

6 One way to indicate the end of a codeword is to use a specific delimiting sequence
7 which is not part of any of the codewords. While this is an obvious solution, it is
8 also wasteful, as these sequences will not contain any information regarding the orig-
9 inal source. Prefix-free codes were developed to be able to omit these sequences, while
10 still allowing unique decodability, making them very popular. This is achieved by mak-
11 ing sure that once a codeword was assigned, no other codeword would start with that
12 sequence. In other words, no codeword is a prefix of another codeword, hence the name
13 of the technique. During decoding the bits are read until a valid codeword is matched.
14 Since it is guaranteed that no other codeword will start with the same sequence, the
15 decoder can record the symbol corresponding for that codeword, and continue reading
16 the compressed stream.

17 Let's take the example in Table 2.1. Five letters are coded in binary code by Code
18 #1 and by Code #2. Code #1 is not a prefix code, and because of this when reading the
19 encoded sequence we can not be sure when we reach the end of a codeword. Decoding
20 the sequence 0000 for example could be interpreted as 4 letters of a_1 or 2 letters of a_3 .
21 For Code #2 it is clear that the sequence decodes to 2 letters of a_3 .

22 Huffman coding

23 Huffman coding is a prefix-free, optimal code that is widely used in data compression.
24 It was developed by David A. Huffman as a course assignment in the first ever course
25 on information theory at MIT, and was published shortly afterwards [100]. It is able to
26 achieve optimal compression in a sense that no other prefix-free codes will produce a
27 shorter output. Due to its simplicity combined with a good compression ratio it is still

Table 2.2: Letters to be Huffman coded and their probabilities.

Letter	Probability	Codeword
a_2	0.4	$c(a_2)$
a_1	0.2	$c(a_2)$
a_3	0.2	$c(a_2)$
a_4	0.1	$c(a_2)$
a_5	0.1	$c(a_2)$

¹ widely used in many compression algorithms, such as in JPEG, bzip2, or DEFLATE
² (zip) [94].

³ The Huffman coding procedure is based on two observations regarding optimal and
⁴ prefix-free codes:

- ⁵ 1. For a letter with higher frequency the code should produce shorter codewords, and
⁶ for letters with lower frequency it should produce longer codewords.
- ⁷ 2. In an optimum code, the two least frequent codewords should have the same
⁸ lengths.

⁹ From these statements it is trivial to see that the first is correct. If the more frequent
¹⁰ letters would have longer codewords than the less frequent letters, the average codeword
¹¹ length (weighted by the probabilities) would be larger than in the opposite case. Thus,
¹² more frequent letters must not have longer codewords than less frequent letter.

¹³ The second statement at first glance might not be so intuitive, so let's consider the
¹⁴ following situation. Let's assume that the two least frequent codewords do not have the
¹⁵ same lengths, and the least frequent is longer. However, because this is a prefix code,
¹⁶ the second longest codeword is not a prefix of the longest codeword. This means that
¹⁷ if we truncate the longest codeword to the same length as the second longest, they will
¹⁸ still be distinct codes and uniquely decodable. This way we have a new coding scheme
¹⁹ which requires less space on average to code the same sequence as the original code, from
²⁰ which we can conclude the original code was not optimal. Therefore, for an optimal code,
²¹ statement 2 must be true.

To construct such a code, the following iterative procedure can be used. Let's consider an alphabet with five letters $A = [a_1, a_2, a_3, a_4, a_5]$ with $P(a_1) = P(a_3) = 0.2$, $P(a_2) = 0.4$ and $P(a_4) = P(a_5) = 0.1$ (Table 2.2). This distribution represents a source with 2.122 bits/symbol. Let's order the letters by probability, and consider the two least frequent. Since the codewords assigned to these should have the same lengths, they can be assigned as

$$c(a_4) = \alpha_1 * 0 \\ c(a_5) = \alpha_1 * 1$$

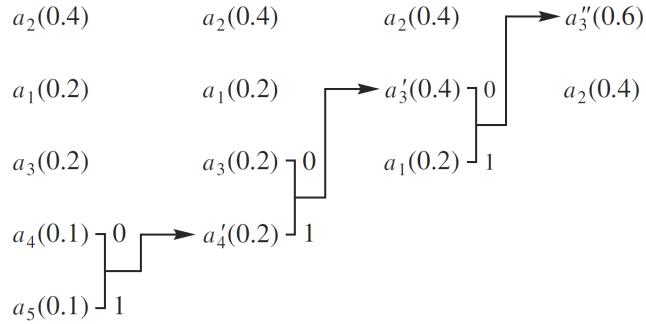


Figure 2.1: Building the binary Huffman tree. The letters are ordered by probability, these will be the final leaves of the tree. To create the branches, at every iteration we join the two nodes with the smallest probability, and create a new common node with the sum of the probabilities. This process is continued until all nodes are joined in a root node with probability of 1. Now, if we traverse down the tree to each leaf, the codeword will be defined by their position.

Table 2.3: Huffman code table.

Letter	Probability	Codeword
a_2	0.4	1
a_1	0.2	01
a_3	0.2	000
a_4	0.1	0010
a_5	0.1	0011

where $c(a_i)$ is the assigned codeword for letter a_i and $*$ denotes concatenation. Now we define a new alphabet A' with only four letters a_1, a_2, a_3, a'_4 , where a'_4 is a merged letter for a_4 and a_5 with the probability $P(a'_4) = P(a_4) + P(a_5) = 0.2$. We can continue this process of merging the letters until all of them are merged and we have only one letter left. Since this contains all of the original letters, its probability is 1. We can represent the end result in a binary tree (see Figure 2.1), where the leaves are the letter of the alphabet, nodes are the merged letters, and the codewords are represented by the path from the root node to each leaf (compare with Table 2.3). The average length of this code is

$$l = 0.4 \times 1 + 0.2 \times 2 + 0.2 \times 3 + 0.1 \times 4 + 0.1 \times 4 = 2.2 \text{ bits/symbol} \quad (2.3)$$

10 The efficiency of a code can be measured by comparing the average code size to the
11 entropy of the source. For this example, the difference is 0.078 bits/symbols. As the
12 Huffman code operates in base 2, it will produce a code with zero redundancy when the
13 probabilities are negative powers of 2.

1 Limitations of Huffman coding

2 Although the Huffman code is optimal among variable-length codes, for certain types of
3 data its compression ratio may be low. This limit lies in the fact that the length of the
4 shortest code assignable is one. For certain types of data with very low entropy this will
5 result in a relatively high redundancy. Arithmetic coding offers an alternative here, as
6 contrary to Huffman coding, it does not rely on substituting symbols with codewords, and
7 thus can compress very low entropy data with minimal redundancy. This coding method
8 maps the whole message to a single number with arbitrary precision in the interval of 0
9 to 1. A more detailed description of arithmetic coding is given in [93].

10 Another alternative for compressing very low entropy data is run-length encoding.
11 This coding method assumes that the same character appears in the message many times
12 consecutively, and instead of storing each of these individually it counts the number of
13 identical symbols in a single run, and encodes the run-length together with the symbol.
14 As this strategy is only efficient for very low entropy data, it is a common practice to
15 combine run-length encoding with Huffman coding to construct a low redundancy coder
16 for a wide range of input data.

17 2.3 Decorrelation

18 Since entropy coding doesn't assume anything about the data structure, it is not capable
19 of recognizing and compressing any regular patterns or correlations between the consec-
20 utive data points. To maximize the efficiency of compression, any correlations should be
21 removed from the data before it is fed to the entropy coder. Here we will briefly discuss
22 some decorrelation strategies for image compression that aim to improve the performance
23 of a successive entropy coder.

24 Transform coding

25 Very popular methods for decorrelation are the transformations that represent the data
26 as a decomposition of orthonormal functions. One of the most common of these trans-
27 formations is the Fourier transform, that represents a periodic signal as a sum of sine
28 and cosine functions. As most natural images are of continuous tone, the idea behind this
29 is that most of the information will be captured by just a few Fourier coefficients. As
30 a result, compressing the coefficients with an entropy coder should result in a smaller
31 output than encoding the original data.

32 For practical purposes, instead of the discrete Fourier transform (DFT), the discrete
33 cosine transform (DCT) [101] is usually used in image compression applications. This
34 is very similar to DFT, the difference being the periodic extension of the signal. In the
35 DCT transform the image is mirrored on the sides instead of just repeated, to avoid large

1 jumps at the boundaries that may introduce unwanted high-frequency components in the
 2 Fourier transform. If the image is extended in a symmetric way, the Fourier transform will
 3 be able to represent the data with only using cosine functions, hence the name discrete
 4 cosine transformations.

5 One drawback of frequency-based transformations, is that they can't capture any
 6 spatial information, as their base functions are periodic for the whole domain. This can
 7 have a negative impact on image compression ratio. For example, if the image contains
 8 a single sharp edge, a high frequency base function will need to have a large coefficient.
 9 Since this will affect the entire image, other, lower frequency bases will also need to have
 10 increased coefficients to negate the unwanted effects. To overcome this limitation, the
 11 DCT is usually performed on small chunks of the image, such as on 8×8 blocks in the
 12 JPEG compression. The other option is to use non-periodic base functions to represent
 13 the signal. One good example for this is the use of wavelets, and based on this, the
 14 discrete wavelet transform (DWT) [102, 103].

15 As both DCT and DWT operate with floating-point coefficients (although DWT has
 16 integer-based variants), the inverse transformation is not guaranteed to exactly recon-
 17 struct the original data. This is due to the finite machine precision of floating-point
 18 numbers. Due to this fact these transformations are generally used in lossy compression
 19 algorithms and the coefficients are quantized based on the required image quality.

20 Predictive decorrelation

21 Another family of decorrelation methods is based on predicting the values of each data
 22 point (or pixel) depending on the context of the neighboring values. These techniques are
 23 based on differential pulse code modulation (DPCM), a decorrelation method originally
 24 used for audio compression. Here, before the data stream is entropy coded, a prediction
 25 is made for each value, which is equal to the preceding value. The difference to the
 26 prediction, called the prediction error, or prediction residual (ε) is passed to the entropy
 27 coder.

28 To see how why such an algorithm can increase compression ratio, let's consider the
 29 following sequence:

30	37	38	39	38	36	37	39	38	40	42	44	46	48
----	----	----	----	----	----	----	----	----	----	----	----	----	----

31 Although there is no obvious pattern in this sequence, there are no sharp jumps between
 32 the consecutive values, and this can be exploited by using a prediction scheme. Let's
 33 define the prediction $\text{Pred}(\cdot)$ for each element X_k to be equal to the preceding element.
 34 The prediction error would be $\varepsilon_k = X_k - \text{Pred}(X_k) = X_k - X_{k-1}$:

35	37	1	1	-1	-2	1	2	-1	2	2	2	2	2
----	----	---	---	----	----	---	---	----	---	---	---	---	---

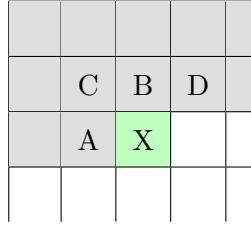


Figure 2.2: Context for pixel prediction. X is the current pixel to be encoded and the previously encoded pixels are indicated with gray background. To allow for decoding, only the already encoded pixels are used for the prediction, typically the nearest neighbors (A, B, C, D).

1 As apparent from this new sequence, the number of distinct values are reduced, which
 2 means that fewer bits can represent this sequence than the original. When running these
 3 two sequences through an entropy coder, the error sequence will be much more com-
 4 pressed due to this property.

5 Using a predictive scheme to remove correlations from neighboring elements is a very
 6 good strategy for various applications, where this signal is only slowly changing. It has
 7 been successfully implemented in audio and image compression algorithms as well, such
 8 as lossless-JPEG [104], JPEG-LS [105], CALIC [106], SFALIC [107] and FLIC [108], just
 9 to name a few.

10 For image compression, as the datasets are inherently multi-dimensional, the predic-
 11 tion rules can also be multi-dimensional, which can better capture the structure of the
 12 data, and achieve better decorrelation. For a typical encoding scenario, where the pixels
 13 are encoded one by one, the prediction can be based on the already encoded values (gray
 14 in Figure 2.2). This causality constraint is necessary in order to be able to decode the
 15 image.

16 Usually the nearest neighbors are used for the prediction, but this is not a necessity;
 17 CALIC, for example, uses the second neighbors [106], and the third dimension can also
 18 be utilized if the data structure allows this, for example when encoding hyperspectral
 19 recordings [109].

The lossless part of the JPEG standard specifies 7 possible rules for the prediction

step [104]:

$$\begin{aligned}\text{Pred}_1(X) &= A \\ \text{Pred}_2(X) &= B \\ \text{Pred}_3(X) &= C \\ \text{Pred}_4(X) &= A + B - C \\ \text{Pred}_5(X) &= A + (B - C)/2 \\ \text{Pred}_6(X) &= B + (A - C)/2 \\ \text{Pred}_7(X) &= (A + B)/2\end{aligned}$$

1 Depending on the patterns of the input image, some predictors can perform better than
 2 others. For general use, predictor 4 or 7 are recommended, as these are not direction
 3 dependent, and usually perform best. Predictor 7 is just the mean of the top and left
 4 neighbors, while predictor 4 assumes that the values for pixels A, B, C and X are on the
 5 same plane, and calculates the prediction based on this.

6 Other algorithms try to improve the prediction by adapting it depending on the local
 7 texture. The LOCO-I algorithm (part of JPEG-LS), for example uses the median edge
 8 detector [105]:

$$\text{Pred}(X) = \begin{cases} \min(A, B) & \text{if } \geq \max(A, B) \\ \max(A, B) & \text{if } \leq \min(A, B) \\ A + B - C & \text{otherwise} \end{cases} \quad (2.4)$$

9 This function tries to estimate if there is an edge close to X. If it detects a vertical edge
 10 to the left of X, it picks B for the prediction, whereas if it detects a horizontal edge above
 11 X it tends to pick A. If no edge is detected, the prediction is the same as predictor 4 of
 12 the lossless JPEG standard.

13 A useful property of the prediction-based methods is, that there is no base change as in
 14 the transform coding methods, and it is possible to construct bounded lossy compression
 15 algorithms. JPEG-LS, for example defines a near-lossless mode of operation, where the
 16 user can select the maximum absolute reconstruction error per pixel. This is achieved by
 17 quantizing the prediction residual ε with a quantization step depending on the allowable
 18 error. Since the decoding algorithm simply calculates $\hat{X} = \text{Pred}(X) + \varepsilon$, any quantization
 19 error of ε is directly reflected in the reconstructed pixel value. Since the quantization
 20 error is known, and bounded by the quantization step size, the maximum reconstruction
 21 error for each pixel is also bounded. This mode is sometimes used for medical imaging
 22 applications, where the preservation of diagnostic quality is required by law, but higher
 23 compression ratios are desired than what is possible by lossless compression.

¹ Chapter 3

² Dual Mouse-SPIM

³ Unraveling the secrets of mammalian development has been a long standing challenge
⁴ for developmental biologists and medical professionals alike. This phase of early life is
⁵ an incredibly complex and dynamic process spanning through large scales in space and
⁶ time. Subcellular processes at the nanoscale are happening in the range of milliseconds
⁷ or shorter, while whole embryo reorganizations and tissue migration events take place
⁸ over the course of hours [110]. Resolving these processes presents a true challenge, since
⁹ to understand the underlying mechanisms, molecular specificity is just as crucial as high
¹⁰ spatial and temporal resolution. As we have seen in Chapter 1, live imaging of mouse
¹¹ embryonic development is an especially challenging task, but light-sheet microscopy can
¹² offer a good solution owing to its gentle optical sectioning and fast image acquisition. The
¹³ Mouse-SPIM [J2] introduced earlier (Section 1.4.1) also solves the issue of live imaging
¹⁴ with its inverted design: the embryos are held by a foil which not only allows easy sample
¹⁵ handling but also acts as a barrier that isolates the embryos from the immersion medium.

¹⁶ As all microscopes, the Mouse-SPIM also had to make a compromise: although its
¹⁷ design allows long-term live imaging, it only has a single detection view, as rotation is
¹⁸ not possible with the sample mounting trays. Due to this configuration, its resolution is
¹⁹ inherently anisotropic, having an axial-to-lateral resolution ratio of around 3. Although
²⁰ this is sufficient for many applications, such as cell tracking, for detecting subcellular
²¹ features it might be limiting. One such example is chromosome tracking, which could shed
²² light on chromosome missegregation mechanisms in the early embryonic development,
²³ which is the cause of many congenital diseases also affecting humans.

²⁴ In this chapter, we present a novel light-sheet microscope developed to address the
²⁵ above challenges by using high NA objectives for subcellular isotropic resolution and low-
²⁶ light imaging, and offering multi-view detection without the need to rotate the samples.
²⁷ The microscope is designed to be live imaging compatible, offering new perspectives
²⁸ in the research of mouse embryonic development. This chapter will describe the design
²⁹ concepts for the microscope, the optical layout, alignment strategies, and the result of

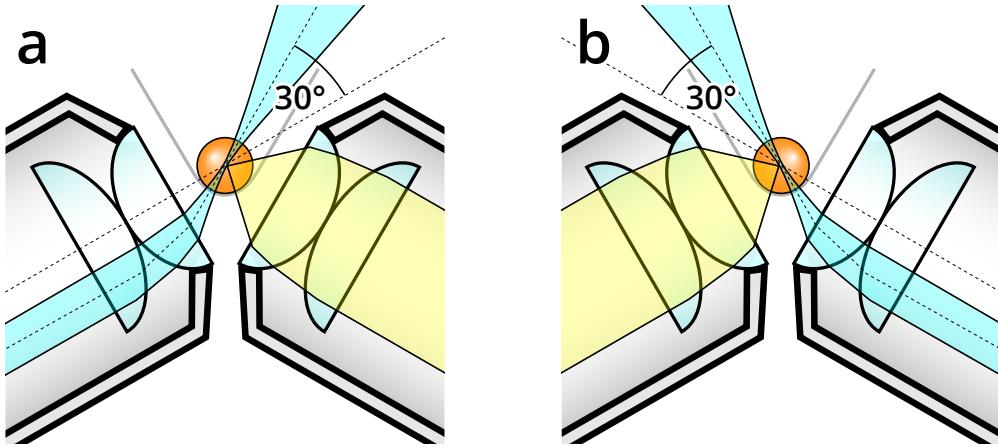


Figure 3.1: Dual view concept with high NA objectives. To achieve multi-view detection while maximizing resolution and light collection efficiency, two high NA objectives are placed in 120° arrangement. The sample (orange) is held from below by a thin FEP foil. To be able to overlap the light-sheet with the focal plane, the light-sheet is tilted by 30° . The objectives are used in an alternating sequence for illumination and detection.

1 various performance measurements, and its multi-view imaging capabilities.

2 3.1 Microscope design concept

3 As the limiting factor for subcellular imaging with the original Mouse-SPIM is the poor
 4 axial resolution relative to the lateral, our first aim was to increase the axial resolution
 5 to ideally reach the lateral resolution. A common way to reach isotropic resolution is
 6 to image a specimen from multiple directions, and combine the resulting images by
 7 multi-view deconvolution [28, 111, 112]. This has the benefit that the high-resolution
 8 information from one view can complement the low axial resolution of the other view,
 9 thus providing better resolution in all three directions.

10 As described in Chapter 1, many SPIM implementations allow for recording mul-
 11 tiple views either by rotating the sample, or by surrounding the sample with multiple
 12 objectives that are used for detection (Figure 1.10). For our setup, following the sam-
 13 ple mounting technique of the original Mouse-SPIM, we wanted to keep the open-top
 14 sample mounting possibility, as this was proven to be highly compatible with mouse
 15 embryo imaging. To achieve multi-view detection in this configuration, we designed a
 16 setup where both objectives can be used for illumination and detection in a sequential
 17 manner, inspired by previous symmetrical SPIM designs [43, 113].

18 To achieve the highest possible resolution from two views, the core of our design is
 19 based on the symmetric arrangement of two Nikon CFI75 Apo LWD 25x water-dipping
 20 objectives, with a numerical aperture of 1.1. Due to the large light collection angle of
 21 these objectives, we arrange them in 120° instead of the conventional 90° used for light-
 22 sheet imaging. As the light-sheet still needs to coincide with the imaging focal plane of

1 the objectives, we tilt the light-sheets by 30° (Figure 3.1). Due to the low NA of the
2 light-sheet, this is possible without affecting illumination quality.

3 This 120° arrangement has several benefits when compared to the traditional 90°
4 configuration. When placing the objectives in 90°, the largest possible light collection
5 half-angle for an objective can be $\alpha_{max,90} = 45^\circ$, and the corresponding NA is $NA_{max,90} =$
6 $n \cdot \sin \alpha_{max,90} = 0.94$, where $n = 1.33$ the refractive index of water. Considering that
7 this is an idealized case, the practically available highest NA is only 0.8. For a 120°
8 arrangement, the theoretical maximum is $NA_{max,120} = n \cdot \sin(120^\circ/2) = 1.15$, with a
9 practical maximum NA of 1.1.

10 Although the resolution won't be completely isotropic when combining the images
11 from two 120° views (Figure 3.2), as it is for 90° views, due to the higher maximum NA
12 possible, the resolution can be higher in the 120° case. When simulating the combined
13 multi-view PSFs (Figure 3.2), for 0.8 NA objectives in 90° the axial and lateral resolutions
14 are both 317 nm; while for two 1.1 NA objectives in 120° the axial resolution will be
15 identical, 317 nm, and the lateral will be better, 193 nm.

16 Although this difference in resolution may seem marginal, the 120° configuration has
17 another advantage in light collection efficiency. Collecting as much of the fluorescence
18 signal as possible is crucial in live imaging applications, due to the limited available
19 photon budget (see Figure 1.1 and [8]). Collecting more light from the sample allows to
20 image faster with the same contrast, or to reduce the illumination power and maintain
21 the imaging speed. As light collection efficiency depends on the solid angle subtended
22 by the detection lens, (see Appendix Section C), a 1.1 NA objective can collect twice as
23 many photons as a 0.8 NA objective, which gives the 120° setup a clear edge in low-light
24 imaging.

25 3.1.1 Light-sheet design

26 To allow for flexibility in the field of view height, even illumination, reduced stripes, and
27 potential for confocal line detection, we opted to use the beam-scanning technique to
28 generate a virtual light-sheet. The effective focal length of the Nikon 25x objective, given
29 the 200 mm focal length tube lens is

$$f_o = \frac{f_{tl}}{M} = \frac{200 \text{ mm}}{25} = 8 \text{ mm}, \quad (3.1)$$

30 and the back aperture diameter is $d = 17.6 \text{ mm}$.

31 To generate the tilted light-sheet as shown on Figure 3.1, the illumination beam will
32 need to be displaced by

$$\delta = f_o \cdot \tan 30^\circ = 4.62 \text{ mm} \quad (3.2)$$

33 Since the Gaussian beam is not uniform, only a smaller portion of it can be used to

3.1 Microscope design concept

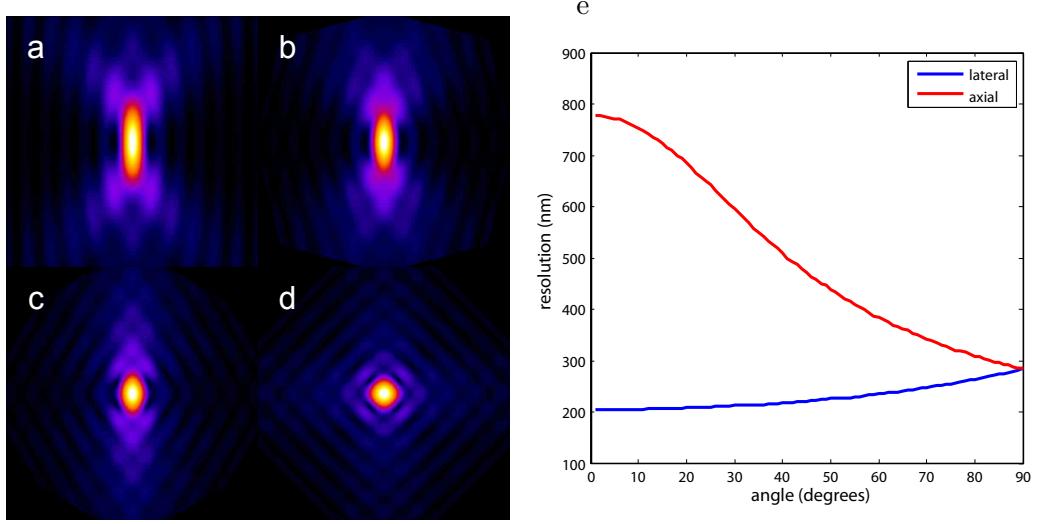


Figure 3.2: Lateral and axial resolution of a multi-view optical system. a) Simulated PSF for a single view. b)-d) Simulated compound PSF of two views aligned in b) 30, c) 60 and d) 90 degrees to each other. e) Axial and lateral resolution of a dual-view setup depending on the rotation angle of the two objectives. Parameters used for calculations: NA=1.1, $\lambda_{ex} = 488\text{nm}$, $\lambda_{det} = 510\text{nm}$, $n = 1.333$ for water immersion.

1 maintain an even illumination (Figure 1.12c). Because the size of an early mouse embryo
2 is around $80\text{ }\mu\text{m}$, we require the length and the height of the light-sheet to be at least
3 $100\text{ }\mu\text{m}$.

4 The length and thickness of the light-sheet

5 As we saw in Section 1.3.1, the length of the light-sheet is determined by the Rayleigh-
6 range of the beam in the zy -plane. Since $l_{FOV} = 2 \cdot z_R = 100\text{ }\mu\text{m}$

$$z_R = 50\text{ }\mu\text{m} \quad (3.3)$$

7 Since the Rayleigh range and the diameter of the beam waist are coupled, the light-sheet
8 thickness can be calculated after rearranging Equation 1.20

$$2 \cdot W_0 = 2 \cdot \sqrt{\frac{z_R \cdot \lambda}{\pi}} = 5.57\text{ }\mu\text{m} \quad (3.4)$$

9 when $\lambda = 488\text{ nm}$ for GFP excitation. As the beam width for these calculations is defined
10 as $1/e^2$ of the peak intensity, we also calculate the more commonly used full width at
11 half maximum (FWHM):

$$\text{FWHM} = W_0 \cdot \sqrt{2 \ln 2} = 3.28\text{ }\mu\text{m.} \quad (3.5)$$

¹ From this, the divergence angle of the beam is

$$\theta_0 = \frac{\lambda}{\pi W_{0,y}} = 55.74 \text{ mrad} = 3.196^\circ \quad (3.6)$$

² This means, the numerical aperture needed to produce this light-sheet is:

$$\text{NA}_{ls} = n \cdot \sin(\theta_0) = 0.0743 \quad (3.7)$$

³ Since $\text{NA} = 1.1$, the diameter of the back aperture is $d = 17.6 \text{ mm}$ and the divergence
⁴ angle $\theta_0 \ll 1$, using paraxial approximation, the necessary beam width at the back focal
⁵ plane in the y direction is

$$b_y = d \cdot \frac{\text{NA}_{ls}}{\text{NA}} = 1.19 \text{ mm} \quad (3.8)$$

⁶ Thus, to generate a light-sheet with appropriate length to cover a whole mouse pre-
⁷ implantation embryo, the laser beam diameter should be $b = 1.19 \text{ mm}$. Larger diameters
⁸ will result in a more focused beam and a shorter light-sheet, while a smaller diameter
⁹ beam will have worse optical sectioning capabilities.

¹⁰ The height of the light-sheet

¹¹ The height of the light-sheet can be adjusted by changing the beam scanning amplitude
¹² with the galvanometric mirror (also referred to as scanner). To scan the entire height of
¹³ the field of view of $h_{\text{FOV}} = 270 \mu\text{m}$, the scanning angle range at the back focal plane of
¹⁴ the objective will need to be $\theta = \tan^{-1}(h_{\text{FOV}}/2/f_o) = \pm 0.967^\circ$.

¹⁵ 3.2 Optical layout

¹⁶ Based on the requirements and other considerations shown in the previous section, the
¹⁷ microscope was designed in three main parts: 1) the core unit, 2) illumination and 3)
¹⁸ detection branches. The aim when integrating these units together was to allow for high
¹⁹ level of flexibility with robust operation, while also keeping efficiency in mind. After
²⁰ finalizing the concept, the optical layout of the microscope was designed in SolidWorks.

²¹ 3.2.1 Core unit

²² As the most important part of the microscope is actually the sample, the design is based
²³ around a core consisting of the imaging chamber and the objectives (Figure 3.3). Also
²⁴ part of the core are two mirror blocks placed at the back of the objectives, and three
²⁵ custom-designed rings to hold the objectives in place. The objectives are pointing slightly
²⁶ upwards, closing a 60° angle with the horizontal plane, and 120° angle with each other.

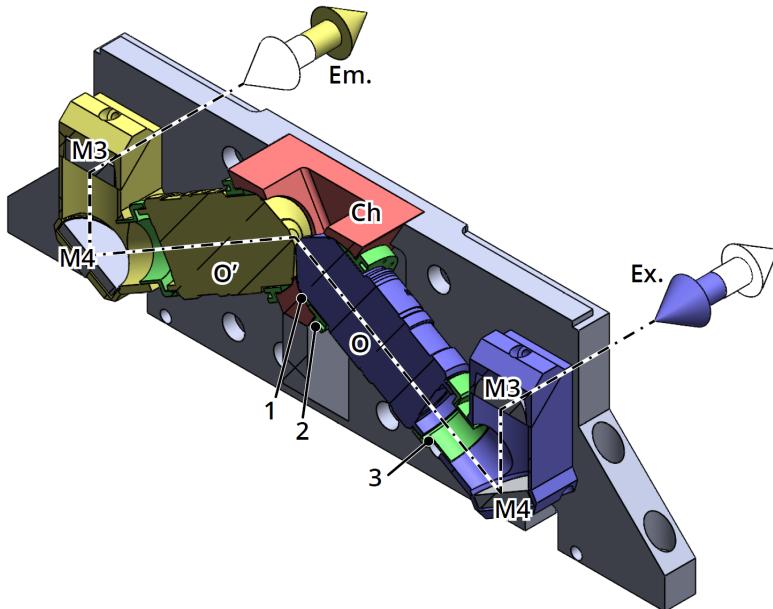


Figure 3.3: The core unit of the microscope. The two objectives (O and O') are mounted on a solid 15 mm thick aluminium plate. Fitting on the objectives, a custom chamber (Ch) is holding the immersion medium for imaging. The mirror block with mirrors $M3$ and $M4$ directs the light to 65 mm optical rails. Excitation (Ex.) and emission (Em.) light paths are indicated by the dash-dot line. Due to the symmetric arrangement, the excitation and illumination paths can be alternated. The objectives are secured with rings 1–3 (green, see main text for details).

¹ Chamber

² The chamber serves two purposes: it holds the immersion liquid necessary for imaging,
³ and it also keeps the objectives in the 120° position. The objectives are held by their
⁴ necks as opposed to the standard mounting method from the back by the threads. The
⁵ advantage of this is that any axial movements due to thermal expansion are greatly
⁶ reduced, thus the focal plane position is more stable even when changing the imaging
⁷ conditions.

⁸ The chamber is machined from a high-performance plastic, polyether ether ketone
⁹ (PEEK). This material has many beneficial properties: it is food safe, and chemically
¹⁰ extremely inert, resistant to most solvents used in a biology laboratory. Due to these
¹¹ properties, PEEK is live imaging compatible, even for sensitive samples, as it can also
¹² be autoclaved. Compared to other plastics its mechanical properties are also superior. It
¹³ has high tensile and compressive strength, comparable to those of aluminium, low ther-
¹⁴ mal expansion and low thermal conductivity. This can be beneficial when implementing
¹⁵ temperature control, as thermal loss is reduced.

¹⁶ The objectives are kept in place by two custom-designed rings (Figure 3.3 1, 2).
¹⁷ The first ring has a cross sectional shape of a wedge, and sits tightly against both the
¹⁸ objective and the wall of the chamber. The second ring can freely slide on the objective,
¹⁹ and has threads matching the chamber. When turned in, the threaded ring pushes the

1 wedge ring further in, which in turn presses against the objective and the chamber wall
2 uniformly, thus preventing the objective from moving, and sealing the chamber at the
3 same time. As the wedge ring is made from a soft plastic (delrin), it will press evenly
4 against the objective preventing any damage. Given the conical shape of the ring, it will
5 also automatically center the objective, ensuring correct positioning.

6 To relieve any rotational stresses from the objective, the back of the objective is also
7 supported by the mirror block. This is not fixed, however. A third ring, made of PEEK is
8 threaded on the objective, and slides into the opening of the mirror block. This reduces
9 the torque on the objectives, while still allowing for some movements that might occur
10 due to thermal expansion.

11 **Mirror blocks**

12 Apart from supporting the objectives from the back, the mirror blocks are housing two
13 broadband dielectric mirrors (Thorlabs, BBE1-E03 and OptoSigma, TFMS-30C05-4/11)
14 to direct the light to and from the objectives on a standard 65 mm height, compatible
15 with the Owis SYS65 rail system. The combination of two mirrors have two benefits
16 compared to using just one. With a single mirror directly reflecting the light to the back,
17 the entire assembly would need to be much higher to reach the desired 65 mm height.
18 This could result in stability problems. Furthermore, due to the 60° rotation angle of the
19 objective, the image of the objective would also be rotated if using only a single mirror.
20 With two mirrors the reflection planes can be kept orthogonal to the optical table, which
21 will result in a straight image after the mirror block. This is not only beneficial when
22 recording the images, but also when aligning the illumination arm. With the use of two
23 mirrors, a convenient vertical scanning is required to produce the light-sheet; with a
24 single mirror, the scanning direction would need to be rotated by 60°.

25 **3.2.2 Illumination**

26 The illumination arm of the microscope directs and shapes the laser beam to generate
27 the proper light-sheet dimension at the sample. As was calculated in Section 3.1.1, a
28 beam diameter of 1.2 mm is ideal for this setup.

29 The illumination arm has three main roles:

- 30 1. expands the laser beam to the calculated 1.2 mm size.
- 31 2. images the galvanometric scanner to the back focal plane of the objective
- 32 3. switches the laser light between the two objectives during imaging

33 To achieve the desired beam diameter, a 1:2 beam expander (Sill Optics, 112751) is
34 used in the reverse direction. As the output of the laser fiber produces a 3 mm diameter

beam, this will reduce it to 1.5 mm. As this is already the required beam diameter, the lenses further in the illumination path will not introduce any magnification.

Switching between the two illumination arms is performed by a custom-designed beam splitter unit (Figure 3.4). Instead of utilizing a 50/50 beam splitter cube and mechanical shutters, we exploit the fact that a galvanometric scanner is needed to generate the light-sheet. As this galvanometric scanner (Cambridge Technology, 6210B) has a relatively large movement range ($\pm 20^\circ$) it is also suitable for diverting the beam from one illumination arm to the other.

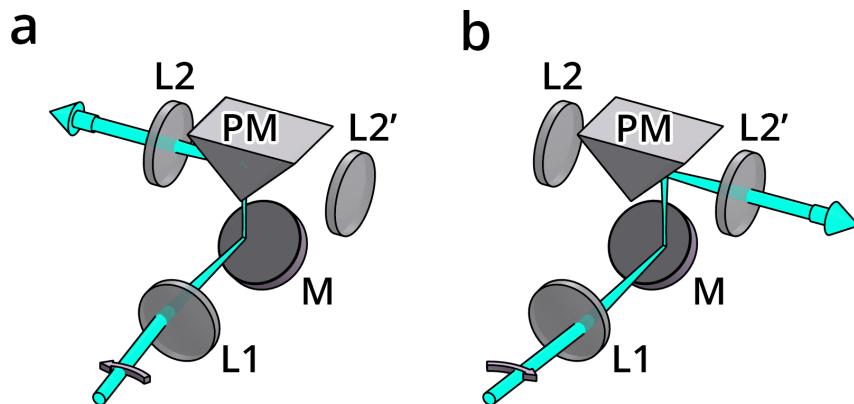


Figure 3.4: Illumination branch splitting unit. To divert the beam to either side, a right angle prism mirror is used in conjunction with a galvanometric scanning mirror. L1 acts as a scan lens, thus the beam is translated on mirror M. Depending on the scanner angle, the beam will be reflected either to the left (a) or to the right (b). L2 and L2' act as relay lenses, and will image the scanner movement to the corresponding intermediate planes.

Switching illumination side is done the following way. As the scanner is positioned at the focus of the first lens (L1, $f_1 = 75$ mm, Edmund Optics, #47-639), the rotational movement will result in a linear scanning movement on mirror M and the prism mirror PM (Figure 3.4). Depending on the lateral position of the beam, it will hit either the left or the right leg of the prism (Thorlabs, MRAK25-E02), and will be reflected to either direction. As the galvanometric mirror can be precisely controlled through our custom software, we can set and save the position when the beam is centered on the left lens L2 ($f_2 = 75$ mm, Edmund Optics, #47-639) (Figure 3.4a) and the position when the beam is centered on the right lens L2' (Figure 3.4b). Lenses L1 and L2(L2') form a 4f system, and are imaging the scanner on mirror M1(M1') (Figure 3.5). This way we can use the same scanner to generate the light-sheet for both directions, depending on the initial offset position. This not only has the advantage of being able to electronically switch the illumination arms, but only requires a single galvanometric scanner instead of one for each arm.

Due to the arrangement of the bottom mirror and the prism mirror, the scanning direction will be rotated by 90° . This will result in a vertical scanning plane, which is

1 exactly what we need to generate the light-sheet on the sample (see Section 3.2.1). Further
2 following the illumination path, two achromatic lenses L3 and L4 ($f_3 = f_4 = 200\text{ mm}$), in
3 a 4f configuration relay the scanning axis to the back focal plane (BFP) of the objective.

4 **3.2.3 Detection**

5 As the emitted light exits the objective and the mirror block, it is spectrally separated
6 from the illumination laser light by a quad band dichroic mirror (DM, Semrock, Di03-
7 R405/488/561/635-t3-25x36) matching the wavelengths of the laser combiner. The light
8 is then focused by a 400 mm achromatic lens (L5, Edmund Optics, #49-281) onto the
9 camera sensor (Andor Zyla 4.2 sCMOS). Just before the camera, a motorized filter wheel
10 (F, LEP 96A361) is placed to discriminate any unwanted wavelengths from the emission
11 light. Although this is not in the infinity space, due to the very small angles after the
12 400 mm tube lens, the maximum axial focal shift is $\sim 50\text{ nm}$ only, which is negligible
13 compared to the axial resolution of $\sim 1.1\text{ }\mu\text{m}$.

14 Similarly to the common scanner in the illumination path, the two detection arms
15 share the same camera. Although two cameras could also be used, due to the operating
16 principle of the microscope, the two objective are not used for imaging at the same time.
17 This means a single camera is capable of acquiring all the images. However, the two
18 distinct detection arms need to be merged to be able to use a single detector.

19 Our solution to this problem is a custom-designed view-switching unit comprised of
20 two broadband dielectric elliptical mirrors (Thorlabs, BBE1-E03) facing opposite direc-
21 tions, mounted on a high precision linear rail (OptoSigma, IPWS-F3090). Depending on
22 the rail position, either the left (Figure 3.6a) or the right (Figure 3.6b) detection path
23 will be reflected upwards, to the camera.

24 Moving the switcher unit is performed by a small, 10 mm diameter pneumatic cylinder
25 (Airtac, HM-10-040) that is actuated by an electronically switchable 5/2 way solenoid
26 valve (Airtac, M-20-510-HN). This solution offers a very fast switching between views,
27 up to 5 Hz, depending on the pressure, and it is extremely simple to control, as only a
28 digital signal is necessary to switch the valve.

29 **3.3 Optical alignment**

30 Precise alignment of the illumination and detection paths are crucial for high quality
31 imaging, and has a pronounced importance for high magnification and high resolution
32 optical systems. Due to the symmetrical setup of the microscope, we will only describe
33 the alignment of one side, as the same procedure is also applicable to the other side.
34 References to optical components will be as defined in Figure 3.5.

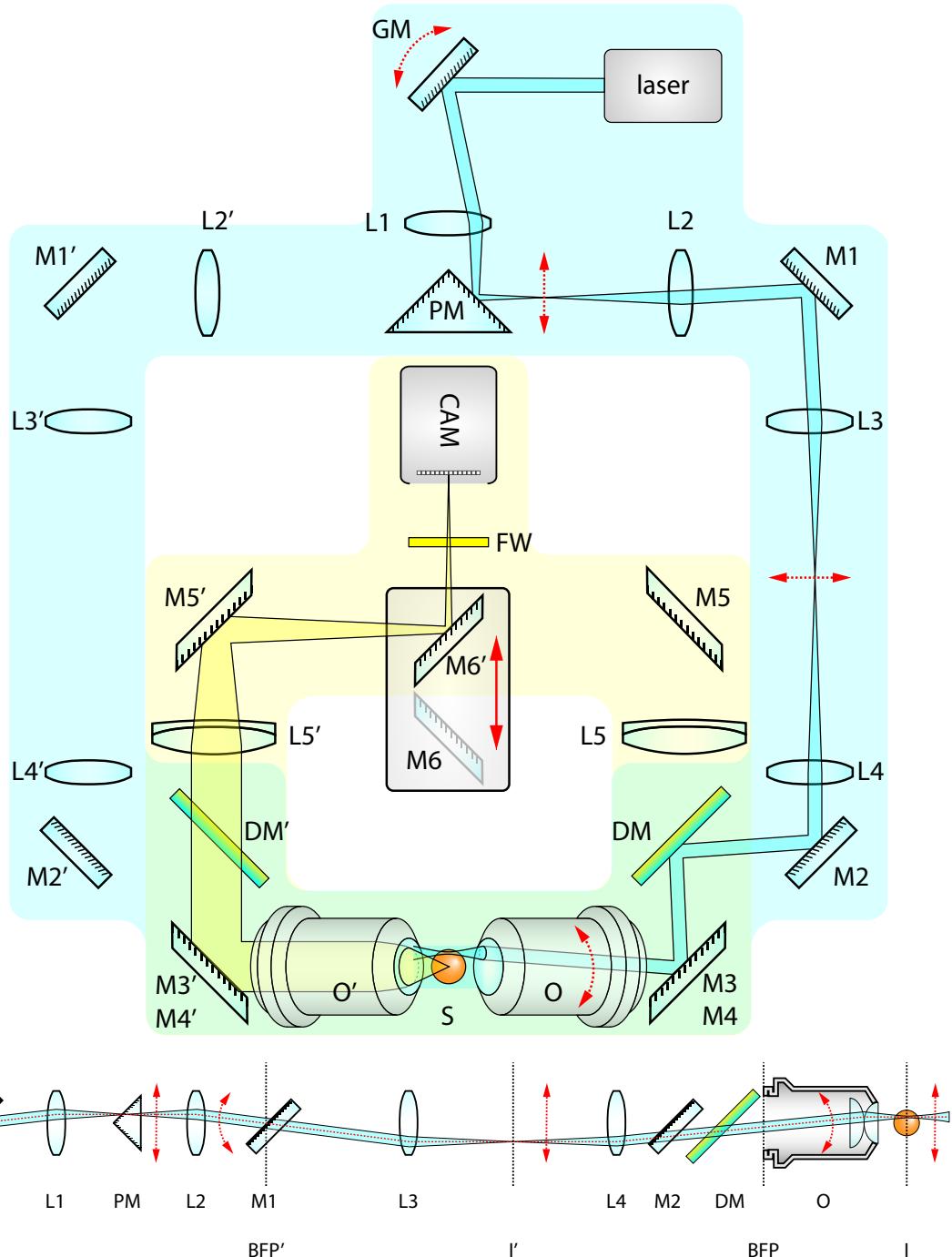


Figure 3.5: Dual Mouse SPIM optical layout. The microscope consists of two main parts, the illumination branches (blue) and detection branches (yellow). For both illumination and detection there are two identical paths implemented. Illumination direction can be changed by applying a different offset to the galvanometric mirror (scanner), which in turn will direct the beam to the opposite face of the prism mirror. L1 and L2 will then image the scanner on M1. Using L3 as a scan lens, and L4 as a tube lens, the scanned beam is coupled into the objective path by a quad band dichroic mirror (DM) CAM – camera, DM – dichroic mirror, FW – filter wheel, L – lens, M – mirror, O – objective, PM – prism mirror, S – sample

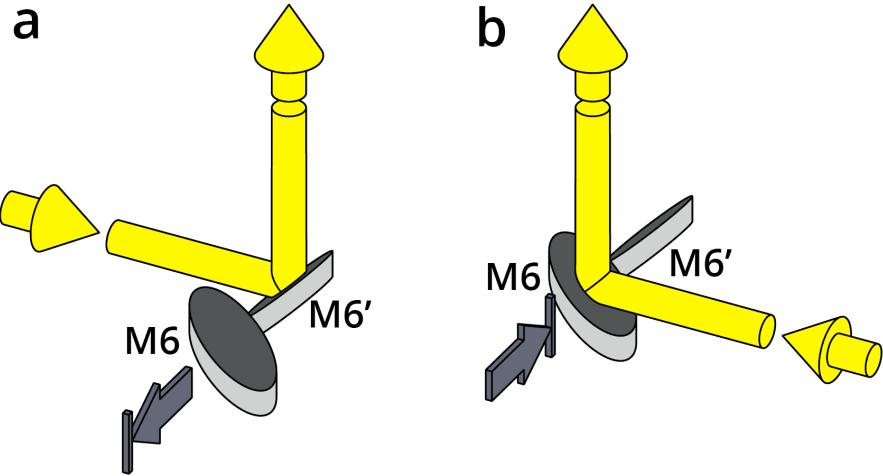


Figure 3.6: Detection branch switching unit. To be able to image both views on the same camera, a moveable mirror unit is introduced. Depending on the imaging direction, the mirror block is either moved backward (a) or forward (b) to reflect the light up to the camera. Since the movement is parallel to the mirrors' surface, the image position on the sensor is not dependent on the exact position of the mirrors.

1 3.3.1 Alignment of illumination branches

2 The two illumination branches start with a common light source, a single-mode fiber
 3 coupled to laser combiner, and they also share a galvanometric mirror that performs
 4 the beam scanning to generate the virtual light-sheet. Likewise shared is a scan lens
 5 focusing on the galvanometric mirror (GM), and the illumination splitter unit (PM, see
 6 item 3.2.2).

7 Alignment of the illumination arms is done in three steps. First the laser beam is
 8 aligned on the rail that holds the scanner, lens L1, and the splitter unit PM. This is
 9 performed by two kinematic mirrors placed between the fiber output and the galvano-
 10 metric mirror (not shown on figure). Using these two mirrors it is possible to freely align
 11 the beam along all four degrees of freedom: translation in two orthogonal directions and
 12 rotation around two orthogonal axes. Beam alignment on the rail is tested by using two
 13 irises at the two ends of the rail, if the beam passes through both of them we consider it
 14 centered and straight on the optical axis.

15 After the beam is aligned on the first rail, lens L1 and the splitter unit PM are placed
 16 in the measured positions to image the galvanometric mirror on mirror M1 using lenses
 17 L1 and L2. Correct positioning of the splitter unit along the rail is crucial, since this
 18 will affect the lateral position and tilt of the beam exiting the unit. To some extent,
 19 this can also be compensated by adjusting the two mirrors before the galvanometric
 20 mirror, but is avoided if possible as this will also displace the beam from the center of
 21 the galvanometric mirror.

22 After the initial alignment of the illumination arms, when the laser is already coupled
 23 in the objective, the fine adjustments are performed based on the image of the beam

1 through the other objective. The beam is visualized by filling the chamber with a 0.1%
2 methylene blue solution. As this solution is fluorescent, and can be excited in a very large
3 range, it is well suited to visualize the beam during adjustment.

4 **Adjusting beam position** Beam position can be adjusted by either translating the
5 beam in a conjugated image plane (I'), or by rotating the beam in a conjugated back
6 focal plane (BFP'). The setup was designed in a way that BFP' coincides with mirror
7 M_1 . This mirror is mounted in a gimbal mirror mount, allowing to rotate the mirror
8 exactly around its center, which avoids unwanted translational movements, and results
9 in pure rotation of the beam. Lens L_3 is positioned exactly 1 focal length away from
10 the mirror, thus acting as a scan lens, and transforming the rotational movements to
11 translation. This translation is further imaged and demagnified by the tube lens L_4 and
12 the objective O onto the sample.

13 **Adjusting beam tilt** Beam tilt can be adjusted by either rotating the beam in an
14 intermediate image plane (I'), or translating it at the back focal plane (BFP). As mirror
15 M_2 is relatively far from the back focal plane, adjusting it will mostly result in translation
16 that will rotate the beam in the image plane. This movement, however, will also introduce
17 translations, and has to be compensated by adjusting mirror M_1 . As the light-sheet
18 needs to be tilted by 30° to coincide with the focal plane of the other objective, this
19 level of adjustment is not possible with M_2 . In order to allow for a pure rotation of
20 the light-sheet, we mounted the dichroic mirrors on linear stages (OptoSigma, TSDH-
21 251C). By translating the dichroic mirror, the illumination laser beam gets translated
22 at the back focal plane, which will result in a pure rotational movement at the sample.
23 Coarse alignment of the light-sheet is performed by adjusting the dichroic position while
24 inspecting the light-sheet through a glass window in the chamber. Precise alignment is
25 done afterwards based on the image of the beam visualized in a fluorescent medium.

26 **Adjusting the scanning-plane angle** After the beam is properly aligned, *i.e.*, it is
27 in focus and in the center of field of view, it is still necessary to check if the scanning
28 direction is parallel to the imaging plane. It is possible that the beam is in focus in the
29 center position, but when moved vertically it drifts out of focus due to a tilted scanning
30 angle. This tilt can be compensated by mirror M_1 , that is placed at the conjugate back
31 focal plane BFP' . Between lenses L_3 and L_4 a magnified version of the light-sheet will
32 be visible, and the tilt can be checked by placing an alignment target in the optical path
33 while scanning the beam. By tilting mirror M_1 up or down, the scanning pattern not only
34 moves up or down, but is also rotated if the mirror surface is not exactly vertical. Since
35 M_1 and GM are in conjugated planes, the tilt and offset can be performed independently.
36 The tilt is first fixed by M_1 while inspecting the target, and the beam is re-centered by

1 changing the offset on the galvanometric mirror. Moving the galvanometric mirror will
2 not introduce tilt, since in this case rotation axis is perpendicular to the reflection plane.

3 **3.3.2 Alignment of the detection branches**

4 Since the detection path is equivalent to a wide-field detection scheme, its alignment is
5 much simpler than that of the illumination branches. The only difference is the detection
6 branch merging unit (see Section 3.2.3.) which features two moving mirrors. This, how-
7 ever, doesn't affect the alignment procedure, since the movement direction is parallel to
8 both mirrors' surfaces, meaning that the exact position of the mirrors will not affect the
9 image quality, as long as the mirrors are not clipping the image itself. A stability test
10 was performed to confirm the consistent switching performance of the mirror unit before
11 the final alignment took place (see Section 3.5.1).

12 **Positioning the tube lens** The position of the tube lens determines the focal plane
13 that is being imaged on the camera sensor. Ideally, the tube lens' distance from the
14 camera sensor is exactly the tube lens' focal length, which will ensure the best imaging
15 performance. If the tube lens' distance is not correct, the focal plane will be slightly
16 shifted in the axial direction. Although small shifts will not necessarily have detrimen-
17 tal effect on the image quality, because the light-sheet can also be shifted accordingly.
18 Because of the shifted focal and image planes, however, the magnification of the system
19 will be affected, and will change depending on the amount of defocus. For this reason we
20 aim for positioning the tube lens as close to the theoretical position as possible.

21 Our tube lens is a compound, achromatic lens with a center thickness of 12.5 mm,
22 and edge thickness of 11.3 mm. Its effective focal length is 400 mm which will produce a
23 50x magnified image. The back focal length is 394.33 mm which we measured from the
24 camera chip, and the lens was positioned at this theoretically optimal position.

25 **Adjusting the correction collar** The Nikon 25x objectives used for this setup have
26 a built in correction ring that can be used to correct spherical aberrations resulting
27 from refractive index differences when imaging samples behind a coverslip. This can
28 be also effectively used to correct for any spherical aberrations occurring from imaging
29 through the FEP foil. Although these aberrations are expected to be extremely low, due
30 to the relatively thin, 50 μm foil thickness, and the close matching of refractive index
31 ($n_{FEP} = 1.344$, $n_{H_2O} = 1.333$), for optimal, aberration free image quality it can't be
32 neglected.

33 The correction collars are adjusted by inspecting a gel-suspended fluorescent bead
34 specimen with the microscope, where the beads can act as a reporter of the point spread
35 function of the microscope. The alignment can be performed "live" by inspecting the

1 bead image quality for aberrations. By gradually changing the correction collar, the ring
2 artifacts are minimized on out-of-focus beads, and the peak intensity is maximized for in-
3 focus beads. By moving the correction ring, the focal plane is also slightly shifted, which
4 has to be compensated by shifting the light-sheet correspondingly to coincide with the
5 correct imaging plane.

6 **Adjusting the field of view** To allow for proper sampling of the image, we use
7 $50\times$ magnification, which combined with the $6.5\text{ }\mu\text{m}$ pixel pitch of our sCMOS cam-
8 era, will result in a $0.13\text{ }\mu\text{m}$ pixel size. The full field of view recorded by the camera
9 is $2048 \times 0.13\text{ }\mu\text{m} = 266.24\text{ }\mu\text{m}$. To ensure the best image quality, we align the center of
10 the objective field of view on the camera sensor, since this region has the best optical
11 properties in terms of numerical aperture, aberration correction and flatness of field.

12 Field of view alignment can be performed by using mirror M4 just before the detection
13 merging unit. To identify the center region of the field of view, diffuse white light is used
14 to illuminate the entire sample chamber and is imaged on the camera. Then, mirror
15 M4 is adjusted until the top edge of the field of view becomes visible, *i.e.*, where the
16 illumination from the chamber is clipped. This will have a circular shape. Afterwards,
17 adjusting the mirror in the orthogonal direction, the left-right position of the field of
18 view can be adjusted, by centering the visible arc on the camera sensor.

19 After the horizontal direction is centered, vertical centering is performed. This, how-
20 ever can not be centered the same way as the horizontal direction, since for that we
21 would have to misalign the already aligned horizontal position. To determine the center,
22 we move the field of view from the topmost position to the bottom. During this process
23 the number of turns of the adjustment screw is counted (this can be done accurately by
24 using a hex key). After reaching the far end of the field of view, the mirror movement is
25 reversed, and the screw is turned halfway to reach the middle.

26 3.4 Control unit

27 The microscope's control and automation is performed by an in-house designed modular
28 microscope control system developed in LabVIEW [113]. The core of the system is a
29 National Instruments cRIO-9068 embedded system that features an ARM Cortex A9
30 processor, and a Xilinx Zynq 7020 FPGA. Having both chips in the same device is a
31 great advantage, since the main processor can be used to run most of the microscope
32 control software, while the FPGA can be used to generate the necessary output signals
33 in real time and with high precision.

34 The embedded system is complemented by a high-performance workstation that is
35 used to display the user interface of the microscope, and to record the images of the
36 high-speed sCMOS camera.

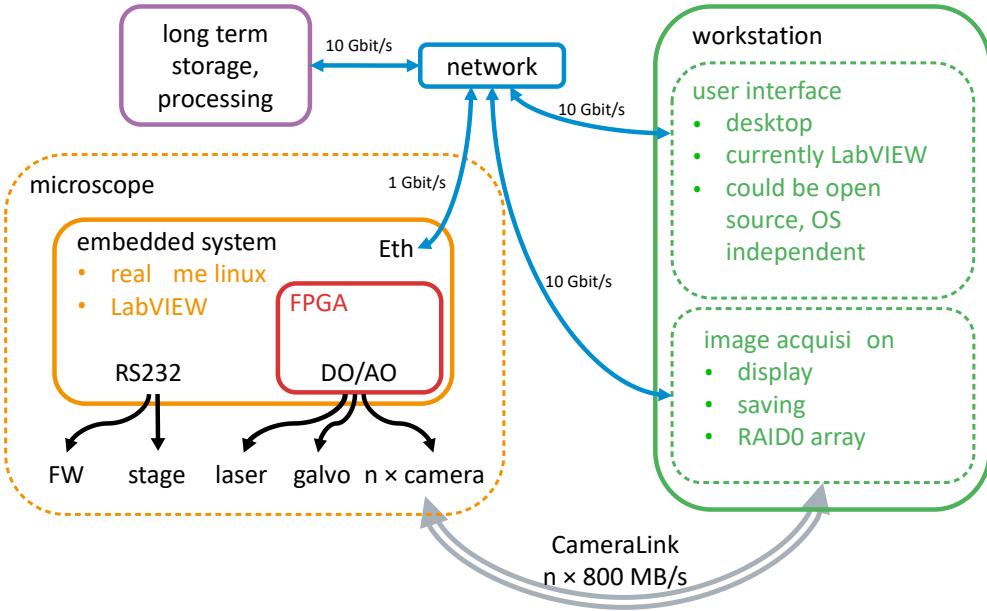


Figure 3.7: Microscope control hardware and software architecture. The embedded system responsible for the hardware control and the workstation are communicating through the network using the WebSocket protocol. The electronic devices of the microscope are either controlled through digital/analog signals, or through serial communication. The camera is also connected to the workstation with a CameraLink connection, which transmits the recorded images.

3.4.1 Hardware

Various components need to be synchronized with high precision to operate the microscope: a laser combiner to illuminate the sample; a galvanometric scanner to generate the light-sheet; stages to move the samples; filter wheel to select the imaging wavelengths; and a camera to detect the fluorescence signal. For high speed image acquisition, all of these devices have to be precisely synchronized in the millisecond range, and some even in the microsecond range. Although they require different signals to control them, we can split them into three main categories:

digital input	analog input	serial communication
camera exposure	galvanometric position	filter wheel
laser on/off ($\times 3$)	laser intensity ($\times 3$)	stages ($\times 2$)

All devices are connected to the NI cRIO 9068 embedded system, either to the built-in RS232 serial port, or to the digital and analog outputs implemented by C-series expansion modules (NI 9401, NI 9263, NI 9264). The workstation with the user interface is communicating with the embedded system through the network. The only device with a connection to both systems is the camera: the embedded system triggers the image acquisition, while the images are piped to the workstation through a dual CameraLink interface, capable of a sustained 800 MB/s data transfer rate (Figure 3.7).

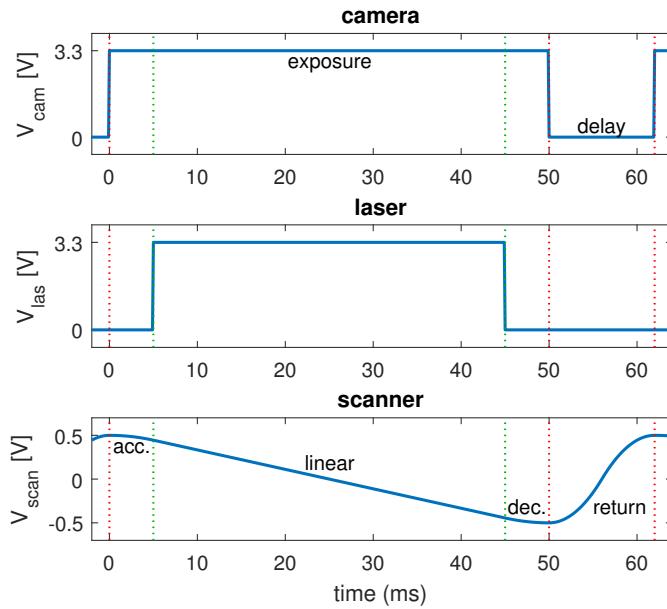


Figure 3.8: Digital and analog control signals. Example traces for recording a single plane with 50 ms exposure time and 12 ms delay. During the exposure of the camera the scanner has 3 sections: acceleration (acc.), linear, and deceleration (dec.). The laser line is synchronized with the scanner linear section to ensure even illumination.

1 3.4.2 Software

2 Being able to precisely control all of the instruments not only relies on the selected hardware,
 3 but just as much on the software. Our custom software is developed in LabVIEW,
 4 using an object-oriented approach with the Actor Framework. The embedded system is
 5 responsible for the low-level hardware control, for keeping track of the state of all devices,
 6 saving the user configurations, and automating and scheduling the experiments. It also
 7 offers a Javascript Object Notation (JSON) based application programming interface
 8 (API) through WebSocket communication. This is mainly used to communicate with the
 9 user interface, however it also offers the possibility of automated control by an external
 10 software.

11 FPGA software

12 The on-board FPGA is responsible for generating the digital and analog output signals
 13 based on the microscope settings (Figure 3.8). To avoid having to calculate all the traces
 14 for a whole stack, the main software only calculates a few key parameters of the traces
 15 that are necessary to completely describe them. The FPGA then calculates the signals
 16 in real time, and outputs them with microsecond precision.

17 To describe the traces, we define them as a concatenation of *sections*. Each section has
 18 3 or 5 parameters, depending on whether they are digital or analog. Both types have three
 19 common properties: `value`, `length`, and `type`. The analog sections additionally contain a

1 dValue and a ddValue element describing the velocity and the acceleration of the signal.
2 This allows us to generate piecewise functions made of second-order polynomials.
3 The type element contains information on which value should be updated for the
4 current section. Setting the lowest bit high will update the value, setting the second bit
5 high will update the dValue, and setting the third bit high will update the ddValue.
6 This feature allows to define smooth transitions between the sections, and also to define
7 more complex signals, as long as they are periodic in the second derivative.

8 3.5 Results

9 Following the design phase, all custom parts were manufactured by the EMBL mechanical
10 workshop, and the microscope was assembled on a MellesGriot optical table (Figure 3.9
11 and Figure D1). The microscope was equipped with an Andor Zyla 4.2 sCMOS camera
12 that offers a large field of view of 2048×2048 pixels with a pixel pitch of $6.5 \mu\text{m}$. It offers
13 a high dynamic range of 1:30,000, and high frame rate at 100 frames per second (fps),
14 while readout noise is minimal (0.9 e^-).

15 To evaluate the performance of the microscope, we conducted various measurements,
16 concerning the stability and the resolution of the system. The methods and results of
17 these measurements will be presented in this section.

18 3.5.1 Stability of view switcher unit

19 As the view switcher unit is a custom-designed solution for this microscope, it is necessary
20 to evaluate the effect of the switching on the field of view. Given that this is a moving
21 unit, many mechanical imprecisions can introduce a drift in the final image.

22 To assess the reproducibility in the movement of the detection branch switching unit,
23 a long-term stability test was conducted. To exclude all other factors (such as sample
24 drift), we imaged the opening of two closed irises that were mounted on the detection
25 optical rail. Image formation was done by two achromatic lenses ($f = 75 \text{ mm}$) positioned
26 directly after mirrors M5 and M5'.

27 The apertures were imaged from both views every 10 seconds, for 4 h, for a total
28 of 1440 images per view, and 2880 switches. The center of the aperture was segmented
29 on all images using Matlab, and tracked to assess any drift occurring during the 4 h
30 time lapse (Figure 3.10). To visualize the uncertainty of the aperture's position, we
31 performed principal component analysis (PCA) on the positions and visualized the results
32 by plotting the 95% confidence ellipse on the images. Although the right view had a
33 significant spread with a standard deviation of 4.37 px and 2.26 px along the long and
34 short axes respectively, the left view was extremely stable, and the standard deviation
35 was only 0.07 px and 0.04 px for the long and short axes respectively. This result implies

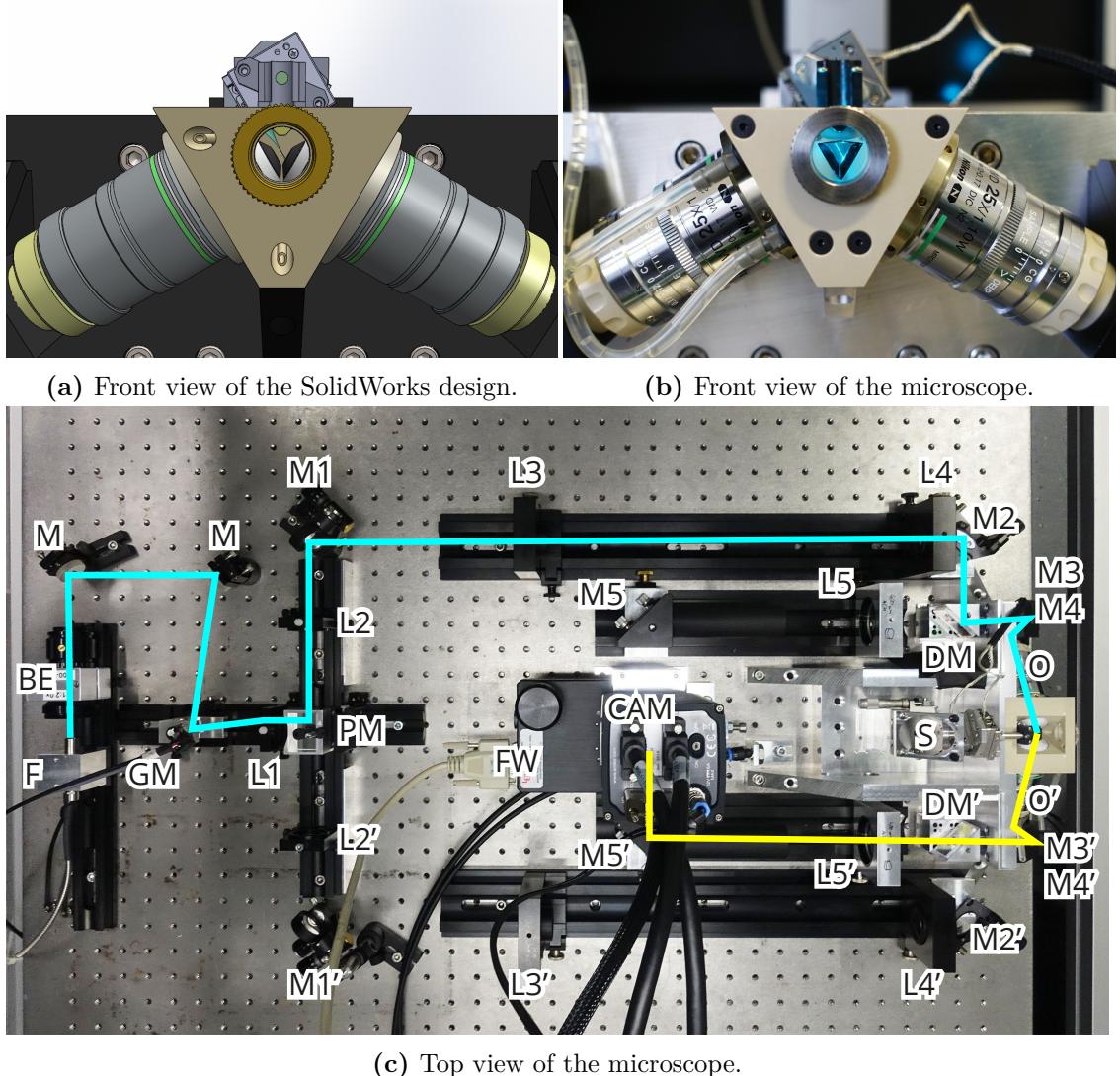


Figure 3.9: Completed DualMouse-SPIM. The excitation and emission light paths are depicted with blue and yellow lines respectively. Component numbering corresponds to Figure 3.5. Detection branch merging unit, and mirrors M6 and M6' are underneath the camera and filter wheel. BE – beam expander, CAM – camera, DM – dichroic mirror, F – fiber, FW – filter wheel, L – lens, M – mirror, O – objective, PM – prism mirror, S – stage

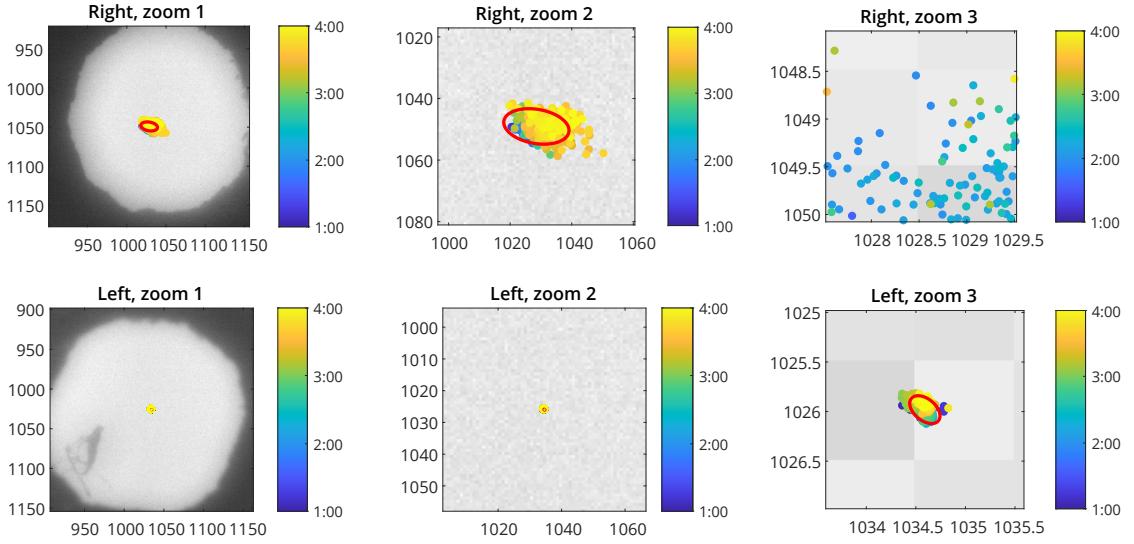


Figure 3.10: Stability measurements of view switcher unit. A closed aperture was imaged on the camera from both views, every 10s, for 4h. The dots represent the center of each frame, color coded with the time. The red ellipse represents the 95% confidence interval of the measured positions based on principal component analysis. Standard deviations for right view: $\sigma_1 = 4.37 \text{ px}$, $\sigma_2 = 2.26 \text{ px}$; for left view: $\sigma_1 = 0.07 \text{ px}$, $\sigma_2 = 0.04 \text{ px}$.

- 1 that there is no conceptual limitation in reaching sub-pixel reproducibility with the view
- 2 switching unit, although some optimization is still needed for the right view to reach the
- 3 same stability as the left view.

4 3.5.2 Characterizing the illumination profile

- 5 Since a Gaussian beam is used for illumination, its intensity profile depends on the axial
- 6 position (Equation 1.18). Although the total intensity at any cross section of the beam
- 7 is constant, because of the divergent properties, the peak intensity varies. To assess
- 8 any non-uniformities in the illumination pattern, we measured the illumination beam
- 9 intensity for each view.

10 To visualize the beam, the chamber was filled with a fluorescent solution (0.1 %
 11 methylene blue in distilled water). In order to increase the signal-to-noise ratio of these
 12 images, a long exposure time of 200 ms was used, and 500 images of each beam was
 13 averaged. Background images were acquired by repeating the image acquisition with
 14 the laser turned off. The average of the background images were subtracted from the
 15 averaged beam images, resulting in the beam intensity profile (Figure 3.11).

16 To determine the beam waist position, we measured the beam thickness (FWHM)
 17 for each column of the averaged images, and located the position of the minimal width.
 18 The beam width at the waist position for the left view was $3.70 \mu\text{m}$, and for the right
 19 view it was $3.58 \mu\text{m}$ (Figure 3.11). We also measured the usable length of the beams
 20 (Section 1.3.1). The distance between the points where the beam has expanded by a

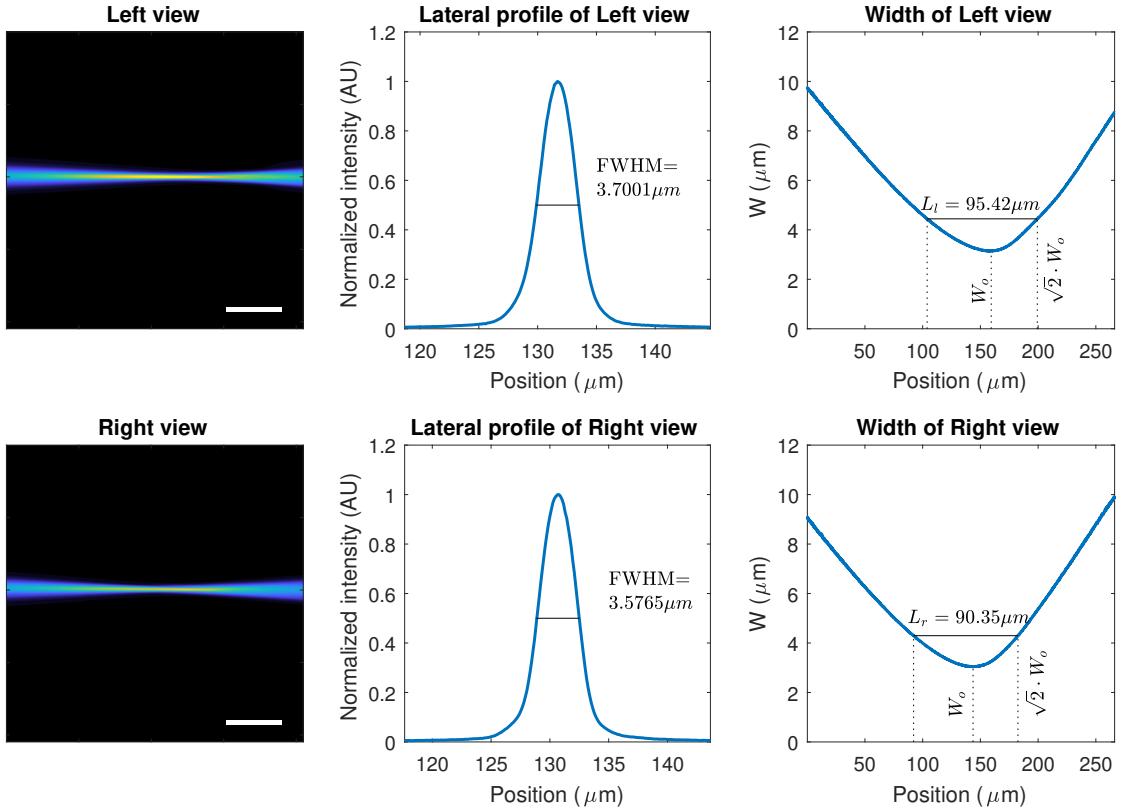


Figure 3.11: Illumination beam profile. Average of 500 stationary beam images for both left (top left) and right objectives (bottom left). Beam intensity profile along the waist of each beam is plotted in the center column. The right column shows the beam width profile of each beam ($1/e^2$).

1 factor of $\sqrt{2}$ relative to the waist is $95.42 \mu\text{m}$ for the left view, and $90.35 \mu\text{m}$ for the
 2 right view. This corresponds well to the original requirement of a minimal field of view
 3 of $100 \mu\text{m}$. Depending on the sample and the required level of optical sectioning, the
 4 practical field of view can be larger than this, as the camera sensor allows for a field of
 5 view of $266 \mu\text{m}$.

6 3.5.3 Resolution and point spread function measurement

7 In order to establish an ideal, reference PSF, we simulated the theoretical PSF of the
 8 microscope with the Gibson-Lanni model [114], using the MicroscPSF Matlab implemen-
 9 tation [115]. The advantage of this model is that it accounts for any differences between
 10 the experimental conditions and the design parameters of the objective, and thus it can
 11 simulate any possible aberrations that may arise. The adjustable parameters are the
 12 thicknesses (t) and refractive indices (n) of the immersion medium (i), coverslip (g),
 13 and sample (s). To simulate the reference PSF, we accounted for the slight mismatch in
 14 refractive index between the FEP foil ($n_g = 1.344$) and water ($n_i = 1.33$). The working
 15 distance of the objective is 2 mm , while the thickness of the foil is $50 \mu\text{m}$.

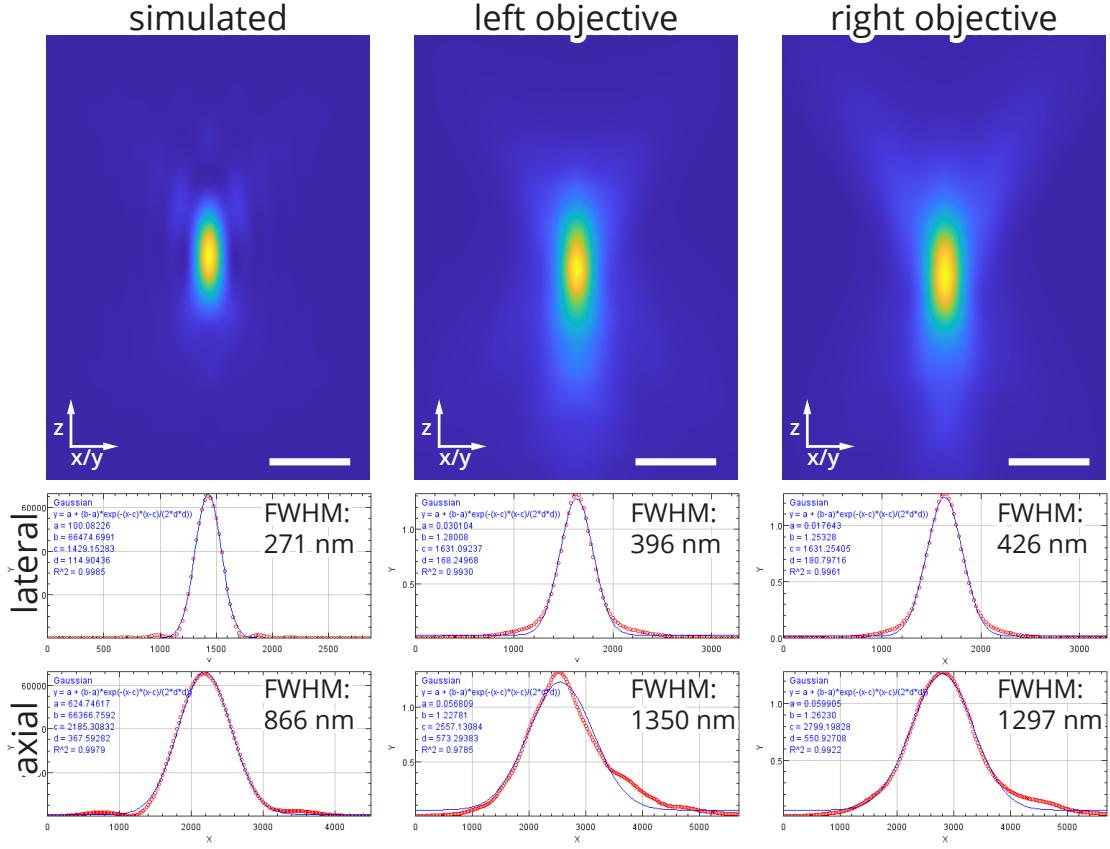


Figure 3.12: Simulated and measured PSF of Dual Mouse-SPIM. Top row: axial sections of simulated and measured point spread functions. Middle row: lateral intensity profile and Gaussian fit. Bottom row: Axial intensity profile and Gaussian fit. Simulations were performed based on the Gibson-Lanni model. Immersion medium and sample refractive index: 1.330, coverslip (FEP foil) refractive index: 1.344, coverslip distance: 1900 μm , coverslip thickness: 50 μm . Excitation wavelength: $\lambda_{ex} = 561 \text{ nm}$. Emission wavelength: $\lambda_{em} = 600 \text{ nm}$. Scale bar: 1 μm .

1 It is apparent from the simulations that even though the FEP refractive index is al-
 2 most identical to the refractive index of water, a slight spherical aberration is still present
 3 even for the ideal case (Figure 3.12, left). The resolution measured as the FWHM of the
 4 intensity profile through the lateral and axial cross sections is 271 nm and 866 nm respec-
 5 tively. The FWHM was measured in Fiji by fitting a Gaussian curve, and multiplying
 6 the standard deviation of the resulting fit by $2\sqrt{2 \ln 2}$.

7 To experimentally determine the resolution of the microscope and characterize its
 8 optical performance, we measured the point spread function using fluorescently labeled
 9 beads suspended in 0.8% GelRite (Section 3.6). The gel was loaded in glass capillaries
 10 and allowed to cool. After the gel solidified, a $\sim 1 \text{ mm}$ piece was cut off and placed in
 11 the microscope sample holder. The beads were imaged from both views using the 561 nm
 12 laser line with the 561 long-pass filter (Semrock, BLP02-561R-25).

13 From both views 12 beads were averaged using Fiji [116] and the 3D PSF estimator
 14 of the MOSAIC suite [117]. In order to acquire a more accurate PSF, the averaged

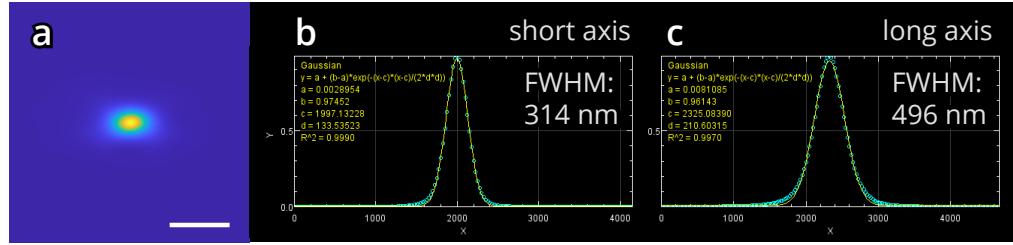


Figure 3.13: Combined PSF of 2 views. (a) The measured PSFs (Figure 3.12) were rotated to their corresponding orientations and multiplied. (b) Gaussian fit along the short axis of the combined PSF; FWHM=314 nm. (c) Gaussian fit along the long axis of the combined PSF; FWHM=496 nm. Scale bar, 1 μm

1 bead images were deconvolved with the ideal image of the bead (uniform sphere with a
 2 diameter of 500 nm) using the DeconvolutionLab2 Fiji plugin [118]. The results of the
 3 deconvolution are shown on Figure 3.12. Similarly to the simulation, we measured the
 4 axial and lateral resolutions on the experimental PSFs. For the left view, the measured
 5 resolutions were 386 nm in the lateral direction, and 1350 nm in the axial direction. For
 6 the right view the lateral resolution was 326 nm, and the axial was 1297 nm.

7 To estimate the achievable multi-view resolution of the system, we combined the two
 8 measured PSFs. First, the PSFs were rotated by ±60° to correspond to the objective
 9 orientations. The PSF images were then normalized by scaling the maximum to 1, and
 10 the two rotated and normalized PSFs were multiplied (Figure 3.13). Resolution of the
 11 combined PSF is much improved in all directions: along its shortest axis the FWHM
 12 is 314 nm, and along the longest axis the FWHM is 496 nm (Figure 3.13). Both are
 13 better than the corresponding resolutions for a single view, especially along the long
 14 axis, where the FWHM is 2.67 times smaller. This is almost perfectly matching the
 15 theoretically expected increase in the axial direction (compare with Section 3.1).

16 3.5.4 Resolution inside a *Drosophila melanogaster* embryo

17 Apart from measuring the point spread function and resolution in an ideal bead sample,
 18 we also wanted to know the resolution inside a biological sample, as the tissues usually
 19 introduce some aberrations to the light path. Although these aberrations highly depend
 20 on the sample, as a worst case scenario, we measured the point spread function inside
 21 a *Drosophila melanogaster* embryo. As this specimen is highly opaque and scattering, it
 22 gives a good estimate for the upper bound of the resolution.

23 Instead of the fluorescent beads, we imaged a *Drosophila* embryo expressing H2A-
 24 mCherry marking the nuclei, and ASL-YFP marking the centrosomes (Figure 3.14a, and
 25 Section 3.6). As the centrosomal protein ASL is diffraction limited in size [119], its image
 26 gives a good estimate for the point spread function inside the specimen.

27 Similarly to the bead recordings, 15 centriole images were averaged with the 3D PSF

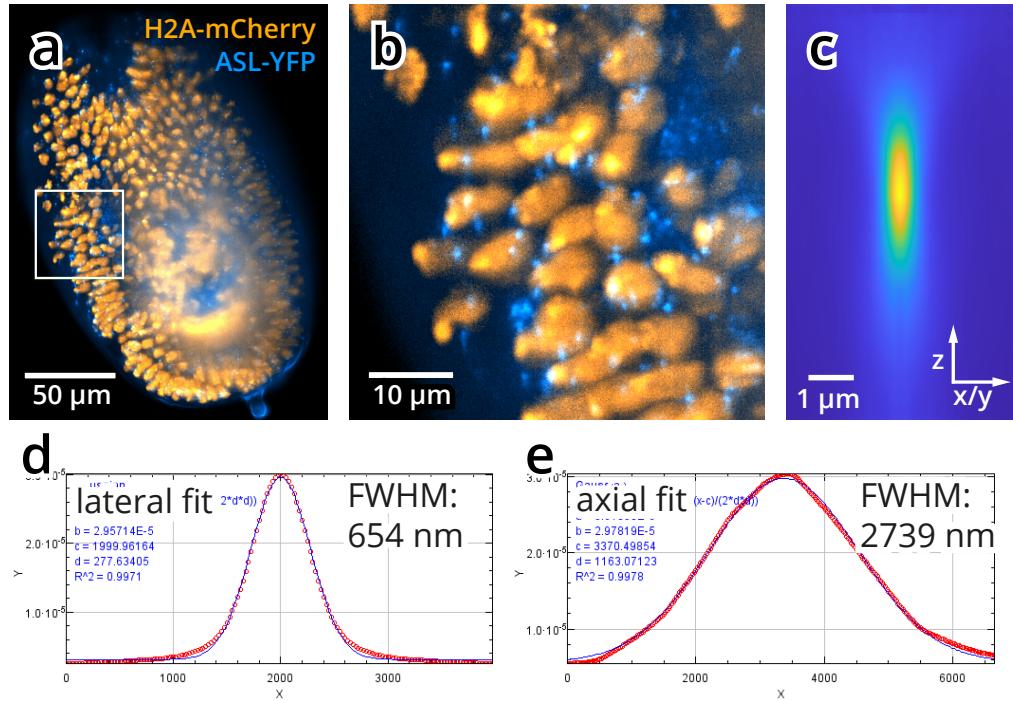


Figure 3.14: Maximum intensity projection of a *Drosophila melanogaster* embryo recording. Maximum intensity projection of a 30 µm thick substack. Orange: nuclei (H2A-mCherry), blue: centrioles (ASL-YFP). 50x magnification. (a) Overview of the embryo. (b) Zoomed in view to the region marked in (a). (c) Average image of 15 centrioles distributed evenly in the embryo. (d) Gaussian fit on the lateral intensity profile of the averaged centrioles; FWHM=654 nm. (e) Gaussian fit on the axial intensity profile of the averaged centrioles; FWHM=2739 nm.

1 tool of the MOSAIC plugin in Fiji (Figure 3.14c). On the averaged image we measured
 2 the resolution by fitting a Gaussian function on the lateral and axial intensity profiles
 3 (Figure 3.14d,e), and calculating the FWHM of the fitted Gaussian. The size of the
 4 averaged centrioles was 654 nm in the lateral, and 2739 nm in the axial direction.

5 3.5.5 Multi-view imaging of a mouse zygote

6 To demonstrate the multi-view capabilities and improved image quality of the micro-
 7 scope, we performed dual-view imaging of a fixed mouse zygote and combined the images
 8 using the Multiview Reconstruction [112] plugin of Fiji (Figure 3.15). The microtubules
 9 were stained with Alexa Fluor 488 coupled secondary antibodies, and the zygote was
 10 imaged from both views with 0.5 µm inter-plane distance.

11 Anisotropy in the resolution becomes apparent in the single-view recordings, when
 12 the stacks are resliced from a different direction than the imaging plane. Even though
 13 the native view of each objective (Figure 3.15a,d) shows good contrast, and easy to rec-
 14 ognize features, when rotated and sliced corresponding to the view of the other objective,
 15 the contrast is almost completely gone, and the resolution is decreased (Figure 3.15b,e).
 16 The fused stack, after the multi-view deconvolution contains the high resolution infor-

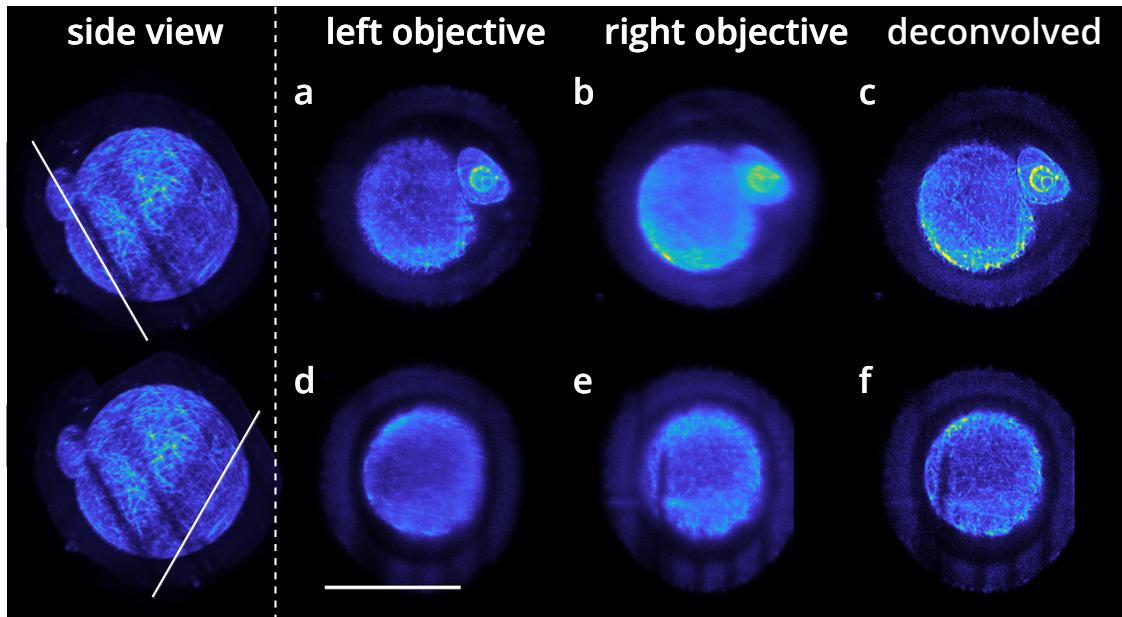


Figure 3.15: Multi-view recording of a mouse zygote. Dual-view imaging was performed on a fixed mouse zygote. Microtubules were stained with Alexa Fluor 488 coupled secondary antibodies. Top row: view of Left objective. Bottom row: view of Right objective. (a) Raw image from left objective. (b) Rotated view of Right objective. (c) Multi-view deconvolution of stacks (a) and (b). (d) Rotated slice from left objective. (e) Raw image from right objective. (f) Multi-view deconvolution of stacks (d) and (e). Scale bar: 50 µm. The darker banding artifacts visible on the side view are due to bleaching.

1 mation from both views, and thus both directions show good quality and high contrast
 2 (Figure 3.15c,f).

3 3.6 Methods

4 3.6.1 Preparation of fluorescent bead samples

5 For registration and resolution measurements we used TetraSpeck 0.5 µm diameter fluo-
 6 rescently labeled beads (ThermoFisher, T7281). The stock bead solution was thoroughly
 7 vortexed and sonicated for 5 min before diluting it 1:100 in distilled water. The diluted
 8 bead solution was stored at 4 °C until use. GelRite (Sigma-Aldrich, G1910) gel was pre-
 9 pared in distilled water at 0.8% concentration with 0.1% MgSO₄ · 7H₂O and kept at
 10 70 °C until use. 50 µl of the diluted bead solution was added with a heated pipette tip to
 11 450 µl of gel solution at 70° to prevent polymerization. The gel was thoroughly vortexed,
 12 and loaded to glass micropipettes (Brand 100 µl). The gel was allowed to cool to room
 13 temperature and stored in a petri dish under dH₂O at 4° until use. For imaging, a small
 14 piece of gel was extruded from the capillary, cut off, and placed in the sample holder.
 15 After positioning the gel to the bottom of the sample holder, the holder was filled with
 16 200 µl dH₂O.

1 3.6.2 Preparing the sample holder

2 The sample holder was lined with 12.5 μm (PSF measurements) or 50 μm (mouse zygote
3 and *Drosophila* embryo imaging) thin FEP foil (Lohmann, RD-FEP050A-610). The FEP
4 foils were cut to size, washed with 70% ethanol followed by a second wash with dH₂O.
5 After washing, both surfaces of the foils were chemically activated by a 20 s plasma
6 treatment (PlasmaPrep2, Gala Instrumente). The foils were then stored in a petri dish
7 until further use separated by lens cleaning tissues (Whatman, 2105-841). Before imaging,
8 the sample holder was cleaned with dish soap, rinsed with tap water, rinsed with 70%
9 ethanol, and rinsed with dH₂O. The prepared foils were glued to the inside of the cleaned
10 sample holder with medical grade silicon glue (twinsil speed, picodent). During the 5 min
11 curing process the foil was pressed against the sample holder by a custom made press
12 fitting the shape of the sample holder. Final dilution of the stock bead solution in the
13 gel is 1:1000.

14 3.6.3 Drosophila embryo imaging

15 *Drosophila melanogaster* embryos (fly stock AH1) expressing fluorescent nuclear (H2A-
16 mCherry) and centriole (ASL-YFP) markers were collected on apple juice agar plates and
17 dechorionated in 50% bleach solution for 1 min. After rinsing the bleach with deionized
18 water, the embryos were placed in the sample holder under PBS solution. A small piece of
19 gel containing fluorescent beads were also placed next to the samples to aid in multi-view
20 registration.

21 3.6.4 Mouse zygote imaging

22 Fixed mouse zygotes labeled with Alexa Fluor 488 (microtubules) and Alexa Fluor 647
23 (kinetochores) coupled secondary antibodies were kindly provided by Judith Reichmann.
24 Zygotes were transferred to the sample holder, and imaged in PBS. To allow for multi-
25 view reconstruction, a small piece of gel containing fluorescent beads was placed next to
26 the zygote in the sample holder. After imaging the zygote from both views, the beads
27 were also recorded using the same stack definitions. After data acquisition, the multi-view
28 datasets were registered and deconvolved in Fiji [116] using the Multiview Reconstruction
29 Plugin [112, 120]. The deconvolution was based on a simulated PSF generated in Fiji
30 with the PSF Generator plugin [121] using the Born-Wolf PSF model [24]. NA_{ex} = 0.1,
31 NA_{em} = 1.1, n=1.33, λ_{ex} = 488 nm, λ_{em} = 510 nm.

1 3.7 Conclusions

2 In this chapter we have presented a novel light-sheet microscope designed for subcellular
3 imaging of mouse embryos. The microscope has two identical, high NA (1.1) objectives,
4 positioned in a 120° angle, and operates with a 30° tilted light-sheet. Due to the symmet-
5 rical design both objectives can be used for detection and illumination as well, providing
6 multi-view capabilities without sample rotation.

7 We have characterized the optical properties of the microscope by measuring the light-
8 sheet dimensions (average length: 93 μm , average thickness: 3.6 μm), and by measuring
9 the point spread function. When combining the two views, the resolution is much closer
10 to an ideal isotropic shape, being 496 nm along the long axis and 314 nm along the
11 short axis as opposed to 1323 nm and 411 nm measured for a single view. This 2.67 fold
12 increase in axial resolution can potentially make the difference in being able to track all
13 chromosomes in a developing mouse embryo.

14 To be able to surpass the proof-of-concept state, the microscope still needs some im-
15 provements. The detection view switching unit needs to be improved to minimize field of
16 view drift of both views. Data processing for long-term experiments would be extremely
17 challenging otherwise. Another necessity for long-term experiments is the implementa-
18 tion of an environmental chamber to provide the appropriate conditions for live embryo
19 imaging.

20 The use of high-NA objectives also keeps the door open for future upgrades, such
21 as the addition of structured illumination [55, 56], or coupling in a high-power pulsed
22 laser for photomanipulation capabilities [122]. Another natural extension to the system
23 would be the addition of a third objective that would allow for truly isotropic imaging
24 and two-sided sample illumination.

25 Single-view solutions for isotropic imaging only allow a very limited field of view. For
26 diffraction-limited optical sectioning a Gaussian beam is diverging too fast [123], while
27 the use of other, non-diffractive beams rely on self-interference [7], which breaks down
28 after a few tens of micrometers inside the sample. Our dual-view high NA solution allows
29 for near isotropic resolution even for large fields of view, such as for entire mouse zygotes
30 or embryos (120 $\mu\text{m} \times 120 \mu\text{m} \times 120 \mu\text{m}$).

¹ Chapter 4

² GPU-accelerated image
³ processing and compression

⁴ 4.1 Challenges in data handling for light-sheet microscopy

⁵ When using any kind of microscopy in research, image processing is a crucial part of
⁶ the workflow. This is especially true for light-sheet microscopy, since it is capable of
⁷ imaging the same specimen for multiple days, producing immense amounts of data. A
⁸ single overnight experiment of *Drosophila* development (which is a very typical use-case
⁹ for light-sheet microscopy) can produce multiple terabytes of data.

¹⁰ Apart from light-sheet microscopy, many other microscopy modalities also suffer from
¹¹ this problem. Methods, such as high content screening [124–126], where tens of thousands
¹² of different genotypes are imaged generating millions of images; and single molecule
¹³ localization microscopy (SMLM) [127–129], where just a single plane of a sample is
¹⁴ imaged hundreds of thousands of times to acquire super-resolved images.

¹⁵ Not only these methods are capable of generating data extremely fast, but with
¹⁶ the sustained high data rate a single experiment can easily reach multiples of terabytes
¹⁷ (Figure 4.1). Handling this amount of data can quickly become the bottleneck for many
¹⁸ discoveries, which is a more and more common issue in biological research [130–132].

¹⁹ This chapter will focus on addressing these challenges by presenting a real-time,
²⁰ GPU-based image preprocessing pipeline developed in CUDA [133] consisting of two
²¹ parts (Figure 4.2). The first part is a fast image fusion method for our workhorse light-
²² sheet microscope, the MuVi-SPIM [45], that enables live fusion of the images arriving
²³ from two opposing cameras. The second part of the pipeline, which can also be used
²⁴ in a standalone way, is a real-time image compression library that allows for lossless
²⁵ and noise-dependent lossy compression of the data directly during acquisition even for
²⁶ high-speed sCMOS cameras.

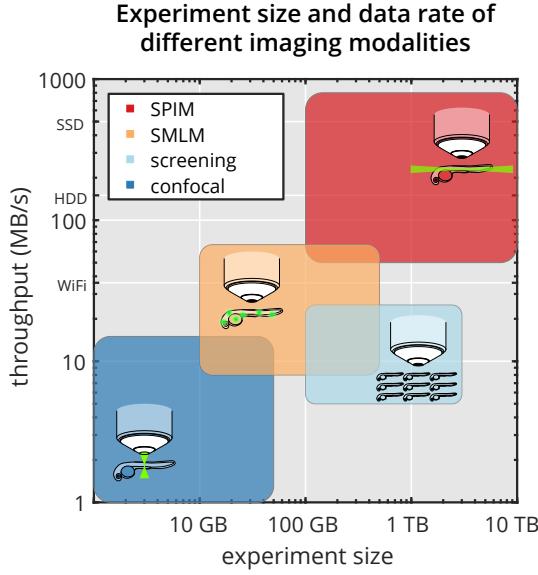


Figure 4.1: Experiment sizes and data rate of different imaging modalities. Comparison of single-plane illumination microscopy (SPIM, red), high-content screening (light blue), single molecule localization microscopy (SMLM, orange) and confocal microscopy (blue) by typical experiment size and data production rate (see also Table B1 in Appendix B).

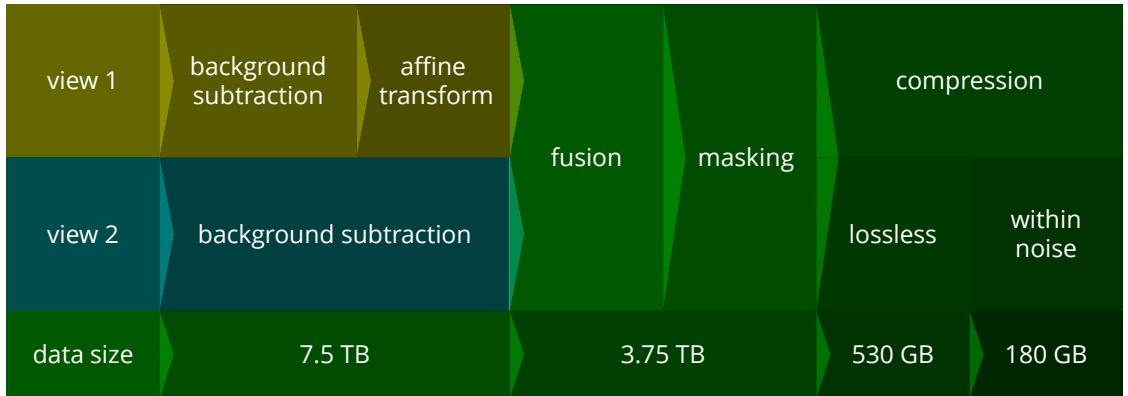


Figure 4.2: Real-time image processing pipeline for multi-view light-sheet microscopy.

4.2 Real-time preprocessing pipeline

Similarly to the DualMouse-SPIM, our currently used production microscope, the Multiview-SPIM (MuVi-SPIM) [45] also uses multiple imaging directions to improve the image quality; especially for opaque specimens. In this case, however, the aim is completeness rather than increasing the resolution. As the MuVi-SPIM is capable of imaging much larger specimens, such as entire *Drosophila* embryos, the sample size itself can present some challenges, especially for opaque specimens. As light scattering and absorption impact both the illumination and detection optics, the negative effects for SPIM are more pronounced compared to single-lens systems [134].

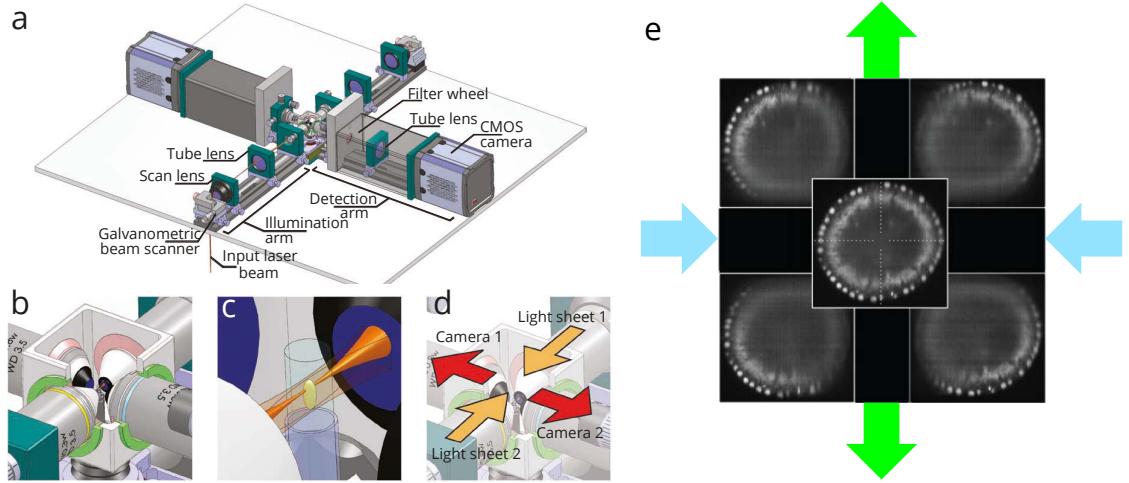


Figure 4.3: Operating principle of MuVi-SPIM. (a) The microscope consists of two illumination and two detection arms for simultaneous multi-view illumination and detection. (b) The 4 arms meet in the imaging chamber that is filled with water, and contains the sample. (c) The sample is held by a glass capillary, in a GelRite cylinder. Optical sectioning is achieved by a virtual light-sheet. (d) The light-sheets can be generated from two sides (Light sheet 1 and 2), and detection is also double sided (Camera 1 and 2). Adapted from [45].

1 4.2.1 Multiview SPIM for *in toto* imaging

2 MuVi-SPIM provides an elegant solution for multi-view imaging. A standard SPIM setup
 3 with a single detection and a single illumination lens would rotate the sample to acquire
 4 images from multiple directions. MuVi-SPIM, on the other hand, utilizes two opposing
 5 objectives for illumination and two opposing objectives for detection (Figure 4.3a). As
 6 the sample is held by an aqueous gel inside the imaging chamber, all objectives have
 7 unobstructed view of it from multiple directions (Figure 4.3b,c).

8 Data acquisition is done in two steps: the sample is illuminated by Light sheet 1, and
 9 fluorescence is collected by both detection objectives at the same time, after which Light
 10 sheet 2 is activated, and both cameras record the fluorescence again (Figure 4.3d). This
 11 process will result in 4 datasets, all with partial information due to scattering effects.
 12 The 4 views are later fused to a single, high quality dataset (Figure 4.3e). This fusion
 13 process is necessary before any further analysis steps can be performed. However, due to
 14 the sheer size of the data, it takes a considerable amount of time after the acquisition.

15 By combining scanned light-sheet [49] with confocal slit detection on the camera chip
 16 [135], it is possible to exclude out-of-focus, scattered illumination light. This way it is
 17 possible to illuminate simultaneously with both light-sheets, which leaves us with only
 18 two views, the views of the two opposing cameras [75].

1 **4.2.2 Image registration**

2 To perform image fusion of multiple views, first image registration is necessary: the
 3 coordinate systems of the views have to be properly overlapped. Ideally a single mirroring
 4 transformation would be enough to superpose the two camera images, however in practice
 5 the microscope can never be aligned with such precision. Other types of transformations
 6 are also necessary: translation to account for offsets in the field of view; scaling in case of
 7 slightly different magnifications; and also shearing if the detection plane is not perfectly
 8 perpendicular to the sample movement direction [136]. To combine all of these effects,
 9 a full, 3D affine transformation is necessary to properly align the two camera images
 10 (Figure 4.4 a). This transformation can be represented by a matrix multiplication with
 11 12 different parameters:

$$\begin{pmatrix} x, \\ y, \\ z, \\ 1 \end{pmatrix} = \begin{pmatrix} a & b & c & d \\ e & f & g & h \\ i & j & k & l \\ 0 & 0 & 0 & 1 \end{pmatrix} \times \begin{pmatrix} x \\ y \\ z \\ 1 \end{pmatrix} = \begin{pmatrix} ax + by + cz + d \\ ex + fy + gz + h \\ ix + jy + kz + l \\ 1 \end{pmatrix}$$

12 where x, y, z are the coordinates in the original 3D image, x', y', z' are coordinates in the
 13 transformed image, and a, b, \dots, l are the affine transformation parameters.

14 These parameters are traditionally acquired by a bead based registration algorithm
 15 after imaging fluorescent beads from each view of the microscope [120, 137]. The beads
 16 are segmented by using a difference of Gaussian filter, and the registration param-
 17 eters are acquired by matching the segmented bead coordinates in each view. Identifying
 18 the corresponding beads is done by a translation and rotation invariant local geomet-
 19 ric descriptor. This matches the beads based on their relative position to their nearest
 20 neighbors. After the matching beads are identified, the affine transformation parameters
 21 are calculated by minimizing the global displacement for each pair of beads.

22 For the two opposing views of MuVi-SPIM, these parameters are only dependent
 23 on the optical setup itself, and not on the sample or on the experiment. Because of
 24 this, it is sufficient to determine the transformation parameters only after modifying the
 25 microscope (*e.g.*, after realignment).

26 In our currently used fusion pipeline, after the parameters are acquired, the 3D stacks
 27 are fused by transforming one of the views to the coordinate system of the other (using
 28 the affine transformation parameters from the bead based registration), and fusing the
 29 two stack by applying a sigmoidal weighted average (4.4a). The weights are determined
 30 in a way to exclude the parts of the stacks that have worse image quality and comple-
 31 ment these from the other view's high quality regions. When using the electronic confocal slit
 32 detection (eCSD) weighting is not necessary, as the scattered light is already rejected in
 33 these recordings, and a simple sum of the two stacks gives the best results regardless of

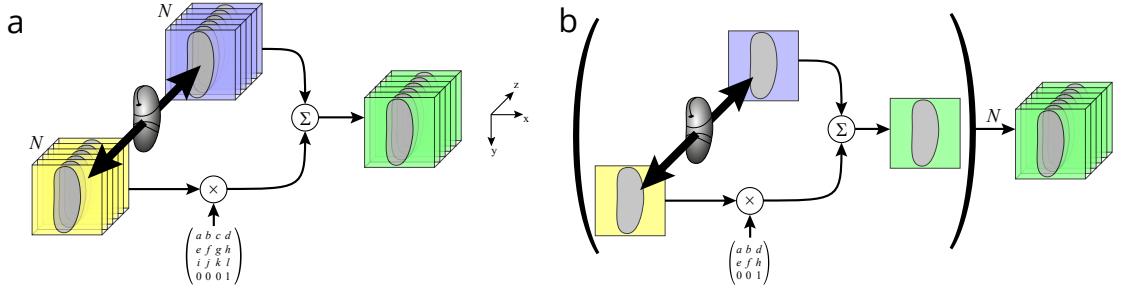


Figure 4.4: Multi-view fusion methods for light-sheet microscopy. a) Full 3D stacks are acquired from the opposing views (yellow and blue), which are then registered in 3D space using previously acquired affine transformation parameters. Registered stacks are then weighted averaged to create the final fused stack (green). b) Images from opposing views are directly fused plane by plane. Registration takes place in 2D space thus reducing computational effort and memory requirements. The registered planes are then weighted averaged to create the final fused image.

1 the sample [75].
 2 The fusion process itself can be very resource intensive and take a considerable
 3 amount of time. This is simply due to the size of the 3D stacks: a single stack is usually
 4 between 2 and 4 GB in size. Thus, the necessary memory requirement to fuse 2 of these
 5 stacks is $3 \cdot 4 \text{ GB} = 12 \text{ GB}$, as the result will also take up the same space. Just reading
 6 and writing this amount of information to the hard disk takes a considerable amount of
 7 time. When using an SSD drive for example, with a 500 MB/s read and write speed, just
 8 the read/write operations will take around $\frac{12 \text{ GB}}{500 \text{ MB/s}} = 24 \text{ s}$.

9 4.2.3 2D fusion of opposing views

10 To speed up the image processing, we take a new approach to fusing the images. As the
 11 two objectives of MuVi-SPIM ideally image the same \$z\$ plane, it should be possible to
 12 reduce the alignment problem to a 2D affine transformation:

$$\begin{pmatrix} x' \\ y' \\ 1 \end{pmatrix} = \begin{pmatrix} a & b & d \\ e & f & h \\ 0 & 0 & 1 \end{pmatrix} \times \begin{pmatrix} x \\ y \\ 1 \end{pmatrix} = \begin{pmatrix} ax + by + d \\ ex + fy + h \\ 1 \end{pmatrix}$$

13 The advantage of a simplified transformation is not only the reduced computational
 14 complexity, but also the massively reduced memory requirement, as in this case it is
 15 sufficient to store 3 planes in memory instead of 3 entire stacks (Figure 4.4 b). Performing
 16 the 2D direct fusion on the GPU immediately after acquisition has two benefits: first,
 17 only the fused images are stored to the hard drive, which directly results in 50% savings
 18 in storage space. Second, since the fusion is faster than the camera acquisition speed, the
 19 fused image can be displayed for the users instead of the 2 raw images. This would greatly
 20 improve user experience and speed up the initial, set-up phase of each experiment.

In order to allow for the direct, 2D fusion, the microscope needs to be very well

4.2 Real-time preprocessing pipeline

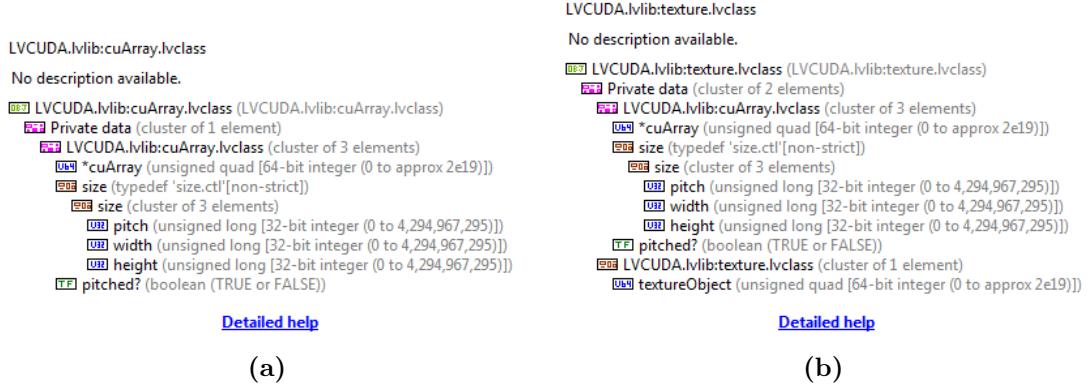


Figure 4.5: Classes of LVCUDA library. (a) `CuArray` class, wrapping a 1D or 2D pitched device memory for use in LabVIEW. (b) `Texture` class, wrapping a CUDA texture object for use in LabVIEW.

aligned to minimize any adverse effects arising from discarding some of the transformation parameters. The requirements for this are the following:

$$|cz| < \sigma_{xy} \quad \forall z \quad (4.1)$$

$$|gz| < \sigma_{xy} \quad \forall z \quad (4.2)$$

$$|ix + jy + (k - 1)z + l| < \sigma_z \quad \forall x, y, z \quad (4.3)$$

1 where σ_{xy} is the lateral resolution, and σ_z is the axial resolution of the microscope, which
 2 are 277 nm and 1099 nm respectively.

3 If these conditions are fulfilled (*i.e.*, the microscope is properly aligned), direct plane
 4 by plane fusion will not result in any loss of information compared to the full 3D image
 5 fusion.

6 4.2.4 CUDA implementation of direct fusion

7 Our custom microscope control software (Section 3.4.2) is developed in LabVIEW and
 8 we implemented the pipeline as a combination of a CUDA and a LabVIEW library. The
 9 CUDA library implements all the necessary low level functions and exposes these in a
 10 dynamically linked library (dll). The LabVIEW library, `LVCUDA.lvlib` implements two
 11 high level classes: `cuArray`, and `Texture` (Figure 4.5). These classes interface with the
 12 CUDA dll, and allow to easily build a flexible CUDA-based image processing pipeline in
 13 LabVIEW.

14 As the pipeline operates on 2D images, both classes support 1D or 2D arrays in a
 15 pitched configuration. `CuArray`, as the parent class, stores the pointer to the array, as
 16 well as the pitch, width and height. LabVIEW natively doesn't support pointer opera-
 17 tions, therefore the pointer is simply cast to a 64 bit unsigned integer internally. As this
 18 is a private member of the class there is no danger of accidental modification. Being a

1 descendent of `cuArray` class, the `Texture` class adds a further member to store the refer-
2 ence to a CUDA texture object. This class is used to implement the affine transformation,
3 as it supports floating point indexing and automatic linear interpolation.

4 Since the processing is actually bottlenecked by the data transfer rate between the
5 main memory and GPU memory, we implemented multiple commonly used image prepro-
6 cessing operations for our pipeline to maximize the utility of the GPU. These functions
7 include background subtraction, background masking and image compression. We will
8 discuss these features in the following sections of the chapter.

9 4.2.5 Background subtraction and masking

10 As an additional step of the preprocessing pipeline, background subtraction and back-
11 ground masking was also implemented. Background subtraction can improve image qual-
12 ity as it removes any system-specific signal, while background masking is beneficial for
13 the consecutive compression step.

14 To perform the background subtraction, dark images are recorder without the laser
15 turned on. Typically 500–2000 images are taken which are averaged, to capture the pixel
16 non-uniformities of the camera sensors. As this is camera specific, the step is repeated
17 for all cameras. If necessary, the recordings can be repeated under different conditions,
18 such as different exposure times, or different sensor modes (such as global shutter and
19 rolling shutter). The averaged background images are saved in a custom file format in an
20 HDF5 container [138] together with the camera serial number. The microscope software
21 can read these files and applies the background subtraction to the appropriate cameras
22 during imaging.

23 Background masking is a useful step to increase the efficiency of image compression.
24 For light-sheet microscopy, where typically an entire embryo is imaged, the boundary
25 of the specimen, and thus the area of interest is well defined. Anything outside the
26 embryo is just noise which does not contribute to any useful information. For image
27 compression, however, these regions are extremely difficult to compress, as due to the
28 random nature of noise, almost no reduction is achievable (see 2.1). To circumvent this
29 issue, the background pixels outside the specimen can be set to 0, which will greatly
30 increase the compression ratio without compromising any data of interest [139].

31 The masking is performed in the following way. As the sample has much higher signal
32 compared to the background, a thresholding operation is sufficient. In order to have a
33 smooth mask, the images are first blurred by a Gaussian kernel, and the thresholding
34 is done on the blurred image. This will define a binary mask with smooth boundaries
35 which is applied to the original image (Figure 4.6). The threshold can be calculated
36 automatically by the Triangle method [140], or can also be user specified.

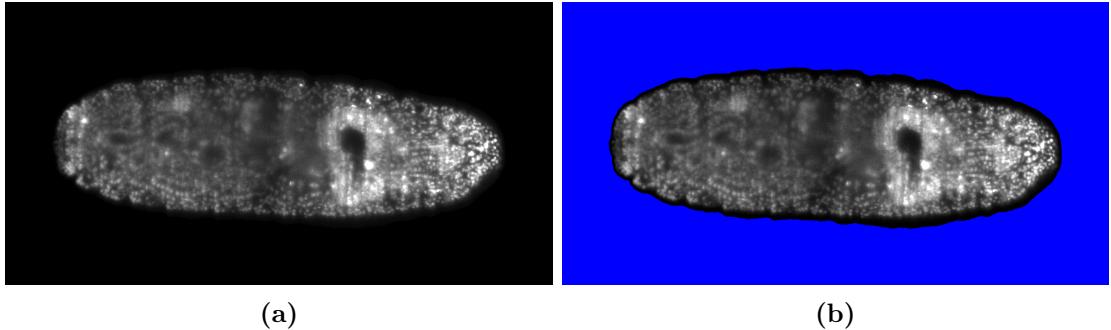


Figure 4.6: Applying mask to remove background. (a) Original image of a *Drosophila* embryo. (b) Background selected by thresholding the blurred image.

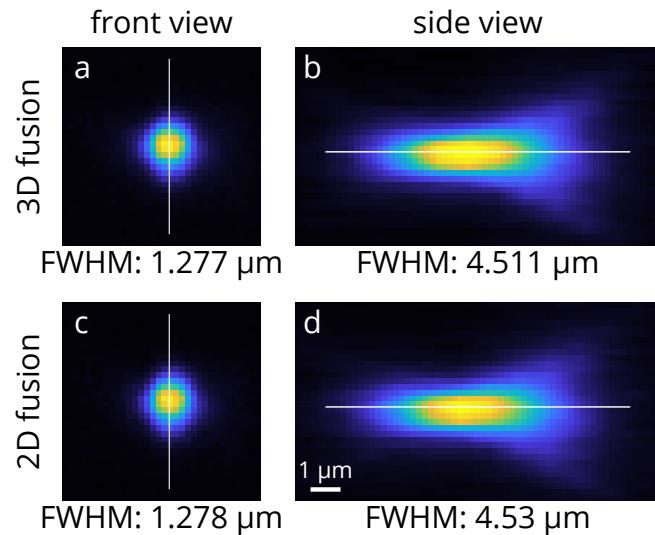


Figure 4.7: Comparison of 3D and 2D fusion of beads. Front and side views of a representative bead after 3D full affine fusion (a, b) and after 2D planewise fusion (c,d). FWHM was measured along the indicated lines, through the center of the bead image. Scale bar: 1 μm .

4.2.6 Results

The image preprocessing pipeline was tested on our MuVi-SPIM microscope [45]. For the background subtraction we recorded 1500 dark images with each camera and averaged them, in order to obtain the camera specific background images. Prior to image acquisition these were uploaded to the GPU memory, and were readily available for the pipeline. The implemented CUDA pipeline with background subtraction, 2D fusion and masking reached a throughput of 135.8 frames per second (fps), while a single-threaded CPU implementation could only process 7.4 images per second on average.

Following careful alignment of the microscope to meet the previously discussed requirements (Eqs. 4.1 – 4.3), we imaged fluorescent beads in a gel suspension to obtain the affine transformation parameters. The measured 3D transformation parameters were the following:

$$\begin{pmatrix} 1.013 & 2.494 \times 10^{-3} & \mathbf{7.182 \times 10^{-3}} & -21.67 \\ -1.697 \times 10^{-3} & 1.014 & -\mathbf{3.192 \times 10^{-5}} & -4.161 \\ -\mathbf{9.694 \times 10^{-5}} & \mathbf{1.937 \times 10^{-7}} & \mathbf{1.003} & -\mathbf{0.433} \\ 0 & 0 & 0 & 1 \end{pmatrix}$$

Substituting these values to Eqs. 4.1 – 4.3 and evaluating for the full range of x, y, z , the maximum contribution of the discarded coefficients for each coordinate will be:

$$\begin{aligned} \max |cz| &= 0.070 \mu\text{m} \\ \max |gz| &= 3.11 \times 10^{-4} \mu\text{m} \\ \max |ix + jy + (k - 1)z + l| &= 0.7789 \mu\text{m} \end{aligned}$$

As these are below the resolution of the microscope, discarding the coefficients and reducing the 3D transformation to a 2D transformation will not have any negative effects on the image quality.

To validate our hypotheses, we fused bead stacks that were acquired for the calibration using both the 3D and the 2D transformations. The 2D, planewise fused beads do not exhibit any pathological morphologies compared to the 3D fused beads (Figure 4.7). When measuring the size of the bead images (FWHM) along the lateral and axial directions, the difference is less than 0.5% compared to the 3D fused dataset. This is well below any resolvable features.

We applied the direct fusion method to various biological specimens, such as *Drosophila melanogaster* embryos, zebrafish larvae, *Phallusia mammillata* embryos and

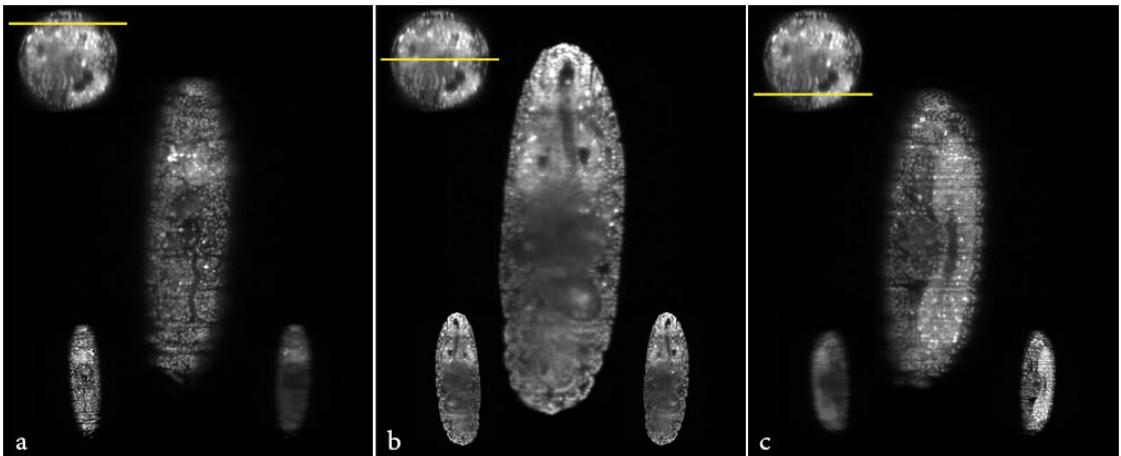


Figure 4.8: GPU fused images of a *Drosophila melanogaster* embryo. Two stacks were taken in quick succession first without fusion, then with fusion enabled. Fused images are shown in the middle of each subfigure, while the individual camera images are in the bottom insets. The top-left inset depicts the z-position of the shown images. (a) Image from closer to the left camera. (b) Image from the center of the embryo. (c) Image from closer to the right camera.

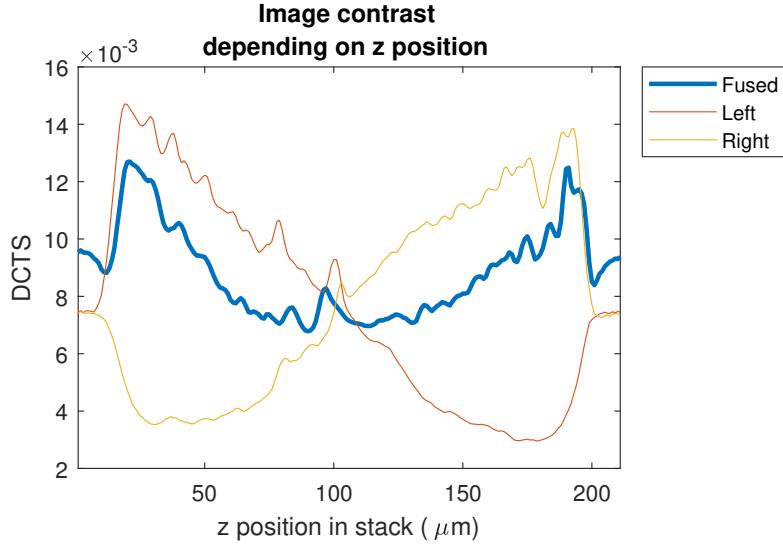


Figure 4.9: Image contrast depending on z position for raw and fused stacks. Image contrast was measured as the Shannon entropy of the normalized Discrete Cosine Transform of the sections [141]. Contrast measurements were performed on the images shown on Figure 4.8.

1 *Volvox* algae. As a demonstration, here we show the results of imaging *Drosophila* embryos expressing H2Av-mCherry histone marker. The embryos were imaged first without
 2 direct fusion enabled, and immediately afterwards with direct fusion enabled (Figure 4.8).
 3 Image quality dependency on the depth of the imaging plane is especially apparent in
 4 single-view stacks. Planes closer to the objective give a sharp, high contrast image, while
 5 planes deeper inside the embryo are severely degraded due to scattering (Figure 4.9).

6 Stacks obtained with the live fusion enabled show a consistently high image quality
 7 throughout the entire stack, independent of the depth (Figure 4.9). This allows us to
 8 keep only the already fused data, thus effectively reducing the storage requirement by
 9 half, and facilitating further data processing steps.

10 **4.2.7 Methods**

11 **Speed measurements**

12 Processing speed of the CPU and GPU implementation of the processing pipeline was
 13 measured with the profiling tool of LabVIEW, and are an average of 10 runs. The bench-
 14 marking computer had 2 Intel Xeon E5620 processors with 8 processing cores running at
 15 2.4 GHz, 24 GB RAM and an Nvidia GeForce GTX 750 graphics card. Test images were
 16 2048×2048 pixels at 16 bit depth.

1 4.3 B³D image compression

2 The second part of our GPU-based image preprocessing pipeline is a new image compression
3 algorithm that allows for extremely fast image compression to efficiently reduce data
4 sizes already during acquisition. Although this would not reduce the requirements for
5 image processing power and time, it still has a big impact on the necessary background
6 infrastructure. By reducing the data size, not only the cost for storage can be reduced,
7 but the time it takes to transfer the data during the various steps of data processing. A
8 fast compression method would also greatly improve 3D data browsing possibilities, as
9 more data could be piped to the rendering software.

10 Despite the advantages, real-time compression for high-speed microscopy has not
11 been available, and this is mostly due to the lack of appropriate compression methods
12 suitable for scientific imaging that also offers the high throughput demanded by these
13 applications. While typically used lossless compression methods, such as JPEG2000 [142]
14 offer good compression ratios, they are very slow in processing speed, at least compared
15 to the data rate of a modern microscope ($\sim 1 \text{ GB/s}$, Figure 4.1). High-speed compression
16 methods that could deal with this data rate have been developed for ultra-high-definition
17 4K and 8K digital cameras, such as the high efficiency video codec (HEVC) [143]. These
18 methods, however, have been optimized for lossy image compression which is generally
19 not acceptable for scientific data [95], and rarely support compression of high bit rate
20 originals, which is typically the case for modern sCMOS sensors. Although the HEVC
21 recommendation does specify bit rates up to 16 bits, and lossless compression, the open
22 source version, x265 only supports bit rates up to 12 bit [144].

23 To address these issues, we developed B³D, a GPU based image compression library
24 capable of high-speed compression of microscopy images during the image acquisition
25 process. By utilizing the massively parallel processing architecture of a GPU, we were not
26 only able to reach a compression and decompression speed of 1 GB/s, but our algorithm
27 also keeps the load off the CPU, making it available for other computing tasks related
28 to operating the microscope itself.

29 A second feature of our compression library is a novel algorithm for noise-dependent
30 lossy compression of scientific images. Although most lossy compression methods are not
31 suitable for scientific data, our method allows for a deterministic way to control the exact
32 amount of loss. The algorithm was designed in a way that any modification of a single
33 pixel is proportional to the inherent image noise accounting for shot noise and camera
34 read noise. Since this noise already introduces some uncertainty to the raw data, if any
35 changes made are smaller than this, the extractable information by further data analysis
36 steps should not be affected, while the compression ratio is massively increased.

¹ **4.3.1 Compression algorithm**

² **Lossless compression**

³ The algorithm design initially had three main requirements to ensure suitability for a
⁴ high-speed microscopy environment: 1) low complexity for fast execution, 2) no data
⁵ dependencies to enable parallel execution, and 3) full reversibility to ensure lossless com-
⁶ pression. Since existing image compression standards such as JPEG2000 [142] or JPEG-
⁷ LS [105] were designed to maximize compression ratio even at the expense of compression
⁸ speed, these were not suitable for our needs. Furthermore, most steps inherently require
⁹ sequential execution, thus preventing efficient parallelization. Promising efforts have been
¹⁰ made to enable parallel compression by simplifying JPEG-LS, by removing the limiting
¹¹ steps from the algorithm (such as Golomb parameter adaptation or context modeling)
¹² [107, 108]. Although some of these methods could reach a significant speedup with min-
¹³ imal expense in compression ratio, their performance was still not enough for real-time
¹⁴ compression of high-speed microscopy data.

¹⁵ As in the case of other image compression methods, our algorithm has two main
¹⁶ components: decorrelation and entropy coding (Figure 4.10a). In this work we focused
¹⁷ on implementing a parallel encoder and decoder for the decorrelation part that would
¹⁸ also be compatible with the noise-dependent lossy compression, as efficient CUDA-based
¹⁹ entropy coders are already available [145]. To decorrelate the data, similarly to JPEG-
²⁰ LS and lossless-JPEG [104], a prediction is performed for each pixel by calculating the
²¹ average of the top and left neighbors (Figure 4.10b). The prediction is subtracted from
²² the real pixel value, resulting in the prediction error term:

$$\varepsilon = X - \text{Pred}(X). \quad (4.4)$$

²³ Since the neighboring pixels have a high chance of correlation, these error terms will
²⁴ typically be very small compared to the raw intensity values. This step is similar to
²⁵ predictor 7 of lossless JPEG (see 2.3).

²⁶ The pixel prediction method is especially well suited for parallel compression, as it can
²⁷ be performed independently for all pixels. For decompression, the top and left neighbor
²⁸ of a pixel need to be first reconstructed, thus parallelization needs some adjustments. An
²⁹ obvious choice is to perform the reverse prediction in tiles, where the number of threads
³⁰ will equal the number of tiles. Parallel execution can be further scaled up if the decoding
³¹ is done in diagonals, starting from the top left corner (Figure 4.10c). With this method
³² the average number of parallel threads will be

$$N_t h = N_t * a_t / 2, \quad (4.5)$$

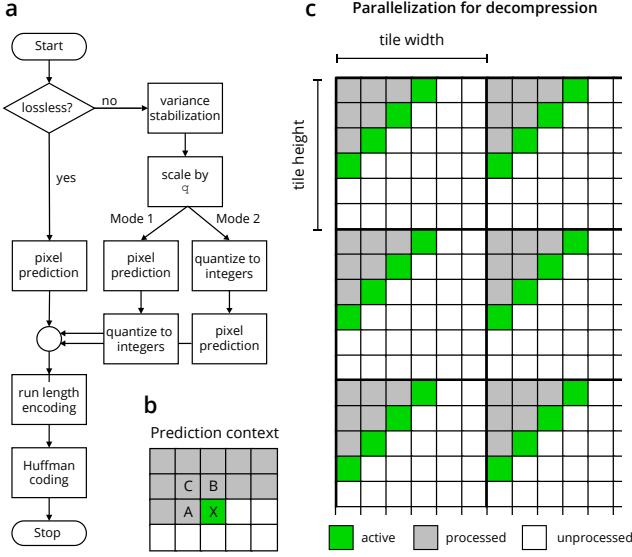


Figure 4.10: B³D algorithm schematics.

(a) Complete algorithm flowchart depicting the main stages of the compression. (b) Prediction context for pixel X, the next sample to be encoded. Three neighboring pixels are considered: left (A), top (B) and top left (C) neighbors. (c) Parallelization scheme for decompression and lossy compression. Already processed pixels are in gray, unprocessed pixels are in white, active pixels are in green.

1 where N_{th} is the number of threads, N_t is the number of tiles, a_t is the tile width and
 2 height for a square tile. Although the threads can be maximized by making smaller tiles,
 3 the compression ratio will be affected, since the corner pixel can't be predicted, and for
 4 the edges only one neighbor can be used instead of 2. We find square tile between 16 and
 5 64 pixels a good choice between decompression speed and compression ratio.

6 The prediction error terms are subsequently run-length encoded and entropy coded
 7 by a fast, CUDA-enabled Huffman coder [145, 146] to effectively reduce data size. The
 8 library is making extensive use of parallel prefix sum, which can be used for the effective
 9 parallel execution for both run-length encoding and Huffman coding. For decompression,
 10 the Huffman coding and run-length encoding are first reversed, then each pixel value is
 11 reconstructed from the neighboring values and the prediction errors. Although this im-
 12 plementation sacrifices a little in compression ratio compared to size-optimized methods,
 13 it can surpass a compression speed of 1 GB/s (see Section 4.3.2), which is sufficient even
 14 for the dual camera confocal MuVi-SPIM setup [75].

15 Within-noise-level compression

16 Noise-dependent lossy compression relies on the fact that microscopy images naturally
 17 contain photon shot noise. To increase compression ratio, this method of compression
 18 allows some differences in the pixel values, as long as they are within a predefined pro-
 19 portion of the inherent noise. A special case for this mode is when all compression errors

1 are smaller than the standard deviation of the noise (σ), which we call within-noise-level
2 (WNL) compression.

3 Our algorithm is inspired by both the near-lossless mode of JPEG-LS [105], where
4 the maximum compression error can be set to a predefined value, and the square root
5 compression scheme [147, 148], that quantizes its input relative to its square. We combine
6 the two methods to achieve noise-dependent lossy compression while still retaining good
7 image quality and avoiding banding artifacts.

8 Before the prediction errors are sent to the entropy coder, a quantization can take
9 place that reduces the number of symbols that need to be encoded in order to increase
10 compression ratio. By changing the quantization step, the maximum absolute reconstruc-
11 tion error can be defined, such as when using JPEG-LS. For noise dependent compression,
12 however, the quantization step should be proportional to the image noise, which can be
13 achieved by the following transformation introduced by Bernstein *et al.* [148]:

$$T = 2\sqrt{e + \sigma_{RN}^2}, \quad (4.6)$$

14 where e is the intensity scaled to photoelectrons, and σ_{RN} is the camera read noise. To
15 perform the scaling of digital numbers (DN) to photoelectrons, it's necessary to know
16 the camera offset and conversion parameters: $e = (I - \text{offset}) \cdot g$, where g is the gain
17 in photoelectrons/DN, and I is the original pixel intensity. This transformation assumes
18 a Poisson-Gaussian noise, incorporating the photon shot noise and the camera readout
19 noise, which is a good model for most scientific imaging sensors, such as (EM)CCD or
20 sCMOS.

21 To accommodate for all imaging needs, two options are available in the noise-
22 dependent lossy mode. In Mode 1, after the stabilizing transform (Equation 4.6), the
23 prediction is performed first, followed by quantization of the prediction errors. This
24 mode reaches a better compression ratio and higher peak signal-to-noise ratio (PSNR)
25 for the same quantization step compared to Mode 2 (Figure 4.11 a, b), and is recom-
26 mended for most use cases. For larger quantization steps ($q \geq 3\sigma$), however, Mode 1
27 might introduce some spatial correlations, because the prediction errors that are being
28 quantized depend on multiple adjacent pixels (Figure 4.11 c, d). Mode 2 excludes this
29 possibility by swapping the quantization and prediction (Figure 4.11 c, d) at the expense
30 of compression ratio. Since no correlations are introduced, Mode 2 is suitable for highly
31 sensitive image analysis tasks, such as single-molecule localization. In this chapter, we
32 use Mode 2 for all single-molecule localization data, and Mode 1 for all other datasets.

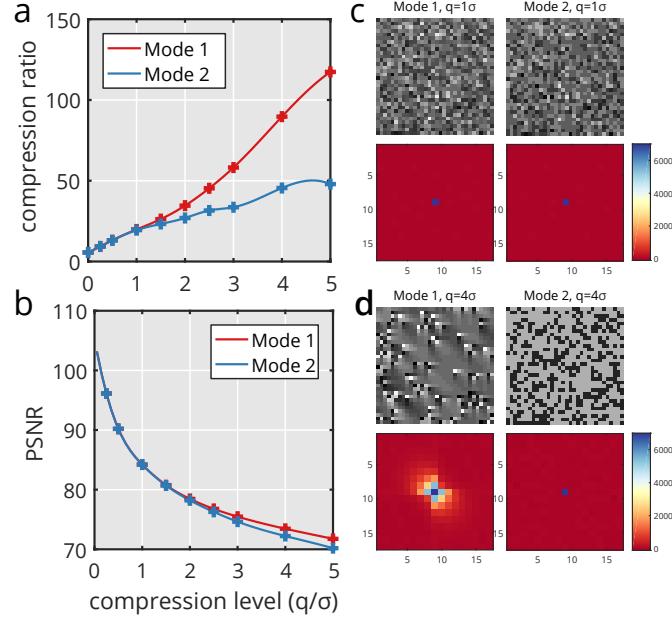


Figure 4.11: Options for noise-dependent lossy compression. Comparing Mode 1 (prediction then quantization) and Mode 2 (quantization then prediction) of noise-dependent lossy compression in terms of compression ratio, peak signal-to-noise ratio (PSNR) and spatial correlations introduced to random noise. (a) Compression ratio as a function of the quantization step for Mode 1 and Mode 2. (b) PSNR as a function of the quantization step for Mode 1 and Mode 2. (c, d) Random noise was compressed at various quantization steps both for Mode 1 and Mode 2. Autocorrelation was calculated for the compressed images to see whether the compression introduces any spatial correlation between the pixels. For $q=1\sigma$ both modes are free of correlation (c, top: compressed images, bottom: autocorrelation), however, for $q=2\sigma$ Mode 1 exhibits a correlation pattern (d, top left: compressed image, bottom left: autocorrelation) that is not present in Mode 2 (d, top right compressed image, bottom right: autocorrelation). For more discussion, see Section 4.3.1.

1 Additional noise introduced by compression

- 2 When applying quantization to any kind of data, the quantization error can be modeled as
- 3 an additional uniform noise with standard deviation of $\frac{\delta}{\sqrt{12}}$, where δ is the quantization
- 4 step size [149]. During compression the quantization is proportional to the standard
- 5 deviation of the original image noise, thus the noise on the uncompressed image will be:

$$\sigma_{out} = \sigma_{in} \cdot \sqrt{1 + \frac{q^2}{12}} \quad (4.7)$$

- 6 For the WNL case, when $q = 1\sigma$ this means an increase of 4%, which also coincides with
- 7 the measured increase of localization error for single molecule localization (Figure 4.18a).
- 8 However, this is not the only source of additional noise we have to consider, because at the
- 9 decompression step there is a possibility of getting floating point results after applying
- 10 the inverse of the compression steps, and for most applications these have to be coerced
- 11 to integer numbers. This effectively adds a second quantization, but now at a constant

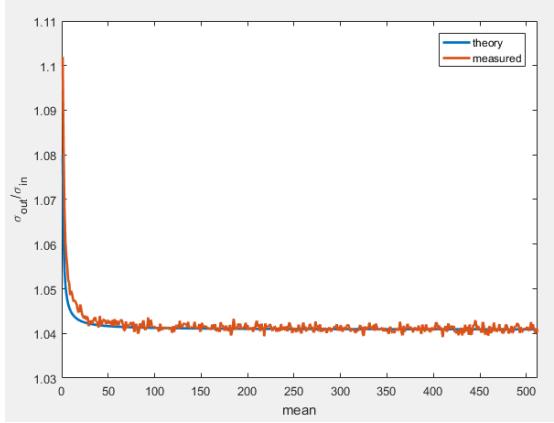


Figure 4.12: Theoretical and measured increase in image noise for WNL compression. Validating Equation 4.8, we plot the relative increase in image noise as a function of the mean. Blue line: plot of Equation 4.8 Red line: plot of measured data. To obtain the measurement, we compressed a stack of 512 images consisting of Poisson noise, each with a different mean. After WNL compression the dataset was read in Matlab, and the standard deviation was calculated for each frame. The ratio σ_{in}/σ_{out} is plotted as a function of the mean.

1 quantization step of 1. This results in an additional uniform noise with variance of $\frac{1}{12}$:

$$\sigma_{out} = \sigma_{in} \cdot \sqrt{1 + \frac{q^2}{12}} + \frac{1}{12} \quad (4.8)$$

2 We verified this by compressing 512 images (1024×1024 pixels) of random Poisson noise
3 with different means (1..512), and calculated the standard deviation for each frame after
4 the decompression step. Finally, we plotted the ratio σ_{out}/σ_{in} as a function of the mean
5 m (Figure 4.12), and we found that it is in very good agreement with the theory outlined
6 above.

7 4.3.2 Evaluation of the compression algorithm

8 We compared our algorithm's performance with some of the most commonly used image
9 formats in the scientific field: TIFF (LZW) and JPEG2000. Furthermore, we also in-
10 cluded the state of the art KLB compression [139], which was especially designed for fast
11 compression of large light-sheet datasets. We measured compression speed, decompres-
12 sion speed and resulting file size for all algorithms (Figure 4.13a). Only B³D is capable
13 of handling the sustained high data rate of modern sCMOS cameras typically used in
14 light-sheet microscopy, while still maintaining compression ratios comparable to more
15 complex, but much slower algorithms (Table B2 in Appendix B).

16 Using the noise-dependent lossy mode with $q = 1\sigma$ (WNL), the compression ra-
17 tio massively increases for all imaging modalities compared to the lossless mode (Fig-
18 ure 4.13b) without any apparent loss in image quality (Figure 4.14). Furthermore, the av-
19 erage compression error is considerably smaller than the image noise itself (Figure 4.15).

4.3 B³D image compression

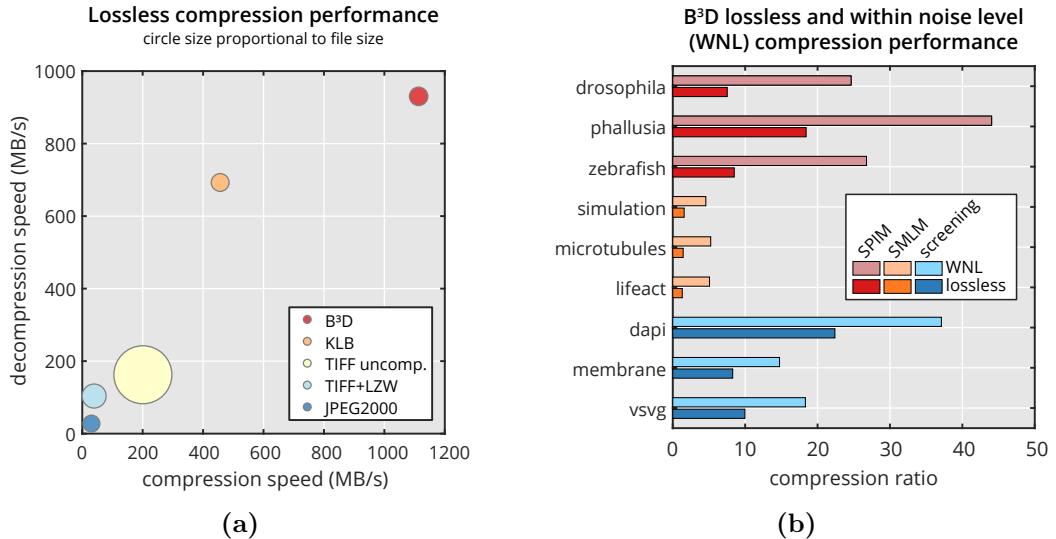


Figure 4.13: Compression performance. (a) Performance comparison of our B³D compression algorithm (red circle) vs. KLB (orange), uncompressed TIFF (light yellow), LZW compressed TIFF (light blue) and JPEG2000 (blue) regarding write speed (horizontal axis), read speed (vertical axis) and file size (circle size). (see also Table B2). (b) WNL compression performance compared with lossless performance for 9 different dataset representing 3 imaging modalities (SPIM, SMLM, screening). Compression ratio = original size / compressed size. For description of datasets see Table B3 in Appendix B.

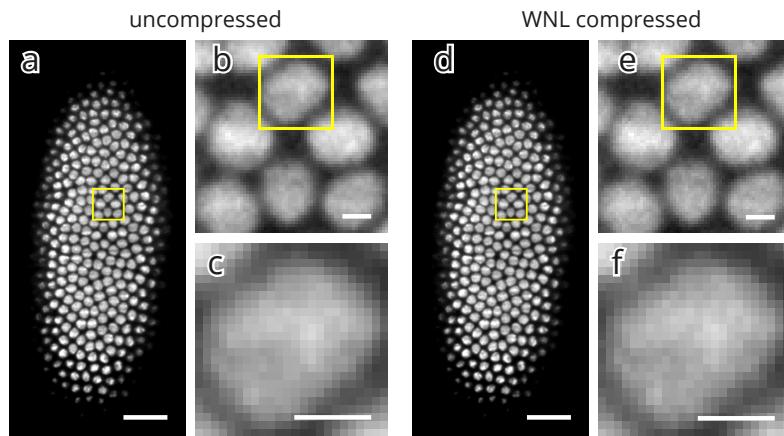


Figure 4.14: Image quality of a WNL compressed dataset. WNL compression of a *Drosophila* recording taken in the MuVi-SPIM setup. Compression ratio: 19.83. (a–c) Uncompressed image of the whole field of view (a), and zoomed in smaller regions (b, c). (d–f) WNL compressed image of the whole field of view (d), and zoomed in smaller regions (e, f). Scale bars: 25 µm (a, e); 2.5 µm (b, c, e, f).

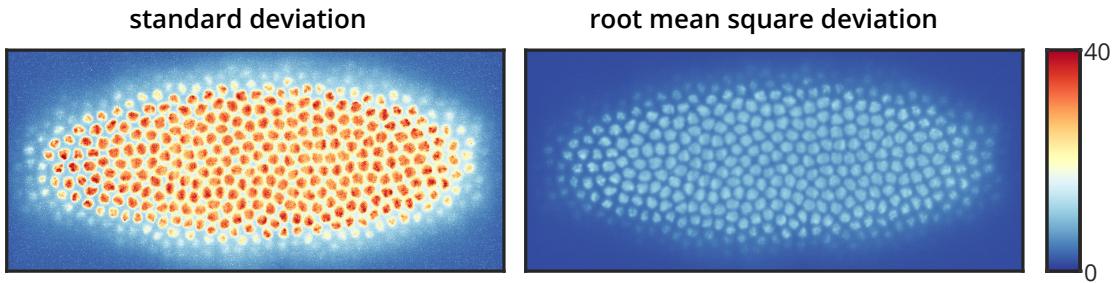


Figure 4.15: Compression error compared to image noise. To compare the difference arising from WNL compression to image noise, we imaged a single plane 100 times in a *Drosophila melanogaster* embryo expressing H2Av-mCherry nuclear marker at 38 ms intervals. The whole acquisition took 3.8 s, for which the sample can be considered stationary. To visualize image noise, the standard deviation was calculated for the uncompressed images (left). All images were then WNL compressed, and the root mean square deviation was calculated compared to the uncompressed images (right). The root mean square deviation on average is 3.18 times smaller than the standard deviation of the uncompressed images.

1 To see how the noise-dependent compression affects common imaging pipelines, we
 2 tested the effect of different levels of compression on 3D nucleus and membrane segmen-
 3 tation in light-sheet microscopy, and on single-molecule localization accuracy in super-
 4 resolution microscopy. First, we imaged a *Drosophila melanogaster* embryo expressing
 5 an H2Av-mCherry nuclear marker in the MuVi-SPIM and segmented the nuclei with
 6 Ilastik [150] (Figure 4.16a and Section 4.3.4). Then we performed noise dependent com-
 7 pression at various quality levels and calculated the segmentation overlap compared to
 8 the uncompressed stack (Section 4.3.4). At WNL compression ($q = 1\sigma$) the segmenta-
 9 tion overlap is almost perfect (Figure 4.16b) with an overlap score of 0.996. Even when
 10 increasing the quantization step to 4σ (Figure 4.16c) the overlap score stays at 0.98 and
 11 only drops below 0.97 when the compression ratio is already above 120 (quantization
 12 step of 5σ , Figure 4.16d). We got similar results for a membrane segmentation pipeline
 13 that is used with *Phallusia mammillata* embryos (Figure 4.17 and Section 4.3.4).

14 Next, we evaluated our compression algorithm in the context of single molecule lo-
 15 calization microscopy (SMLM), and measured how the localization precision is affected
 16 when compressing the raw images by an increasing compression ratio. We compressed
 17 an SMLM dataset of immuno-detected microtubules (Figure 4.18a) with increasing com-
 18 pression levels. For WNL compression ($q = 1\sigma$) no deterioration of the image was visible
 19 (Figure 4.18b), and even for the case of $q = 4\sigma$ the compression induced errors were much
 20 smaller than the resolvable features (Figure 4.18c). To quantify the impact of compres-
 21 sion on the localization error, we used a simulated dataset (Section 4.3.4) and compared
 22 the localization output of different compression levels to the ground truth (Figure 4.18d).
 23 Lossless compression resulted in a compression ratio of 2.7, whereas WNL compression
 24 reached a compression ratio of 5.0, while increasing the localization error by only 4%.
 25 This also coincides with the theoretical increase of image noise (Section 4.3.1). Further-
 26 more, the increase in localization error was not dependent on the signal to background

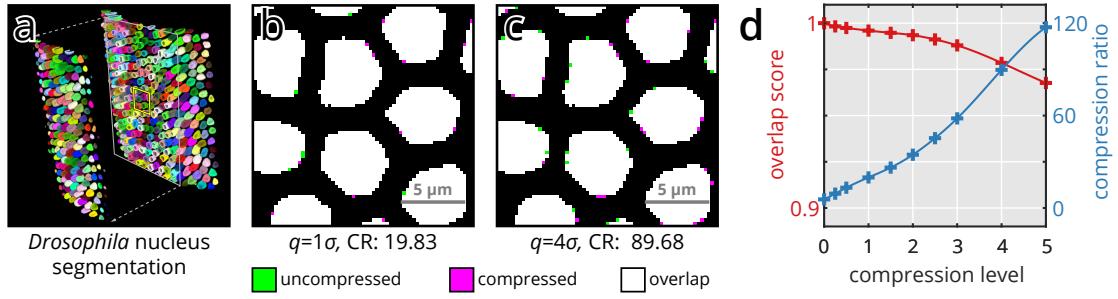


Figure 4.16: Influence of noise-dependent lossy compression on 3D nucleus segmentation. A *Drosophila melanogaster* embryo expressing H2Av-mCherry nuclear marker was imaged in MuVi-SPIM [45], and 3D nucleus segmentation was performed (Section 4.3.4) (a). The raw dataset was subsequently compressed at increasingly higher compression levels, and segmented based on the training of the uncompressed data. To visualize segmentation mismatch, the results of the uncompressed (green) and compressed (magenta) datasets are overlaid in a single image (b, c; overlap in white). Representative compression levels were chosen at two different multiples of the photon shot noise, at $q=1\sigma$ (b) and $q=4\sigma$ (c). For all compression levels the segmentation overlap score (Section 4.3.4) was calculated and is plotted in (d) along with the achieved compression ratios.

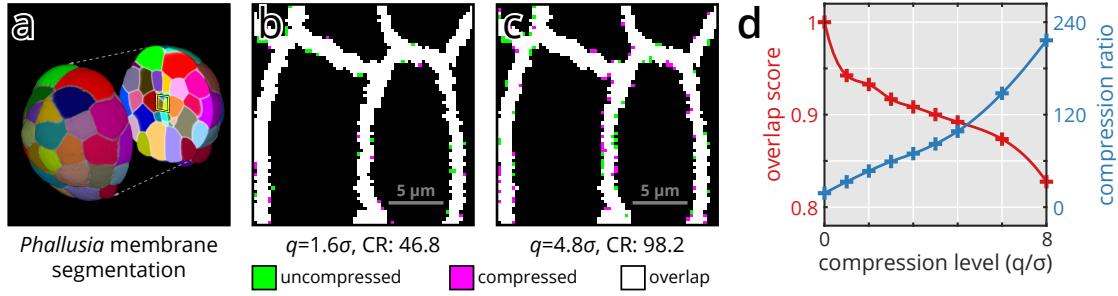


Figure 4.17: Influence of noise-dependent lossy compression on 3D membrane segmentation. A *Phallusia mammillata* embryo expressing PH-citrine membrane marker was imaged in MuVi-SPIM [45], and 3D membrane segmentation was performed (Section 4.3.4) (a). The raw dataset was subsequently compressed at increasingly higher compression levels, and segmented using the same settings as the uncompressed data. To visualize segmentation mismatch, the results of the uncompressed (green) and compressed (magenta) datasets are overlaid in a single image (b, c; overlap in white). Representative compression levels were chosen at two different multiples of the photon shot noise, at $q=1.6\sigma$ (b) and $q=4.8\sigma$ (c). For all compression levels the segmentation overlap score (Section 4.3.4) was calculated and is plotted in (d) along with the achieved compression ratios.

1 noise ratio (Figure 4.19).

2 4.3.3 HDF5 integration

- 3 To make our compression method more accessible, we developed a filter plugin for HDF5.
- 4 Because of its versatility, HDF5 has emerged as the de facto standard in the open source
- 5 light sheet microscopy field, and is also the basis for the widely used BigDataViewer
- 6 [151] in Fiji [116]. Starting from version 1.8.11 (May 2013), HDF5 supports dynamically
- 7 loaded filters, and is able to load third party components without having to modify
- 8 any already installed files. When loading a B³D compressed image in an HDF5 enabled
- 9 application, the library automatically calls our filter plugin, decompresses the image on
- 10 the GPU, and copies it back into CPU memory.

4.3 B³D image compression

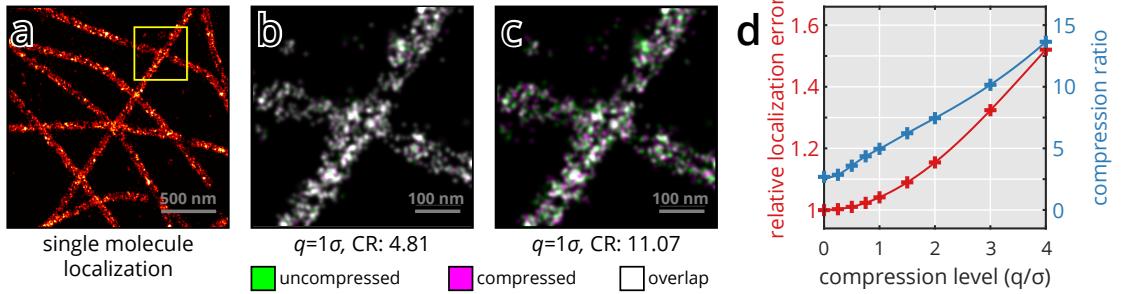


Figure 4.18: Influence of noise-dependent lossy compression on single-molecule localization. Microtubules, immunolabeled with Alexa Fluor 647 were imaged by SMLM (a). The raw dataset was compressed at increasingly higher compression levels, and localized using the same settings as the uncompressed data. To visualize localization mismatch, the results of the uncompressed (green) and compressed (magenta) datasets are overlaid in a single image (b, c; overlap in white). Two representative compression levels were chosen at $q=1\sigma$ (b) and $q=4\sigma$ (c). To assess the effects of compression on localization precision, a simulated dataset with known emitter positions was compressed at various levels. For all compression levels the relative localization error (normalized to the Cramér–Rao lower bound) was calculated and is plotted in (d) along with the achieved compression factors.

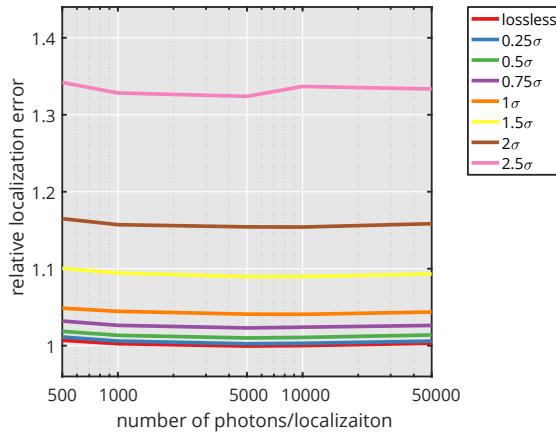


Figure 4.19: Change in localization error only depends on selected quantization step. We simulated multiple datasets (Section 4.3.4) with different average photon numbers per localization. Background was kept at a constant average of 20 photons/pixel. Datasets were compressed at multiple compression levels (see legend), and localization error relative to the Cramér–Rao lower bound was calculated. The relative localization error only depends on the compression level, and not on the signal to background illumination ratio.

1 Since HDF5 is extensively used in many scientific/image analysis software, this plugin
2 format is especially suitable to equip existing software with compression/decompression
3 capabilities. To read compressed files no modification is required, and any software sup-
4 porting HDF5 should be compatible. The following software have been tested with our
5 filter plugin and are able to read B³D compressed files: Fiji (also with BigDataViewer),
6 Imaris 8.4.1, Ilastik 1.2.0, Matlab R2016a, Python 2.7.10 and 3.5.3, LabVIEW 2015,
7 C++.

8 Writing compressed HDF5 files is possible if the user has direct control over the
9 file writing routine, as an additional command is required to set up compression. This
10 functionality has been tested in the following environments: C++, Python 2.7.10 and
11 3.5.3, LabVIEW 2015.

12 4.3.4 Methods

13 Compression benchmarking

14 For all presented benchmarks, TIFF and JPEG2000 performance was measured through
15 MATLAB's imwrite and imread functions, while KLB and B³D performance was mea-
16 sured in C++. All benchmarks were run on a computer featuring 32 processing cores
17 (2×Intel Xeon E5-2620 v4), 128 GB RAM and an NVIDIA GeForce GTX 970 graph-
18 ics processing unit. Read and write measurements were performed in RAM to minimize
19 I/O overhead, and are an average of 5 runs. SMLM datasets were provided by Joran De-
20 schamps (EMBL, Heidelberg), and were originally published in [152] and [153]. Screening
21 datasets were provided by Jean-Karim Hériché (EMBL, Heidelberg), and were originally
22 published in [154]

23 Light-sheet imaging

24 *Drosophila* embryos were imaged in the MuVi-SPIM setup [45] using the electronic con-
25 focal slit detection (eCSD) [75]. Embryos were collected on an agar juice plate, and
26 dechorionated in 50% bleach solution for 1 min. The embryos were then mounted in a
27 shortened glass capillary (Brand 100 µl) filled with 0.8% GelRite (Sigma-Aldrich), and
28 pushed out of the capillary to be supported only by the gel.

29 3D nucleus segmentation

30 3D nucleus segmentation of *Drosophila* embryos was performed using Ilastik [150]. The
31 original dataset was compressed at different quantization levels, then upscaled in z to ob-
32 tain isotropic resolution. To identify the nuclei, we used the pixel classification workflow,
33 and trained it on the uncompressed dataset. This training was then used to segment the
34 compressed datasets as well. Segmentation overlap was calculated in Matlab using the

1 Sørensen–Dice index [155, 156]:

$$QS = 2 |A \cap B| / (|A| + |B|) \quad (4.9)$$

2 where the sets A and B represent the pixels included in two different segmentations.

3 **3D membrane segmentation**

4 Raw MuVi-SPIM recordings of *Phallusia mammillata* embryos expressing PH-citrine
5 membrane marker were kindly provided by Ulla-Maj Fiuba (EMBL, Heidelberg). Each
6 recording consisted of 4 views at 90 degree rotations. The views were fused using an
7 image based registration algorithm followed by a sigmoidal blending of the 4 views. The
8 fused stack was then segmented using the MARS algorithm [157] with an hmin parameter
9 of 10. The raw data (all 4 views) was compressed at different levels, and segmented using
10 the same pipeline. Segmentation results were then processed in Matlab to calculate the
11 overlap score for the membranes using the Sørensen–Dice index.

12 **Single-molecule localization imaging**

13 In order to visualize microtubules, U2OS cells were treated as in [152] and imaged in a
14 dSTORM buffer [158]. In brief, the cells were permeabilized and fixed with glutaralde-
15 hyde, washed, then incubated with primary tubulin antibodies and finally stained with
16 Alexa Fluor 647 coupled secondary antibodies. The images were recorded on a home-built
17 microscope previously described [152], in its 2D single-channel mode.

18 **Single-molecule localization data analysis**

19 Analysis of single-molecule localization data was performed on a custom-written MAT-
20 LAB software as in [153]. Pixel values were converted to photon counts according to
21 measured offset and calibrated gain of the camera (EMCCD iXon, Andor). The back-
22 ground was estimated with a wavelet filter [159], background-subtracted images were
23 thresholded and local maxima were detected on the same images. 7-pixel ROIs around
24 the detected local maxima were extracted from the raw images and fitted with a GPU
25 based MLE fitter [160]. Drift correction was performed based on cross-correlation. Fi-
26 nally, images were reconstructed by filtering out localizations with a high uncertainty
27 (>30 nm and large PSF (>150 nm) and Gaussian rendering.

28 **Simulation of single-molecule localization data**

29 Single molecule localization datasets were simulated in Matlab by generating a grid of
30 pixelated Gaussian spots with standard deviation of 1 pixel. With a pixel size of a 100

1 nm, this corresponds to a FWHM of 235.48 nm. The center of each spot was slightly
2 offset from the pixel grid at 0.1 pixel increments in both x and y directions. To this
3 ground truth image a constant value was added to simulate illumination background,
4 and finally Poisson noise was applied to the image. This process was repeated 10,000
5 times to obtain enough images for adequate accuracy.

6 **Code availability**

7 Code used for analyzing data, B³D source code and compiled binaries, including a filter
8 plugin for HDF5, is available for download at <https://git.embl.de/balazs/B3D>.

9 **4.4 Conclusions**

10 Our algorithm is implemented in C++, and allows for easy integration through an API
11 with various programming languages. The library was tested on Linux (Ubuntu 16.04)
12 and Windows (10). Additionally, we implemented a filter plugin for HDF5 which enables
13 a seamless integration in all software packages that are supporting the native HDF5
14 library, such as Matlab, Python, Imaris, or Ilastik. Due to B³D's efficient compression
15 ratio and its high decompression speed, loading data is often accelerated: For a state-of-
16 the-art hard drive with 200 MB/s bandwidth, loading a 2 GB uncompressed 3D stack of
17 images takes about 10 seconds. With an average compression ratio of 20 fold in the WNL
18 mode, the loading time is reduced to 0.5 seconds followed by 2 seconds of decompression,
19 which yields a factor of four speed-up. It is also worth to note, that the achieved WNL
20 compression reduces the camera data rate to below 40 MB/s, well below the 1 Gb/s
21 Ethernet standard. This enables to use current network infrastructure to move data to
22 long term storage and even makes the use of cloud services possible. Altogether, B³D, our
23 efficient GPU-based image compression library allows for exceptionally fast compression
24 speed and greatly increases compression ratio with its WNL scheme, offering a versatile
25 tool that can be easily tailored to any high-speed microscopy environment.

¹ **Chapter 5**

² **Discussion**

³ **5.1 New scientific results**

⁴ **Thesis I.** *I have designed and constructed a new light-sheet microscope suitable for*
⁵ *high resolution imaging of delicate samples. A novel arrangement of two high numeri-*
⁶ *cal aperture objectives in 120 degrees combined with a tilted light-sheet allows for near*
⁷ *isotropic resolution while increasing light collection efficiency by a factor of two.*

⁸ Corresponding publications: [J3],[J2], [J1]

⁹ Dual Mouse-SPIM is a novel design for symmetric light-sheet microscopy. The use
¹⁰ of high NA objectives in 120° not only increases volumetric resolution compared to the
¹¹ conventional 90° setup, but due to the larger detection angle, light collection efficiency
¹² is doubled. This is especially beneficial for delicate, light-sensitive specimens, such as
¹³ mouse embryos, since phototoxic effects are reduced while the contrast is preserved.

¹⁴ As part of the microscope I have designed a custom beam splitter unit that allows
¹⁵ the use of a single galvo scanner to generate the light-sheet for both objectives. I have
¹⁶ also designed a custom detection merging unit that allows the use of a single camera for
¹⁷ both detection views.

¹⁸ I have characterized the optical properties of the microscope, measured the illumina-
¹⁹ tion profile and point spread function. With a 3.6 µm thick light-sheet, a 95 µm field of
²⁰ view is evenly illuminated. Dual view imaging of bead samples revealed a lateral resolu-
²¹ tion of 314 nm, and axial resolution of 496 nm. This is a 2.67× improvement compared
²² to the axial resolution of a single lens. I have also demonstrated the imaging capabilities
²³ of the microscope on *Drosophila* embryos and mouse zygotes.

²⁴ **Thesis II.** *I have developed a GPU-based image processing pipeline for multi-view*
²⁵ *light-sheet microscopy that enables real time fusion of opposing views.*

²⁶ Corresponding publications: [C1], [C2], [C3]

I have developed a GPU-based image preprocessing pipeline, which integrates directly to our universal microscope control software in LabVIEW. The pipeline currently supports background subtraction and background masking, furthermore it is capable of fusing opposing views of the same plane faster than real-time. I have shown that it is possible to reduce the registration of opposing camera views from a 3D alignment to a 2D alignment without any negative effects in image quality and resolution. This massively reduces the necessary computing resources, and allows the use of CUDA textures for faster than real-time image fusion. Processing speed of this implementation is 138 fps, a $18.3\times$ increase compared to a single threaded CPU implementation.

Thesis III. *I have developed a new image compression algorithm that enables noise dependent lossy compression of light microscopy images, and can reach a compression ratio of 100 fold while preserving the results of downstream data analysis steps. A fast CUDA implementation allows for real-time image compression of high-speed microscopy images.*

Corresponding publications: [J4], [C1], [C2], [C3]

Since many high-speed microscopy methods generate immense amounts of data, easily reaching terabytes per experiment, image compression is especially important to efficiently deal with such datasets. Existing compression methods suitable for microscopy images are not able to deal with the high data rate of modern sCMOS cameras ($\sim 800 \text{ MB/s}$).

I developed a GPU-based parallel image compression algorithm called B³D, capable of over 1 GB/s throughput, allowing live image compression. To further reduce the data size, I developed a noise dependent lossy compression that only modifies the data in a deterministic manner. The allowed differences for each pixel can be specified as a proportion of the inherent image noise, accounting for photon shot noise and camera readout noise. Due to the use of pixel prediction, the subjective image quality is higher than for other methods that simply quantize the square root of the images.

Thesis IV. *I have shown that within noise level compression does not significantly affect the results of most commonly used image processing tasks, and it allows a $3.32\times$ average increase in compression ratio compared to lossless mode.*

Corresponding publications: [J4], [C1], [C2], [C3]

As data integrity in microscopy is paramount for drawing the right conclusions from the experiments, using a lossy compression algorithm might be controversial. I have shown that the within noise level (WNL) mode of B³D does not significantly affect the results of several commonly used image analysis tasks. For light-sheet microscopy data I have shown that WNL compression introduces less variation to the image than the photon shot noise. When segmenting nuclei of *Drosophila* embryos and membranes of *Phallusia*

1 embryos, the overlap of the segmented regions of uncompressed and WNL compressed
2 datasets were 99.6% and 94.5% respectively, while compression ratios were 19.83 for
3 *Drosophila* and 40.01 for *Phallusia* embryos. For single molecule localization microscopy
4 data I have shown that WNL compression only introduces 4% increase in localization
5 uncertainty, while the average compression ratio is increased from 1.44 (lossless) to 4.96
6 (WNL). I have also shown that change in localization error due to the compression does
7 not depend on the SNR of the input images.

8 5.2 Application of the results

9 Both the new Dual Mouse-SPIM microscope and the GPU-based image processing and
10 compression pipeline have direct applications in light-sheet imaging of embryonic devel-
11 opment.

12 Multiple potential collaborators indicated their interest in using the Dual Mouse-
13 SPIM for their studies in mouse embryonic development. The Hiiragi group, focusing
14 on symmetry breaking events in the pre-implantation and early post-implantation stages
15 would like to use this system for imaging larger specimens from multiple direction, which
16 is not possible on their current microscopes, and could allow them to observe previously
17 unknown mechanisms. The Ellenberg group is interested in investigating chromosome
18 missesgregation mechanisms in the first few divisions during embryonic development. The
19 increased axial resolution of this system will allow to track each individual chromosome
20 during the division process, which was not possible on their current setup due to the
21 insufficient axial resolution.

22 The GPU-based image processing pipeline, especially the 2D fusion of opposing
23 views is already being used on our lab's workhorse microscope, the MuVi-SPIM. Being
24 able to fuse the two views of the opposing objectives during imaging not only results in
25 considerable storage space savings, but significantly speeds up the data analysis as well.

26 The image compression algorithm, B³D, although was developed with light-sheet
27 microscopy in mind, has a more wide-spread use-case. Any kind of high-speed, high-
28 throughput light-microscopy experiment can benefit form the massive data reduction
29 offered by the within noise level mode. Since the compression can also be done imme-
30 diately during imaging, not only the storage requirements, but the data bandwidth is
31 reduced as well, which renders the use of high performance RAID arrays and 10 Gbit
32 networks unnecessary, further reducing costs. Due to the similarly high decompression
33 speed, reading the data is also accelerated, which can be beneficial for data browsing
34 and 3D rendering applications. Several companies of different fields already expressed
35 their interest in the compression library, including Bitplane AG (3D data analysis and
36 visualisation), Luxendo GmbH (light-sheet microscopy), and Hamamatsu Photonics K.K

5.2 Application of the results

¹ (camera and sensor manufacturing).

¹ Appendix

² A Bill of materials

³ Optical components

- ⁴ • Lenses
 - ⁵ – 2×Nikon CFI75 Apo LWD 25x/1.10w water dipping objectives
 - ⁶ – 2×Nikon ITL200 200 mm tube lens
 - ⁷ – 2×200 mm x 25 mm Dia. achromatic lens (Edmund Optics, #47-645)
 - ⁸ – 2×400 mm x 40 mm Dia. achromatic lens (Edmund Optics, #49-281)
 - ⁹ – 3×75 mm x 25 mm Dia. achromatic lens (Edmund Optics, #47-639)
 - ¹⁰ – SILL 112751 1:2 beam expander
- ¹¹ • Filters
 - ¹² – 2×BrightLine quad-edge dichroic beam splitter (Semrock, Di03-R405/488/561/635-t3-25x36)
 - ¹³ – EdgeBasic 488 nm long pass filter (Semrock, BLP01-488R-25)
 - ¹⁴ – BrightLine 525/50 band pass filter (Semrock, FF03-525/50-25)
 - ¹⁵ – EdgeBasic 561 nm long pass filter (Semrock, BLP02-561R-25)
 - ¹⁶ – RazorEdge 647 nm long pass filter (Semrock, LP02-647RU-25)
- ¹⁷ • Mirrors
 - ¹⁸ – 6×1” Broadband Dielectric Elliptical Mirror (Thorlabs, BBE1-E03)
 - ¹⁹ – 2×30 mm Broadband 1/10λ Mirror (OptoSigma, TFMS-30C05-4/11)
 - ²⁰ – 10 Pack of 1” Protected Silver Mirrors (Thorlabs, PF10-03-P01-10)
 - ²¹ – Knife-Edge Right-Angle Prism Dielectric Mirror (Thorlabs, MRAK25-E02)

²³ Mechanical components

- ²⁴ • 40 mm travel range pneumatic cylinder (Airtac, HM-10-040)
- ²⁵ • 5/2-way electric valve (Airtac, M-20-510-HN)
- ²⁶ • 2×25 mm extended contact bearing steel stage (OptoSigma, TSDH-251C)
- ²⁷ • 30x90 mm stainless steel slide (OptoSigma, IPWS-F3090)
- ²⁸ • close proximity gimbal mirror mount (Thorlabs, GMB1/M)
- ²⁹ • 30 mm cage elliptical mirror mount (Thorlabs, KCB1E)
- ³⁰ • Melles Griot Performance Plus Optical Breadboard
- ³¹ • 4×Newport Stabilizer, High Performance laminar Flow Isolator, I-2000 Series

1 Custom parts

2 All parts are (anodized) aluminium, unless stated otherwise.

- 3 • 2×mirror holder block**
- 4 • Front plate for objective, chamber and mirror mounting**
- 5 • Imaging chamber (PEEK)**
- 6 • Wedge ring and matching threaded ring to fasten objectives**
- 7 • Camera bridge**
- 8 • Illumination splitter unit**
- 9 • adapter plates to mount stages**

10 Electronics

- 11 • Embedded system (National Instruments, cRIO-9068) equipped with:**
 - 12 – 2×C series digital I/O card (NI 9401)**
 - 13 – 1×C Series 100 kS/s 4-channel Voltage Output Module (NI 9263)**
 - 14 – 1×C Series 25 kS/s 16-channel Voltage Output Module (NI 9264)**
- 15 • Omicron SOLE-3 laser combiner, with 488, 561 and 638 nm laser lines**
- 16 • Andor Zyla 4.2 sCMOS Camera**
- 17 • Galvanometric scanner mirror (Cambridge Technology, 6210B)**
- 18 • 6 position filter wheel (Ludl Electronic Products, 96A361)**
- 19 • Filter wheel controller unit (Ludl Electronic Products, MAC5000)**
- 20 • 2 piezoelectric stages (Nanos Instruments, LPS-30-30-1-V2_61-S-N)**
- 21 • 2 stage controller boards (Nanos Instruments, BMC101)**

B Supplementary Tables

¹ B Supplementary Tables

Table B1: Data sizes in microscopy. Typical devices used for confocal microscopy, high-content screening, single-molecule localization microscopy and light-sheet microscopy and their data production characteristics. Data visualized on Figure 4.1

	imaging device	image size	frame rate	data rate	data size
SPIM	2x sCMOS camera (e.g. Hamamatsu ORCA Flash4.0)	2048x2048	50/s	800 MB/s	10 TB
SMLM	2x EMCCD camera (e.g. Andor iXon Ultra 897)	512x512	56/s	56 MB/s	500 GB
screening	CCD camera (e.g. Hamamatsu ORCA-R2)	1344x1024	8.5s/	22 MB/s	5 TB
confocal	Zeiss LSM 880, 10 channels	512x512	5/s	12.5 MB/s	50 GB

Table B2: Lossless compression performance. B³D is compared with various popular lossless image compression methods regarding write speed, read speed and compression ratio (original size / compressed size). Data visualized on Figure 4.13.

	write speed	read speed	CR	file size
B³D	1,115.08 MB/s	928.97 MB/s	9.861	100%
KLB	283.19 MB/s	619.95 MB/s	10.571	93.28%
JPEG2000	31.94 MB/s	26.38 MB/s	11.782	83.69%
TIFF uncompressed	202.32 MB/s	161.08 MB/s	1.00	986.1%
TIFF + LZW	40.85 MB/s	102.37 MB/s	5.822	169.37%

B Supplementary Tables

Table B3: Datasets used for benchmarking compression performance.

Dataset name	Imaging modality	Description	Size (MB)
drosophila	SPIM	dataset acquired in MuVi-SPIM of a <i>Drosophila melanogaster</i> embryo expressing H2Av-mCherry nuclear marker	494.53
zebrafish	SPIM	dataset acquired in MuVi-SPIM of a zebrafish embryo expressing b-actin::GCaMP6f calcium sensor	2,408.00
phallusia	SPIM	dataset acquired in MuVi-SPIM of a <i>Phallusia mammillata</i> embryo expressing PH-citrine membrane marker	1,323.88
simulation	SMLM	MT0.N1.LD-2D simulated dataset of microtubules labeled with Alexa Fluor 647 from SMLMS 2016 challenge	156.22
microtubules	SMLM	microtubules immuno-labeled with Alexa Fluor 674-bound antibodies in U2OS cells	1,643.86
lifeact	SMLM	actin network labeled with LifeAct-tdEOS in U2OS cells	3,316.15
dapi	screening	wide field fluorescence images of DAPI stained HeLa Kyoto cells [154]	1,005.38
vsvg	screening	wide field fluorescence images of CFP-tsO45G proteins in HeLa Kyoto cells [154]	1,005.38
membrane	screening	wide field fluorescence images of membrane localized CFP-tsO45G proteins labeled with AlexaFluor647 in HeLa Kyoto cells [154]	1,005.38

¹ C Light collection efficiency of an objective

² Let's define light collection efficiency η as the ratio of collected photons and all emitted
³ photons:

$$\eta = \frac{N_{collected}}{N_{emitted}}$$

⁴ Since we can assume that the direction of photons emitted from a fluorescent molecule
⁵ are random, the light collection efficiency will correspond to the solid angle subtended
⁶ by the objective front lens at the focal point. To calculate this, let's consider the unit
⁷ sphere centered at the focal point, and calculate the surface area of the spherical cap
⁸ corresponding to the objective acceptance angle α (Fig. C1a). The area of the cap can
⁹ be expressed as a function of the angle:

$$A_{cap} = 2\pi r^2(1 - \cos \alpha)$$

¹⁰ The surface area of the full sphere is calculated as:

$$A_{sph} = 4\pi r^2$$

¹¹ For both equations r is the radius of the sphere. From here, the light collection efficiency
¹² can be calculated as:

$$\eta = \frac{N_{collected}}{N_{emitted}} = \frac{A_{cap}}{A_{sph}} = \frac{1 - \cos \alpha}{2}$$

¹³ As most objectives are characterized by the numerical aperture, we also plot η as a
¹⁴ function of the NA on Figure C1b.

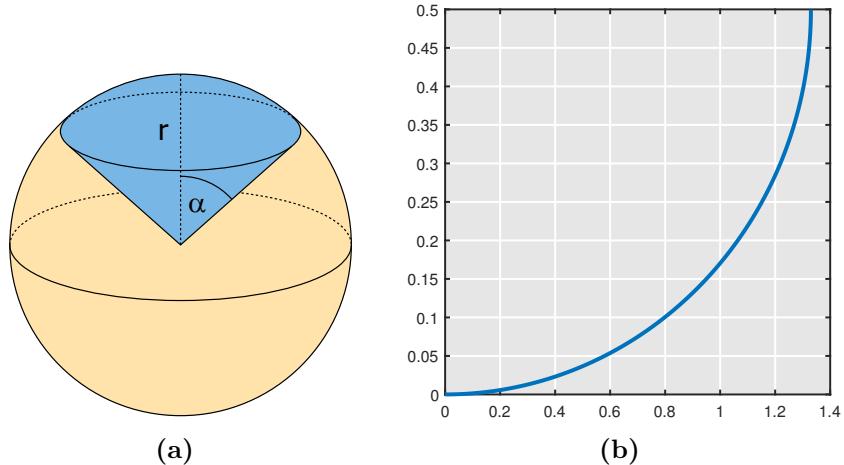


Figure C1: Light collection efficiency of an objective. (a) Light collection efficiency is the ratio of photons collected by the objective and all emitted photons. If the fluorophores are emitted randomly in all directions, it will be the surface ratio of the conical section (blue) to the whole sphere. (b) Light collection efficiency (η) as a function of the numerical aperture (NA).

1 D 3D model of Dual Mouse-SPIM

**2 To view the 3D figure on the next page, Adobe Reader is required. Click on the figure
3 to activate the 3D view.**

4 Navigation:

- 5 • zoom: mouse wheel / right click and drag vertically**
- 6 • rotate: left click and drag**
- 7 • pan: left + right click and drag / Ctrl + click and drag**

Interactive 3D figure
Click to activate

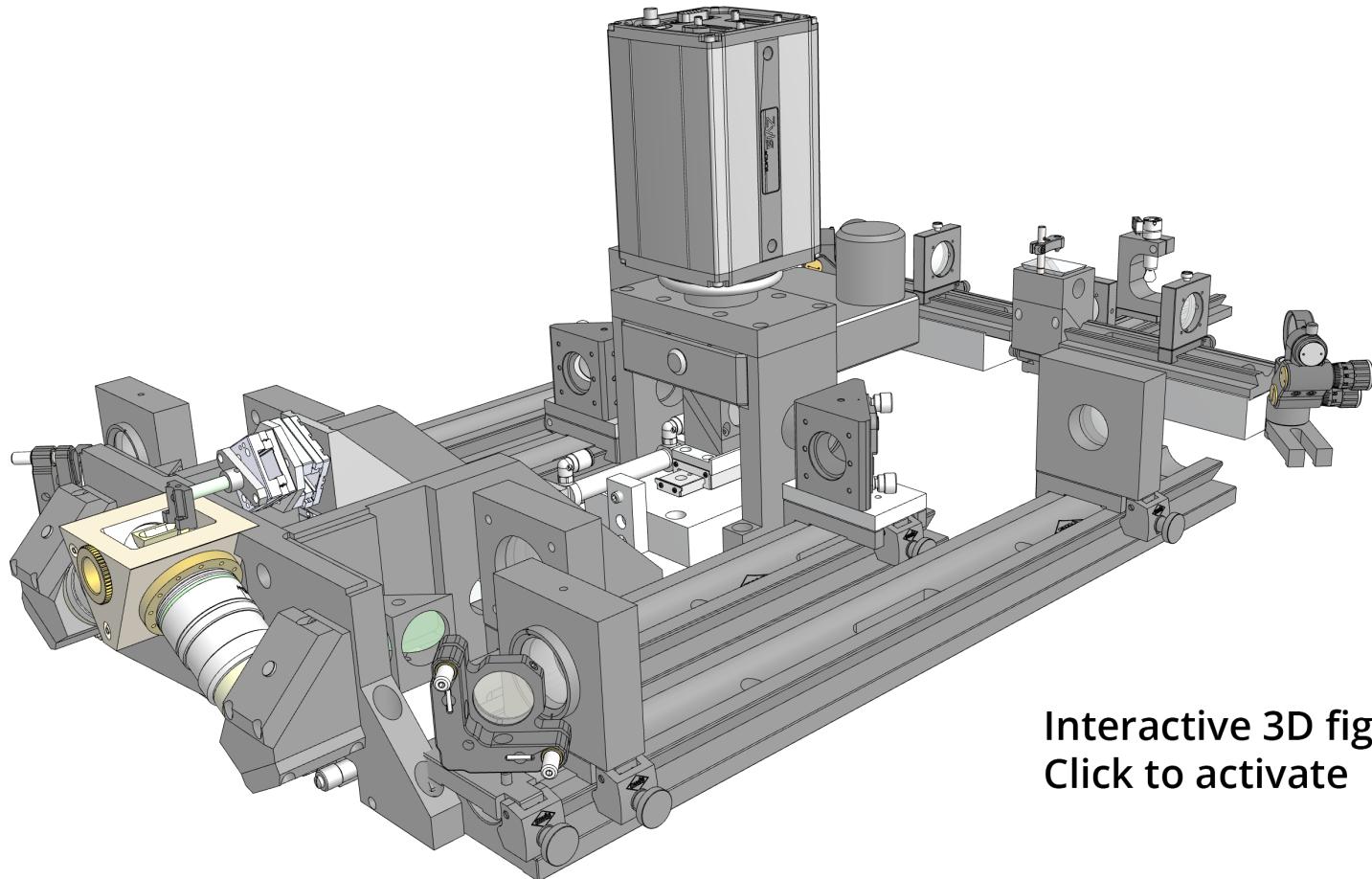


Figure D1: 3D model of DualMouse-SPIM.

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