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¹ Abstract

² Abstract in English.

¹ Tartalmi kivonat

² Absztrakt magyarul.

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¹ Introduction

² Coming soon.

INTRODUCTION

¹ Chapter 1

² Live imaging in three dimensions

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 faster, while whole embryo reorganizations and tissue migration events take place
⁸ over the course of hours [1]. 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.

¹¹ Light microscopy is one of the oldest methods that is still widely used today to
¹² investigate the inner workings of microscopic life. A particularly important branch is
¹³ fluorescence microscopy [2], that relies on using fluorescent labels to mark specific regions
¹⁴ inside the specimens. Light-sheet microscopy is a relatively new addition to the arsenal of
¹⁵ tools that comprise light microscopy methods, and is especially suitable for live imaging
¹⁶ of embryonic samples over extended periods of time [3–6]. It is also easily adapted to
¹⁷ the sample, allowing to image a large variety of specimens, from entire organs, such as
¹⁸ cleared mouse brains [7], to the subcellular processes occurring inside cultured cells [8].

¹⁹ In an ideal setting, the ultimate microscope would be able to record a continuos,
²⁰ 3D, and multicolor dataset of any biological process of interest with the highest possible
²¹ resolution. Unfortunately this is not possible; due to several limitations in physics and
²² biology, a compromise is necessary. The diffractive nature of light, the lifetime of fluo-
²³ rescent probes and the photosensitivity of biological specimens all require microscopy to
²⁴ be able to adapt to answer the question at hand. In order to acquire useful data one has
²⁵ to choose a tradeoff between spatial and temporal resolution, and signal contrast, while
²⁶ making sure the biology is not affected by the imaging process itself (Fig. 1.1) [9].

1. LIVE IMAGING IN THREE DIMENSIONS

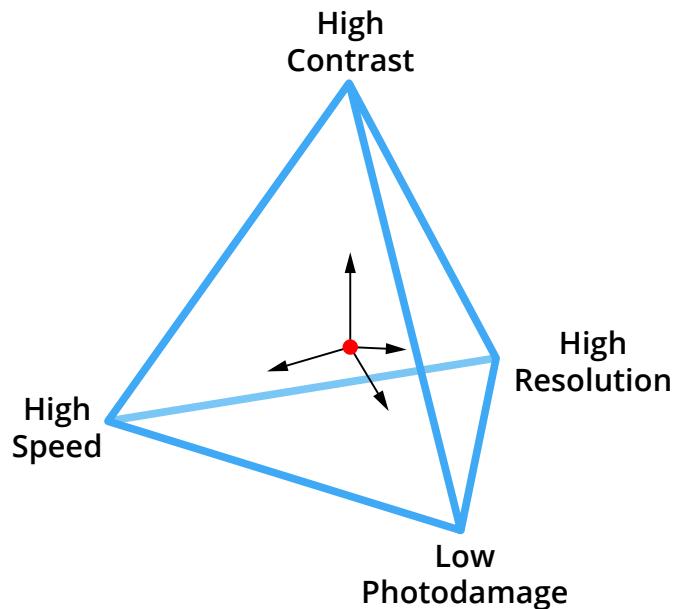


Figure 1.1: Tradeoffs in fluorescence microscopy for live imaging. Also called the “pyramid of frustration”. When optimizing the imaging conditions (red dot), a tradeoff has to be made between resolution, contrast, and imaging speed, while avoiding photodamage. One can only be improved at the expense of the others due to the limited photon budget of the fluorescent molecules. Adapted from [9].

¹ 1.1 Wide-field fluorescence microscopy

² Fluorescence microscopy [2, 10], as a subset of light microscopy is one of the few methods
³ that allows subcellular imaging of live specimens. The first use of the term fluorescence is
⁴ credited to George Gabriel Stokes [11], and it refers to the phenomenon when a molecule
⁵ emits light after absorbing light or other electromagnetic radiation. As the name of the
⁶ technique suggests, this method collects fluorescent light from the specimens which has
⁷ numerous advantages, but also some drawbacks. Since biological tissue is usually not
⁸ fluorescent, except for some autofluorescence at shorter wavelengths, fluorescent dyes or
⁹ proteins have to be introduced to the system in order to be able to collect the necessary
¹⁰ information. The advantage of this is that the signal of the labeled structures will be of
¹¹ very high ratio compared to the background.

¹² A fluorescent molecule is capable of absorbing photons in a given range (excitation
¹³ spectrum) and temporarily store its energy by having an electron in a higher energy
¹⁴ orbital, *i.e.* in an excited state. This excited state, however is not stable, and the electron
¹⁵ quickly jumps back to the ground state while emitting a photon with equal energy to the
¹⁶ energy difference between the excited and ground states. The energy of the absorbed and
¹⁷ emitted photons are not the same, as energy loss occurs due to internal relaxation events,
¹⁸ and the emitted photon has lower energy than the absorbed photon. This phenomenon
¹⁹ is called the Stokes shift, or red shift, and can be exploited in microscopy to drastically
²⁰ increase the signal to noise ratio by filtering out the illumination light (Fig. 1.2).

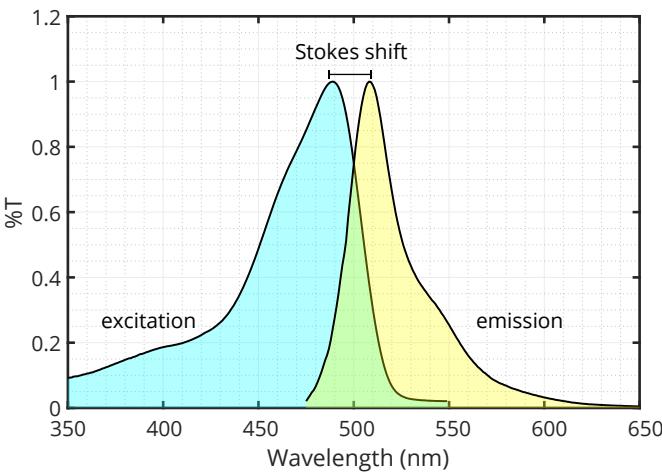


Figure 1.2: Excitation and emission spectrum of enhanced green fluorescent protein (EGFP). Excitation spectrum in blue, emission spectrum in yellow. The separation between the two spectra is due to the Stokes shift, which is 19 nm for EGFP. Emitted and excitation light can be separated by a long-pass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

¹ 1.1.1 Fluorescent proteins

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

⁸ The discovery of fluorescent proteins have 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], the first of its kind, genetic
¹² engineering techniques evolved since then [15], and not only has it been successfully
¹³ integrated in the genome of nematodes [16], zebrafish [17], and mice [18], but many
¹⁴ variants have been also engineered by introducing mutations to increase fluorescence
¹⁵ intensity, and to change the fluorescence spectrum to allow multicolor imaging [18–21].
¹⁶ The usefulness and impact of these proteins are so profound, that in 2008 the Nobel
¹⁷ Prize in chemistry was awarded to Osamu Shimomura, Martin Chalfie, and Roger Tsien
¹⁸ “for the discovery and development 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 fluorescent emitted light due to the
²² Stokes shift described in the previous section. The microscope’s operating principle is

1. LIVE IMAGING IN THREE DIMENSIONS

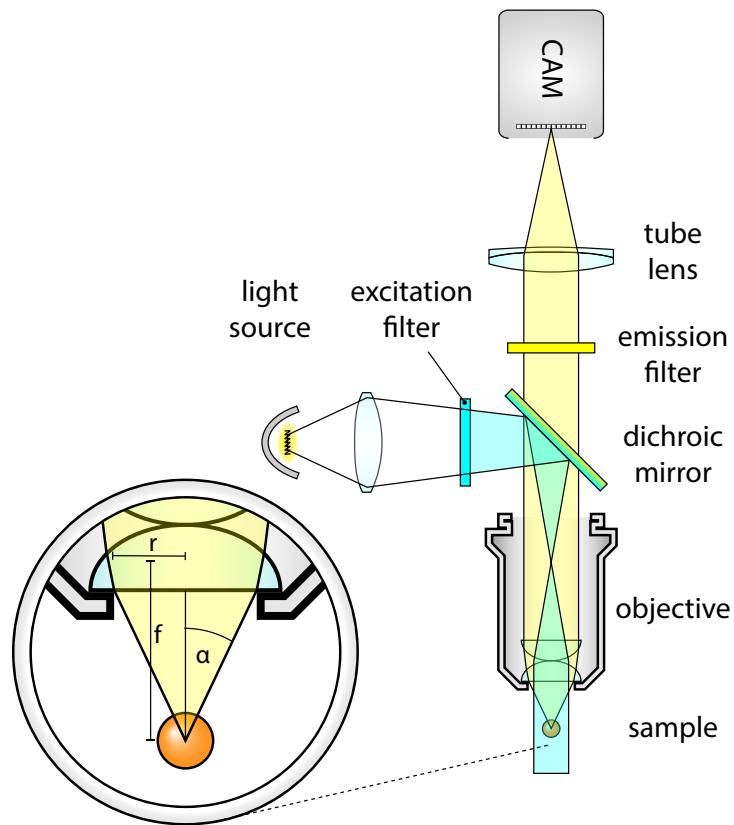


Figure 1.3: Wide-field fluorescence microscope. (a) The light source is focused on the back focal plane of the objective to provide an even illumination to the sample. Emitted photons are collected by the objective, and are separated from the illumination light by a dichroic mirror. (b), α

1 depicted in Figure 1.3.

2 A light source, typically a mercury lamp is focused on the back focal plane of the
3 objective to create even illumination at the sample. Before entering the objective, the
4 light is filtered, so only the wavelengths that correspond to the excitation properties
5 of the observed fluorophores are transmitted. Since the same objective is used for both
6 illumination and detection, a dichroic mirror is utilized to decouple the illumination
7 and detection paths. The emitted light is filtered again to make sure any reflected and
8 scattered light from the illumination source is blocked to increase signal to noise ratio.

9 Finally, the light is focused by a tube lens to create a magnified image in the cam-
10 era sensor. This type of imaging is called infinity corrected optics, since the back focal
11 point of the objective is in “infinity”, meaning that the light exiting the back aperture is
12 parallel. This is achieved by placing the sample exactly at the focal point of the objec-
13 tive. Infinity corrected optics has the advantage that it allows placing various additional
14 optical elements in the infinity space (*i.e.* the space between the objective and the tube
15 lens) without affecting the image quality. In this example such elements are the dichroic
16 mirror and the emission filter.

¹ The combination of the objective and tube lens together will determine the magnification of the system, it will be the ratio of the focal lengths of these lenses:

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

³ The final field of view (FOV) of the microscope will depend on the magnification, and
⁴ also on the size of the imaging sensor:

$$FOV = \frac{A}{M}, \quad (1.2)$$

⁵ where A is the area of the sensor.

⁶ Apart from magnification, the most important property of the objective is the half-angle of the light acceptance cone, α . This not only determines the amount of collected
⁷ light, but also the achievable resolution of the system (see next section). This angle
⁸ depends on the size of the lens relative to its focal length. In other words depends on
⁹ the aperture of the lens, which is why the expression *numerical aperture* (NA) is more
¹⁰ commonly used to express this property of the objective:
¹¹

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

¹² For small α angles, the following approximation holds true: $\sin \alpha \approx \tan \alpha \approx \alpha$. Thus,
¹³ the numerical aperture can also be expressed as a ratio of the radius of the lens and the
¹⁴ focal length:

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

¹⁵ 1.1.3 Resolution of a wide-field microscope

¹⁶ The resolution of an optical systems is defined by the size of the smallest distinguishable
¹⁷ feature on the image. Practically this means the minimum distance between two point-like objects so that the two objects can still be resolved. This mainly depends on two
¹⁸ factors: the NA of the objective, and the pixel size of the imaging sensor.
¹⁹

²⁰ Even if the imaging sensor would have infinitely fine resolution, it is not possible to
²¹ reach arbitrary high resolutions due to the wave nature of light and diffraction effects
²² that occur at the aperture of the objective. This means that depending on the wavelength
²³ of the light, any point source will have a finite size on the image, it will be spread out,
²⁴ that will limit the resolution. The shape of this image is called the *point spread function*,
²⁵ or PSF (Fig. 1.4), as this function describes the behavior of the optical system when
²⁶ imaging a point like source. This property of lenses was already discovered by Abbe in

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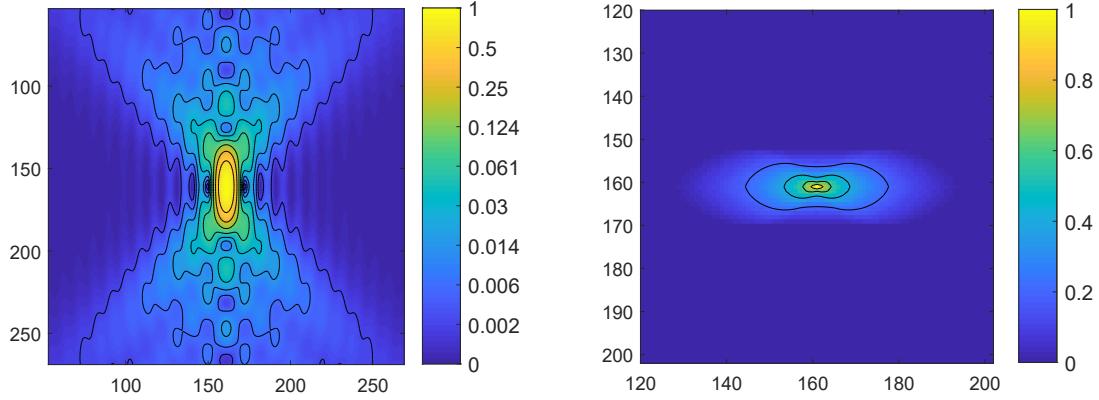


Figure 1.4: Axial cross section of the PSF and OTF of a wide-field microscope. Simulated PSF and OTF for a wide-field microscope with a water immersion objective ($n = 1.33$). $NA = 1.1$, $\lambda = 510\text{ nm}$

¹ 1873 [23], when he constructed his famous formula for resolution:

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

² where d is the smallest distance between two distinguishable features.

³ Another representation of the optical performance, is the *optical transfer function*,
⁴ or OTF (Fig. 1.4), which is the Fourier transform of the PSF:

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

⁵ As this function operates in the frequency space, it describes how the different frequencies
⁶ are affected by the system. The resolution can also be defined as the support of the OTF,
⁷ since this describes the highest frequency that is still transmitted through the optical
⁸ system. Any pattern with higher frequency will be lost, thus beyond the resolution limit.
⁹ For circularly symmetric PSFs, the OTF will be real valued, however if this is not the
¹⁰ case, the Fourier transform also introduces complex components.

¹¹ Abbe's formula can be derived from the scalar theory of diffraction using a paraxial
¹² approximation (Fraunhofer diffraction, [24]) that describes the intensity of the electric
¹³ field in the focus of a lens [25]:

$$H(u, v) = C_0 \left| \int_0^1 J_0(vr) e^{-i\frac{1}{2} \cdot ur^2} r dr \right|^2, \quad (1.7)$$

¹⁴ where C_0 is a normalization constant, and J_0 is the zero order Bessel function of the first
¹⁵ kind. Furthermore, instead of the commonly used Cartesian coordinates x , y and z , the

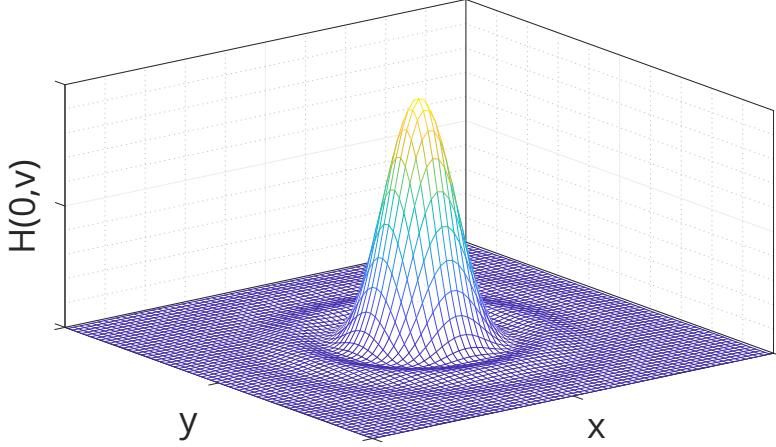


Figure 1.5: Airy pattern. Airy pattern calculated in Matlab based on Eq. 1.9

1 following optical coordinates are defined:

$$v = \frac{2\pi nr}{\lambda_0} \sin \alpha, \quad u = \frac{8\pi nz}{\lambda_0} \sin^2 \frac{\alpha}{2} \quad (1.8)$$

2 where $r = \sqrt{x^2 + y^2}$ is the distance from the optical axis, and α is the light collection
3 angle as shown on Fig. 1.3.

4 To determine the lateral resolution of the system, let's substitute $u = 0$ as the axial
5 optical coordinate, and evaluate Eq. 1.7 which will give the intensity distribution in the
6 focal plane:

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

7

8 where J_1 is the first order Bessel function of the first kind. This equation describes
9 the famous Airy pattern (Fig. 1.5) which will be the shape of the PSF in the focal plane.
10 The width of this pattern is the resolution, and although there are multiple definitions
11 for this, the most commonly accepted is the Rayleigh criterion [24, 26] which defines the
12 resolution as the distance between the central peak and the first local minimum. As this
13 lies at $v = 3.83$, the resolution can be expressed by substituting this value to Eq. 1.8 and
14 calculating the real distance (r):

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

15 which is equivalent to Abbe's original formula (Eq. 1.5). The only difference is the scaling
16 factor which is due to the slightly different interpretations of width of the Airy disk as
17 mentioned earlier.

1. LIVE IMAGING IN THREE DIMENSIONS

1 Similarly, to calculate the intensity distribution along the axial direction, let's sub-
2 stitute $v = 0$ to Eq. 1.7:

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

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

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

5 So far we only considered a single, point-like emitter. As the intensity function de-
6 scribes how an optical system “spreads out” the image of a point, it is also called the
7 Point Spread Function (PSF, Fig. 1.4). In a more realistic scenario, however the emitters
8 are neither point-like, nor single. Effectively, however, for every emitter the PSF would
9 be imaged on the sensor, and this creates the final image. In mathematical terms, this can
10 be expressed as a convolution operation between the underlying fluorophore distribution
11 of the object (O) and the PSF (H):

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

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

14 The PSF is further affected by the illumination pattern as well. Since the number of
15 emitted fluorescent photons are roughly proportional to the illumination intensity, if the
16 illumination has any structure at the order of the detection resolution, it will have an
17 effect on the overall PSF of the system, which can be expressed as:

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

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

20 1.2 Point scanning methods

21 In most cases, a wide-field microscope is used to image a section of a tissue, thus axial
22 resolution is not a concern. Imaging live specimens, however is not so straightforward,
23 as these samples are usually much thicker than a typical section. For these samples 3-
24 dimensional (3D) imaging is highly beneficial, which necessitates some kind of optical
25 sectioning technique to be able to discriminate the features at different depths.

26 Due to the design of the wide-field microscope, any photons emitted from outside the
27 focal plane will also be detected by the sensor, however as these are not originating from

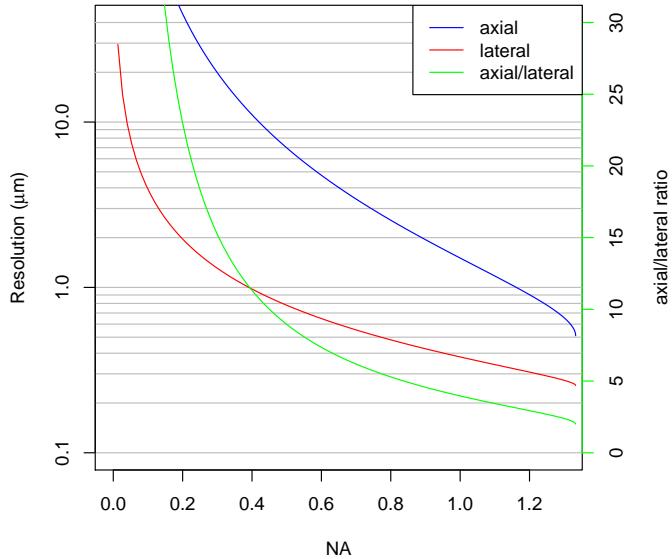


Figure 1.6: Resolution of a wide-field microscope. Axial (blue) and lateral (red) resolutions of a wide-field microscope are shown with respect to the numerical aperture (NA). Resolutions are calculated with $\lambda = 510\text{nm}$, the emission maximum of GFP and $n = 1.33$, the refractive index of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

the focus, only a blur will be visible. This blur potentially degrades image quality and signal-to-noise ratio to such extent that makes imaging thick sample 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 (Fig. 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.

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 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 result, 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

1. LIVE IMAGING IN THREE DIMENSIONS

- 1 extremely difficult sample handling, since the sample was completely surrounded by
2 objectives from all directions.

3 **1.2.1 Confocal laser scanning microscopy**

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

9 This is achieved by two significant modifications compared to the wide-field optical
10 path. To be able to reject the out of focus light, an adjustable pinhole is placed at the focus
11 of the tube lens. Light rays originating from the focal point will meet at this position,
12 and are able to pass through the pinhole, however out-of-focus light will converge either
13 before or after the aperture, and thus the aperture blocks these rays. To maximize the
14 fluorescence readout efficiency for the single focal point, a photomultiplier tube is used
15 instead of an area sensor (Fig. 1.7a).

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

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

23 where δ_{ill} and δ_{det} are the resolutions for the illumination and detection, respectively.
24 Since the same objective is used for both illumination and detection, and the difference
25 in wavelength is almost negligible, $\delta_{ill} = \delta_{det} = \delta$, the final system resolution will be:

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

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

28 Because of the different detection method in a confocal microscope, direct image
29 formation on an area sensor is not possible, since at any given time, only a single point is
30 interrogated in the sample. Instead, it is necessary to move the illumination and detection
31 point in synchrony (or in a simpler, albeit slower solution, to move the sample) to scan
32 the entire field of view. The image can be later computationally reconstructed by a

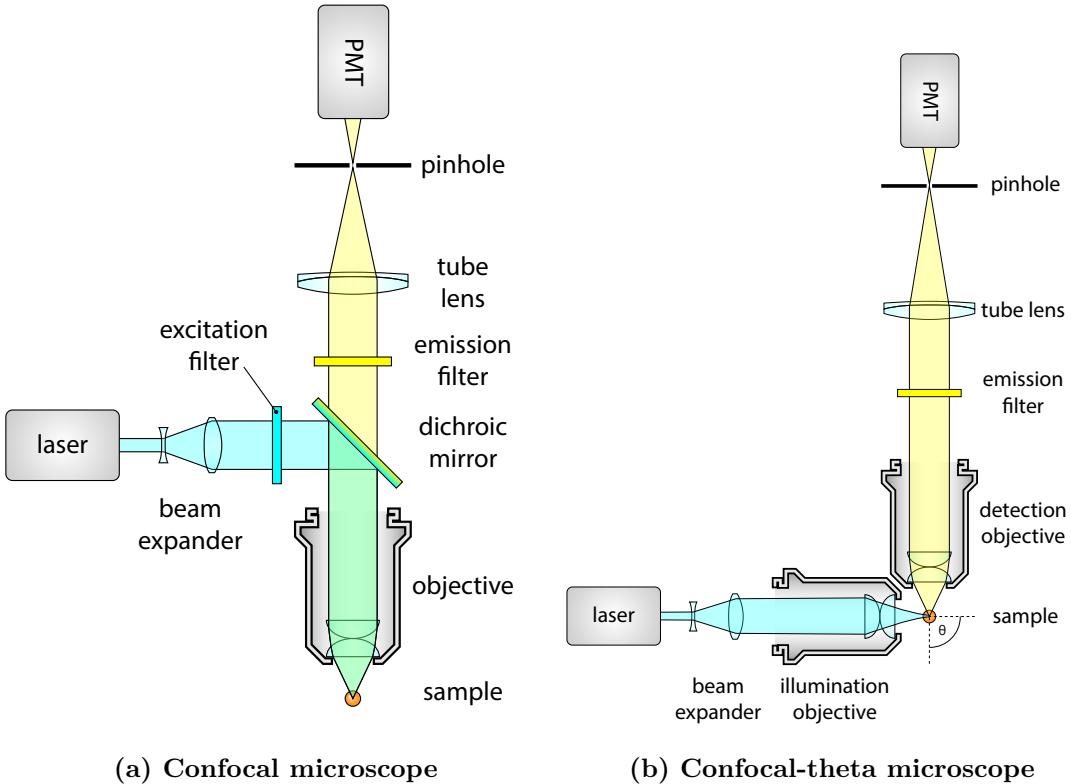


Figure 1.7: Basic optical components of a confocal laser scanning and confocal-theta microscope. Both type of microscopes use confocal images detection, which means that a pinhole is used to exclude light coming from out of focus points. Light intensity is measured by a photomultiplier for every voxel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

- 1 computer program that records the fluorescence intensity of every point of the field of
- 2 view, and displays these values as a raster image.

3 1.2.2 Confocal-theta microscopy

4 Although confocal microscopy already has 3D capabilities, its axial resolution is still lim-
 5 ited compared to the lateral, since it uses only one objective. An alternative realization
 6 of the confocal microscope, the confocal theta microscope [31] introduces a second objec-
 7 tive to the system, that is used to illuminate the sample (Fig. 1.7b). Since this decouples
 8 the illumination and detection, using a filter cube is no longer necessary. The second
 9 objective is rotated by θ around the focus, this is where the name of this setup originates
 10 from.

11 As in the case of standard confocal microscopy, the system PSF is improved by the
 12 illumination pattern. Here, however, the axial direction of the detection coincides with
 13 the lateral direction of the illumination, which results in a dramatic improvement of

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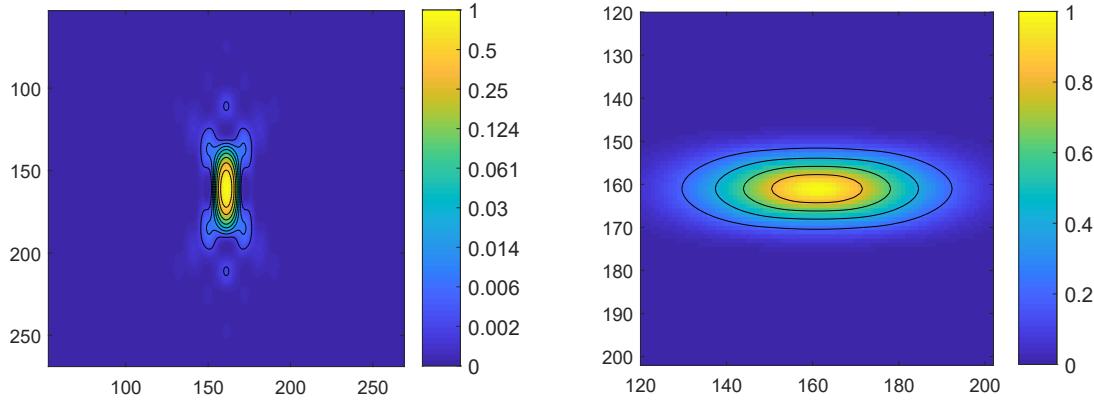


Figure 1.8: Axial cross section of the PSF and OTF of a confocal laser scanning microscope. Simulated PSF and OTF for a laser scanning confocal microscope with a water immersion objective ($n = 1.33$). $NA = 1.1$, $\lambda = 510\text{ nm}$

1 axial resolution compared to standard confocal microscopy. Lateral resolution will also
2 be increased, but by a smaller extent, resulting in an almost isotropic PSF, and equal
3 axial and lateral resolutions (Fig. ??).

4 Although this is a big improvement to confocal microscopy in terms of resolution,
5 this technique did not reach a widespread adoption as it complicates sample handling,
6 while still suffering from two big drawbacks of confocal microscopy techniques that limits
7 its live imaging capabilities.

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

18 1.3 Light-sheet microscopy

19 A selective-plane illumination microscope (SPIM) uses a light-sheet to illuminate only
20 a thin section of the sample (Fig. 1.9). This illumination plane is perpendicular to the
21 imaging axis of the detection objective and coincides with the focal plane. This way, only
22 the section in focus will be illuminated, thus providing much better signal to noise ratio.

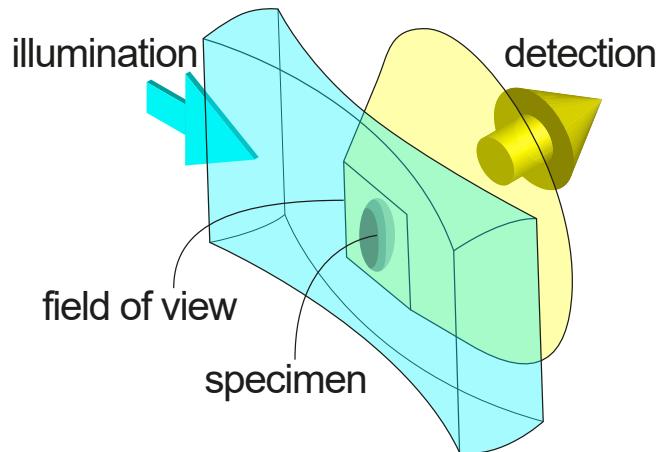


Figure 1.9: Basic concept of single-plane illumination microscopy. The sample is illuminated from the side by laser light shaped to a light-sheet (blue). This illuminates the focal plane of the detection lens, that collects light in a wide-field mode (yellow). The image is recorded, and the sample is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

1 In case of conventional wide-field fluorescence microscopy, where the whole specimen is
 2 illuminated, light scattering from different regions contributes to a significant background
 3 noise. With selective-plane illumination, this problem is intrinsically solved, and it also
 4 provides a true sectioning capability. This makes SPIM especially suitable for 3D imaging.

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

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

22 Later, in 2002, Fuchs et al. developed Thin Light-Sheet Microscopy (TLSM) [39]
 23 and used this technique to investigate the microbial life in seawater samples without
 24 disturbing their natural environment (by e.g. placing them on a coverslip). Their light-

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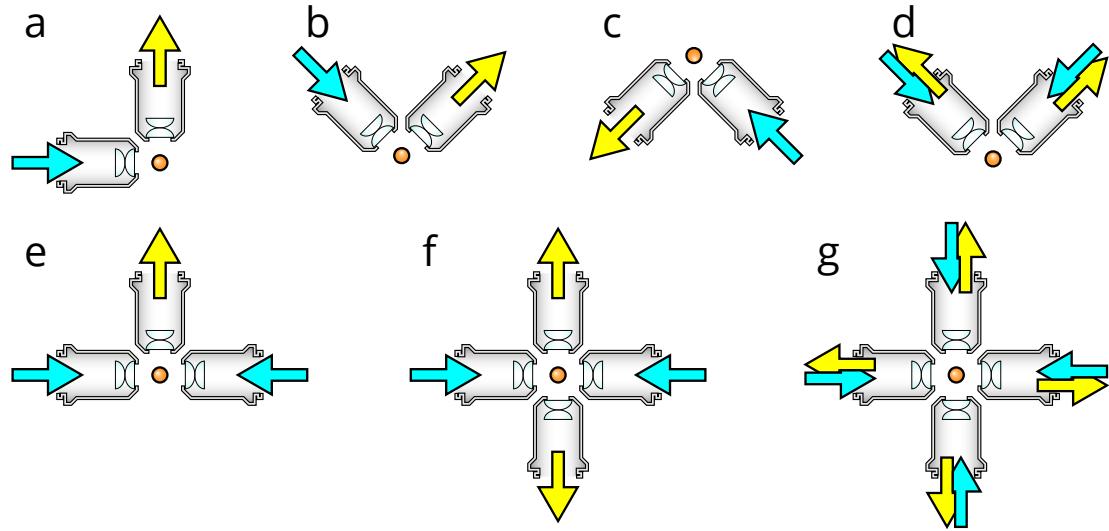


Figure 1.10: Different optical arrangements for light-sheet microscopy. (a) Original SPIM design with a single lens for detection and illumination. [40] (b) Upright SPIM to allow for easier sample mounting such as using a petri dish (iSPIM, [41, 42, J1]). (c) Inverted SPIM, where the objectives are below 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 (disSPIM, [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]).

1 sheet was similar to the one utilized in OPFOS, being $23\text{ }\mu\text{m}$ thin, and providing a
 2 $1\text{ mm} \times 1\text{ mm}$ field of view.

3 Despite these early efforts, the method did not gain larger momentum. The real
 4 breakthrough in light-sheet imaging happened at EMBL in 2004, where Huisken *et al.* [40]
 5 combined the advantages of endogenous fluorescent proteins and the optical sectioning
 6 capability of light-sheet illumination to image Medaka fish embryos, and the complete
 7 embryonic development of a *Drosophila melanogaster* embryo. They called this Selective-
 8 Plane Illumination Microscopy (SPIM), and it quickly became popular to investigate
 9 developmental biological questions.

10 Since then, light-sheet based imaging has gained more and more popularity, as it can
 11 be adapted and applied to a wide variety of problems. Although sample mounting can
 12 be challenging because of the objective arrangement, this can also be an advantage, since
 13 new microscopes can be designed with the sample in mind [4, J3] (Fig. 1.10). This made
 14 it possible to adapt the technique for numerous other specimens, such as zebrafish larvae
 15 [49], *C. elegans* embryos [42], mouse brain [7], and even mouse embryos [J2, 50, 51].

16 As many of these specimens require very different conditions and mounting tech-
 17 niques, these microscopes have been adapted to best accommodate them. An upright
 18 objective arrangement for example (Fig 1.10b) allows imaging samples on a coverslip,

1 while its inverted version is well suited for mouse embryos, where a foil is separating them
2 from the immersion medium (Fig. 1.10c). A modified version of the upright arrangement
3 allows for multi-view imaging using both objectives for illumination and detection in a
4 sequential manner (Fig. 1.10d) [52].

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

11 Furthermore, because of the wide-field detection scheme it is possible to combine
12 SPIM with many superresolution techniques, such as single molecule localization [53],
13 STED [54], RESOLFT [J1], or structured illumination [8, 55, 56].

14 1.3.1 Optics of light-sheet microscopy

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

17 The detection unit of a SPIM is basically equivalent to a detection unit of a wide-field
18 microscope, without a dichroic mirror (Fig. 1.11). The most important components are
19 the objective together with the tube lens, filter wheel, and a sensor, typically a CCD or
20 sCMOS camera.

21 One of the most important aspects that determines the resolution of the microscope
22 is the detection objective. Since imaging biological specimens usually requires a water-
23 based solution, the objectives also need to be directly submerged in the medium to
24 minimize spherical aberrations. Since the refraction index of water ($n = 1.33$) is greater
25 than the refraction index of air, these objectives tend to have a higher NA, which results
26 in higher resolution. The final resolution, however, also depends on the pixel size of the
27 sensor which determines the spatial sampling rate of the image.

28 Although image quality and resolution greatly depends on the detection optics, the
29 real strength of light-sheet microscopy, the inherent optical sectioning is due to the
30 specially designed illumination pattern, that confines light to the vicinity of the detection
31 focal plane.

32 There are two most commonly used options to generate a light-sheet: either by using
33 a cylindrical lens, to illuminate the whole field of view with a static light-sheet, as in the
34 original SPIM concept [40]; or by quickly scanning a thin laser beam through the focal
35 plane, thus resulting in a virtual light-sheet [49].

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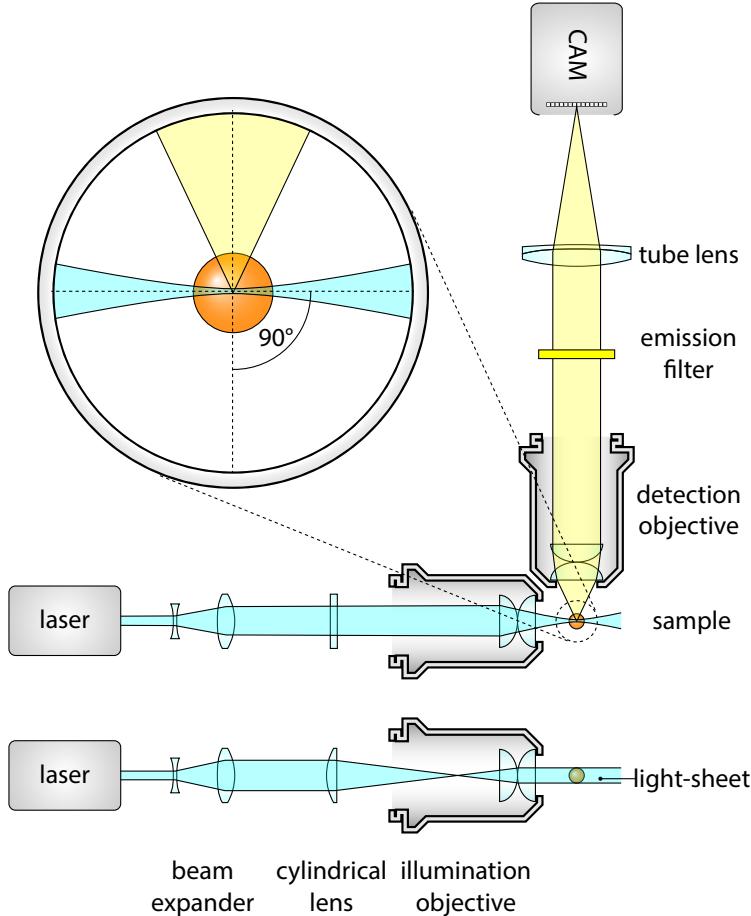


Figure 1.11: Basic optical components of a SPIM. A dedicated illumination objective is used to generate the light sheet, which is an astigmatic Gaussian beam, focuses along one direction. Astigmatism is introduced by placing a cylindrical lens focusing on the back focal plane of the objective. Detection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

1.3.2 Static light-sheet illumination

- 2 For a static light-sheet, the normally circular Gaussian laser beam needs to be shaped
 - 3 in an astigmatic manner, i.e. either expanded or squeezed along one direction, to shape
 - 4 it into a sheet instead of a beam. This effect can be achieved by using a cylindrical lens,
 - 5 which as the name suggests has a curvature in one direction, but is flat in the other, thus
 - 6 focusing a circular beam to a sheet.
- 7 However, to achieve light-sheets that are thin enough, one would need to use cylindrical lenses 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

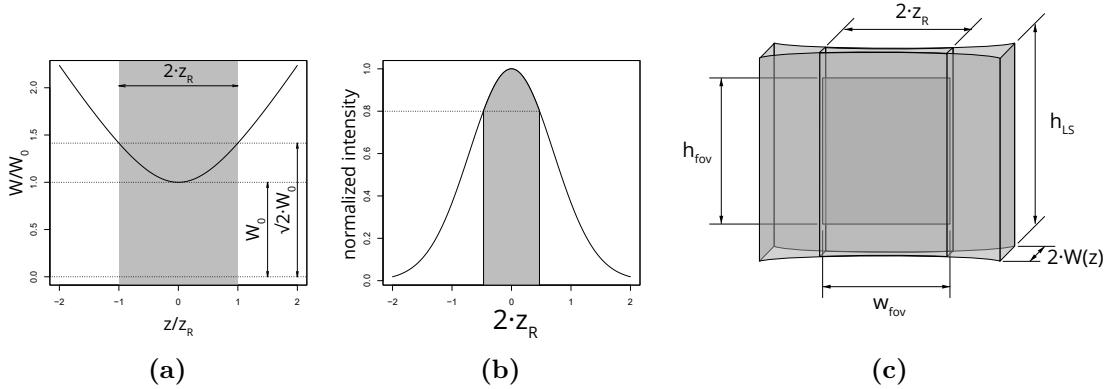


Figure 1.12: Light-sheet dimensions. 1.12a The width and thickness of the field of view depends on the Rayleigh length of the beam ($z_{R,y}$) and the beam waist (W_0). 1.12b Height of the field of view is determined by the Gaussian profile of the astigmatic beam. 1.12c shows a light sheet, with the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

1 adjusted for the specific imaging tasks.

2 Light-sheet dimensions

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

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

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

9 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
 10 the direction of the light propagation.

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

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

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

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

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

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

2 Apart from the circular Gaussian beam, the elliptical Gaussian beam is also an eigen-
3 function of Helmholtz equation (1.17) which describes the beam shape after a cylindrical
4 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 be now 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:

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

5 Based on these equations, the light-sheet dimensions and usable field of view (fov)
6 can be specified (Fig. 1.12c). The light-sheet thickness will depend on the beam waist,
7 $W_{0,y}$ (if we assume the cylindrical lens is focusing along y), and the length of the light-
8 sheet can be defined as twice the Rayleigh range, $2 \cdot z_{R,y}$. As these are coupled (see Eq.
9 1.23), having a thin light-sheet for better sectioning also means the length of it will be
10 relatively short. Fortunately, because of the quadratic relation, to increase the field of
11 view by a factor of two, the light-sheet thickness only needs to increase by a factor of
12 $\sqrt{2}$.

13 Light-sheet height is determined by the intensity profile of the beam along the vertical
14 axis (Fig. 1.12b). Since this is a Gaussian function (see Eq. 1.18), only a small part in
15 the middle can be used for imaging, because towards the sides the intensity dramatically
16 drops. To allow a maximum 20% drop of intensity at the edges, the light-sheet height is
17 $h_{fov} = 2 \cdot 0.472 \cdot W_{x,0}$.

18 1.3.3 Digitally scanned light-sheet illumination

19 Although generating a static light-sheet is relatively straightforward, with the simple
20 addition of a cylindrical lens to the light path, it has some drawbacks. As already men-

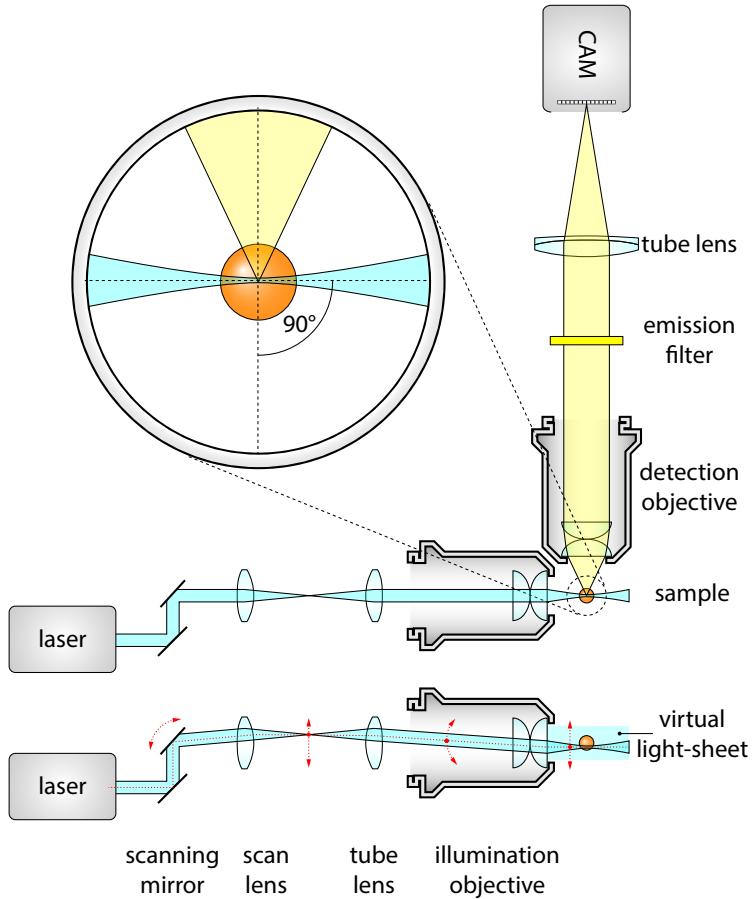


Figure 1.13: DSLM illumination. DSLM illuminates a specimen by a circularly-symmetric beam that is scanned over the field of view. This creates a virtual light-sheet, which illuminates a section of a specimen just like the SPIM. Light-sheet in DSLM is uniform over the whole field of view and its height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

tioned 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 Fig. 1.13. A galvanometer controlled

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

10 1.4 Light-sheet microscopy for mouse imaging

11 Because of its optical sectioning capabilities, combined with the high specificity of fluo-
12 rescent labels, confocal microscopy had an immense influence on biological research, and
13 has been the go-to technique for decades [59–61]. Live imaging capabilities of confocal
14 microscopy are, however, limited. This is mostly due to the illumination scheme: to col-
15 lect information from a single point, almost the whole embryo has to be illuminated.
16 This unwanted illumination can easily lead to bleaching of the fluorophores, and also to
17 phototoxic effects that can prevent further embryonic development. The point scanning
18 nature of image acquisition also implies an inherent constraint to the maximum imaging
19 speed, as it can be only increased at the expense of reducing signal intensity (Fig. 1.1).

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

26 Although out-of-focus illumination is still an issue, spinning disk confocal microscopy
27 was successfully used to investigate the mechanisms of symmetry breaking in early mouse
28 embryonic development [64], and the mechanisms leading to the first cell fate specification
29 that will differentiate extraembryonic tissue from the embryo proper [33, 65, 66]. Even
30 though these studies were carried out with live embryos, only a few hours of continuous
31 imaging is possible before the accumulation of phototoxic effects arrests development
32 [67]. Compared to this, just the pre-implantation phase of mouse embryonic development
33 spans 3 days, and the full development is 18 days [68].

1 1.4.1 Imaging pre-implantation

2 Pre-implantation is the first phase of mouse embryonic development, that starts right
3 after fertilization. The embryo in this phase is still in the oviduct, travelling towards
4 the uterus, where it will implant to the uterine wall. The developmental stage between
5 fertilization and the implantation is called the pre-implantation stage. Here the embryo
6 divides, and already the first cell fate specifications start when forming the trophoecto-
7 derm (TE) and the inner cell mass (ICM) at the blastocyst stage. ICM cells will form
8 the embryo proper, while TE cell will contribute to the formation of the extraembryonic
9 tissues.

10 During this process the embryo is still self-sufficient, which makes it possible to image
11 this stage in an *ex vivo* embryo culture by providing the proper conditions [69]. Long
12 term imaging, however, is extremely challenging due to the very high light sensitivity of
13 the specimens. Imaging these embryos in a confocal microscope will lead to incomplete
14 development, even if the imaging frequency is minimized to every 15 mins [J2].

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

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

28 As a mouse embryo culture is not compatible with the standard agarose-based sample
29 mounting techniques, a completely new approach was taken, which resulted in a micro-
30 scope designed around the sample. The sample holder forming a V-shape was built with
31 a bottom window, and it is lined with a thin FEP (fluorinated ethylene propylene) foil
32 that supports the embryos (Fig. 1.14A, i). This arrangement allows the utilization of
33 the standard microdrop embryo culture, while providing proper viewing access for the
34 objectives. As the embryos are relatively small (100 µm) and transparent, a single illu-
35 mination and single detection objective arrangement is enough for high quality imaging.
36 A low resolution (NA=0.3) objective is used to generate the scanned light-sheet, and
37 a high resolution (NA=1.1) objective is detecting the fluorescence at 50×magnification
38 (Fig 1.14A, ii). As the foil is curved, it allows unrestricted access to the embryo, while

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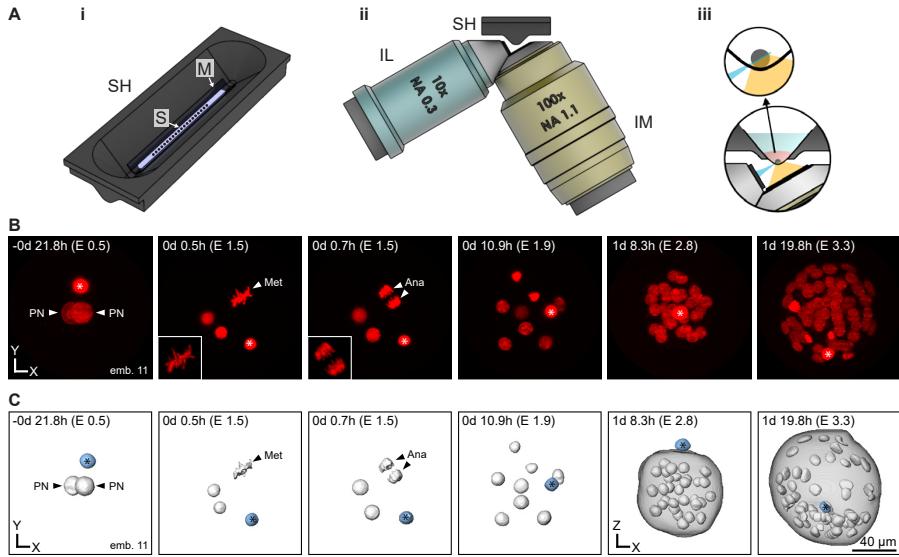


Figure 1.14: Inverted light-sheet microscope for multiple early mouse embryo imaging.. (i) A sample holder (SH), containing a transparent FEP membrane (M) allows multiple embryo samples (S) to be placed in line for multisample imaging. (ii) Inverted objective orientation with side view of the sample holder. One possible configuration is to use a 10×0.3 NA illumination objective (IL) and another 100×1.1 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 separating the imaging medium from the immersion liquid (Fig. 1.14A, iii). Furthermore,
 2 its refractive index is matching the refractive index of water, so optical aberrations are
 3 minimized.

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

12 1.4.2 Imaging post-implantation development

13 After the initial 3 days of pre-implantation, the embryo undergoes the implantation
 14 process, during which it is inaccessible to microscopical investigations. Although a new
 15 method was recently developed that allows *in vitro* culturing of the embryos embedded
 16 in a 3D gel [76], this has not reached wider adoption yet. Because of this, developmental
 17 processes during implantation have only been investigated in fixed embryos.

18 Following the implantation process, at the post-implantation phase, *ex vivo* embryo

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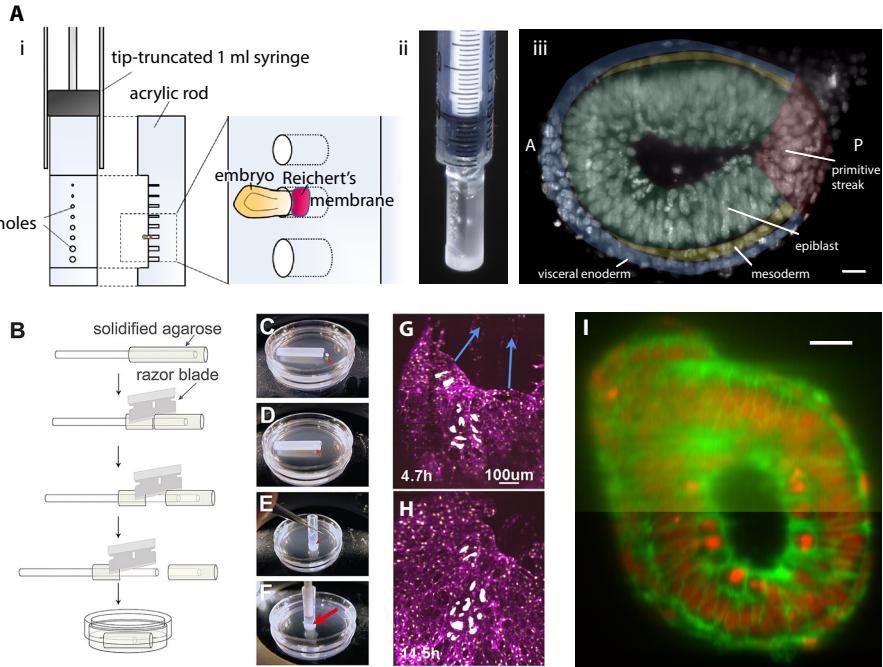


Figure 1.15: Imaging mouse post-implantation development. (A) (i, ii) Mounting technique for E5.5 to E6.5 embryos. A tip-truncated 1 mL syringe holds an acrylic rod, cut and drilled with holes of different size in order to best fit the mouse embryo by its Reichert's membrane, leaving the embryo free inside the medium. (iii) Maximum intensity projection of a 13 μm 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].

1 culturing becomes possible again [77, 78], and these embryos can be kept alive for several
 2 days in an artificial environment. During this process especially interesting stages are the
 3 late blastocyst (\sim E4.5), gastrulation (\sim E6.5), and somite formation (\sim E8.5). Before live
 4 imaging techniques became available, these stages were mostly investigated using *in*
 5 *situ* visualization techniques to shed light on several developmental processes [79]. Many
 6 pathways playing important roles have been identified this way, however live imaging is
 7 still necessary to validate these results, and ensure continuity in the same specimen [80].

8 Light-sheet microscopy is a good choice for imaging these stages, just like in the case
 9 of pre-implantation embryos. These embryos, however, present new challenges for sample
 10 handling and culturing. Owing to their extreme sensitivity, dissection can be difficult,
 11 especially for earlier stages (E4.5). Furthermore, since the embryo is also growing during
 12 development, gel embedding is not an option, as this might constrain proper development.
 13 Thus, special handling and mounting techniques had to be developed in order to allow

1. LIVE IMAGING IN THREE DIMENSIONS

1 live 3D imaging of these specimens.

2 Ichikawa *et al.* [50] designed a custom mounting apparatus manufactured from acrylic
3 in the shape of a rod that fits in a standard 1 mL syringe with its tip truncated (Fig.
4 1.15A, i). In the rod several holes were drilled with different sizes that can accommo-
5 date different sized embryos, which are held by an extraembryonic tissue, the Reichert's
6 membrane (Fig. 1.15A, ii). Mounting this way doesn't disturb the embryo itself, and it
7 can freely develop in the culturing medium, while it is also stationary for the purpose of
8 imaging. Using this technique, Ichikawa *et al.* were able to image through several stages
9 of development, including interkinetic nuclear migration at stages E5.5–6.5 (Fig. 1.15A,
10 iii).

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

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

22 1.4.3 Imaging adult mice

23 Imaging adult mice is especially interesting for answering neurobiological questions. Since
24 development is over at this stage, the use of an environmental chamber is no longer neces-
25 sary. The biggest challenge for imaging these samples is their size, as they are centimeters
26 in size instead of less than a millimeter in the embryonic stage. Furthermore, the tissues
27 of adult mice are much more opaque which severely limits imaging depth. Light-sheet
28 microscopy can already deal with large specimens, however to achieve (sub)cellular res-
29 olution for an entire brain for example, multiple recording have to be stitched together
30 after acquisition [81].

31 Light scattering and absorption depends on the tissue composition, and also imaging
32 depth. Especially the brain with the high concentration of lipids in the myelinated fibers
33 pose a real challenge for imaging. Live imaging is usually performed with 2-photon mi-
34 croscopy which can penetrate the tissue up to 800 µm [82]. Using fixed samples, however,
35 the scattering problem can be eliminated by the use of tissue clearing methods.

36 Tissue clearing is a process that removes and/or substitutes scattering and absorbing
37 molecules by a chemical process while keeping the tissue structure intact and preserving

1.4 Light-sheet microscopy for mouse imaging

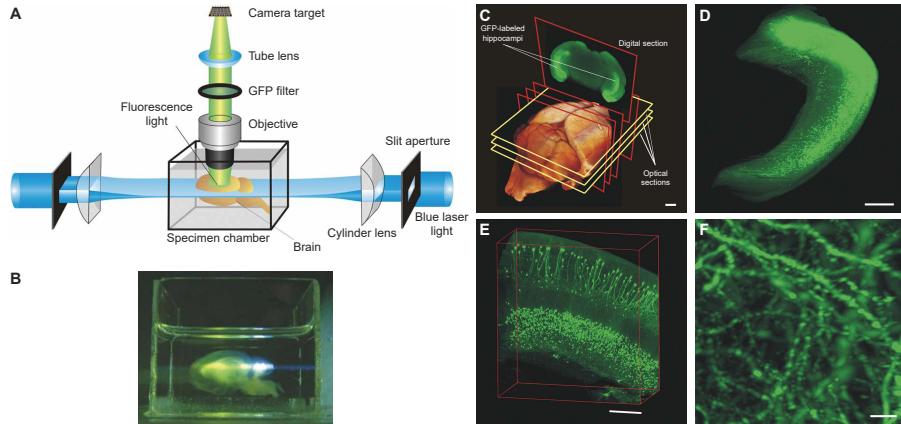


Figure 1.16: Imaging adult mouse brain with light-sheet microscopy.. (A) Schematics of the ultramicroscope for brain imaging. The specimen is embedded in clearing medium to ensure necessary imaging depth. Illumination is applied from two sides to achieve even illumination for the whole field of view. Light-sheet is generated by a slit aperture flowed 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 imaged. Hippocampal pyramidal and granule cell layers are visible in the digital section. Scale bar: 1 mm. Objective: Planapoachromat 0.5 \times . (D) Reconstruction of an excised hippocampus from 410 sections. Note that single cell bodies are visible. Scale bar: 500 μ m. Objective: Fluar 2.5 \times . (E) 3D reconstruction of a smaller region of an excised hippocampus from 132 sections. Scale bar: 200 μ m. Objective: Fluar 5 \times . (F) 3D reconstruction of CA1 pyramidal cells imaged with a higher resolution objective (LD-Plan Neofluar 20 \times 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 μ m. Adapted from Dodt *et al.* [7].

1 fluorescence. The most dominant contributors to these effects are the proteins and lipids.
 2 Proteins in the cells locally change the refractive index of the tissue which leads to
 3 scattering, while lipids absorb the light. Clearing methods tackle these problems by
 4 chemically removing and substituting lipids by certain types of gel, and immersing the
 5 whole sample in a medium with higher refractive index to match the optical properties
 6 of proteins. Numerous methods have been developed for tissue clearing, such as ScaleA2
 7 [83], 3DISCO [84, 85], ClearT2 [86], SeeDB [87], CLARITY [88, 89], CUBIC [90] and
 8 iDISCO [91].

9 The first combination of optical clearing and light-sheet microscopy for whole brain
 10 imaging was performed by Dodt *et al.* using a custom ultramicroscope consisting of two
 11 opposing illumination arms and a single detection with an objective from above (Fig.
 12 1.16A). The light-sheets were positioned horizontally, and the cleared samples could be
 13 placed in a transparent imaging chamber filled with the clearing medium (Fig. 1.16B).
 14 Imaging was performed from both top and bottom after rotating the sample 180°. By
 15 changing the detection lens, it is possible to adapt the system to different samples:
 16 low magnification is capable of imaging the whole brain (Fig. 1.16C), while for smaller,
 17 dissected parts, such as the hippocampus, higher magnification with higher resolution is
 18 more appropriate (Fig. 1.16D). With this configuration individual cell-cell contacts can
 19 be recognized (Fig. 1.16E), and even dendritic spines can be visualized (Fig. 1.16F).

1. LIVE IMAGING IN THREE DIMENSIONS

1 Although light-sheet microscopy is highly suitable for imaging cleared specimens, even
2 entire mice [92], brain imaging in live animals is more challenging due to the standard
3 two-objective setup of a conventional SPIM microscope. Two light-sheet based methods,
4 however offer a solution for this, axial plane optical microscopy (APOM) [93] and swept
5 confocally-aligned planar excitation (SCAPE) [94] both use only a single objective to
6 generate a light-sheet and detect the fluorescence as well. This is done by rotating the
7 detection plane at an intermediate image (APOM), or by rotating both the light-sheet
8 and detection plane simultaneously (SCAPE).

¹ Chapter 2

² Dual Mouse-SPIM

³ mammalian development chromosome segregation cell specification symmetry breaking
⁴ signaling pathways human infertility congenital diseases
⁵ current mouse-SPIM [J2] already is very good for answering many of these questions
⁶ resolution from beads: diSPIM [43] combine these two plus also [95]
⁷ advantages: immersion liquid and culture medium separated open-top sample holder
⁸ allow standard microdrop *in vitro* embryo culture row of embryos - high throughput dual
⁹ color imaging environmental chamber allows live imaging for 3 days, every
¹⁰ follow chromosomes by tracking kinetochores
¹¹ from lattice paper: “because photodamage mechanisms that scale supralinearly with
¹² peak intensity have been identified for both visible [96] and two-photon [97] excitation.”

¹³ 2.1 Microscope design principles

¹⁴ In order to address the challenges outlined in the previous section, we propose a new light-
¹⁵ sheet microscope design for imaging pre-implantation development at high resolution.
¹⁶ The design is guided by the following requirements:

- ¹⁷ 1. subcellular, isotropic resolution
- ¹⁸ 2. high light collection efficiency
- ¹⁹ 3. live embryo compatible

²⁰ design principles: highest isotropic resolution, highest light collection efficiency pos-
²¹ sible 2x0.8 NA water dipping in 90° multi-view deconvolve Gaussian fit $\sigma_{xyz} = 135$ 2x1.1
²² NA water dipping in 120° multi-view deconvolved Gaussian fit $\sigma_z = 135$, and $\sigma_{xy} = 82.2$
²³ simulations from PSF generator! put here

2. DUAL MOUSE-SPIM

1 2.1.1 Light collection efficiency of an objective

2 Resolution is not the only parameter of concern when designing a new microscope setup.
3 We also have to consider light collection efficiency, since many application, especially
4 live imaging applications require a tight photon budget. This means a single fluorophore
5 can only emit so many times before it undergoes an irreversible chemical reaction, i.e. it
6 bleaches. The more we can collect of these photons, the more information we gain, and
7 altogether the efficiency is higher.

8 Photon collection efficiency also defines single molecule localization accuracy, since
9 the signal to noise ratio will depend on the square of the number of collected photons.
10 This is why it's important to also maximize light collection efficiency.

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

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

13 Since we can assume that the direction of photons emitted from a fluorescent molecule
14 are random, the light collection efficiency will correspond to the solid angle subtended
15 by the objective front lens at the focal point. To calculate this, let's consider the unit
16 sphere centered at the focal point, and calculate the surface area of the spherical cap
17 corresponding to the objective acceptance angle α (Fig. 2.1a). The area of the cap can
18 be expressed as a function of the angle:

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

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

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

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

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

22 Some more calculations: (Fig. 2.1b).

23 2.1.2 Multi-view resolution

24 here a figure with rotating 2 psfs

25 In order to maximize light-collection efficiency and resolution, we opted for a high-
26 NA water dipping objective, the Nikon CFI75 Apo LWD 25x/1.1w model. Two of these
27 arranged in 120° allows for twice improved light collection efficiency compared to the 0.8
28 NA configuration in 90° alignment, with further improved lateral resolution along 2 axes

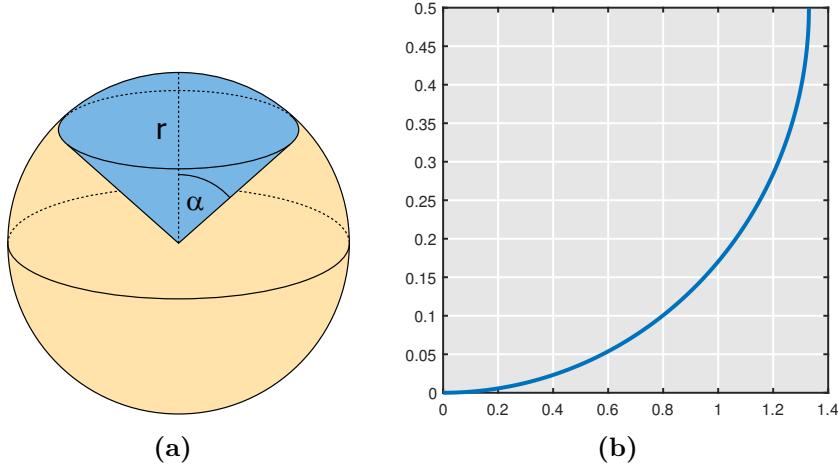


Figure 2.1: Light collection efficiency of an objective. (a) Light collection efficiency is the ratio of photons collected by the objective and all emitted photons. If the fluorophores are emitted randomly in all directions, it will be the surface ratio of the conical section (blue) to the whole sphere. (b) Light collection efficiency (η) as a function of the numerical aperture (NA)

¹ (Figure 2.2). In the next section I will describe the design considerations for illumination
² and detection optics to achieve optimal sample illumination.

³ 2.1.3 Light-sheet design

⁴ To allow for flexibility in the field of view height, even illumination, reduced stripes, and
⁵ potential for confocal line detection, we opted to use the beam scanning technique to
⁶ generate a virtual light-sheet. The effective focal length of the Nikon 25x objective, given
⁷ the 200 mm focal length tube lens is

$$f_o = \frac{f_{tl}}{M} = \frac{200 \text{ mm}}{25} = 8 \text{ mm}, \quad (2.1)$$

⁸ and the back aperture diameter is 17.6 mm.

⁹ To generate the tilted light-sheet as shown on Fig. 2.3, the illumination beam will
¹⁰ need to be displaced by

$$\delta = f_o \cdot \tan 30^\circ = 4.62 \text{ mm} \quad (2.2)$$

¹¹ Since the Gaussian beam is not uniform, only a smaller portion of it can be used to
¹² maintain even illumination (Figure 1.12c). Because the size of an early mouse embryo
¹³ is around 80 μm , we require the length and the height of the light-sheet to be at least
¹⁴ 100 μm .

2. DUAL MOUSE-SPIM

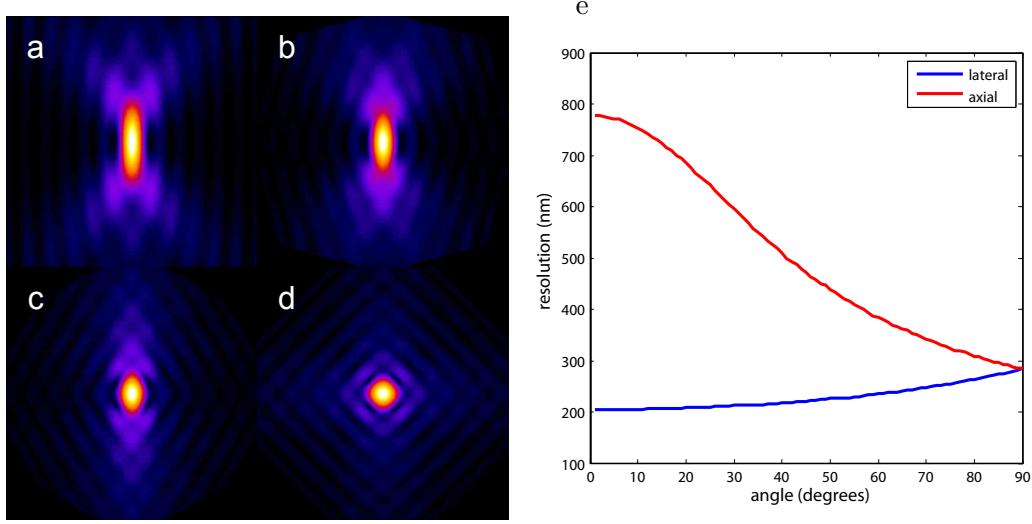


Figure 2.2: Lateral and axial resolution of a multi-view optical system. a) Simulated PSF for a single view. b)-d) Simulated compound PSF of two views aligned in b) 30, c) 60 and d) 90 degrees to each other. e) Axial and lateral resolution of a dual-view setup depending on the rotation angle of the two 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 The length and thickness of the light-sheet

2 As we saw in section 1.3.2, the length of the light-sheet is determined by the Rayleigh-
3 range of the beam in the zy plane. Since $l_{fov} = 2 \cdot z_R = 100\text{ }\mu\text{m}$

$$z_R = 50\text{ }\mu\text{m} \quad (2.3)$$

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

$$2 \cdot W_0 = 2 \cdot \sqrt{\frac{z_R \cdot \lambda}{\pi}} = 5.57\text{ }\mu\text{m} \quad (2.4)$$

6 when $\lambda = 488\text{ nm}$ for GFP excitation. As the beam width for these calculations is defined
7 as $1/e^2$ of the peak intensity, we also calculate the more commonly used FWHM:

$$\text{FWHM} = W_0 \cdot \sqrt{2 \ln 2} = 3.28\text{ }\mu\text{m.} \quad (2.5)$$

8 From this, the divergence angle of the beam is

$$\theta_0 = \frac{\lambda}{\pi W_0, y} = 55.74\text{ mrad} = 3.196^\circ \quad (2.6)$$

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

$$\text{NA}_{ls} = n \cdot \sin(\theta_0) = 0.0743 \quad (2.7)$$

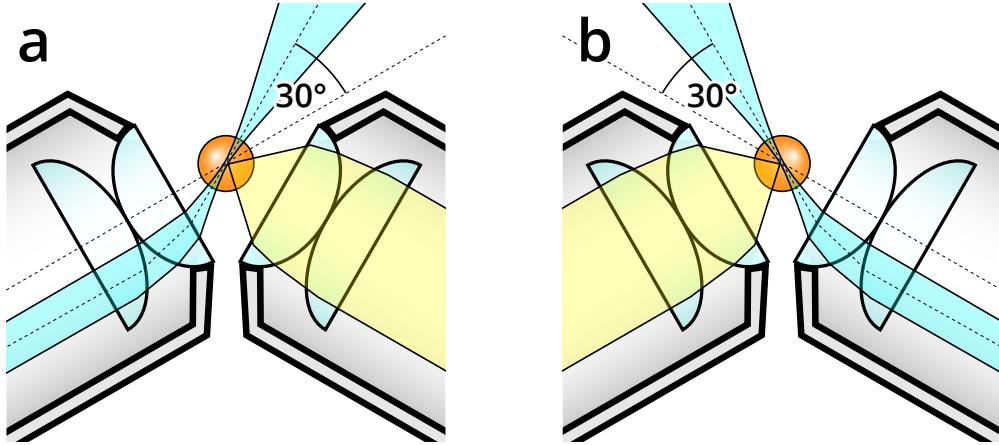


Figure 2.3: Dual view concept with high NA objectives. To achieve multi-view detection while maximizing resolution and light collection efficiency, two high NA objectives are placed in 120° arrangement. To be able to overlap the light-sheet with the focal plane, the light-sheet is tilted 30°. Because of the high NA objectives, this tilt does not effect the illumination quality. The objectives are used in an alternating sequence for illumination and detection.

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

$$b_y = d \cdot \frac{\text{NA}_{ls}}{\text{NA}} = 1.19 \text{ mm} \quad (2.8)$$

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

8 The height of the light-sheet

- 9 The height can be adjusted by changing the beam scanning amplitude with the galvo
- 10 mirror. To scan the entire field of view of $h_{fov} 270 \mu\text{m}$, the scanning angle range at the
- 11 back focal plane of the objective will need to be $\theta = \tan^{-1}(h_{fov}/2/f_o) = \pm 0.967^\circ$.

12 2.2 Optical layout

- 13 Based on the requirements and other considerations layed out in the previous section, the
- 14 microscope was designed based on three main parts: 1) the core unit, 2) illumination and
- 15 3) detection branches. The aim when integrating everything together was to allow for high
- 16 level of flexibility with robust operation, while keeping efficiency at a high level. After
- 17 finalizing the concept, the optical layout of the microscope was designed in SolidWorks.

2. DUAL MOUSE-SPIM

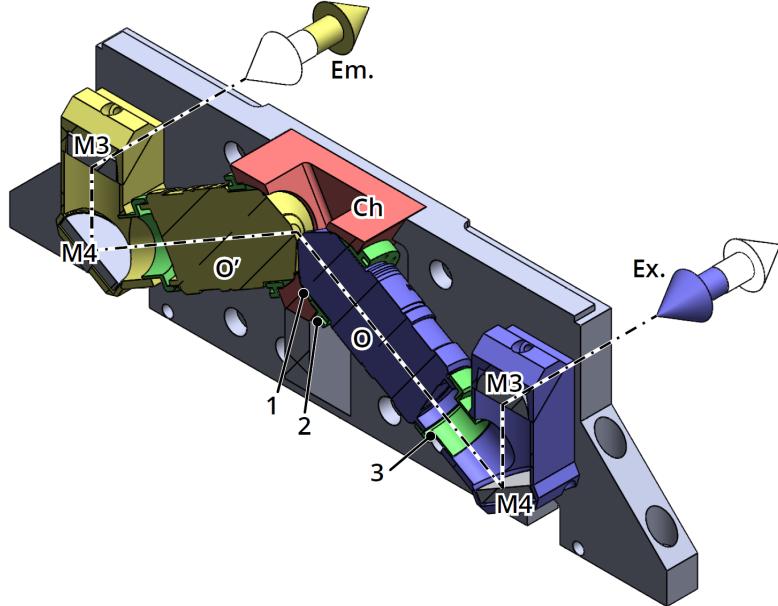


Figure 2.4: The core unit of the microscope. The two objectives (O and O') are mounted on a solid 15 mm thick aluminium plate. The objectives are secured at the chamber (Ch) by two custom made rings (1 and 2, see main text for details), and at the back by a mirror block (M and M'). Excitation (Ex.) and emission (Em.) light paths are indicated by the dash-dot line. Due to the symmetric arrangement, the excitation and illumination paths can be switched around. Objectives are secured with rings 1–3 (green, see main text for details).

¹ 2.2.1 Core unit

² As the most important part of the microscope is actually the sample, the design is based
³ around a core consisting of the imaging chamber and the objectives (Figure 2.4). Also
⁴ part of the core are two mirror blocks placed at the back of the objectives, and three
⁵ custom designed rings to hold the objectives in place. The objectives are pointing slightly
⁶ up, closing a 60° angle with the horizontal plane, and 120° angle with each other.

⁷ Chamber

⁸ The chamber serves two purposes: it holds the immersion liquid necessary for imaging,
⁹ and it also keeps the objectives in the 120° position. The objectives are held by their
¹⁰ necks as opposed to the standard mounting method from the back by the threads. The
¹¹ advantage of this is that any axial movements due to thermal expansion are greatly
¹² reduced, thus the focal plane position is more stable even when changing the imaging
¹³ conditions.

¹⁴ The chamber is machined from a high performance plastic, poly(ether-ether-ketone)
¹⁵ (PEEK). This material has many beneficial properties: it is food safe, and chemically
¹⁶ extremely inert, resisting to most solvents used in a biology laboratory. It can also be
¹⁷ autoclaved. Compared to other plastics its mechanical properties are also superior. It has

1 high tensile and compressive strength, comparable to aluminium, low thermal expansion
2 and low thermal conductivity. This can be beneficial when implementing temperature
3 control, as thermal loss is reduced.

4 The objectives are kept in place by two custom designed rings (Figure 2.4 1, 2).
5 The first ring has a cross sectional shape of a wedge, and sits tightly against both the
6 objective and the wall of the chamber. The second ring can freely slide on the objective,
7 and has threads matching the chamber. When turned in, the threaded ring pushes the
8 wedge ring further in, which in turn presses against the objective and the chamber wall
9 uniformly, thus preventing the objective from moving, and sealing the chamber at the
10 same time. As the wedge ring is made from a soft plastic (delrin), it will press evenly
11 against the objective preventing any damage. Because of the conical shape of the ring,
12 it will also automatically center the objective, ensuring correct positioning.

13 To relieve any rotational stresses from the objective, the back of the objective is also
14 supported by the mirror block, this is not fixed, however. A third ring, made of PEEK
15 is screwed on the thread of the objective, and slides in the opening of the mirror block.
16 This reduces the torque on the objectives, while still allowing for some movements that
17 might occur due to thermal expansion.

18 **Mirror blocks**

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

32 **2.2.2 Illumination**

33 The illumination arm of the microscope directs and shapes the laser beam to generate
34 the proper light-sheet dimension at the sample. As was calculated in subsection 2.1.3, a
35 beam diameter of 1.2 mm is ideal for this setup.

36 The illumination arm has 3 main roles:

2. DUAL MOUSE-SPIM

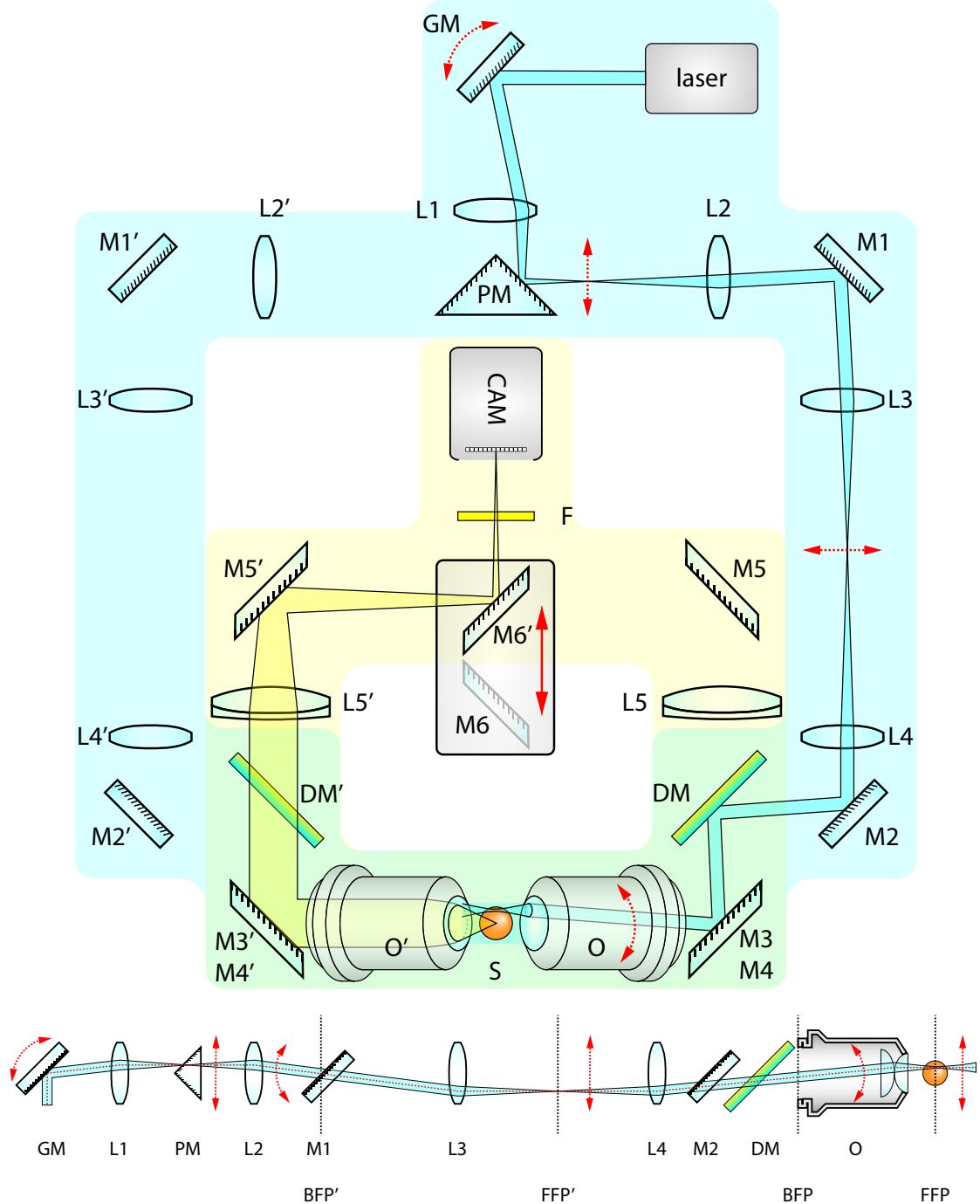


Figure 2.5: Dual Mouse SPIM optical layout. The microscope consists of two main parts, the illumination branches (blue) and detection branches (yellow). For both illumination and detection there are two identical paths implemented. Illumination direction can be changed by applying a different offset to the galvo mirror, which in turn will direct the beam to the opposite face of the prism mirror. L1 and L2 will then image the galvo on M1. Using L3 as a scan lens, and L4 as a tube lens, the scanned beam is coupled to the objective path by quad band dichroic mirror (DM) CAM – camera, DM – dichroic mirror, F – filter wheel, L – lens, M – mirror, O – objective, PM – prism mirror, S – sample

- 1 1. expands the laser beam to the calculated 1.2 mm size.
 - 2 2. images the galvo scanner to the back focal plane of the objective
 - 3 3. switches the laser light between the two objectives during imaging
- 4 To achieve the desired beam diameter, a 1:2 beam expander is used in the reversed
 5 direction. As the output of the laser fiber produces a 3 mm diameter beam, this will
 6 reduce it to 1.5 mm. As this is already the required beam diameter, the lenses further in
 7 the illumination path will not introduce any magnification.
- 8 Switching between the two illumination arms is performed by a custom designed beam
 9 splitter unit (Figure 2.6). Instead of utilizing a 50/50 beam splitter cube and mechanical
 10 shutters, we exploit the fact that a galvo scanner is needed to generate the light-sheet.
 11 As this galvo scanner has a relatively large movement range (20°) it is also suitable for
 12 diverting the beam from one illumination arm to the other.

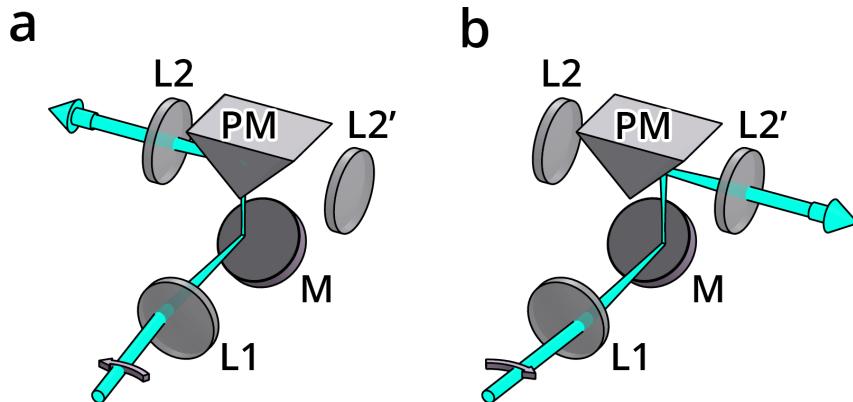


Figure 2.6: Illumination branch splitting unit. To divert the beam to either side, a right angle prism mirror is used in conjunction with a galvanometric scanning mirror. L1 acts as a scan lens, thus the beam is translated on mirror M. Depending on the galvo angle, the beam will be reflected either to the left (a) or to the right (b). L2 and L2' act as relay lenses, and will image the galvo movement to intermediate planes.

13 Switching illumination side is done the following way. As the galvo is positioned at
 14 the focus of the first lens (L1, $f_1 = 75$ mm, Edmunds Optics, #47-639), the rotational
 15 movement will result in a linear scanning movement on mirror M and the prism mirror
 16 PM. Depending on the lateral position of the beam, it will hit either the left or the
 17 right leg of the prism, and will be reflected to either direction. As the galvo mirror can
 18 be precisely controlled through our custom software, we can set and save the position
 19 when the beam is centered on the left lens L2 ($f_2 = 75$ mm, Edmunds Optics, #47-
 20 639) (Figure 2.6a) and the position when the beam is centered on the right lens L2'
 21 (Figure 2.6b). Lenses L1 and L2(L2') form a 4f system, and are imaging the galvo scanner
 22 on mirror M1(M1') (Figure 2.5). This way we can use the same galvo to generate the
 23 light-sheet for both directions, depending on the initial offset position. This not only
 24 has the advantage of electronically switching the illumination arms, but only requires a

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¹ single galvo scanner instead of one for each arm.

² Due to the arrangement of the bottom mirror and the prism mirror, the scanning
³ direction will be rotated by 90°. This will result in a vertical scanning plane, which is
⁴ exactly what we need to generate the light-sheet on the sample (see section 2.2.1).

⁵ Further following the illumination path, two lenses L3 and L4 ($f_3 = f_4 = 200\text{ mm}$),
⁶ again in a 4f configuration relay the scanning axis to the back focal plane (BFP) of
⁷ the objective. To couple in the laser with the imaging path, a quad-band dichroic mir-
⁸ ror (DM) is used, matching the wavelengths of the laser combiner (Semrock, Di03-
⁹ R405/488/561/635-t3-25x36).

10 2.2.3 Detection

¹¹ As the emitted light exits the objective and the mirror block, it is decoupled from the
¹² illumination path by the dichroic mirror. The light is then focused by a 400 mm achrho-
¹³ matic lens (L5, Edmunds Optics, #49-281) onto the camera (Andor Zyla 4.2 sCMOS)
¹⁴ sensor. Just before the camera, a motorized filter wheel (F, LEP 96A361) is placed to
¹⁵ filter out any unwanted wavelength from the emission light. Although this is not in the
¹⁶ infinity space, due to the very small angles after the 400 mm tube lens, the maximum
¹⁷ axial focal shift is $\sim 50\text{ nm}$ only, which is negligible compared to the axial resolution of
¹⁸ $\sim 1.1\text{ }\mu\text{m}$.

¹⁹ Similarly to the common galvo in the illumination path, the two detection arms
²⁰ share the same camera. Although two cameras could also be used, due to the operating
²¹ principle of the microscope the two objective are not used for imaging at the same time.
²² This means a single camera is capable of acquiring all the images, however the two
²³ distinct detection arm need to be merged to be able to use a single camera.

²⁴ Our solution to this problem is a custom designed view switching unit comprised
²⁵ of two broadband dielectric elliptical mirrors (Thorlabs,BBE1-E03)) facing opposite
²⁶ directions, mounted on a high precision linear rail (OptoSigma, IPWS-F3090). Depending
²⁷ on the rail position either the left (Figure 2.7a) or the right (Figure 2.7b) detection path
²⁸ will be reflected up, towards the camera.

²⁹ Moving the switcher unit is performed by a small, 10 mm diameter pneumatic cylinder
³⁰ (Airtac, HM-10-040) that is actuated by an electronically switchable 5/2 way solenoid
³¹ valve (Airtac, M-20-510-HN). This solution offers a very fast switching between views,
³² up to 5 Hz, depending on the pressure, and it is extremely simple to control, as only a
³³ digital signal is necessary to switch the valve.

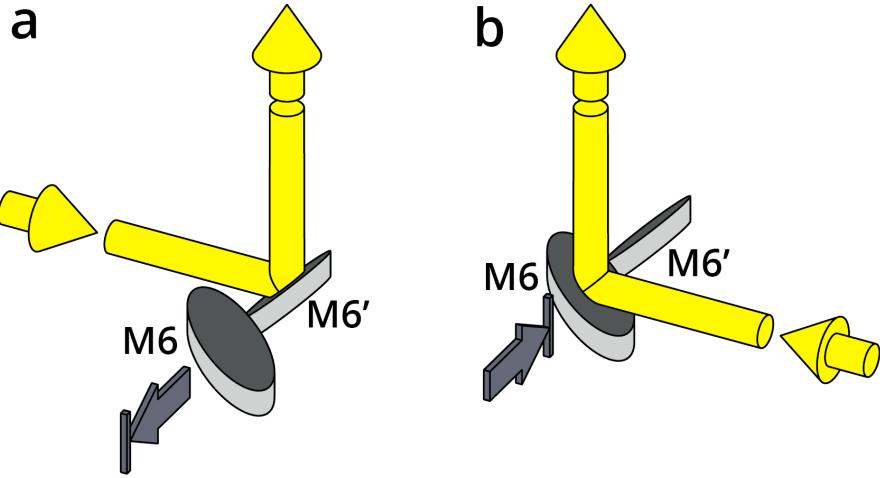


Figure 2.7: Detection branch switching unit. To be able to image both views on the same camera, a moveable mirror unit is introduced. Depending on the imaging direction, the mirror block is either moved backward (a) or forward (b) to reflect the light up to the camera. Since the movement is parallel to the mirrors' surface, the image position on the sensor is not dependent on the exact position of the mirrors.

1 2.3 Optical alignment

2 Precise alignment of the illumination and detection paths are crucial for high quality
 3 imaging, and has a pronounced importance for high magnification and high resolution
 4 optical systems. The DualMouse SPIM contains two illumination and detection paths
 5 that share many common elements, which makes alignment a *important?* process. The
 6 final aim for the alignment is to perfectly overlap the light-sheets with the detection focal
 7 planes, and to optimize ...

8 2.3.1 Alignment of illumination branches

9 The two illumination branches start with a common light source, a single mode fiber
 10 coupled to laser combiner, and they also share a galvanometric mirror that performs the
 11 beam scanning to generate the virtual light-sheet. Likewise shared is a scan lens focusing
 12 on the galvo mirror (GM), and the illumination splitter unit (PM, see Section 2.2.2).

13 Alignment for the illumination arms are done in three steps. First the laser beam is
 14 aligned on the first rail that holds the galvo, lens L1, and the splitter unit PM. This is
 15 performed by two kinematic mirrors placed between the fiber output and the galvo mirror
 16 (not shown on figure). Using these two mirrors it's possible to freely align the beam along
 17 all 4 degrees of freedom: translation in two orthogonal directions, and rotation around
 18 two orthogonal axes. Beam alignment on the rail is tested by two irises at the two ends
 19 of the rail, if the beam passes through both of them we consider it centered and straight
 20 on the optical axis.

21 After the beam is aligned on the first rail, lens L1 and the splitter unit PM are placed

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1 in the measured positions to image the galvo mirror on mirror M1 using lenses L1 and
2 L2. Correct positioning of the splitter unit along the rail is crucial, since this will affect
3 the lateral position and tilt of the beam exiting the unit. To some extent, this can also
4 be compensated by adjusting the two mirrors before the galvo mirror, but is avoided if
5 possible as this will also displace the beam from the center of the galvo mirror.

6 **Adjusting beam position.**

7 Beam position can be adjusted by either translating the beam in a conjugated image
8 plane (I'), or by rotating the beam in a conjugated back focal plane (BFP'). The setup
9 was designed in a way, that BFP' coincides with mirror M2. This mirror is mounted in
10 a gimbal mirror mount, allowing to rotate the mirror exactly around its center, which
11 avoids unwanted translational movements, and results in pure rotation of the beam. Lens
12 L3 is positioned exactly 1 focal length from the mirror, thus acting as a scan lens, and
13 transforming the rotational movements to translation. This translation is further imaged
14 and demagnified by the tube lens L4 and the objective O.

15 **Adjusting beam tilt** Beam tilt can be adjusted by

16 **Adjusting beam axial position.**

17 **Adjusting scanning plane angle.** After the beam is properly aligned, i.e. it is in
18 focus, and in the center of field of view, it is still necessary to check if the scanning
19 direction is parallel to the imaging plane. It is possible that the beam is in focus in the
20 center position, but when moved up or down it drifts out of focus due to a tilted scanning
21 angle. This tilt can be compensated by mirror M1, that is placed at the conjugated back
22 focal plane BFP' . Between lenses L3 and L4 a magnified version of the light-sheet will
23 be visible, and the tilt can be checked by placing an alignment target in the optical path
24 while scanning the beam. By tilting mirror M1 up or down, the scanning pattern not only
25 moves up or down, but is also rotated if the mirror surface is not exactly vertical. Since
26 M1 and GM are in conjugated planes, the tilt and offset can be performed independently.
27 The tilt is first fixed by M1 while inspecting the target, and the beam is re-centered by
28 changing the offset on the galvo mirror. Moving the galvo mirror will not introduce tilt,
29 since in this case rotation axis is perpendicular to the reflection plane.

30 **2.3.2 Alignment of detection branches**

31 Since the detection path is equivalent to a wide-field detection scheme, its alignment is
32 much simpler than that of the illumination branches. The only difference is the detection
33 branch merging unit (see Sec. 2.2.3.) that features two moving mirrors. This, however

1 doesn't effect the alignment procedure, since the movement direction is parallel to both
2 mirror's surface, meaning that the exact position of the mirrors will not affect the im-
3 age quality, as long as the mirrors are not clipping the image itself. Stability test were
4 performed to confirm the consistent switching performance of the mirror unit before the
5 final alignment took place (see Sec. 2.5.2).

6 The final alignment procedure

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

16 Our tube lens is a compound, achromatic lens with a center thickness of 12.5 mm, and
17 edge thickness of 11.3 mm. Its effective focal length is 400 mm which will produce a 50x
18 magnified image. Back focal length is 394.33 mm which we measured form the camera
19 chip, and the lens was positioned at this theoretically optimal position.

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

28 The correction collars are adjusted by inspecting a gel suspended fluorescent bead
29 specimen with the microscope, where the beads can act as a reporter of the point spread
30 function of the microscope. The alignment can be performed "live" by inspecting the
31 bead image quality for any aberrations. By gradually changing the correction collar, the
32 ring are minimized on out of focus beads, and the peak intensity is maximized for in
33 focus beads. By moving the correction ring, the focal plane is also slightly shifted, which
34 has to be compensated by shifting the light-sheet correspondingly to coincide with the
35 correct imaging plane.

2. DUAL MOUSE-SPIM

¹ **Adjusting field of view.** To allow for proper sampling of the image, we use 50x
² magnification, which, combined with the 6.5 μm pixel pitch of our sCMOS camera will
³ result in a 0.13 μm pixel size. The full field of view with this magnification is $2048 \times 0.13 =$
⁴ 266.24 μm . The full field of view the objective provide, are larger than this, at 800 μm .
⁵ To ensure the best image quality, we align the center of the objective field of view on
⁶ the camera sensor, since this region has the best optical properties in term of numerical
⁷ aperture, aberration correction and field flatness.

⁸ Field of view alignment can be performed using mirror M4 just before the detection
⁹ merging unit. To identify the center region of the field of view, diffuse white light is used
¹⁰ to illuminate the entire sample chamber, and is imaged on the camera. Then, mirror M4 is
¹¹ adjusted until the top edge of the field of view becomes visible, *i.e.* where the illumination
¹² from the chamber is clipped. This will have a circular shape. Then, adjusting the mirror
¹³ in the orthogonal direction, the left-right position of the field of view can be adjusted,
¹⁴ by centering the visible arc on the camera sensor.

¹⁵ After the horizontal direction is centered, vertical centering is performed. This, how-
¹⁶ ever can't be centered the same way as the horizontal direction, since for that we would
¹⁷ have to misalign the already aligned horizontal position. To determine the center, we
¹⁸ move the field of view from the topmost position to the bottom. During this process
¹⁹ the number of turns of the adjustment screw is counted (this can be done accurately by
²⁰ using a hex key). After reaching the far end of the field of view, the mirror movement is
²¹ reversed, and the screw is turned halfway to reach the middle.

²² 2.4 Control unit

²³ Apart from the optics design, automation hardware and software is also a custom design.
²⁴ To be able to conduct high speed experiments and time-lapse recordings, a high level
²⁵ of automation, hardware and software integration is necessary. Our system comprises
²⁶ of two main components: a National Instruments embedded system (cRIO 9068), and a
²⁷ high performance workstation.

²⁸ 2.4.1 Hardware

²⁹ Various components need to be properly synchronized to operate the microscope. Illu-
³⁰ mination is performed with a multi-line laser combiner, light-sheet is generated by a
³¹ galvo scanner. To discriminate fluorescent light from th excitation lihgt, a motorized
³² filter wheel is used with appropriate long pass and band pass filters (see Appendix A).
³³ For more details, see Appendix section A.

³⁴ Equation 1.5

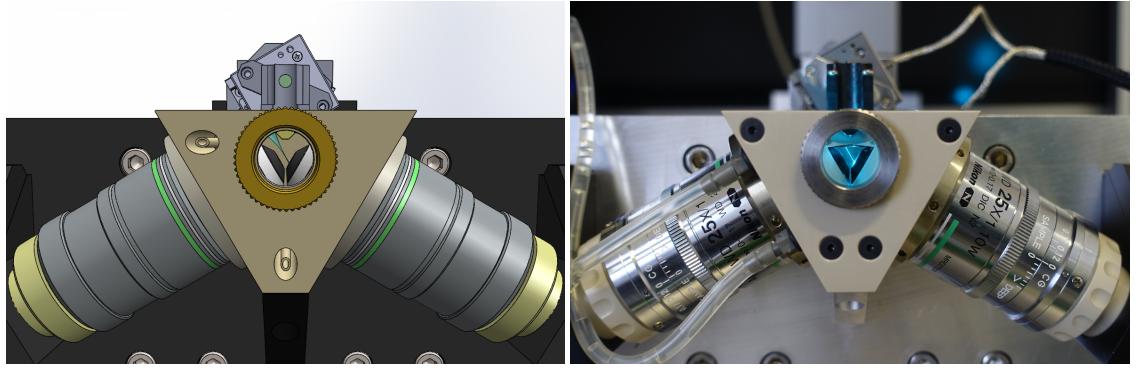
³⁵ Figure 1.16

1 subsection 2.2.3

2 Table 3.4

3 **2.4.2 Software**

4 **2.5 Results**



(a) Rendering of SolidWorks design.

(b) Photo of constructed microscope.

Figure 2.8: Front view of the Dual Mouse-SPIM.

5 Resolution for Lattice light-sheet [8]: 230 nm in x and 370 nm in z [48]

6 After the design phase, all custom parts were manufactured at the EMBL workshop,
7 and I assembled the microscope on a NewPort X by X optical table (Fig. 2.8).

8 **2.5.1 Characterizing illumination profile**

9 Since a Gaussian beam is used for illumination, its intensity profile is dependent on the
10 axial position (Eq. 1.18). Although the total intensity at any cross section of the beam
11 is constant, because of the divergent properties, the peak intensity varies. To assess any
12 non-uniformities in the illumination pattern, I measured the illumination beam intensity
13 for each view.

14 To visualize the beam, the chamber was filled with a fluorescent solution (methylene
15 blue solution in distilled water). In order to increase the signal to noise ratio of these
16 images, a long exposure time of 200 ms were used, and 500 images of each beam was
17 averaged (Fig. 2.9). The measure Rayleigh range of the left and right beams respectively:
18 "666 µm" and "666 µm". The width of the beam, *i.e.* the thickness of the light-sheet are
19 "666 µm" and "666 µm" for the left and right beams respectively (Figure 2.9).

20 **2.5.2 Stability of view switcher unit**

21 To assess the reproducibility in the movement of the detection merging unit, a long
22 term stability test was conducted. To exclude all other factors (such as sample drift), we

2. DUAL MOUSE-SPIM

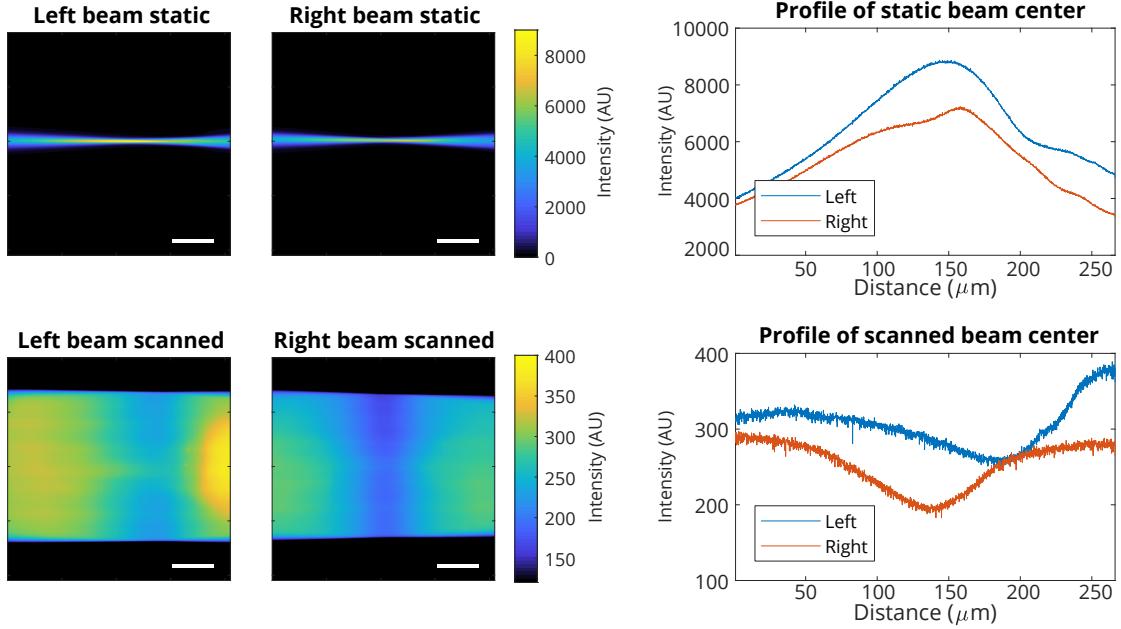


Figure 2.9: Illumination beam profile. Average of 50 stationary beam images for both left and right objectives (top left). Beam intensity profile along the center of each beam is plotted (top right). To simulate scanned beam illumination intensity, each beam image was averaged along the vertical axis (bottom left). Intensity profile of the projections are plotted (bottom right).

1 replaced lenses L5 and L5' with a short focal length achromatic lenses, and used these to
 2 image a closed aperture placed on the rail. The apertures were imaged from both views
 3 every 10 seconds, for 4 h. The center of the aperture was segmented on all images, and
 4 tracked to assess any drift occurring during the 4 h time lapse (??). Although there was
 5 a small shift in the first 10 minutes, the system stabilized afterwards, and no further
 6 drift was detected (??).

7 2.5.3 Resolution and point spread function measurement

8 To measure the resolution of the microscope, and characterize its optical perfor-
 9 mance I performed point spread function measurement using fluorescently labeled beads
 10 (TetraSpeck 500 nm, ThermoFisher). The beads were sonicated, and mixed with 0.8%
 11 GelRite solution at a final dilution of 1:1000. The gel was loaded in glass capillaries and
 12 allowed to cool. After the gel solidified, a ~1 mm piece was cut off and placed in the
 13 microscope sample holder.

14 In order to establish an ideal, reference PSF, we simulated the theoretical PSF of the
 15 microscope with the Gibson-Lanni model [98], using the MicroscPSF Matlab implemen-
 16 tation [99]. The advantage of this model is that it accounts for any differences between
 17 the experimental conditions and the design parameters of the objective, and thus it can
 18 simulate any possible aberrations that should arise. The adjustable parameters are the

2.6 Methods

1 thicknesses and refractive indices of the immersion medium, coverslip, and sample.

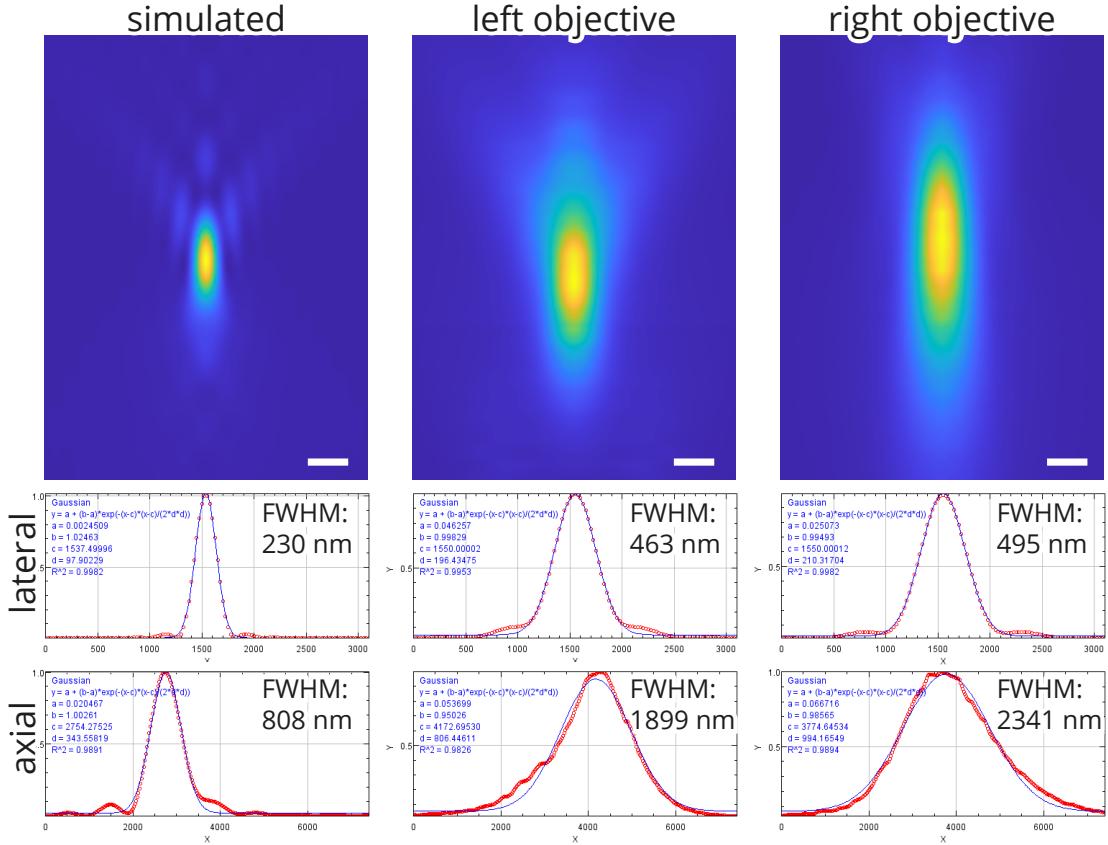


Figure 2.10: Simulated and measured PSF of Dual Mouse-SPIM. Top row: axial sections of simulated and measured point spread functions. Middle row: lateral intensity profile and Gaussian fit. Bottom row: Axial intensity profile and Gaussian fit. Simulations were performed based on the Gibson-Lanni model. Immersion medium and sample refractive index: 1.330, coverslip (FEP foil) refractive index: 1.344, coverslip distance: 1900 μm , coverslip thickness: 50 μm . Scale bar: 500 nm.

2 **2.5.4 Multi-view imaging of a mouse zygote**

3 2.6 Methods

4 2.6.1 Bead imaging

5 For registration and resolution measurements we used TetraSpeck (ThermoFisher, T7279
6 and T7281) 0.1 μm and 0.5 μm diameter beads. The bead solutions were thoroughly
7 vortexed and sonicated for 5 mins before diluting them 1:100 in distilled water. GelRite
8 (Sigma-Aldrich, G1910) gel was prepared in distilled water at 0.8% concentration with
9 0.1% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and kept at 70 °C until use.

2. DUAL MOUSE-SPIM

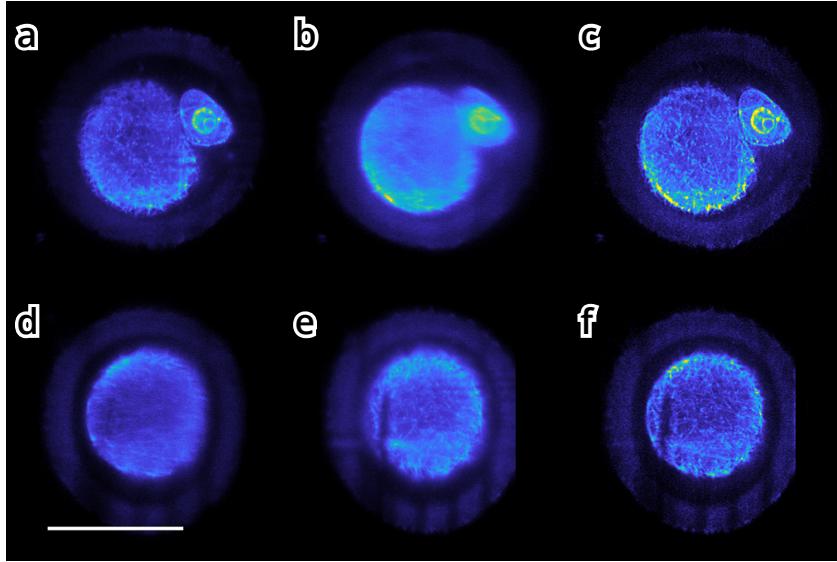


Figure 2.11: Multi-view recording of a mouse zygote. Dual-view imaging was performed on a fixed mouse zygote. Microtubules were stained with Alexa Fluor 488. Top row: view of Left objective. Bottom row: view of Right objective. (a) Raw image from left objective. (b) Rotated view of Right objective. (c) 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 μ m.

¹ 2.6.2 Mouse zygote imaging

² Fixed mouse zygotes labeled with Alexa-488 (microtubules) and Alexa-647 (kinetochores)
³ were kindly provided by Judith Reichmann. Zygotes were transferred to the sample
⁴ holder, and imaged in PBS. To allow for multi-view reconstruction, a fluorescent beads
⁵ (TetraSpeck 0.5 μ m suspended in GelRite were placed next to the zygote in the sample
⁶ holder. After imaging the zygote from both views, the beads were also recorded using the
⁷ same stack definitions. After data acquisition, the multi-view datasets were registered
⁸ and deconvolved in Fiji [100] using the Multiview Reconstruction Plugin [101, 102]. The
⁹ deconvolution was based on a simulated PSF generated in Fiji using the PSF Generator
¹⁰ plugin [103] using the Born-Wolf PSF model. $NA_{ex} = 0.1$, $NA_{em} = 1.1$, $n=1.33$, $\lambda_{ex} =$
¹¹ 488 nm, $\lambda_{em} = 510$ nm.

¹² 2.6.3 Drosophila embryo imaging

¹³ *Drosophila melanogaster* embryos expressing fluorescent nuclear (H2A-mCherry) and
¹⁴ centriole (ASL-YFP) markers were collected on an agar juice plate and dechorionated in
¹⁵ 50% bleach solution for 1 min. After rinsing the bleach with deionized water, the embryos
¹⁶ were placed in the sample holder under PBS solution. A small piece of gel containing
¹⁷ fluorescent beads were also placed next to the samples to aid in multi-view registration.

1 2.6.4 Drosophila salivary gland imaging

2 Salivary glands from third instar larvae expressing fluorescent nuclear (H2A-mCherry)
3 and centriole (ASL-YFP) markers were kindly provided by Ulla-Maj Fiuza. The salivary
4 glands were imaged in PBS, together with a small piece of gel with fluorescent beads.

2. DUAL MOUSE-SPIM

¹ Chapter 3

² Image processing for multi-view
³ microscopy

⁴ When using any kind of microscopy in research, image processing is a crucial part of the
⁵ workflow. This is especially true for light-sheet microscopy, since it's capable imaging the
⁶ same specimen for multiple days, producing immense amounts of data. A single overnight
⁷ experiment of *Drosophila* development (which is a very typical use-case for light-sheet)
⁸ can produce multiple terabytes of data. To see the real scales, let's consider the following
⁹ imaging conditions:

	camera	Hamamatsu Orca Flash 4
	image size	8 MB
¹⁰	1 stack = 250 planes	2 GB
	2 views	4 GB
	Time-lapse: 16 h @ 2/min	7.5 TB
	n colors	$n \cdot 7.5$ TB

¹¹ As it is apparent from this table,

3. IMAGE PROCESSING FOR MULTI-VIEW MICROSCOPY

1 3.1 Multi-view image fusion

2 3.1.1 Registration

3 Image based registration

4 Bead based registration

5 3.1.2 Transformation

6 Rigid

7 Affine

8 Elastic

9 3.1.3 Image fusion

10 Average

11 Sigmoidal weighted average

12 Fourier mixing

13 Huisken had something like this

14 Wavelet-based fusion

15 Multi-view deconvolution

16 [104] Uros thesis [105], [106] Spatially variant deconvolution

17 3.2 Image compression

18 Image compression is an important tool for everyday life, however it's rarely used in the
19 context of scientific imaging because of fear of information loss. This preconception is
20 mainly due to the famous blocking artifacts found in many highly compressed JPEG
21 images, however not all image compression algorithms introduce artifacts, and in fact
22 many lossless algorithms exist that would be suitable for such images. Nowadays, when
23 data production is in an exponential growth compression is again in highlight, without it
24 it would be extremely difficult to maintain many scientific projects that produce images
25 at a high data rate.

26 In this paper I will review the basics of image compression based on Sayood's text-
27 book, Introduction to Data Compression [107]. I will introduce some basics of information
28 theory and entropy, followed by discussing two widely used entropy coding algorithms,

1 Huffman coding and arithmetic coding. Section 3 will be about transform coding, specifically
 2 Discrete Cosine Transform (DCT) and wavelet transform while also touching upon
 3 techniques based on differential pulse code modulation. Finally I will show how some of
 4 the most widely used image compression standards use these methods to achieve effective
 5 image compression.

6 **3.2.1 Entropy coding**

7 **Information and Entropy**

8 "For the purpose of data compression it is useful to quantify the amount of *information*
 9 contained within a piece of data. The first rigorous definition of information was presented
 10 in an extremely influential paper by Shannon, published in two parts in 1948 [108, 109]."

11 "First, let's define the amount of self-information contained in the outcome of a
 12 random experiment:"

$$I(A) = \log_b \frac{1}{P(A)} = -\log_b P(A) \quad (3.1)$$

13 2 independent events:

$$P(A, B) = P(A) \cdot P(B) \quad (3.2)$$

self-information is additive:

$$I(A, B) = \log_b \frac{1}{P(A, B)} \quad (3.3)$$

$$= \log_b \frac{1}{P(A) \cdot P(B)} \quad (3.4)$$

$$= \log_b \frac{1}{P(A)} + \log_b \frac{1}{P(B)} \quad (3.5)$$

14 entropy for random variable X average or expected self-information for the random
 15 variable

$$H(X) = \sum_i P(A_i)I(A_i) = -\sum_i P(A_i) \log_b P(A_i) \quad (3.6)$$

16 entropy rate for data source S average information output by the data source

17 **Huffman coding**

18 Huffman coding is a prefix-free, optimal code that is widely used in data compression. It
 19 was developed by David A. Huffman as a course assignment on the first ever course on
 20 information theory at MIT, and was published shortly afterwards [110]. It is a variable
 21 length binary code which assigns different length codewords to letters of different prob-
 22 abilities. It is able to achieve optimal compression, which means the total length of the
 23 coded sequence will be minimal.

3. IMAGE PROCESSING FOR MULTI-VIEW MICROSCOPY

Table 3.1: Examples of a random binary code (#1) and a prefix-free binary code (#2). Code #2 is uniquely decodable, while for code #1 it's 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 Although it produces a variable length code which can introduce some issues with
 2 decoding, it is still uniquely decodable. It achieves this property by using prefix-free
 3 codewords, meaning that none of the codewords are prefixes of any other codewords.
 4 This property can be exploited when decoding the codeword, since during this procedure
 5 the number of bits for the next codeword can not be determined in advance. However if
 6 no codeword is a prefix of another codeword, by simply reading the successive bits one
 7 by one until we reach a valid codeword, it's possible to uniquely decode the message.

8 Let's take the example in Table 3.2. Five letters are coded in binary code by Code
 9 #1 and by Code #2. Code 1 is not a prefix code, and because of this when reading the
 10 encoded sequence we can not be sure when we reach the end of a codeword. Decoding
 11 the sequence 0000 for example could be interpreted as 4 letters of a_1 or 2 letters of a_3 .

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

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

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

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

Table 3.2: Huffman code table.

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	$c(a_2)$

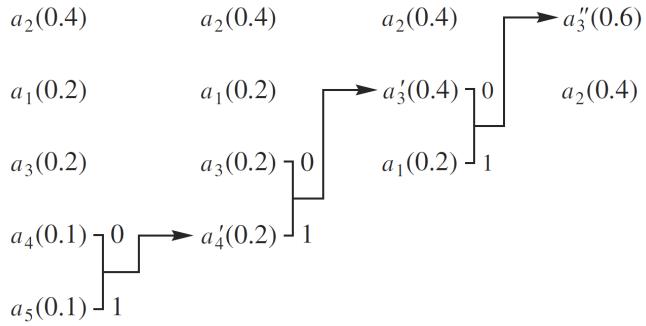


Figure 3.1: Building the binary Huffman tree. The letters are ordered by probability, these will be the final leave of the tree. To join the to branches at every iteration we join the to nodes with the smallest probability, and create a new common node with the sum of the probabilities. This process 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.

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 3.2). "The entropy for this source is 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, "we can assign their codewords as"

$$\begin{aligned} c(a_4) &= \alpha_1 * 0 \\ c(a_5) &= \alpha_1 * 1 \end{aligned}$$

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 3.1), 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 3.3). "The average length of this

3. IMAGE PROCESSING FOR MULTI-VIEW MICROSCOPY

Table 3.3: 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

1 code is”

$$l = 0.4 \times 1 + 0.2 \times 2 + 0.2 \times 3 + 0.1 \times 4 + 0.1 \times 4 = 2.2 \text{ bits/symbol} \quad (3.7)$$

2 ”A measure of the efficiency of this code is its redundancy—the difference between the
3 entropy and the average length. In this case, the redundancy is 0.078 bits/symbol. The
4 redundancy is zero when the probabilities are negative powers of two.”

5 Arithmetic coding

6 Although in this case the redundancy of the Huffman code is minimal, however in cases
7 where a few symbols have vary high probability compared to the rest, the redundancy
8 increases. This is simply because even for the most frequent letter the shortest codeword
9 the Huffman code can produce is of length 1.

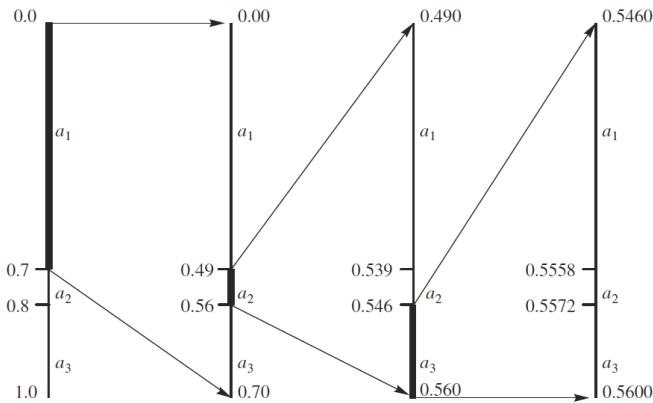
10 Let’s consider the following example: take alphabet $A = [a_1, a_2]$ with $P(a_1) = 0.95$
11 and $P(a_2) = 0.05$. The first order entropy for this source is $-0.95 \log 0.95 - 0.05 \log 0.05 =$
12 0.2864 bits/symbol, however if we assign the Huffman code we will have to use $c(a_1) = 0$
13 and $c(a_2) = 1$, which means the average length will be 1 bit/symbol. This means in order
14 to code this sequence using a Huffman code, we will need more than 3 times the number
15 of bits promised by the entropy.

16 To get around this fundamental limitation, a coding scheme must be used which does
17 not use discrete codewords at all. Arithmetic coding is the most well-known example of
18 such a scheme. The idea is due to Peter Elias. He developed it during the same course
19 on information theory in which Huffman developed his coding method, but he never
20 published it.

21 ”In order to distinguish a sequence of symbols from another sequence of symbols we
22 need to tag it with a unique identifier. One possible set of tags for representing sequences
23 of symbols are the numbers in the unit interval $[0,1]$. Because the number of numbers in
24 the unit interval is infinite, it should be possible to assign a unique tag to each distinct
25 sequence of symbols. In order to do this, we need a function that will map sequences of
26 symbols into the unit interval.”

Table 3.4: Example alphabet for arithmetic coding.

Letter	Probability
a_1	0.7
a_2	0.1
a_3	0.2


 Figure 3.2: Arithmetic coding scheme in practice with the alphabet from Table 3.4 on the sequence a_1, a_2, a_3 .

1 "A straightforward algorithm to arithmetically encode a given input string is the
 2 following: Partition the unit interval $[0, 1)$ into sub-intervals and assign one subinterval
 3 to each symbol in the input alphabet. The sizes of the sub-intervals are chosen to be
 4 proportional to the symbol frequencies. A string of symbols can be encoded by applying
 5 this process recursively: The sub-interval from the previous step is subdivided again
 6 using the same proportions. Figure 3.2. shows an example of arithmetic coding using the
 7 symbol frequencies given in Table 3.4."

8 This kind of coding although easy to understand, it's actually quite cumbersome to
 9 directly implement on a computer, where only a certain floating point precision can be
 10 achieved. For real life use this precision is often unsatisfactory, so some extra steps are
 11 involved in the coding.

12 Since after each step the next subinterval is a subset of the previous interval, if the
 13 coding interval after a certain number of steps is contained in either the upper or lower
 14 half of the unit interval it will remain there for the rest of the coding. We can exploit
 15 this fact by rescaling that interval to the unit interval and writing either a 0 or 1 bit to
 16 the output depending on the position of the subinterval. By continuing this scheme it's
 17 possible to reach arbitrary precision even using a computer.

18 The decoding process is analogous to encoding. The decoder keeps track of the current
 19 lower and upper bounds. It mimics the rescaling operations of the encoder based on the
 20 bits of the encoded binary number.

3. IMAGE PROCESSING FOR MULTI-VIEW MICROSCOPY

1 3.2.2 Transform coding

2 The methods outlined in the previous section are effective at compressing the data in an
3 optimal way and reaching the first order entropy, however they assume nothing about the
4 structure of the data. For image compression it's important to also consider this, since
5 most images have a relatively high level of autocorrelation, meaning that neighboring
6 values have a high chance of being similar, although not necessarily equal. Transform
7 coding is a technique that by itself does not compress the data, however by exploiting
8 some knowledge of the structure it transforms the data effectively reducing its first or-
9 der entropy. When regular entropy coding is then performed on the transformed data,
10 because of the reduced first-order entropy, these techniques can achieve a higher rate
11 of compression. At decompression, after decoding the entropy coder, the reverse trans-
12 formation is applied to reveal the original data. This section will introduce two of the
13 most important algorithms for transform coding, Discrete Cosine Transform and Discrete
14 Wavelet Transform.

15 **Discrete Cosine Transform**

16 Discrete Cosine Transform [111] is closely related to the well known Fourier transform.
17 The main idea behind this is to represent a function by a weighted sum of different sine
18 and cosine functions. This provides a different view, instead of looking at the data in the
19 time domain, we gain information about the frequencies that compose the signal.

20 "Discrete Cosine Transform is a variant of the discrete Fourier transformation", how-
21 ever instead of making the signal periodic which can introduce large jumps at the edges,
22 the signal is extended in a symmetric way. Since this will give a smooth transition even at
23 the boundaries, the transform does not have to include so many high frequency compo-
24 nents. Also, because of the symmetric extension, it's possible to represent the functions
25 only by using the cosine bases, resulting in fewer coefficients. Overall, the DCT is much
26 better suited for compression, than DFT.

27 The DCT base functions are defined in the following way:

$$c_{i,j} = s_i \cdot \cos \frac{(2j+1)i\pi}{2n} \quad \text{with } s_i = \begin{cases} \sqrt{\frac{1}{n}} & \text{if } i = 0 \\ \sqrt{\frac{2}{n}} & \text{otherwise} \end{cases} \quad (3.8)$$

28 "The scaling factors are chosen so that the L_2 norm of each basis vector is 1 and so
29 the transform is orthonormal. The inverse transform can therefore be found by simply
30 transposing the transform matrix."

31 Applying the DCT to two dimensional functions, i.e. images is very similar to the
32 two dimensional Fourier transform. The base functions are the outer products of the 1D
33 base functions (Figure 3.3.), and the transform can actually be performed separately for

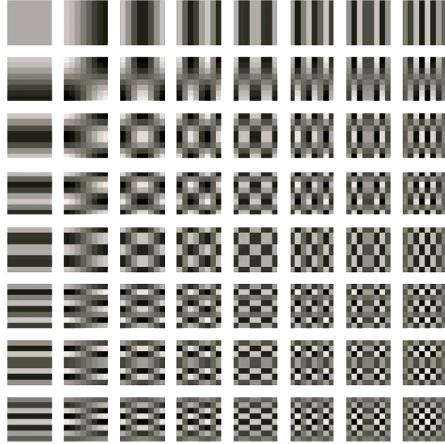


Figure 3.3: Basis functions of the 8×8 2D DCT, computed as the outer product of the 1D basis vectors..

1 each dimension.

2 Computation effort in a naive implementation is $O(n^2)$ but since DCT is based on the
 3 Fourier transform, an $O(n \log n)$ algorithm is also possible, analogous to the fast Fourier
 4 transform. This is still larger than a linear scaling with the data size, which can be very
 5 inconvenient. Therefore, in practice the DCT is usually applied to smaller blocks of the
 6 image, such as 4×4 , 8 or 16×16 .

7 Although DCT is an effective way of reducing the first-order entropy, it has a potential
 8 shortcoming by its use of floating point arithmetic. Because of the finite machine precision
 9 inherent rounding errors will occur, which means the reverse transformation can not
 10 generate the exact original data. For many application, such as photography, this still can
 11 be acceptable, but for scientific image data lossy compression is generally not accepted.

12 DCT-like integer-based variants in JPEG-XR, and H.264

13 Discrete Wavelet Transform

14 ”!!CHANGE THIS!!Methods based on Fourier analysis, such as the DCT introduced in
 15 the previous section, give excellent localization in frequency space: They tell us exactly
 16 which frequencies occur in the data, which is very useful for data compression. However,
 17 they give no spatial localization: They do not tell us where in the signal these frequencies
 18 occur. Every DCT base function affect the whole image domain!!UNTIL HERE!!”, which
 19 means distinct local structures can have a global effect on the final outcome. In case of
 20 an edge for example, it’s necessary to include a high frequency component with a large
 21 coefficient, but since every DCT base function has an impact on the whole domain, this
 22 will have to be compensated on smoother regions by also increasing the coefficients of
 23 other factors. This can negatively impact compression performance.

24 A solution for this is to use different base functions, namely ones with finite support.

3. IMAGE PROCESSING FOR MULTI-VIEW MICROSCOPY

¹ This way we will not only be able to get information about the frequency, but also about
² the localization of that frequency in some extent.

³ ”!!!!One option for such a set of local basis functions, and certainly the most popular one, is the multi-resolution analysis based on wavelets. The term “multi-resolution analysis” in the context of wavelets was introduced in the late 1980s by Stéphane Mallat [112], though research on wavelets had been ongoing for several years before that.!!!!”

⁷ ”The idea behind the wavelet multi-resolution analysis is to build a basis out of
⁸ translated and scaled versions of one underlying function called the *mother wavelet* ψ .
⁹ The mother wavelet is non-zero only in a small region, leading to the locality properties.
¹⁰ It is translated to cover the whole domain. It also covers only a small frequency band,
¹¹ and is scaled to cover higher or lower frequencies. The family of translated and scaled
¹² functions $\psi_{l,i}$ is generated from according to”

$$\psi_{l,i}(t) = \sqrt{2^l} \psi(2^l t - i), \quad l, i \in \mathbb{Z} \quad (3.9)$$

¹³ ”Incrementing l halves the width of the resulting function, which thus corresponds to
¹⁴ a higher frequency band. Changing i moves the function along the x axis. The size of
¹⁵ each step scales with the width of the function, defined by l . The normalization factor
¹⁶ $\sqrt{2^l}$ is chosen so that the L_2 norm stays constant. The mother wavelet can be chosen
¹⁷ so that the $\psi_{l,i}$ are pairwise orthogonal and thus form a basis of some function space.
¹⁸ However, representing a function in this basis will generally require an infinite number
¹⁹ of basis functions $\psi_{l,i}$: To represent a constant component, i.e. content of frequency zero,
²⁰ the wavelet must be infinitely scaled. To address this, it is necessary to introduce an
²¹ additional scaling function ϕ which complements the wavelet. It is scaled and translated
²² the same way as the mother wavelet.”

²³ The oldest wavelets are the Haar wavelets, and because of their simplicity, they
²⁴ provide a good example on how wavelet transformation works. The Haar wavelet scaling
²⁵ ϕ and wavelet ψ functions are the following:

$$\phi(t) = \begin{cases} 1 & \text{if } 0 \leq t < 1 \\ 0 & \text{otherwise} \end{cases} \quad (3.10)$$

$$\psi(t) = \begin{cases} -1 & \text{if } 0 \leq t < \frac{1}{2} \\ 1 & \text{if } \frac{1}{2} \leq t < 1 \\ 0 & \text{otherwise} \end{cases} \quad (3.11)$$

²⁶ ”Clearly, all wavelet functions $\psi_{l,i}$ are orthogonal. Additionally, the scaling functions $\phi_{l,i}$
²⁷ at a fixed level i are orthogonal. The scaling functions $\phi_{l,i}$ are also orthogonal to the
²⁸ wavelet functions $\psi_{k,i}$, $k \geq l$ at the same and all finer levels.”

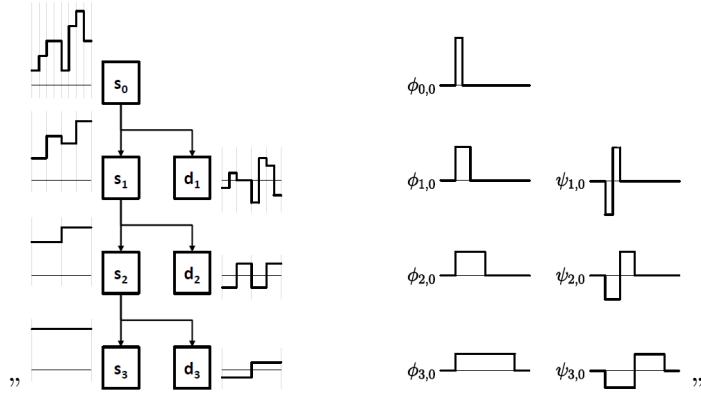


Figure 3.4: Multi-resolution wavelet decomposition, using the Haar wavelets as an example. Left: decomposition to multiple levels of low pass and high pass coefficients corresponding to the scaling and the wavelet functions respectively. Right: some of the base wavelet functions used for the decomposition.

1 "Figure 3.4. (left) schematically shows the decomposition of a signal into low-pass
 2 components corresponding to the scaling function, and high-pass components correspond-
 3 ing to the wavelet. The signal s_0 can be represented by the translated scaling function
 4 at a finer scale l_0 , which can be decomposed to the sum of a coarser approximation
 5 s_1 corresponding to the scaling function and the detail part d_1 corresponding to the
 6 wavelet functions. This decomposition to a sum of coarser approximation and detail can
 7 be continued multiple times, thus resulting in the previously mentioned multi-resolution
 8 analysis.

9 Of course, for image compression applications, it is desirable to extend this trans-
 10 formation to a 2D version, that can be applied to the images before performing the
 11 arithmetic coding. Similarly to the DCT, the wavelet transform is also separable, and
 12 can be performed as a sequence of 1D transforms along different directions. Separable in
 13 a sense, that it does not matter in which order the individual 1D DWTs are applied to
 14 get the same 2D transformation.

15 JPEG [113], JPEG-LS [114] and JPEG2000 [115]

3. IMAGE PROCESSING FOR MULTI-VIEW MICROSCOPY

¹ **3.2.3 Differential pulse code modulation / LOCO-I?**

² **3.3 Noise in light microscopy images**

³ **3.3.1 Photon shot noise**

⁴ **3.3.2 Camera noise**

⁵ **CCD**

⁶ **EM-CCD**

⁷ **sCMOS**

⁸ **3.3.3 Variance stabilization**

¹ Chapter 4

² Real-time, GPU accelerated
³ image processing pipeline

⁴ 4.1 GPU architecture

⁵ CUDA [116]

⁶ 4.2 Live fusion

⁷ 4.2.1 MuVi-SPIM

⁸ 4.2.2 Bead based registration

⁹ 4.3 B³D image compression

¹⁰ B³D image compression high content screening [117–119] light-sheet (Sec. 1.3) single
¹¹ molecule localization microscopy [120–122] data handling is bottleneck [123–125] lossy
¹² compression not recommended for microscopy [126] KLB [127] jpeg compression so loss
¹³ is not perceptible [107] big data viewer [128] Fiji [100]

¹⁴ FLIC [129] SFALIC [130] FELICS [131] Treib terrain editing [132] Treib turbulence
¹⁵ [133] square root compression [134] noise and bias in square root compression [135]
¹⁶ quantization [136] Anscombe [137] optimal inverse Anscombe [138, 139] optimal inverse
¹⁷ generalized Anscombe [140]

4. REAL-TIME, GPU ACCELERATED IMAGE PROCESSING PIPELINE

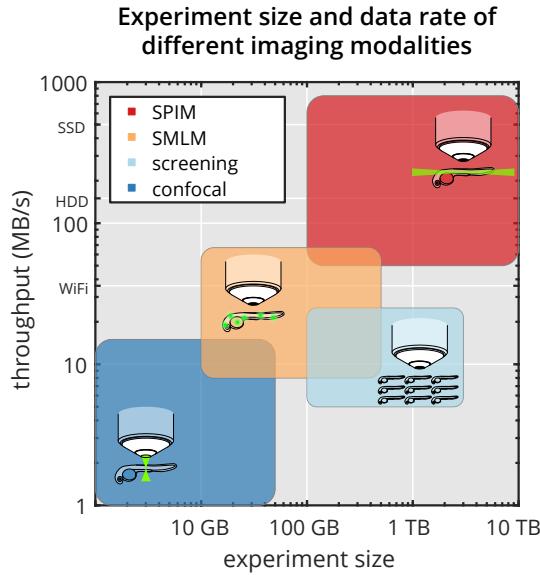


Figure 4.1: Experiment sizes and data rate of different imaging modalities. Comparison of single-plane illumination microscopy (SPIM, red rectangle), high-content screening (light blue), single molecule localization microscopy (SMLM, orange) and confocal microscopy (blue) by typical experiment size and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

¹ 4.3.1 Data sizes in microscopy

² 4.3.2 Compression algorithm

³ 4.3.3 Lossless compression performance

⁴ 4.3.4 Swapping for WNL

⁵ 4.3.5 Benchmarking

⁶ 4.4 Noise dependent lossy compression

⁷ 4.5 Methods

⁸ Compression benchmarking

⁹ For all presented benchmarks, TIFF and JPEG2000 performance was measured through
¹⁰ MATLAB's imwrite and imread functions, while KLB and B³D performance was mea-
¹¹ sured in C++. All benchmarks were run on a computer featuring 32 processing cores
¹² (2×Intel Xeon E5-2620 v4), 128 GB RAM and an NVIDIA GeForce GTX 970 graphics
¹³ processing unit. Read and write measurements were performed in RAM to minimize I/O
¹⁴ overhead, and are an average of 5 runs.

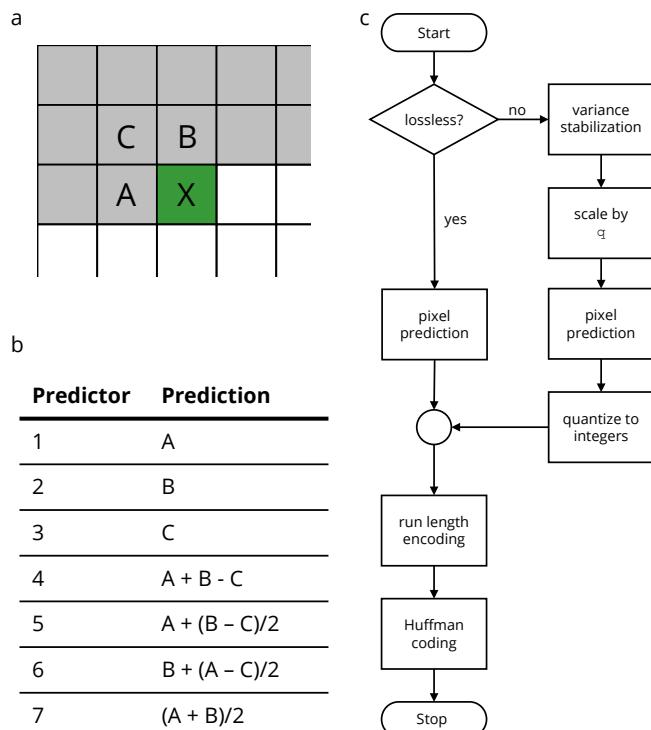


Figure 4.2: B³D algorithm schematics. (a) Prediction context for pixel X, the next sample to be encoded. Three neighboring pixels are considered: left (A), top (B) and top left (C) neighbors. (b) Lossless JPEG predictors for X, based on the context showed in (a). The first three predictors are one-dimensional, while the rest are two-dimensional. Using a two dimensional predictor while increases complexity slightly, also increases the achievable compression ratio. We found that for fluorescence microscopy images predictor 7 performs best, hence its inclusion in the B³D algorithm. (c) Complete algorithm flowchart depicting the main stages of the compression. First, if the result should be lossless, pixel prediction is performed on the original image values. If within noise level mode is selected, the image noise is stabilized first (see "Supplementary Note"), which is then scaled by the quantization step q. Prediction is performed, and the prediction errors are rounded to the nearest integer. The prediction errors for both lossless and lossy modes are then run-length encoded, and finally Huffman coding is applied to effectively reduce data size. The output of the Huffman coder is saved as the compressed file.

4. REAL-TIME, GPU ACCELERATED IMAGE PROCESSING PIPELINE

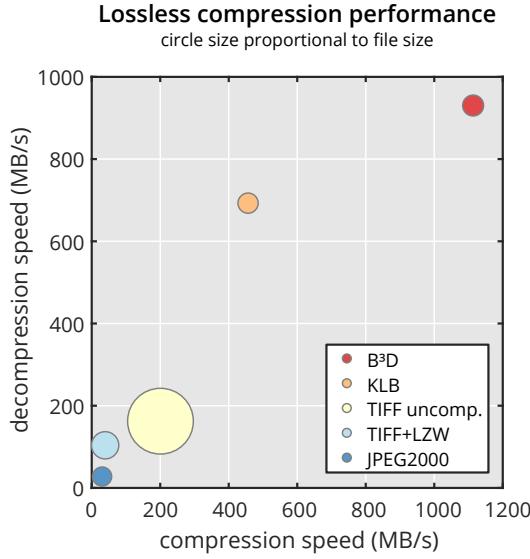


Figure 4.3: Lossless compression performance. Performance comparison of our B³D B³D compression algorithm (red circle) vs. KLB (orange), uncompressed TIFF (light yellow), LZW compressed TIFF (light blue) and JPEG2000 (blue) regarding write speed (horizontal axis), read speed (vertical axis) and file size (circle size). (see also Table B2).

1 Light-sheet imaging

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

7 3D nucleus segmentation

8 3D nucleus segmentation of *Drosophila* embryos was performed using Ilastik [141]. The
 9 original dataset was compressed at different quantization levels, then upsampled in z to ob-
 10 tain isotropic resolution. To identify the nuclei, we used the pixel classification workflow,
 11 and trained it on the uncompressed dataset. This training was then used to segment the
 12 compressed datasets as well. Segmentation overlap was calculated in Matlab ("Supple-
 13 mentary Code") using the Sørensen–Dice index [142, 143]:

$$QS = 2 |A \cap B| / (|A| + |B|) \quad (4.1)$$

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

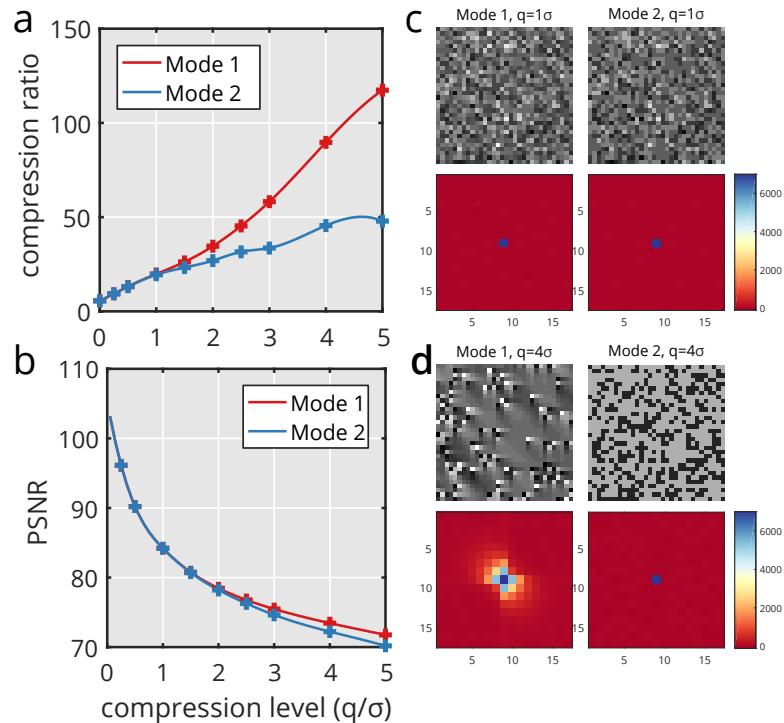


Figure 4.4: Options for noise dependent lossy compression. Comparing Mode 1 (prediction then quantization) and Mode 2 (quantization then prediction) of noise dependent lossy compression in terms of compression ratio, peak signal to noise ratio (PSNR) and spatial correlations introduced to random noise. (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 “Supplementary Note”.

4. REAL-TIME, GPU ACCELERATED IMAGE PROCESSING PIPELINE

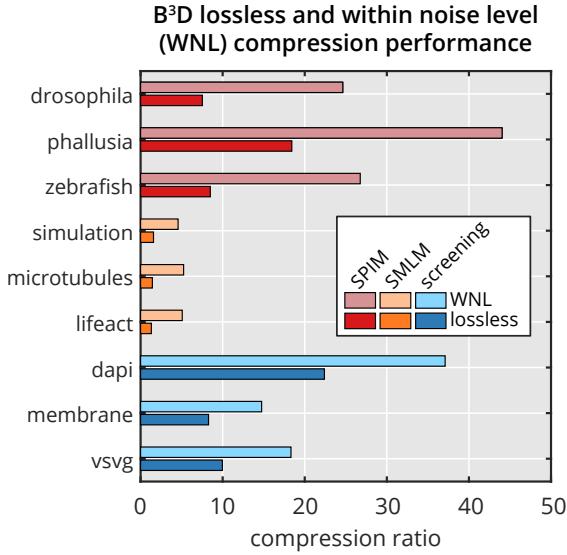


Figure 4.5: Within noise level compression performance. WNL compression performance compared with lossless performance for 9 different dataset representing 3 imaging modalities (SPIM, SMLM, screening). Compression ratio = original size / compressed size. For description of datasets see Table B3 in Appendix B.

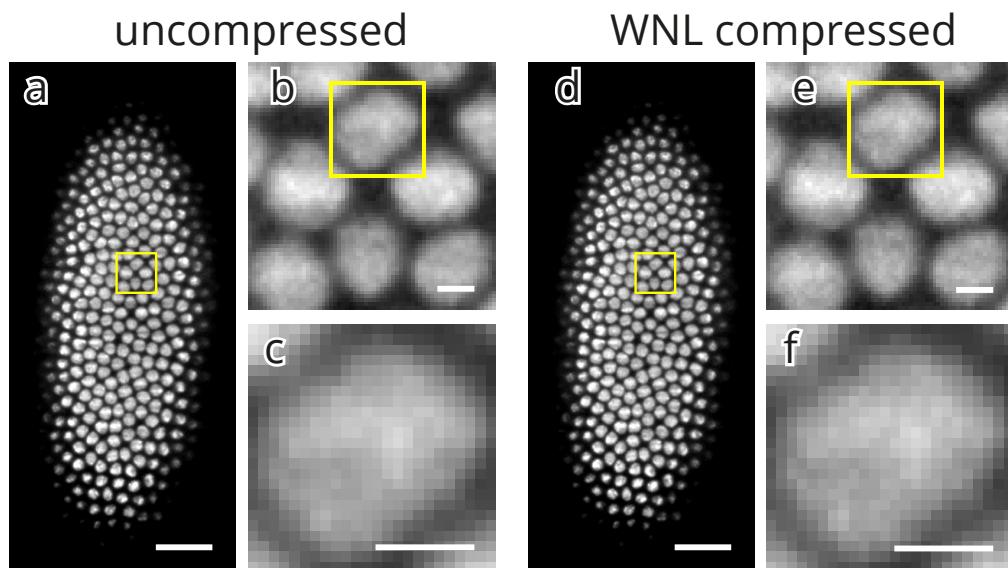


Figure 4.6: Image quality of a WNL compressed dataset. WNL compression performance compared with lossless performance for 9 different dataset representing 3 imaging modalities (SPIM, SMLM, screening). Compression ratio = original size / compressed size. For description of datasets see Table B3.

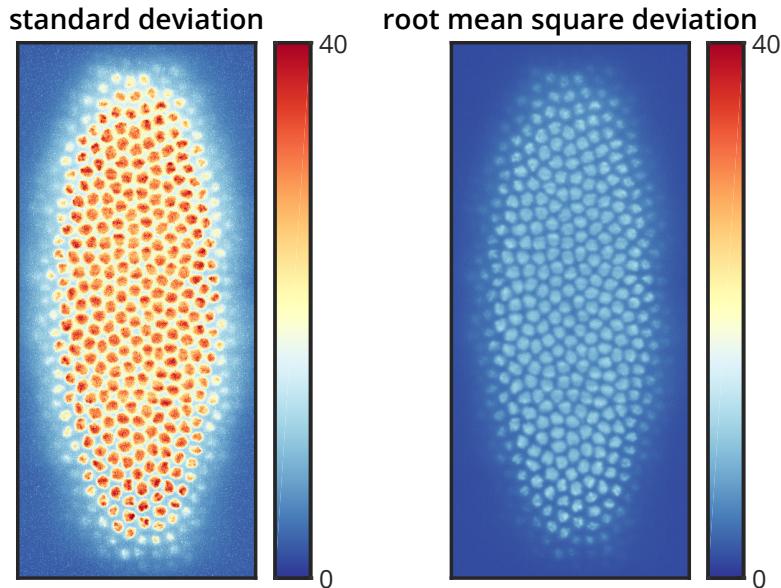


Figure 4.7: Compression error compared to image noise. To compare the difference arising from WNL compression to image noise, we imaged a single plane 100 times in a *Drosophila melanogaster* embryo expressing H2Av-mCherry nuclear marker at 38 ms intervals. The whole acquisition took 3.8 s, for which 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.

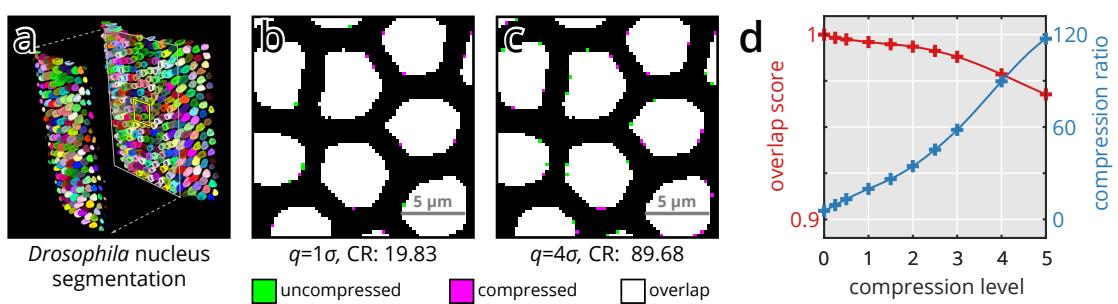


Figure 4.8: Influence of noise dependent lossy compression on 3D nucleus segmentation. A *Drosophila melanogaster* embryo expressing H2Av-mCherry nuclear marker was imaged in MuVi-SPIM [45], and 3D nucleus segmentation was performed ("Online Methods") (a). The raw data was subsequently compressed at 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 ("Online Methods") was calculated and is plotted in (g) along with the achieved compression ratios.

4. REAL-TIME, GPU ACCELERATED IMAGE PROCESSING PIPELINE

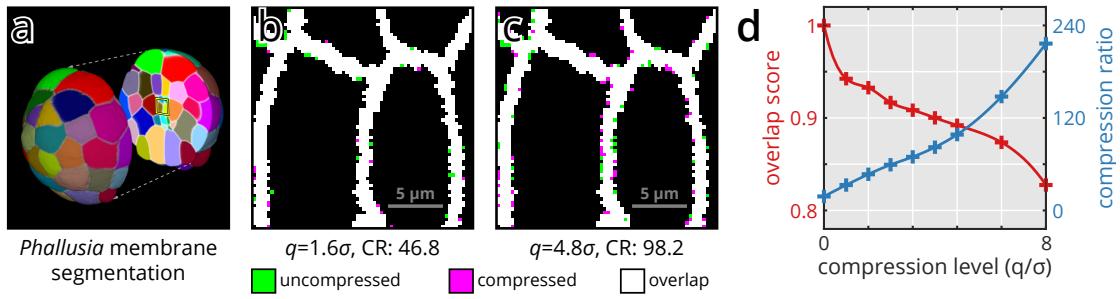


Figure 4.9: Influence of noise dependent lossy compression on 3D membrane segmentation. A *Phallusia mammillata* embryo expressing PH-citrine membrane marker was imaged in MuVi-SPIM [45], and 3D membrane segmentation was performed (“Supplementary Methods”) (a). The raw data was subsequently compressed 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 (“Supplementary Methods”) was calculated and is plotted in (d) along with the achieved compression ratios.

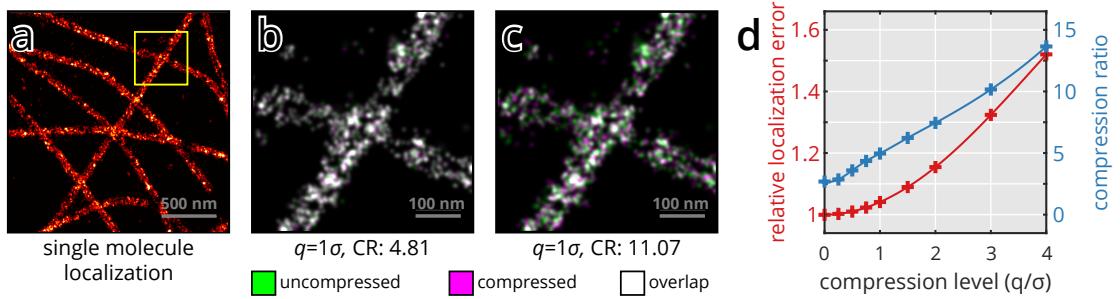


Figure 4.10: Influence of noise dependent lossy compression on single-molecule localization. Microtubules, immunolabeled with Alexa Fluor 647 were imaged by SMLM (a). The raw data was compressed at increasingly higher compression levels, and localized using the same settings as the uncompressed data. 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.

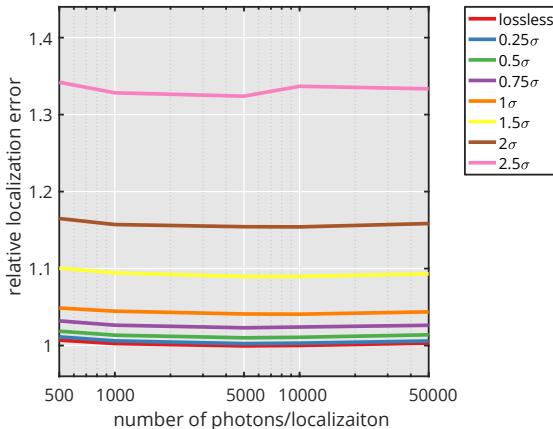


Figure 4.11: Change in localization error only depends on selected quantization step. We simulated multiple datasets ("Supplementary Methods") with different average photon numbers per localization. Background was kept at a constant average of 20 photons/pixel. Datasets were compressed at multiple compression 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 3D membrane segmentation

² Raw MuVi-SPIM recordings of *Phallusia mammillata* embryos expressing PH-citrine
³ membrane marker were kindly provided by Ulla-Maj Fiura (EMBL, Heidelberg). Each
⁴ recording consisted of 4 views at 90 degree rotations. The views were fused using an image
⁵ based registration algorithm followed by a sigmoidal blending of the 4 views. The fused
⁶ stack was then segmented using the MARS algorithm [144] with an hmin parameter
⁷ of 10. The raw data (all 4 views) was compressed at different levels, and segmented
⁸ using the same pipeline. Segmentation results were then processed in Matlab to calculate
⁹ the overlap score for the membranes using the Sørensen–Dice index ("Supplementary
¹⁰ Code").

¹¹ Single-molecule localization imaging

¹² In order to visualize microtubules, U2OS cells were treated as in [145] and imaged in a
¹³ dSTORM buffer [146]. In brief, the cells were permeabilized and fixed with glutaralde-
¹⁴ hyde, washed, then incubated with primary tubulin antibodies and finally stained with
¹⁵ Alexa Fluor 647 coupled secondary antibodies. The images were recorded on a home-built
¹⁶ microscope previously described [145], in its 2D single-channel mode.

¹⁷ Single-molecule localization data analysis

¹⁸ Analysis of single-molecule localization data was performed on a custom-written MAT-
¹⁹ LAB software as in [147]. Pixel values were converted to photon counts according to
²⁰ measured offset and calibrated gain of the camera (EMCCD iXon, Andor). The back-

4. REAL-TIME, GPU ACCELERATED IMAGE PROCESSING PIPELINE

1 ground was estimated with a wavelet filter [148], background-subtracted images were
2 thresholded and local maxima were detected on the same images. 7-pixel ROIs around
3 the detected local maxima were extracted from the raw images and fitted with a GPU
4 based MLE fitter [149]. Drift correction was performed based on cross-correlation. Fi-
5 nally, images were reconstructed by filtering out localizations with a high uncertainty
6 (>30 nm and large PSF (>150 nm) and Gaussian rendering.

7 Simulation of single-molecule localization data

8 Single molecule localization data was simulated in Matlab ("Supplementary Code") by
9 generating a grid of pixelated Gaussian spots with standard deviation of 1 pixel. With a
10 pixel size of a 100 nm, this corresponds to a FWHM of 235.48 nm. The center of each spot
11 was slightly offset from the pixel grid at 0.1 pixel increments in both x and y directions.
12 To this ground truth image a constant value was added for illumination background, and
13 finally Poisson noise was applied to the image. This process was repeated 10000 times
14 to obtain enough images for adequate accuracy.

15 Code availability

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

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 sensitivity imaging of delicate samples. A novel arrangement of two high numerical*
⁶ *aperture objectives in 120 degrees allows for near isotropic resolution while increasing*
⁷ *light collection efficiency by a factor of two.*

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

⁹ Live imaging of light sensitive specimens, such as a developing mouse embryo is a chal-
¹⁰ lenging task

¹¹ **Thesis II.** *I have developed a GPU-based image processing pipeline for multi-view light-*
¹² *sheet microscopy that enables real time fusion of opposing views.*

¹³ Corresponding publications: [C1], [C2], [C3]

¹⁴ **Thesis III.** *I have developed a new image compression algorithm that enables noise*
¹⁵ *dependent lossy compression of light microscopy images, and can reach a compression*
¹⁶ *ratio of 100 fold while preserving the results of downstream data analysis steps.*

¹⁷ Corresponding publications: [J4], [C1], [C2], [C3]

¹⁸ All microscopy images contain inherent noise, that is

¹⁹ **Thesis IV.** *I have developed a new GPU-based image compression library, B^3D , that*
²⁰ *implements the algorithm described in Thesis III, and allows for real time compression*
²¹ *of microscopy images with a throughput of up to 1 GB/s.*

²² Corresponding publications: [J4], [C1], [C2], [C3]

²³ B^3D is an efficient, GPU-based image compression library allowing lossless and noise
²⁴ dependent lossy compression of microscopy images. Since many high-speed microscopy

5. DISCUSSION

- ¹ methods generate immense amounts of data, easily reaching terabytes per experiment,
- ² image compression is especially important to efficiently deal with such datasets.

¹ Acknowledgements

ACKNOWLEDGEMENTS

¹ Appendix A

² Bill of materials

³ Bill of Materials

⁴ Optical components

- ⁵ • Lenses

- ⁶ – 2×Nikon CFI75 Apo LWD 25x/1.10w water dipping objectives
- ⁷ – 2×Nikon ITL200 200 mm tube lens
- ⁸ – 2×200 mm x 25 mm Dia. achromatic lens (Edmunds Optics, #47-645)
- ⁹ – 2×400 mm x 40 mm Dia. achromatic lens (Edmunds Optics, #49-281)
- ¹⁰ – 3×75 mm x 25 mm Dia. achromatic lens (Edmunds Optics, #47-639)
- ¹¹ – SILL 112751 1:2 beam expander

- ¹² • Filters

- ¹³ – 2×BrightLine quad-edge dichroic beam splitter (Semrock, Di03-R405/488/561/635-t3-25x36)
- ¹⁴ – EdgeBasic 488 nm long pass filter (Semrock, BLP01-488R-25)
- ¹⁵ – BrightLine 525/50 band pass filter (Semrock, FF03-525/50-25)
- ¹⁶ – EdgeBasic 561 nm long pass filter (Semrock, BLP02-561R-25)
- ¹⁷ – RazorEdge 647 nm long pass filter (Semrock, LP02-647RU-25)

- ¹⁹ • Mirrors

- ²⁰ – 6×1” Broadband Dielectric Elliptical Mirror (Thorlabs, BBE1-E03)
- ²¹ – 2×30 mm Broadband 1/10λ Mirror (OptoSigma, TFMS-30C05-4/11)
- ²² – 10 Pack of 1” Protected Silver Mirrors (Thorlabs, PF10-03-P01-10)
- ²³ – Knife-Edge Right-Angle Prism Dielectric Mirror (Thorlabs, MRAK25-E02)

²⁴ Mechanical components

- ²⁵ • 40 mm travel range pneumatic cylinder (Airtac, HM-10-040)
- ²⁶ • 5/2-way electric valve (Airtac, M-20-510-HN)
- ²⁷ • 2×25 mm extended contact bearing steel stage (OptoSigma, TSDH-251C)
- ²⁸ • 30x90 mm stainless steel slide (OptoSigma, IPWS-F3090)
- ²⁹ • close proximity gimbal mirror mount (Thorlabs, GMB1/M)
- ³⁰ • 30 mm cage elliptical mirror mount (Thorlabs, KCB1E)

APPENDIX A

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)

¹ **Appendix B**

² **Appendix B**

³ **Data sizes in microscopy**

⁴ **Lossless compression performnace**

⁵ **Benchmarking datasets**

APPENDIX B

	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 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

	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%
JPEG2000	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%

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 4.3.

Dataset name	Imaging modality	Description	Size (MB)
drosophila	SPIM	dataset acquired in MuVi-SPIM of a Drosophila melanogaster 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 Phallusia mammillata 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 [150]	1,005.38
vsvg	screening	wide field fluorescence images of CFP-tsO45G proteins in HeLa Kyoto cells [150]	1,005.38
membrane	screening	wide field fluorescence images of membrane localized CFP-tsO45G proteins labeled with AlexaFluor647 in HeLa Kyoto cells [150]	1,005.38

Table B3: Datasets used for benchmarking compression performance.

APPENDIX B

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