

<sup>1</sup>    A new angle on light-sheet microscopy  
<sup>2</sup>    and real-time image processing

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Bálint Balázs

<sup>4</sup>



<sup>5</sup>

Pázmány Péter Catholic University

<sup>6</sup>

Faculty of Information Technology and Bionics

<sup>7</sup>

Roska Tamás Doctoral School of Sciences and Technology

<sup>8</sup>

Advisor: Balázs Rózsa, *M.D., Ph.D.*

<sup>9</sup>

A thesis submitted for the degree of

<sup>10</sup>

*Doctor of Philosophy*

<sup>11</sup>

2017

# <sup>1</sup> Abstract

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

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

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

# <sup>1</sup> Contents

<sup>2</sup> <b>Abstract</b>	<sup>ii</sup>
<sup>3</sup> <b>List of Figures</b>	<sup>vi</sup>
<sup>4</sup> <b>List of Tables</b>	<sup>vii</sup>
<sup>5</sup> <b>List of Abbreviations</b>	<sup>viii</sup>
<sup>6</sup> <b>Introduction</b>	<sup>x</sup>
<sup>7</sup> <b>1 Live imaging in three dimensions</b>	<sup>1</sup>
<sup>8</sup> 1.1 Wide-field fluorescence microscopy . . . . .	<sup>2</sup>
<sup>9</sup> 1.2 Point scanning methods . . . . .	<sup>8</sup>
<sup>10</sup> 1.3 Light-sheet microscopy . . . . .	<sup>12</sup>
<sup>11</sup> 1.4 Light-sheet microscopy for mouse imaging . . . . .	<sup>19</sup>
<sup>12</sup> <b>2 Image compression</b>	<sup>26</sup>
<sup>13</sup> 2.1 Basics of information theory . . . . .	<sup>26</sup>
<sup>14</sup> 2.2 Entropy coding . . . . .	<sup>27</sup>
<sup>15</sup> 2.3 Decorrelation . . . . .	<sup>31</sup>
<sup>16</sup> <b>3 Dual Mouse-SPIM</b>	<sup>35</sup>
<sup>17</sup> 3.1 Microscope design concept . . . . .	<sup>36</sup>
<sup>18</sup> 3.2 Optical layout . . . . .	<sup>39</sup>
<sup>19</sup> 3.3 Optical alignment . . . . .	<sup>43</sup>
<sup>20</sup> 3.4 Control unit . . . . .	<sup>48</sup>
<sup>21</sup> 3.5 Results . . . . .	<sup>51</sup>
<sup>22</sup> 3.6 Methods . . . . .	<sup>58</sup>
<sup>23</sup> 3.7 Conclusions . . . . .	<sup>60</sup>

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1	<b>4 GPU-accelerated image processing and compression</b>	<b>61</b>
2	4.1 Challenges in data handling for light-sheet microscopy . . . . .	61
3	4.2 Real-time preprocessing pipeline . . . . .	62
4	4.3 B <sup>3</sup> D image compression . . . . .	71
5	4.4 Conclusions . . . . .	83
6	<b>5 Discussion</b>	<b>84</b>
7	5.1 New scientific results . . . . .	84
8	5.2 Application of the results . . . . .	86
9	<b>Appendix</b>	<b>88</b>
10	A Bill of materials . . . . .	88
11	B Supplementary Tables . . . . .	90
12	C Light collection efficiency of an objective . . . . .	92
13	D 3D model of Dual Mouse-SPIM . . . . .	93
14	<b>References</b>	<b>95</b>
15	<b>Acknowledgements</b>	<b>107</b>

# <sup>1</sup> List of Figures

2	1.1	Tradeoffs in fluorescence microscopy for live imaging . . . . .	1
3	1.2	Excitation and emission spectrum of enhanced green fluorescent protein (EGFP) . . . . .	2
4	1.3	Wide-field fluorescence microscope . . . . .	4
5	1.4	Axial cross section of the PSF and OTF of a wide-field microscope . . . . .	6
6	1.5	Airy pattern . . . . .	7
7	1.6	Resolution of a wide-field microscope . . . . .	9
8	1.7	Basic optical components of a confocal laser scanning and confocal-theta microscope . . . . .	10
9	1.8	Axial cross section of the PSF and OTF of a confocal laser scanning mi- croscope . . . . .	11
10	1.9	Basic concept of single-plane illumination microscopy . . . . .	12
11	1.10	Different optical arrangements for light-sheet microscopy . . . . .	14
12	1.11	Basic optical components of a SPIM . . . . .	15
13	1.12	Light-sheet dimensions . . . . .	16
14	1.13	DSLM illumination . . . . .	19
15	1.14	Inverted light-sheet microscope for multiple early mouse embryo imaging.	21
16	1.15	Imaging mouse post-implantation development . . . . .	23
17	1.16	Imaging adult mouse brain with light-sheet microscopy. . . . .	24
18	2.1	Building the binary Huffman tree . . . . .	30
19	2.2	Context for pixel prediction . . . . .	33
20	3.1	Dual view concept with high NA objectives . . . . .	36
21	3.2	Lateral and axial resolution of a multi-view optical system. . . . .	38
22	3.3	The core unit of the microscope . . . . .	40
23	3.4	Illumination branch splitting unit . . . . .	42
24	3.5	Dual Mouse SPIM optical layout . . . . .	44
25	3.6	Detection branch switching unit . . . . .	45
26	3.7	Microscope control hardware and software architecture . . . . .	49

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## LIST OF FIGURES

1	3.8	Digital and analog control signals . . . . .	50
2	3.9	Completed DualMouse-SPIM . . . . .	52
3	3.10	Stability measurements of view switcher unit . . . . .	53
4	3.11	Illumination beam profile . . . . .	54
5	3.12	Simulated and measured PSF of Dual Mouse-SPIM . . . . .	55
6	3.13	Combined PSF of 2 views . . . . .	56
7	3.14	Maximum intensity projection of a <i>Drosophila melanogaster</i> embryo recording . . . . .	57
8	3.15	Multi-view recording of a mouse zygote . . . . .	58
10	4.1	Experiment sizes and data rate of different imaging modalities . . . . .	62
11	4.2	Real-time image processing pipeline for multi-view light-sheet microscopy . . . . .	62
12	4.3	Operating principle of MuVi-SPIM . . . . .	63
13	4.4	Multi-view fusion methods for light-sheet microscopy . . . . .	65
14	4.5	Classes of LVCUDA library . . . . .	66
15	4.6	Applying mask to remove background . . . . .	68
16	4.7	Comparison of 3D and 2D fusion of beads . . . . .	68
17	4.8	GPU fused images of a <i>Drosophila melanogaster</i> embryo . . . . .	69
18	4.9	Image contrast depending on z position for raw and fused stacks . . . . .	70
19	4.10	B <sup>3</sup> D algorithm schematics . . . . .	73
20	4.11	Options for noise-dependent lossy compression . . . . .	75
21	4.12	Theoretical and measured increase in image noise for WNL compression . . . . .	76
22	4.13	Compression performance . . . . .	77
23	4.14	Image quality of a WNL compressed dataset . . . . .	77
24	4.15	Compression error compared to image noise . . . . .	78
25	4.16	Influence of noise-dependent lossy compression on 3D nucleus segmentation . . . . .	79
26	4.17	Influence of noise-dependent lossy compression on 3D membrane segmentation . . . . .	79
28	4.18	Influence of noise-dependent lossy compression on single-molecule localization . . . . .	80
30	4.19	Change in localization error only depends on selected quantization step . . . . .	80
31	C1	Light collection efficiency of an objective . . . . .	92
32	D1	3D model of DualMouse-SPIM . . . . .	94

# <sup>1</sup> List of Tables

<sup>2</sup>	2.1 Examples of a random binary code (#1) and a prefix-free binary code (#2)	28
<sup>3</sup>	2.2 Letters to be Huffman coded and their probabilities . . . . .	29
<sup>4</sup>	2.3 Huffman code table . . . . .	30
<sup>5</sup>	B1 Data sizes in microscopy . . . . .	90
<sup>6</sup>	B2 Lossless compression performance . . . . .	90
<sup>7</sup>	B3 Datasets used for benchmarking compression performance . . . . .	91
<sup>8</sup>		

# <sup>1</sup> List of Abbreviations

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

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

# <sup>1</sup> Introduction

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

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

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

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

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

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

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

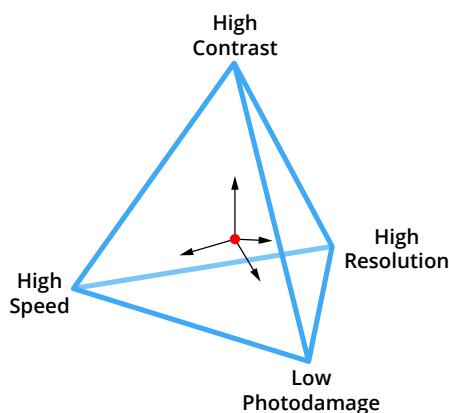
7 In Chapter 4 we present a GPU-based real-time image processing pipeline designed  
8 to efficiently handle large amounts of microscopy data. As 3D imaging is gaining more  
9 and more traction, image datasets are generated at a faster pace than ever. We present  
10 a real-time preprocessing solution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

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

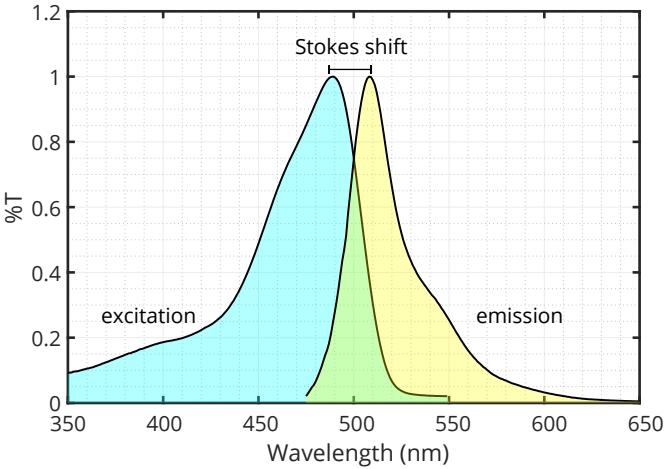
# <sup>1</sup> Chapter 1

## <sup>2</sup> Live imaging in three dimensions

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



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



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

## <sup>1</sup> 1.1 Wide-field fluorescence microscopy

<sup>2</sup> Fluorescence microscopy [9, 10], as a subset of light microscopy, is one of the few methods  
<sup>3</sup> that allow subcellular imaging of live specimens. The first use of the term fluorescence is  
<sup>4</sup> credited to George Gabriel Stokes [11], and it refers to the phenomenon of light emission  
<sup>5</sup> following the absorption of light or other electromagnetic radiation. As the name of  
<sup>6</sup> the technique suggests, this method collects fluorescent light from the specimens. Since  
<sup>7</sup> biological tissues are usually not fluorescent, except for some autofluorescence mostly at  
<sup>8</sup> shorter wavelengths, fluorescent dyes or proteins have to be introduced to the system in  
<sup>9</sup> order to be able to collect the necessary information. The advantage of this is that the  
<sup>10</sup> signal of the labeled structures will be of very high ratio compared to the background.

<sup>11</sup> A fluorescent molecule is capable of absorbing photons in a given wavelength range  
<sup>12</sup> (excitation spectrum) and temporarily store its energy by having an electron in a higher  
<sup>13</sup> energy state, *i.e.*, in an excited state. This excited state, however, is not stable, and the  
<sup>14</sup> electron quickly returns to the ground state while emitting a photon. The energy of the  
<sup>15</sup> absorbed and emitted photons are not the same, as energy loss occurs due to internal  
<sup>16</sup> relaxation events, and the emitted photon has lower energy than the absorbed photon.  
<sup>17</sup> This phenomenon is called the Stokes shift, or red shift, and can be exploited in mi-  
<sup>18</sup> croscopy to drastically increase the signal-to-noise ratio by filtering out the illumination  
<sup>19</sup> light (Figure 1.2).

### <sup>20</sup> 1.1.1 Fluorescent proteins

<sup>21</sup> Traditionally, synthetic fluorescent dyes were used to label certain structures in the speci-  
<sup>22</sup> mens. Some of these directly bind to their target, and others can be used when conjugated

1 to an antibody specific to the structure of interest. A requirement for these methods is  
2 that the fluorescent label has to be added to the sample from an external source, and, in  
3 many cases, this also necessitates sample preparation techniques incompatible with live  
4 imaging, such as fixation [13].

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

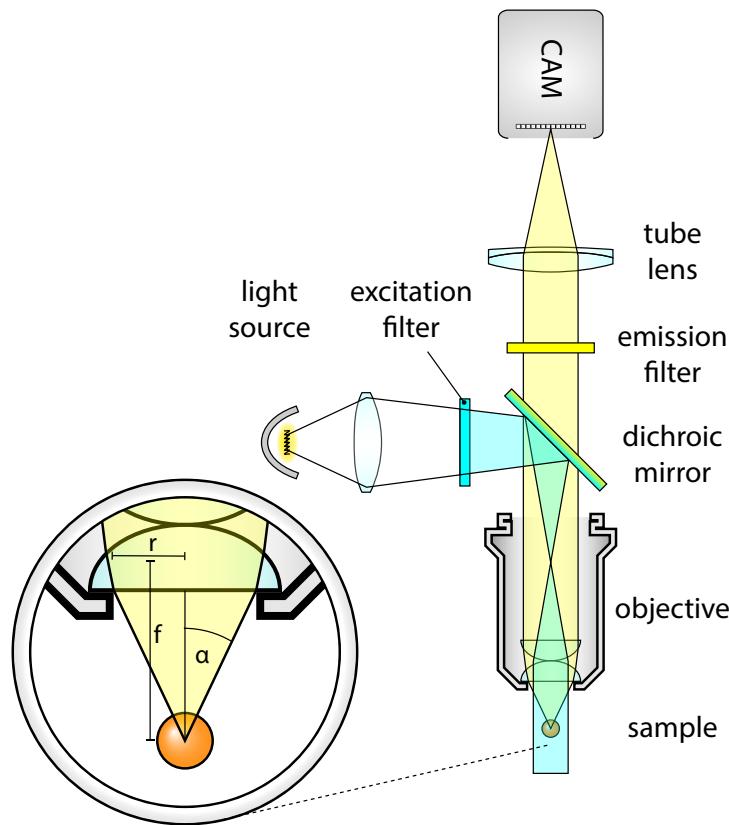
### 16 1.1.2 Wide-field image formation

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

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

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

36 The combination of the objective and tube lens together will determine the optical



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

1 magnification of the system, which will be the ratio of the focal lengths of these lenses:

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

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

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

5 Apart from the magnification, the most important property of the objective is the  
 6 half-angle of the light acceptance cone,  $\alpha$  (Figure 1.3, inset). This not only determines  
 7 the amount of collected light, but also the achievable resolution of the system (see Sec-  
 8 tion 1.1.3). This angle depends on the size of the lens relative to its focal length. In other  
 9 words it depends on the aperture of the lens, which is why the expression *numerical*

<sup>1</sup> aperture (NA) is more commonly used to express this property of the objective:

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

<sup>2</sup> For small  $\alpha$  angles, the following approximation holds true:  $\sin \alpha \approx \tan \alpha \approx \alpha$ . Thus,  
<sup>3</sup> the numerical aperture can also be expressed as a ratio of the radius of the lens and the  
<sup>4</sup> focal length:

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

### <sup>5</sup> 1.1.3 Resolution of a wide-field microscope

<sup>6</sup> The resolution of an optical system depends on the size of the smallest distinguishable  
<sup>7</sup> feature on the image. Practically this means the minimum distance between two point-  
<sup>8</sup> like objects so that the two objects can still be resolved. This mainly depends on two  
<sup>9</sup> factors: the NA of the objective, and the pixel size of the imaging sensor.

<sup>10</sup> Even if the imaging sensor would have infinitely fine resolution, it is not possible to  
<sup>11</sup> reach arbitrarily high resolutions due to the wave nature of light and diffraction effects  
<sup>12</sup> that occur at the aperture of the objective. This means that depending on the wavelength  
<sup>13</sup> of the light, any point source will have a finite size on the image, it will be spread out,  
<sup>14</sup> limiting the resolution. The shape of this image is called the *point spread function*, or  
<sup>15</sup> PSF (Figure 1.4), as this function describes the behavior of the optical system when  
<sup>16</sup> imaging a point like source. This property of lenses was already discovered by Abbe in  
<sup>17</sup> 1873 [23], when he constructed his famous formula:

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

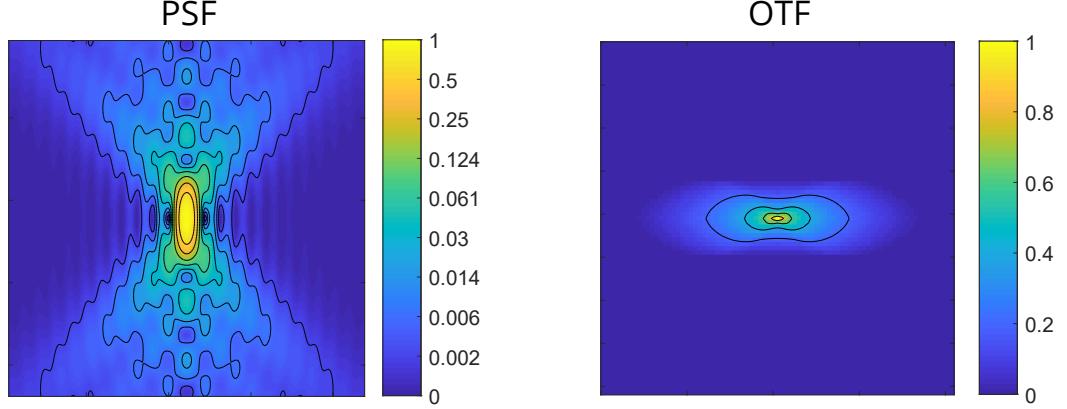
<sup>18</sup> where  $\delta$  is the smallest distance between two distinguishable features.

<sup>19</sup> Another representation of the optical performance, is the *optical transfer function*,  
<sup>20</sup> or OTF (Figure 1.4), which is the Fourier transform of the PSF:

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

<sup>21</sup> As this function operates in the frequency space, it describes how the different frequencies  
<sup>22</sup> are affected by the system. The resolution can also be defined as the support of the OTF,  
<sup>23</sup> since this describes the highest frequency that is still transmitted by the optical system.  
<sup>24</sup> Any pattern with higher frequency will be lost, thus lies beyond the resolution limit. For  
<sup>25</sup> circularly symmetric PSFs, the OTF will have real values. However, if this is not the  
<sup>26</sup> case, the Fourier transform also introduces complex components.

<sup>27</sup> Abbe's formula can be derived from the scalar theory of diffraction using a paraxial  
<sup>28</sup> approximation (Fraunhofer diffraction, [24]). It is useful to define the following optical



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

1 coordinates instead of the commonly used Cartesian coordinates  $x$ ,  $y$  and  $z$ :

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

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

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

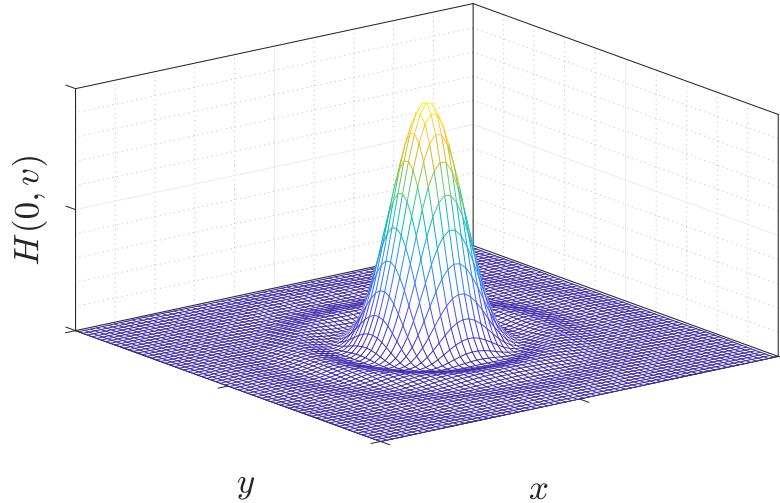
5 where  $C_0$  is a normalization constant, and  $J_0$  is the zero order Bessel function of the first  
6 kind.

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

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

10

11 where  $J_1$  is the first order Bessel function of the first kind. This equation describes the  
12 famous Airy pattern (Figure 1.5) which will be the shape of the PSF in the focal plane.  
13 The width of this pattern is the resolution, and although there are multiple definitions  
14 for this, the most commonly accepted is the Rayleigh criterion [24, 26] which defines  
15 the resolution as the distance between the central peak and the first local minimum.  
16 As this lies at  $v = 3.38$ , the resolution can be expressed by substituting this value into



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

1 Equation 1.7 and solving it for  $r$ :

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

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

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

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

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

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

9 So far we only considered a single, point-like emitter. As the intensity function de-  
10 scribes how an optical system "spreads out" the image of a point, it is also called the  
11 Point Spread Function (PSF, Figure 1.4). In a more realistic scenario, however the emit-  
12 ters are neither point-like, nor single. Effectively, however, for every emitter the PSF  
13 would be imaged on the sensor, and this creates the final image. In mathematical terms,  
14 this can be expressed as a convolution operation between the underlying fluorophore

1 distribution of the object ( $O$ ) and the PSF ( $H$ ):

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

2 The effective result of this kind of diffraction-limited image formation is a blurred  
3 image with a finite resolution of  $\delta_{xy}$  in the lateral direction, and  $\delta_z$  in the axial direction.

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

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

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

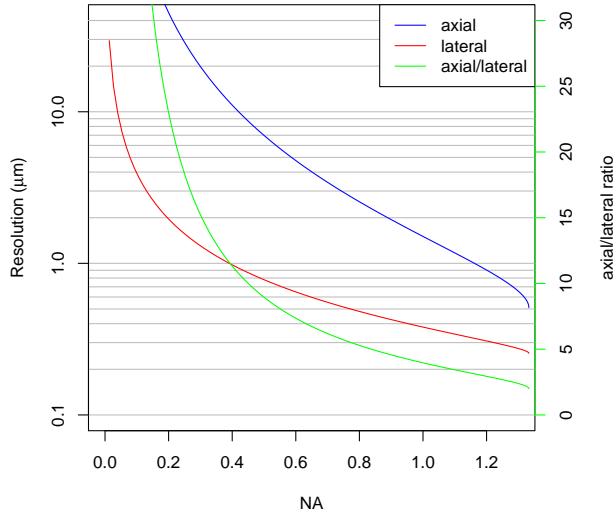
## 10 1.2 Point scanning methods

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

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

22 Evaluating Equations 1.10 and 1.12 for a range of possible numerical apertures reveals  
23 the significant differences in lateral and axial resolution for any objective (Figure 1.6).  
24 Especially for low NAs, this can be significant, a factor of  $\sim 20$  difference. For higher  
25 ( $>0.8$ ) NAs the axial resolution increases faster than the lateral, however they will only  
26 be equal when  $\alpha = 180^\circ$ . This means that isotropic resolution with a single lens is only  
27 possible if the lens is collecting all light emitting from the sample, which seems hardly  
28 possible, and would be highly impractical. For commonly used high NA objectives the  
29 lateral to axial ratio will still be around 3–6.

30 Instead of using a single lens to achieve isotropic resolution, it is more practical to  
31 image the sample from multiple directions to complement the missing information from  
32 different views. When rotating the sample by  $90^\circ$  for example, the lateral direction of



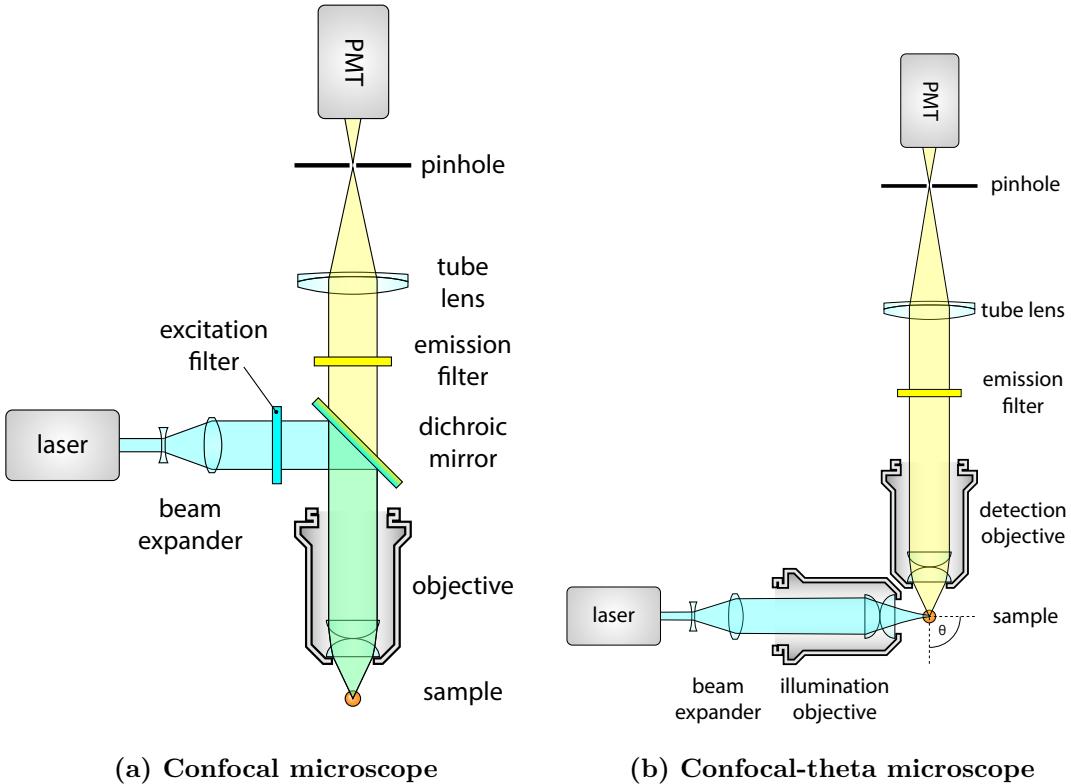
**Figure 1.6: Resolution of a wide-field microscope.** Axial (blue) and lateral (red) resolutions of a wide-field microscope are shown with respect to the numerical aperture (NA). Resolutions are calculated with  $\lambda = 510\text{nm}$ , the emission maximum of GFP and  $n = 1.33$ , the refractive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

the second view will correspond to the axial direction of the first view. If rotation is not possible, using multiple objectives can also achieve similar results, such as in the case of Multi-Imaging Axis Microscopy (MIAM) [27, 28]. This microscope consisted of 4 identical objectives arranged in a tetrahedral fashion to collect as much light as possible from multiple directions, and provide isotropic 3D resolution, albeit at the expense of extremely difficult sample handling, since the sample was surrounded by objectives from all directions.

### 1.2.1 Confocal laser scanning microscopy

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

This is achieved by two significant modifications compared to the wide-field optical path. To be able to reject the out-of-focus light, an adjustable pinhole is placed at the focus of the tube lens. Light rays originating from the focal point will meet here, and are able to pass through the pinhole. However, out-of-focus light will converge either before or after the aperture, and thus the aperture blocks these rays. To maximize the fluorescence readout efficiency for the single focal point, a photomultiplier tube is used

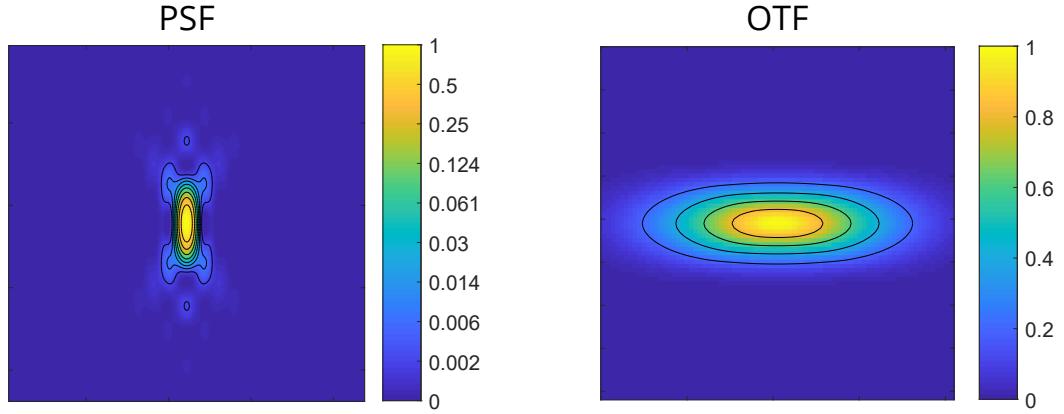


**Figure 1.7: Basic optical components of a confocal laser scanning and confocal-theta microscope.** Both types of microscopes use confocal image detection, which means that a pinhole is used to exclude light coming from out-of-focus points. Light intensity is measured by a photomultiplier for every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

- 1 instead of an area sensor (Figure 1.7a).
- 2 To maximize the signal from the focal point, the illumination light is also focused
- 3 here by coupling an expanded laser beam through the back aperture of the objective.
- 4 This not only increases illumination efficiency (since other, not detected points are not
- 5 illuminated), but has the added benefit of increasing the resolution as well. This is due
- 6 to the combined effect of illumination and detection PSFs as described in Equation 1.14
- 7 (Figure 1.8). For Gaussian-like PSFs, the final resolution (along a single direction) can
- 8 be calculated in the following way:

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

- 9 where  $\delta_{ill}$  and  $\delta_{det}$  are the resolutions for the illumination and detection, respectively.
- 10 Since the same objective is used for both illumination and detection, and the difference



**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 in wavelength is almost negligible,  $\delta_{ill} = \delta_{det} = \delta$ , the final system resolution will be:

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

2 This means that the distinguishable features in a confocal microscope are  $\sim 0.7$  times  
3 smaller than in a wide-field microscope using the same objective.

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

### 11 1.2.2 Confocal-theta microscopy

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

19 As in the case of standard confocal microscopy, the system PSF is improved by the  
20 illumination pattern. Here, however, the axial direction of the detection coincides with



**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.

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

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

### 1.3 Light-sheet microscopy

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

### 1.3 Light-sheet microscopy

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

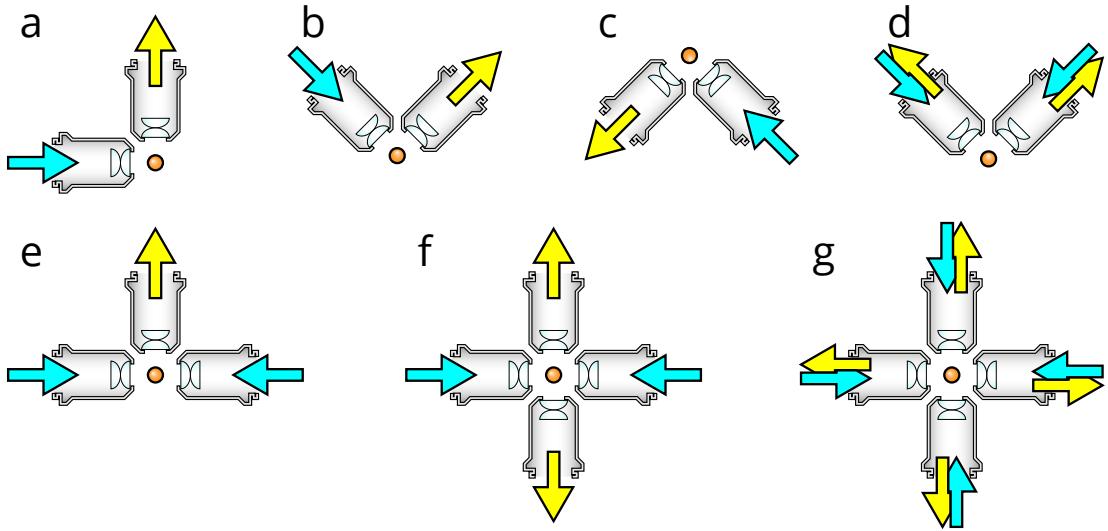
2 The main principle behind single-plane illumination microscopy, that is illuminating  
3 the sample from the side by a thin light-sheet, dates back to the early 20<sup>th</sup> century, when  
4 Siedentopf and Zsigmondy first described the ultramicroscope [36]. This microscope used  
5 sunlight as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

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

18 Later, in 2002, Fuchs *et al.* developed Thin Light-Sheet Microscopy (TLSM) [39]  
19 and used this technique to investigate the microbial life in seawater samples without  
20 disturbing their natural environment (by, *e.g.*, placing them on a coverslip). Their light-  
21 sheet was similar to the one utilized in OPFOS, being 23 µm thin, and providing a  
22 1 mm × 1 mm field of view.

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

30 Since then, light-sheet-based imaging has gained more and more popularity, as it can  
31 be adapted and applied to a wide variety of problems. Although sample mounting can  
32 be challenging because of the objective arrangement, this can also be an advantage, since  
33 new microscopes can be designed with the sample in mind [3, J3] (Figure 1.10). This  
34 made it possible to adapt the technique for numerous other specimens, such as zebrafish  
35 larvae [49], *C. elegans* embryos [42], mouse brain [6], and even mouse embryos [J2, 50,



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

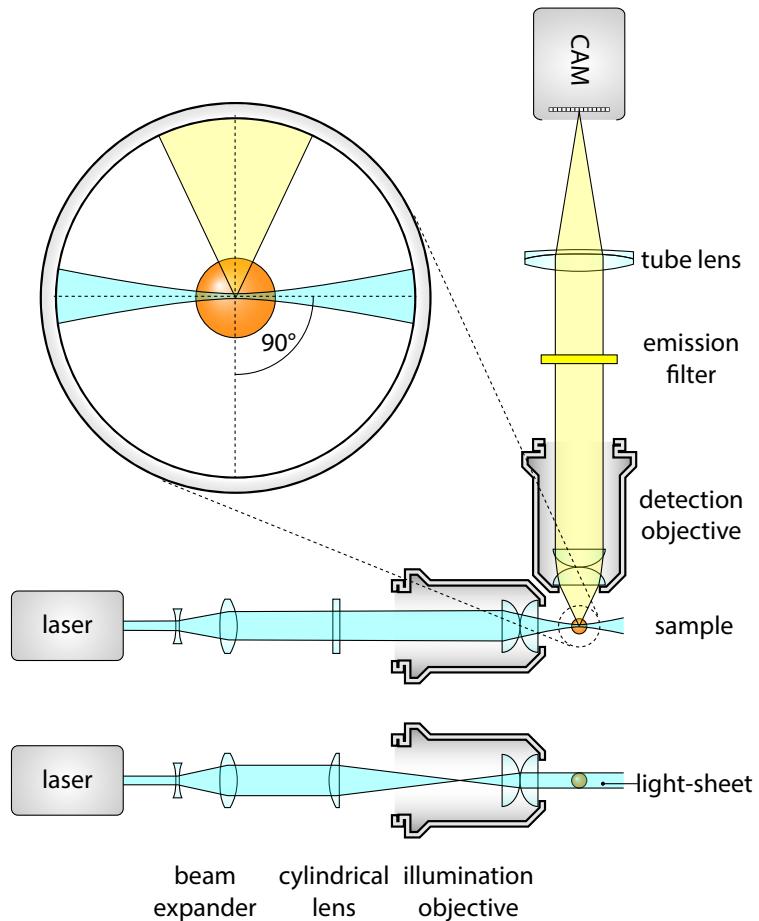
36 51].

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

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

12 Furthermore, because of the wide-field detection scheme it is possible to combine  
13 SPIM with many superresolution techniques, such as single molecule localization [53],  
14 STED [54], RESOLFT [J1], or structured illumination [7, 55, 56].

15 Since illumination and detection for light-sheet microscopy are decoupled, two inde-



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

16 pendent 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 (Figure 1.11). The most important components  
 19 are the objective together with the tube lens, filter wheel, and a sensor, typically a CCD  
 1 or sCMOS camera.

2 One of the most important aspects that determines the resolution of the microscope is  
 3 the detection objective. Since imaging biological specimens usually requires a water-based  
 4 solution, the objectives also need to be directly submerged in the medium to minimize  
 5 spherical aberrations. Since the refraction index of water ( $n = 1.33$ ) is greater than the  
 6 refraction index of air, these objectives tend to have a higher NA, resulting in higher  
 7 resolution. As the illumination is decoupled in this system, the light-sheet thickness also

8 has an influence on the axial resolution. Finally, the resolution also depends on the pixel  
9 size of the sensor which determines the spatial sampling rate of the image.

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

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

#### 8 1.3.1 Static light-sheet illumination

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

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

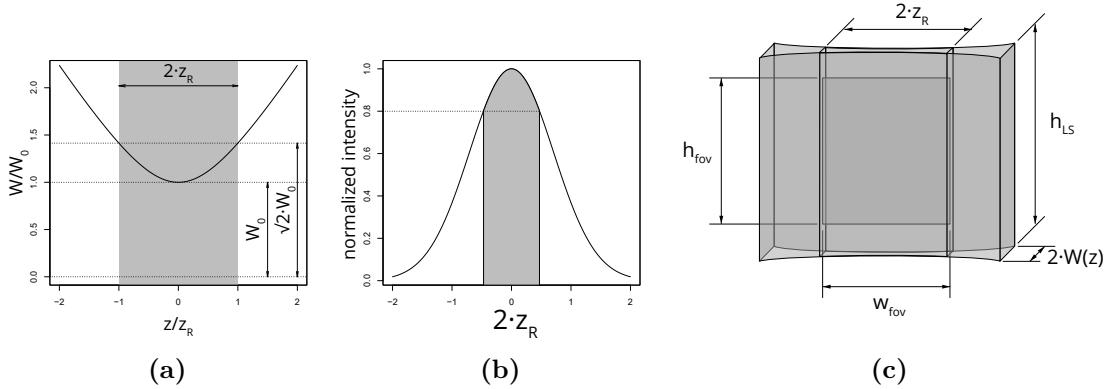
#### 20 Light-sheet dimensions

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

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

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

5 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  
6 the direction of the light propagation.



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

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

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

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

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

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

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

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

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

This beam still has a Gaussian profile along the  $x$  and  $y$  axes, but the radii ( $W_{0,x}$  and  $W_{0,y}$ ), and the beam waist positions ( $z_{0,x}$  and  $z_{0,y}$ ) are uncoupled, which results in an elliptical and astigmatic beam. The beam width can now be described by two independent

equations for the two orthogonal directions:

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

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

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

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

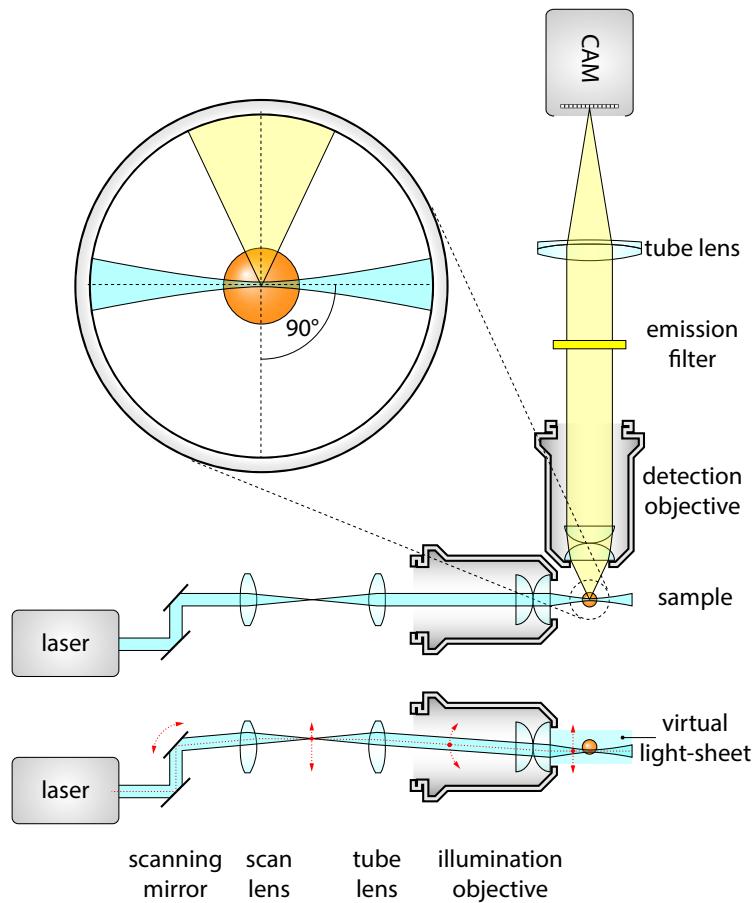
Light-sheet height is determined by the intensity profile of the beam along the vertical axis (Figure 1.12b). Since this is a Gaussian function (see Equation 1.18), only a small part in the middle can be used for imaging, because towards the sides the intensity dramatically drops. To allow a maximum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

### 1.3.2 Digitally scanned light-sheet illumination

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

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

The basic optical layout of a DSLM is shown on Figure 1.13. A galvanometer controlled mirror is used to alter the beam path that can quickly turn around its axis which



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

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

## 7 1.4 Light-sheet microscopy for mouse imaging

8 Because of its optical sectioning capabilities, combined with the high specificity of flu-  
 9 orescent labels, confocal microscopy had an immense influence on biological research,

and has been the go-to technique for decades for many discoveries [59–61]. Live imaging capabilities of confocal microscopy are, however, limited. This is mostly due to the illumination scheme: to collect information from a single point, almost the whole embryo has to be illuminated. This unwanted illumination can easily lead to bleaching of the fluorophores, and also to phototoxic effects that can prevent further embryonic development. The point scanning nature of image acquisition also implies an inherent constraint to the maximum imaging speed, as it can be only increased at the expense of reducing signal intensity (Figure 1.1).

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

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

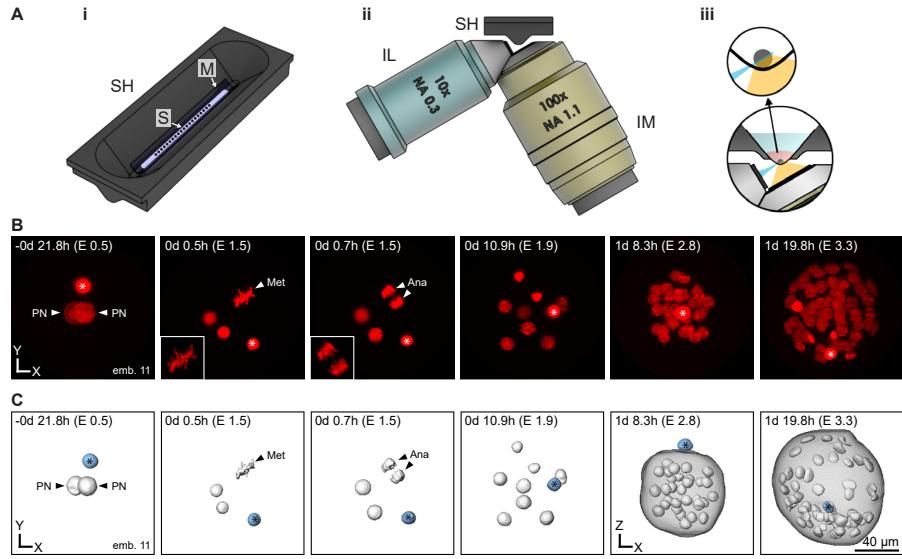
### 1.4.1 Imaging pre-implantation development

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

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

Imaging for just a few hours is already enough to investigate important processes, such

## 1.4 Light-sheet microscopy for mouse imaging



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

as cell fate patterning [70]. Other approaches aim to lower the phototoxicity by either using 2-photon illumination which operates at longer wavelengths [71–73], or by lowering imaging frequency as a compromise [74]. These approaches, however either require highly specialized equipment, such as an ultra-short pulsed laser, or is compromising on the time resolution.

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

As a mouse embryo culture is not compatible with the standard agarose-based sample mounting techniques, a completely new approach was taken, which resulted in a microscope designed around the sample. The sample holder forming a V-shape was built with a bottom window, and it is lined with a thin FEP (fluorinated ethylene propylene) foil that supports the embryos (Figure 1.14A, i). This arrangement allows the utilization of the standard microdrop embryo culture, while providing proper viewing access for the objectives. As the embryos are relatively small (100 μm) and transparent, a single illu-

mination and single detection objective arrangement is enough for high quality imaging. A low resolution ( $NA=0.3$ ) objective is used to generate the scanned light-sheet, and a high resolution ( $NA=1.1$ ) objective is detecting the fluorescence at  $50\times$ magnification (Figure 1.14A, ii). As the foil is curved, it allows unrestricted access to the embryo, while separating the imaging medium from the immersion liquid (Figure 1.14A, iii). Furthermore, its refractive index is matching the refractive index of water, so optical aberrations are minimized.

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

### 1.4.2 Imaging post-implantation development

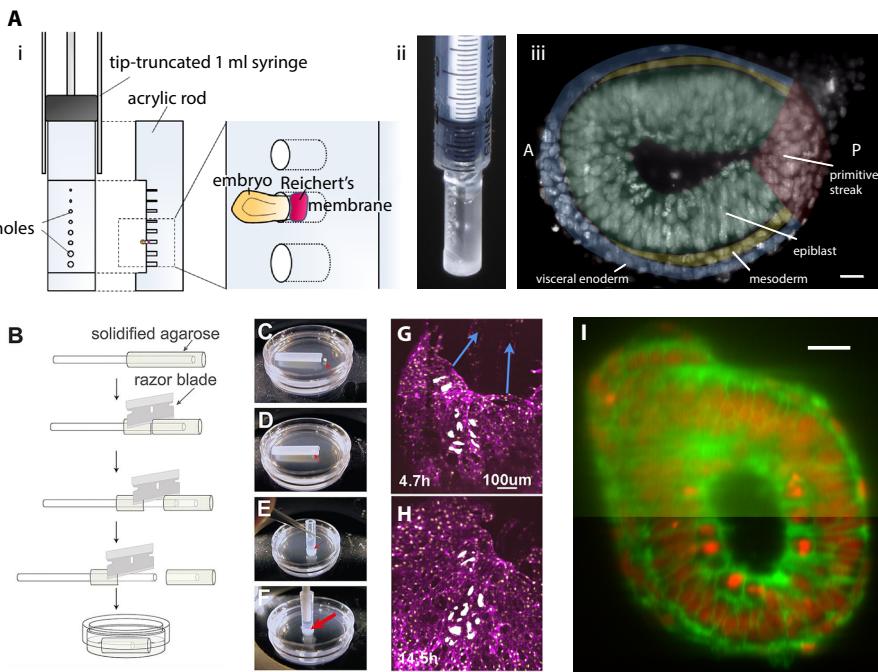
After the initial 3 days of pre-implantation, the embryo undergoes the implantation process, during which it is inaccessible to microscopical investigations. Although a new method was recently developed that allows the *in vitro* culturing of the embryos embedded in a 3D gel [76], this has not reached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

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

Light-sheet microscopy is a good choice for imaging these stages, just like in the case of pre-implantation embryos. These embryos, however, present new challenges for sample handling and culturing. Owing to their extreme sensitivity, dissection can be difficult, especially for earlier stages (E4.5). Furthermore, since the embryo is also growing during development, gel embedding is not an option, as this might constrain proper development. Thus, special handling and mounting techniques had to be developed in order to allow live 3D imaging of these specimens.

Ichikawa *et al.* [50] designed a custom mounting apparatus manufactured from acrylic

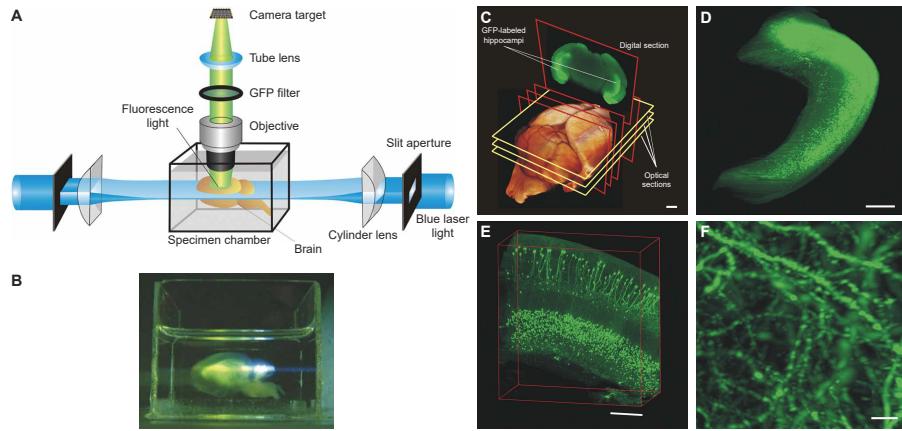
## 1.4 Light-sheet microscopy for mouse imaging



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

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

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



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

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

#### 5 1.4.3 Imaging adult mice

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

14 Light scattering and absorption depend on the tissue composition and imaging depth.  
 15 Especially the brain with a high concentration of lipids in the myelinated fibers pose a  
 16 real challenge for imaging. Live imaging is usually performed with 2-photon microscopy

## 1.4 Light-sheet microscopy for mouse imaging

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17 which can penetrate the tissue up to 800 µm deep [82]. Using fixed samples, however, the  
18 scattering problem can be eliminated by the use of tissue clearing methods.

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

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

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

## <sup>4</sup> Chapter 2

# <sup>5</sup> Image compression

<sup>6</sup> As we will see later (Section 4.1), light-sheet microscopy is capable of generating an  
<sup>7</sup> immense amount of data in a short amount of time. For this reason not only fast data  
<sup>8</sup> processing, but data compression also plays an important role when evaluating, sharing  
<sup>9</sup> and storing the images. In this chapter we will briefly introduce the core concepts of  
<sup>10</sup> image compression and show some examples as a background for Chapter 4. For a more  
<sup>11</sup> comprehensive review on data compression fundamentals, the interested reader is referred  
<sup>12</sup> to the works of Sayood [93], and Salomon and Motta [94].

<sup>13</sup> There are two distinct ways of data compression: lossless and lossy. This is an impor-  
<sup>14</sup> tant difference, and the choice between the two greatly depends on the application itself.  
<sup>15</sup> For some types of data no loss is acceptable, for example computer programs or text.  
<sup>16</sup> Here, even a small change could drastically influence the meaning of the original data.  
<sup>17</sup> For other applications, such as audio and video compression, some loss can be tolerated  
<sup>18</sup> as long as there is no perceived change or distortion. For scientific applications mostly  
<sup>19</sup> lossless compression is used. Even for image data, where lossy compression can signifi-  
<sup>20</sup> cantly increase the compression ratio (original size / compressed size), lossy compression  
<sup>21</sup> is usually not recommended, as it can alter the measurement results in unpredictable  
<sup>22</sup> ways [95].

### <sup>23</sup> 2.1 Basics of information theory

<sup>24</sup> As compression is the art of reducing the size of a message while keeping the same  
<sup>25</sup> amount of information, it is necessary to briefly discuss the theory behind information,  
<sup>26</sup> and how to quantify it. These concepts were first introduced by Shannon, in his highly  
<sup>27</sup> influential papers [96–98]. As a rigorous mathematical explanation is outside the scope  
<sup>1</sup> of this work, we refer the interested reader to the aforementioned works.

<sup>2</sup> Informally, information can be quantified as the amount of surprise. Any statement,  
<sup>3</sup> if it has a high probability carries a low amount of information, while if it has a small

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

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

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

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

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

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

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

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

## 27 2.2 Entropy coding

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

**Table 2.1: Examples of a random binary code (#1) and a prefix-free binary code (#2).** Code #2 is uniquely decodable, while for code #1 it'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

4    **Prefix-free codes**

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

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

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

25    **Huffman coding**

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

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

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

<sup>4</sup> widely used in many compression algorithms, such as in JPEG, bzip2, or DEFLATE  
<sup>5</sup> (zip) [94].

<sup>6</sup> The Huffman coding procedure is based on two observations regarding optimal and  
<sup>7</sup> prefix-free codes:

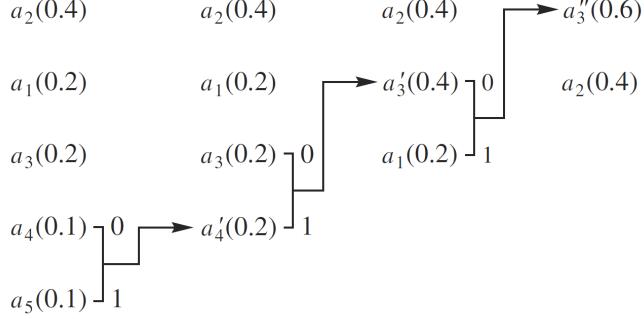
- <sup>8</sup> 1. For a letter with higher frequency the code should produce shorter codewords, and  
<sup>9</sup> for letters with lower frequency it should produce longer codewords.
- <sup>10</sup> 2. In an optimum code, the two least frequent codewords should have the same  
<sup>11</sup> lengths.

<sup>12</sup> From these statements it is trivial to see that the first is correct. If the more frequent  
<sup>13</sup> letters would have longer codewords than the less frequent letters, the average codeword  
<sup>14</sup> length (weighted by the probabilities) would be larger than in the opposite case. Thus,  
<sup>15</sup> more frequent letters must not have longer codewords than less frequent letter.

<sup>16</sup> The second statement at first glance might not be so intuitive, so let's consider the  
<sup>17</sup> following situation. Let's assume that the two least frequent codewords do not have the  
<sup>18</sup> same lengths, and the least frequent is longer. However, because this is a prefix code,  
<sup>19</sup> the second longest codeword is not a prefix of the longest codeword. This means that  
<sup>20</sup> if we truncate the longest codeword to the same length as the second longest, they will  
<sup>21</sup> still be distinct codes and uniquely decodable. This way we have a new coding scheme  
<sup>1</sup> which requires less space on average to code the same sequence as the original code, from  
<sup>2</sup> which we can conclude the original code was not optimal. Therefore, for an optimal code,  
<sup>3</sup> statement 2 must be true.

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

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



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

**Table 2.3: Huffman code table.**

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

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

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

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

4    **Limitations of Huffman coding**

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

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

20    **2.3 Decorrelation**

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

27    **Transform coding**

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

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

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

8 One drawback of frequency-based transformations, is that can't capture the spatial  
 9 To overcome this limitation, the DCT is usually performed on small chunks of the image,  
 10 such as on  $8 \times 8$  blocks in the JPEG compression. The other option is to use non-periodic  
 11 base functions to represent the signal. One good example for this is the use of wavelets,  
 12 and based on this, the discrete wavelet transform (DWT) [102, 103].

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

18 **Predictive decorrelation**

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

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

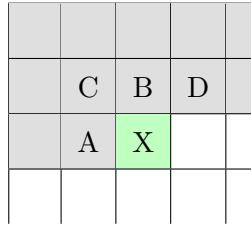
28	37	38	39	38	36	37	39	38	40	42	44	46	48
----	----	----	----	----	----	----	----	----	----	----	----	----	----

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

33	37	1	1	-1	-2	1	2	-1	2	2	2	2	2
----	----	---	---	----	----	---	---	----	---	---	---	---	---

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

2 Using a predictive scheme to remove correlations from neighboring elements is a very  
 3 good strategy for various applications, where this signal is only slowly changing. It has



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

4 been successfully implemented in audio and image compression algorithms as well, such  
 5 as lossless-JPEG [104], JPEG-LS [105], CALIC [106], SFALIC [107] and FLIC [108], just  
 6 to name a few.

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

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

The lossless part of the JPEG standard specifies 7 possible rules for the prediction step [104]:

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

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

<sup>4</sup> same plane, and calculates the prediction based on this.

<sup>5</sup> Other algorithms try to improve the prediction by adapting it depending on the local  
<sup>6</sup> texture. The LOCO-I algorithm (part of JPEG-LS), for example uses the median edge  
<sup>7</sup> detector [105]:

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

<sup>8</sup> This function tries to estimate if there is an edge close to X. If it detects a vertical edge  
<sup>9</sup> to the left of X, it picks B for the prediction, whereas if it detects a horizontal edge above  
<sup>10</sup> X it tends to pick A. If no edge is detected, the prediction is the same as predictor 4 of  
<sup>11</sup> the lossless JPEG standard.

<sup>12</sup> A useful property of the prediction-based methods is, that there is no base change as in  
<sup>13</sup> the transform coding methods, and it is possible to construct bounded lossy compression  
<sup>14</sup> algorithms. JPEG-LS, for example defines a near-lossless mode of operation, where the  
<sup>15</sup> user can select the maximum absolute reconstruction error per pixel. This is achieved by  
<sup>16</sup> quantizing the prediction residual  $\varepsilon$  with a quantization step depending on the allowable  
<sup>17</sup> error. Since the decoding algorithm simply calculates  $\hat{X} = \text{Pred}(X) + \varepsilon$ , any quantization  
<sup>18</sup> error of  $\varepsilon$  is directly reflected in the reconstructed pixel value. Since the quantization  
<sup>19</sup> error is known, and bounded by the quantization step size, the maximum reconstruction  
<sup>1</sup> error for each pixel is also bounded. This mode is sometimes used for medical imaging  
<sup>2</sup> applications, where the preservation of diagnostic quality is required by law, but higher  
<sup>3</sup> compression ratios are desired than what is possible by lossless compression.

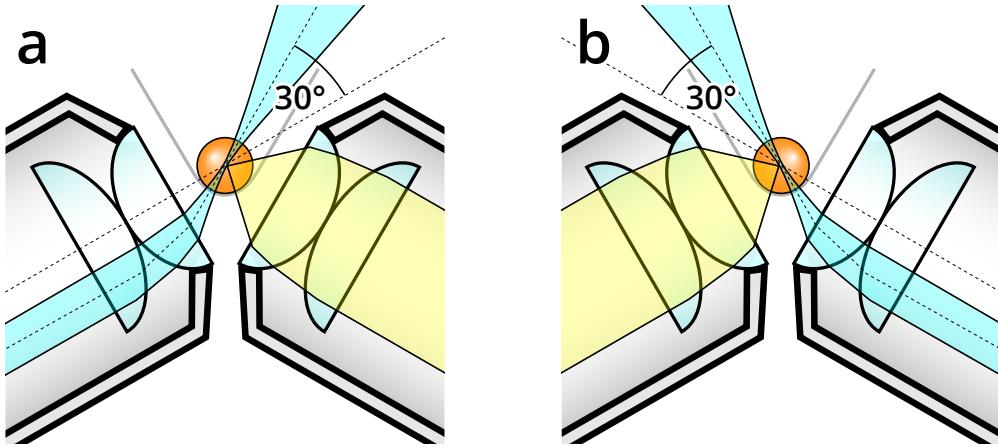
<sup>4</sup> **Chapter 3**

<sup>5</sup> **Dual Mouse-SPIM**

<sup>6</sup> Unraveling the secrets of mammalian development has been a long standing challenge  
<sup>7</sup> for developmental biologists and medical professionals alike. This phase of early life is  
<sup>8</sup> an incredibly complex and dynamic process spanning through large scales in space and  
<sup>9</sup> time. Subcellular processes at the nanoscale are happening in the range of milliseconds  
<sup>10</sup> or shorter, while whole embryo reorganizations and tissue migration events take place  
<sup>11</sup> over the course of hours [110]. Resolving these processes presents a true challenge, since  
<sup>12</sup> to understand the underlying mechanisms, molecular specificity is just as crucial as high  
<sup>13</sup> spatial and temporal resolution. As we have seen in Chapter 1, live imaging of mouse  
<sup>14</sup> embryonic development is an especially challenging task, but light-sheet microscopy can  
<sup>15</sup> offer a good solution owing to its gentle optical sectioning and fast image acquisition. The  
<sup>16</sup> Mouse-SPIM [J2] introduced earlier ( Section 1.4.1) also solves the issue of live imaging  
<sup>17</sup> with its inverted design: the embryos are held by a foil which not only allows easy sample  
<sup>18</sup> handling but also acts as a barrier that isolates the embryos from the immersion medium.

<sup>19</sup> As all microscopes, the Mouse-SPIM also had to make a compromise: although its  
<sup>20</sup> design allows long-term live imaging, it only has a single detection view, as rotation is  
<sup>21</sup> not possible with the sample mounting trays. Due to this configuration, its resolution is  
<sup>22</sup> inherently anisotropic, having an axial-to-lateral resolution ratio of around 3. Although  
<sup>23</sup> this is sufficient for many applications, such as cell tracking, for detecting subcellular  
<sup>24</sup> features it might be limiting. One such example is chromosome tracking, which could shed  
<sup>25</sup> light on chromosome missegregation mechanisms in the early embryonic development,  
<sup>26</sup> which is the cause of many congenital diseases also affecting humans.

<sup>27</sup> In this chapter, we present a novel light-sheet microscope developed to address the  
<sup>28</sup> above challenges by using high NA objectives for subcellular isotropic resolution and low-  
<sup>29</sup> light imaging, and offering multi-view detection without having to rotate the samples.  
<sup>1</sup> The microscope is designed to be live imaging compatible, offering new perspectives  
<sup>2</sup> in the research of mouse embryonic development. This chapter will describe the design  
<sup>3</sup> concepts for the microscope, the optical layout, alignment strategies, and the result of



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

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

## 5 3.1 Microscope design concept

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

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

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

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

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

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

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

#### 3.1.1 Light-sheet design

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

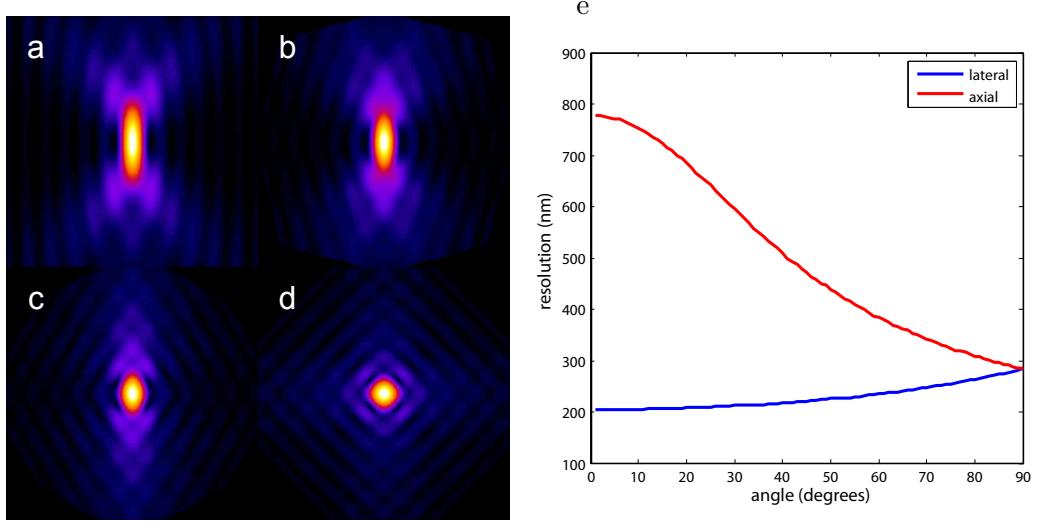
$$f_o = \frac{f_{tl}}{M} = \frac{200 \text{ mm}}{25} = 8 \text{ mm}, \quad (3.1)$$

and the back aperture diameter is 17.6 mm.

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

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

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



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

4 maintain an even illumination (Figure 1.12c). Because the size of an early mouse embryo  
 5 is around  $80\text{ }\mu\text{m}$ , we require the length and the height of the light-sheet to be at least  
 6  $100\text{ }\mu\text{m}$ .

### 7 The length and thickness of the light-sheet

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

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

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

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

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

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

4 From this, the divergence angle of the beam is

$$\theta_0 = \frac{\lambda}{\pi W_0, y} = 55.74 \text{ mrad} = 3.196^\circ \quad (3.6)$$

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

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

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

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

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

13 **The height of the light-sheet**

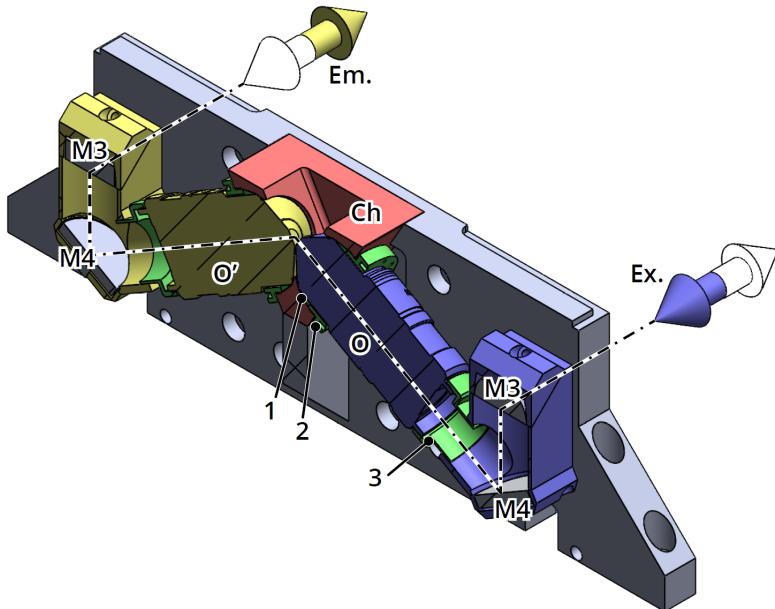
14 The height of the light-sheet can be adjusted by changing the beam scanning am-  
15 plitude with the galvo mirror. To scan the entire field of view of  $h_{FOV} = 270 \mu\text{m}$ ,  
16 the scanning angle range at the back focal plane of the objective will need to be  
17  $\theta = \tan^{-1}(h_{FOV}/2/f_o) = \pm 0.967^\circ$ .

18 **3.2 Optical layout**

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

24 **3.2.1 Core unit**

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



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

#### 4 Chamber

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

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

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

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

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

14 **Mirror blocks**

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

28 **3.2.2 Illumination**

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

32 The illumination arm has three main roles:

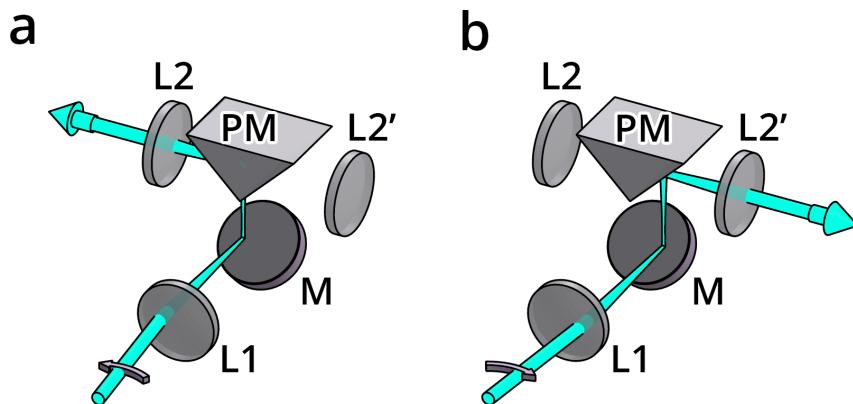
- 33 1. expands the laser beam to the calculated 1.2 mm size.
- 34 2. images the galvo scanner to the back focal plane of the objective
- 1 3. switches the laser light between the two objectives during imaging

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

### 3.2 Optical layout

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

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



**Figure 3.4: Illumination branch splitting unit.** To divert the beam to either side, a right angle prism mirror is used in conjunction with a galvanometric scanning mirror. L1 acts as a scan lens, thus the beam is translated on mirror M. Depending on the 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.

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

Due to the arrangement of the bottom mirror and the prism mirror, the scanning direction will be rotated by  $90^\circ$ . This will result in a vertical scanning plane, which is exactly what we need to generate the light-sheet on the sample (see Section 3.2.1).

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

9     **3.2.3 Detection**

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

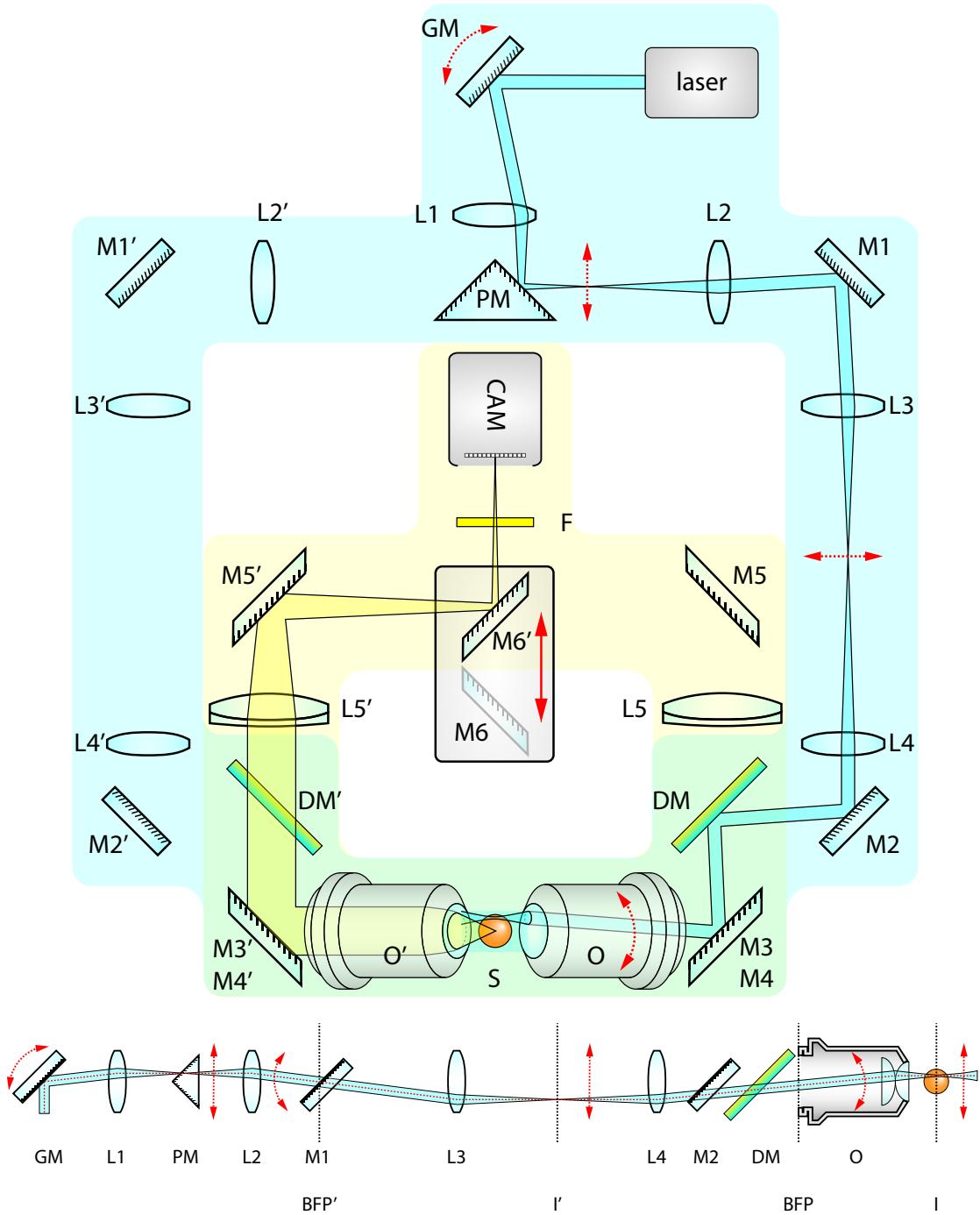
18    Similarly to the common galvo in the illumination path, the two detection arms  
19    share the same camera. Although two cameras could also be used, due to the operating  
20    principle of the microscope, the two objective are not used for imaging at the same time.  
21    This means a single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

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

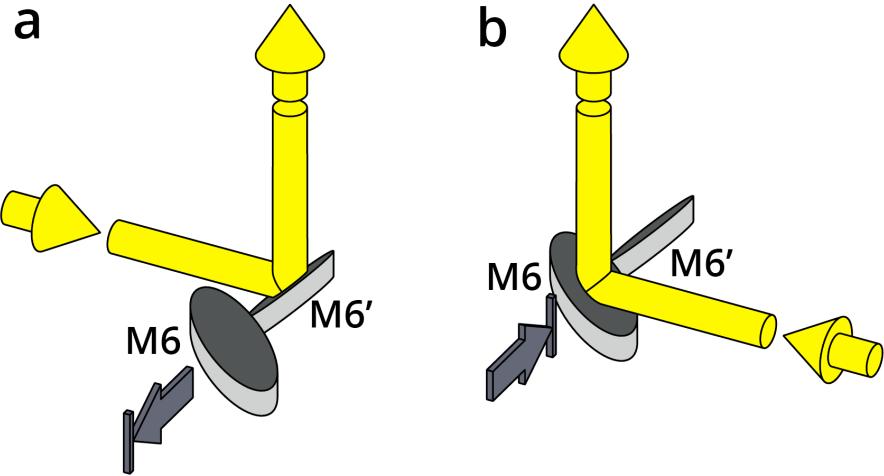
28    Moving the switcher unit is performed by a small, 10 mm diameter pneumatic cylinder  
29    (Airtac, HM-10-040) that is actuated by an electronically switchable 5/2 way solenoid  
30    valve (Airtac, M-20-510-HN). This solution offers a very fast switching between views,  
31    up to 5 Hz, depending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

33    **3.3 Optical alignment**

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



**Figure 3.5: Dual Mouse SPIM optical layout.** The microscope consists of two main parts, the illumination branches (blue) and detection branches (yellow). For both illumination and detection there are two identical paths implemented. Illumination direction can be changed by applying a different offset to the 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



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

#### 4 3.3.1 Alignment of illumination branches

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

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

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

23 After rough alignment of the illumination arms, when the laser is already coupled  
 1 in the objective, the fine adjustments are performed based on the image of the beam  
 2 through the other objective. The beam is visualized by filling the chamber with a 0.1%  
 3 methylene blue solution. As this solution is fluorescent, and can be excited in a very large

4 range, it is well suited to visualize the beam during adjustment.

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

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

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

#### **4 3.3.2 Alignment of the detection branches**

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

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

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

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

34 The correction collars are adjusted by inspecting a gel-suspended fluorescent bead  
35 specimen with the microscope, where the beads can act as a reporter of the point spread  
36 function of the microscope. The alignment can be performed "live" by inspecting the  
1 bead image quality for any aberrations. By gradually changing the correction collar, the  
2 ring artifacts are minimized on out-of-focus beads, and the peak intensity is maximized  
3 for in-focus beads. By moving the correction ring, the focal plane is also slightly shifted,

4 which has to be compensated by shifting the light-sheet correspondingly to coincide with  
5 the correct imaging plane.

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

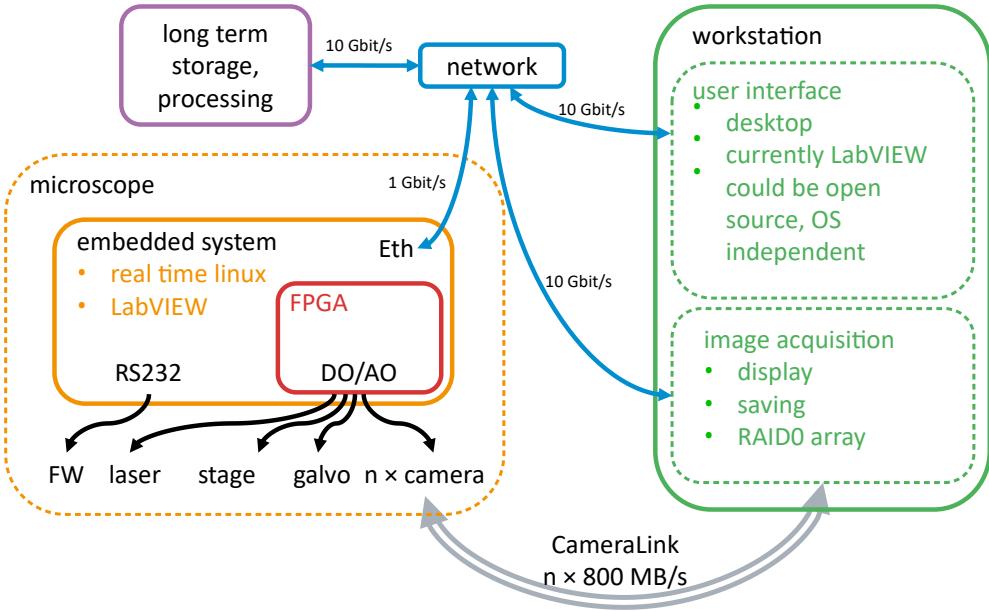
12 Field of view alignment can be performed by using mirror M4 just before the detection  
13 merging unit. To identify the center region of the field of view, diffuse white light is used  
14 to illuminate the entire sample chamber and is imaged on the camera. Then, mirror  
15 M4 is adjusted until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

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

## 26 3.4 Control unit

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

1 The embedded system is complemented by a high-performance workstation that is  
2 used to display the user interface of the microscope, and to record the images of the  
3 high-speed scientific complementary metal–oxide–semiconductor (sCMOS) camera.



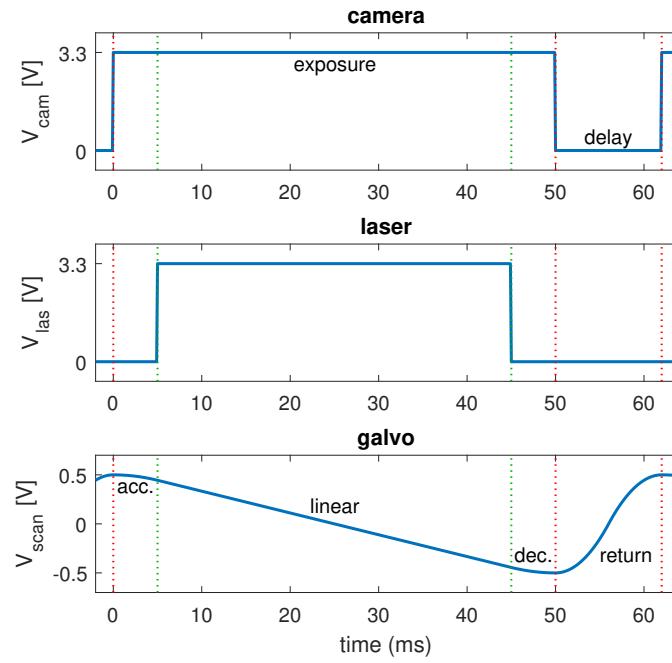
**Figure 3.7: Microscope control hardware and software architecture.** The embedded system responsible for the hardware control and the workstation are communicating through the network using the WebSocket protocol. The electronic devices of the microscope are either controlled through digital/analog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

#### 3.4.1 Hardware

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

	<b>digital input</b>	<b>analog input</b>	<b>serial communication</b>
12	camera exposure	galvo position	filter wheel
	laser on/off ( $\times 3$ )	laser intensity ( $\times 3$ )	stages ( $\times 2$ )

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



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

#### 4 3.4.2 Software

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

#### 14 **FPGA software**

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

2 To describe the traces, we define them as a concatenation of *sections*. Each section has  
 3 3 or 5 parameters, depending on whether they are digital or analog. Both types have three

4 common properties: `value`, `length`, and `type`. The analog sections additionally contain a  
5 `dValue` and a `ddValue` element describing the velocity and the acceleration of the signal.  
6 This allows us to generate piecewise functions made of second-order polynomials.

7 The `type` element contains information on which value should be updated for the  
8 current section. Setting the lowest bit high will update the `value`, setting the second bit  
9 high will update the `dValue`, and setting the third bit high will update the `ddValue`.  
10 This feature allows to define smooth transitions between the sections, and also to define  
11 more complex signals, as long as they are periodic in the second derivative.

## 12 3.5 Results

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

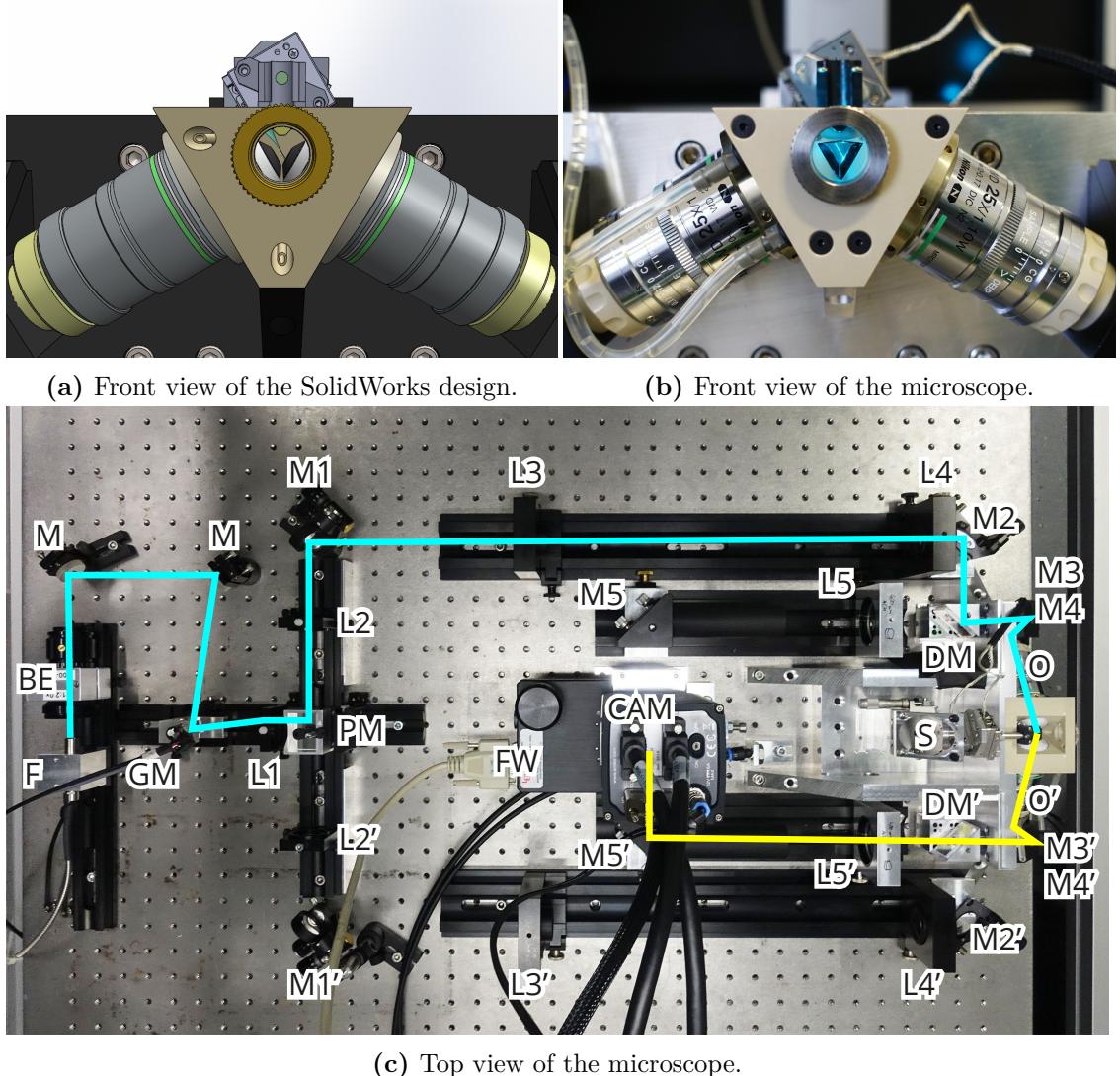
19 To evaluate the performance of the microscope, we conducted various measurements,  
20 concerning the stability and the resolution of the system. The methods and results of  
21 these measurements will be presented in this section.

### 22 3.5.1 Stability of view switcher unit

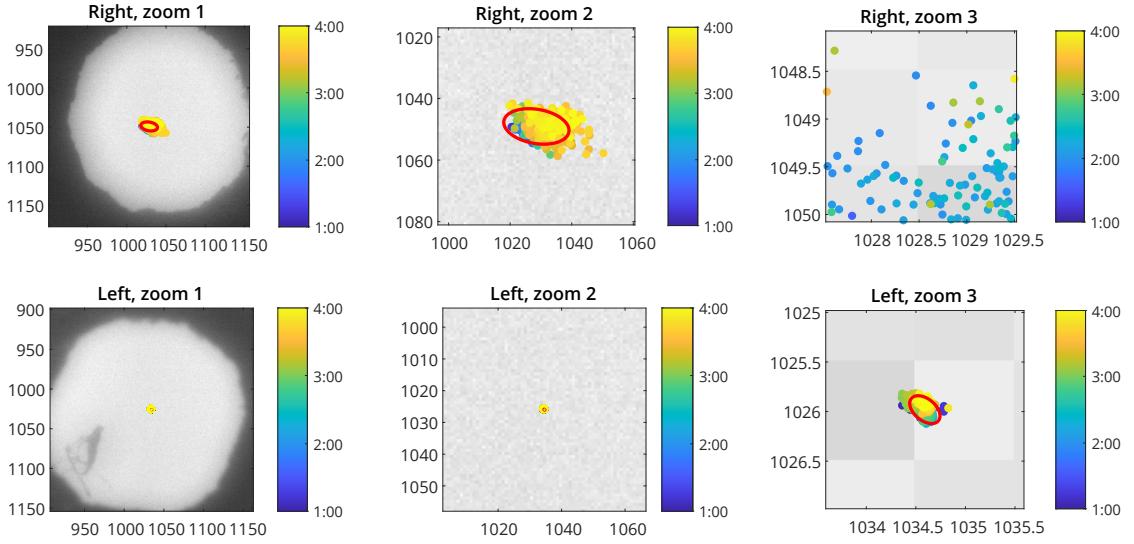
23 As the view switcher unit is a custom-designed solution for this microscope, it is necessary  
24 to evaluate the effect of the switching on the field of view. Given that this is a moving  
25 unit, many mechanical imprecisions can introduce a drift in the final image.

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

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



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



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

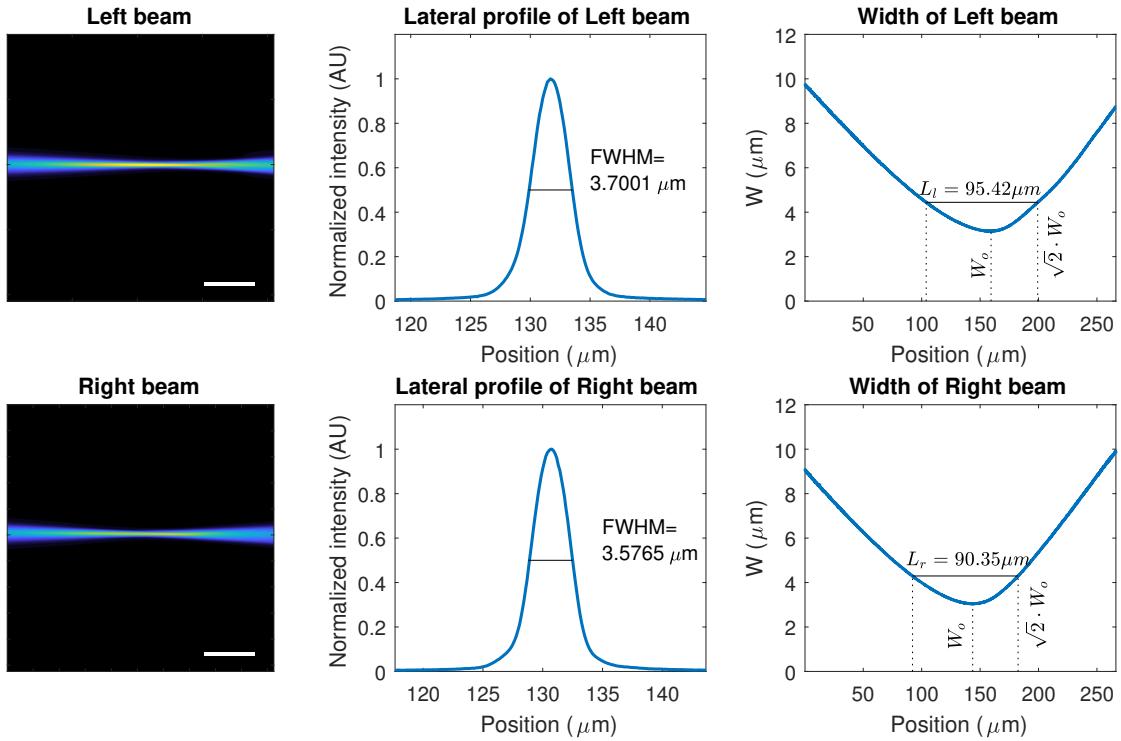
4 was only 0.07 px and 0.04 px for the long and short axes respectively. This result implies  
5 that there is no conceptual limitation in reaching sub-pixel reproducibility with the view  
6 switching unit, although some optimization is still needed for the right view to reach the  
7 same stability as the left view.

#### 8 3.5.2 Characterizing the illumination profile

9 Since a Gaussian beam is used for illumination, its intensity profile depends on the axial  
10 position (Equation 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  
12 any non-uniformities in the illumination pattern, we measured the illumination beam  
13 intensity for each view.

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

20 To determine the beam waist position, we measured the beam thickness (FWHM)  
1 for each column of the averaged images, and located the position of the minimal width.  
2 The beam width at the waist position for the left view was  $3.70 \mu\text{m}$ , and for the right  
3 view it was  $3.58 \mu\text{m}$  (Figure 3.11). We also measured the usable length of the beams



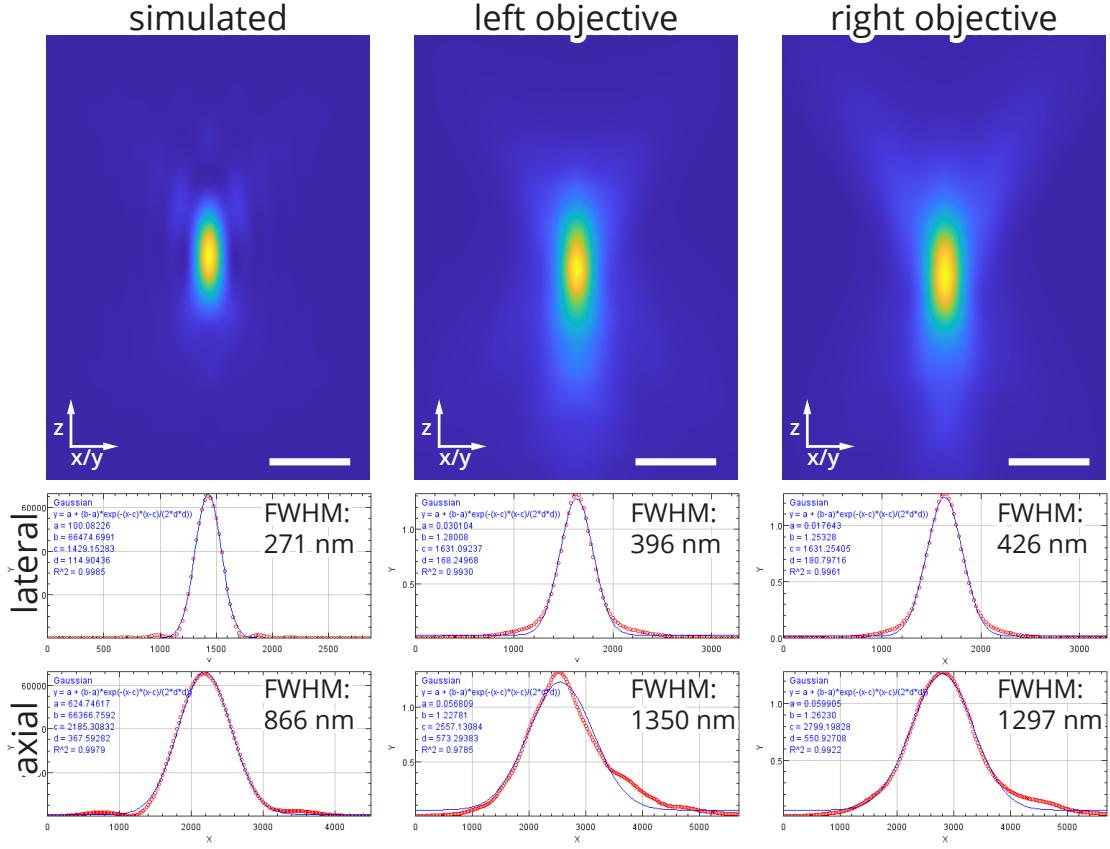
**Figure 3.11: Illumination beam profile.** Average of 500 stationary beam images for both left (top left) and right objectives (bottom left). Beam intensity profile along the waist of each beam is plotted in the center column. The right column shows the beam width profile of each beam ( $1/e^2$ ).

4 (Section 1.3.1). The distance between the points where the beam has expanded by a  
 5 factor of  $\sqrt{2}$  relative to the waist is  $95.42 \mu\text{m}$  for the left view, and  $90.35 \mu\text{m}$  for the  
 6 right view. This corresponds well to the original requirement of a minimal field of view  
 7 of  $100 \mu\text{m}$ . Depending on the sample and the required level of optical sectioning, the  
 8 practical field of view can be larger than this, as the camera sensor allows for a field of  
 9 view of  $266 \mu\text{m}$ .

### 10 3.5.3 Resolution and point spread function measurement

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

3 It is apparent from the simulations that even though the FEP refractive index is

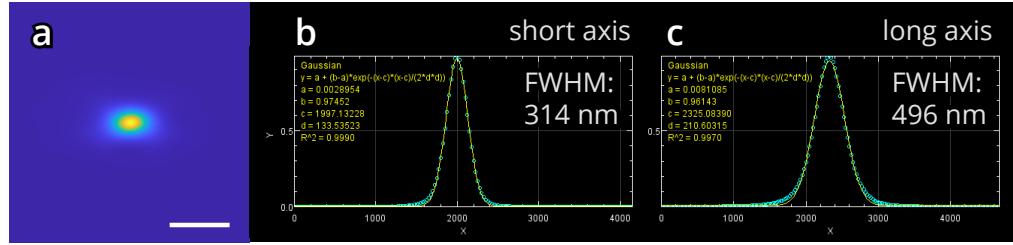


**Figure 3.12: Simulated and measured PSF of Dual Mouse-SPIM.** Top row: axial sections of simulated and measured point spread functions. Middle row: lateral intensity profile and Gaussian fit. Bottom row: Axial intensity profile and Gaussian fit. Simulations were performed based on the Gibson-Lanni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

almost identical to the refractive index of water, a slight spherical aberration is still present even for the ideal case (Figure 3.12, left). The resolution measured as the FWHM of the intensity profile through the lateral and axial cross sections is 271 nm and 866 nm respectively. FWHM was measured in Fiji by fitting a Gaussian curve, and multiplying the standard deviation of the resulting fit by  $2\sqrt{2 \ln 2}$ .

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

From both views 12 beads were averaged using Fiji [116] and the 3D PSF estimator of the MOSAIC suite [117]. In order to acquire a more accurate PSF, the averaged bead images were deconvolved with the ideal image of the bead (uniform sphere with a



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

4 diameter of 500 nm) using the DeconvolutionLab2 Fiji plugin [118]. The results of the  
 5 deconvolution are shown on Figure 3.12. Similarly to the simulation, we measured the  
 6 axial and lateral resolutions on the experimental PSFs. For the left view, the measured  
 7 resolutions were 386 nm in the lateral direction, and 1350 nm in the axial direction. For  
 8 the right view the lateral resolution was 326 nm, and the axial was 1297 nm.

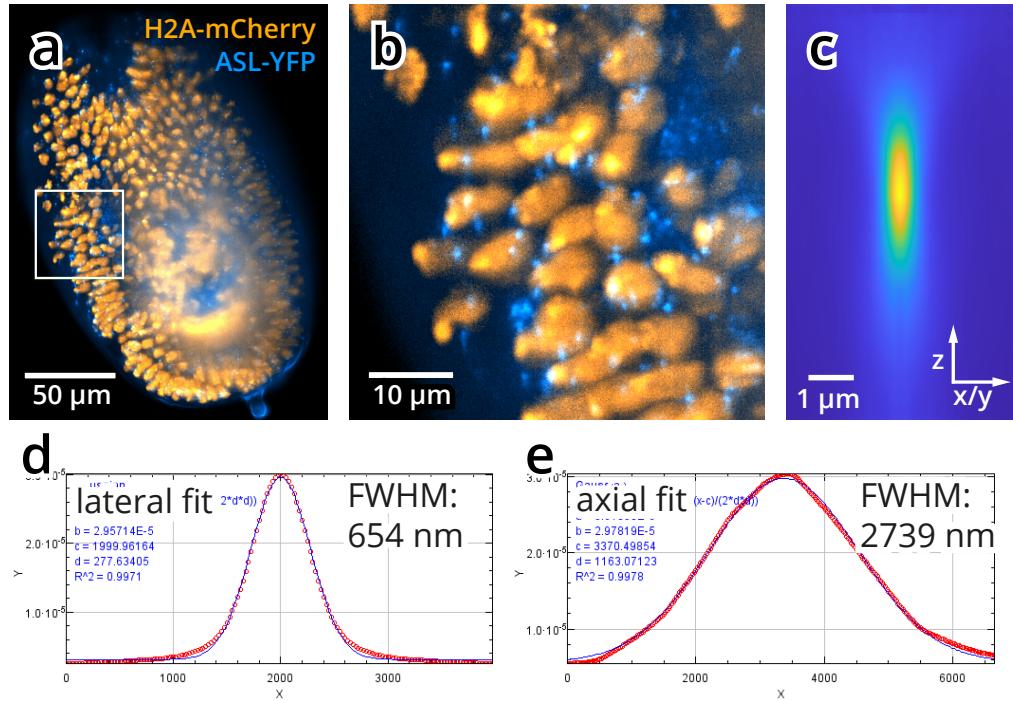
9 To estimate the achievable multi-view resolution of the system, we combined the two  
 10 measured PSFs. First, the PSFs were rotated by  $\pm 60^\circ$  to correspond to the objective  
 11 orientations. The PSF images were then normalized by scaling the maximum to 1, and  
 12 the two rotated and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

#### 18 3.5.4 Resolution inside a *Drosophila melanogaster* embryo

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

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

2 Similarly to the bead recordings, 15 centriole images were averaged with the 3D PSF  
 3 tool of the MOSAIC plugin in Fiji (Figure 3.14c). On the averaged image we measured



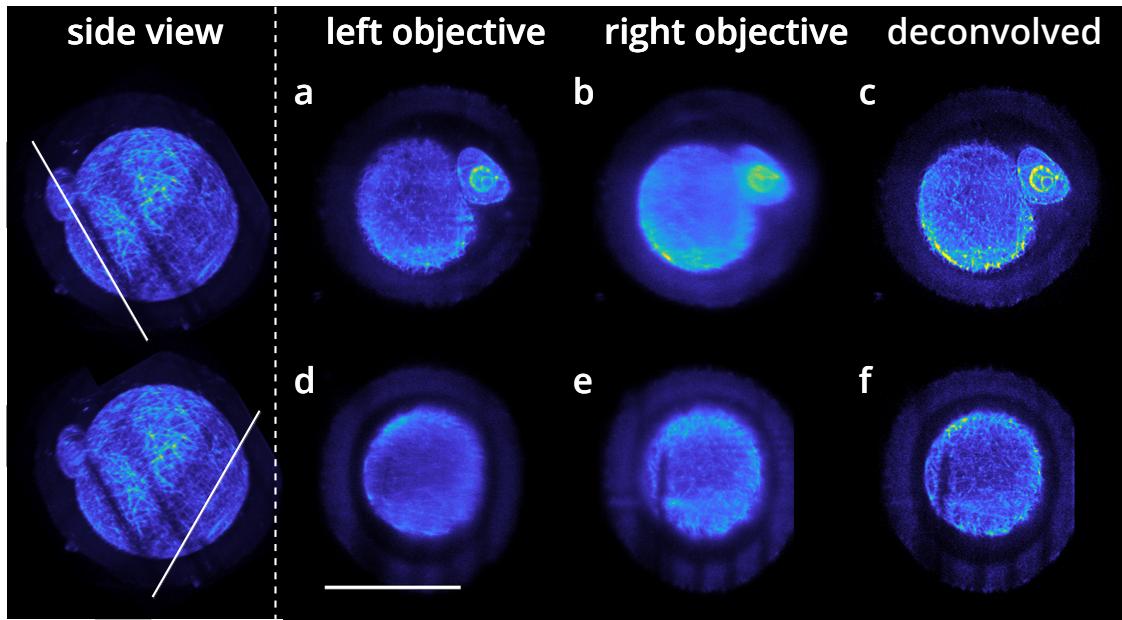
**Figure 3.14: Maximum intensity projection of a *Drosophila melanogaster* embryo recording.** Maximum intensity projection of a 30 μm thick substack. Orange: nuclei (H2A-mCherry), blue: centrioles (ASL-YFP). 50x magnification. (a) Overview of the embryo. (b) Zoomed in view to the region marked in (a).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

the resolution by fitting a Gaussian function on the lateral and axial intensity profiles (Figure 3.14d,e), and calculating the FWHM of the fitted Gaussian. The size of the averaged centrioles was 654 nm in the lateral, and 2739 nm in the axial direction.

### 3.5.5 Multi-view imaging of a mouse zygote

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

Anisotropy in the resolution becomes apparent in the single-view recordings, when the stacks are resliced from a different direction than the imaging plane. Even though the native view of each objective (Figure 3.15a,d) shows good contrast, and easy to recognize features, when rotated and sliced corresponding to the view of the other objective, the contrast is almost completely gone, and resolution is decreased (Figure 3.15b,e). The fused stack, after the multi-view deconvolution contains the high resolution information from both views, and thus both directions show good quality and high contrast



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

<sup>4</sup> (Figure 3.15c,f).

## <sup>5</sup> 3.6 Methods

### <sup>6</sup> 3.6.1 Preparation of fluorescent bead samples

<sup>7</sup> For registration and resolution measurements we used TetraSpeck 0.5 µm diameter fluo-  
<sup>8</sup> rescently labeled beads (ThermoFisher, T7281). The stock bead solution was thoroughly  
<sup>9</sup> vortexed and sonicated for 5 min before diluting it 1:100 in distilled water. The diluted  
<sup>10</sup> bead solution was stored at 4 °C until use. GelRite (Sigma-Aldrich, G1910) gel was pre-  
<sup>11</sup> pared in distilled water at 0.8% concentration with 0.1% MgSO<sub>4</sub> · 7H<sub>2</sub>O and kept at  
<sup>12</sup> 70 °C until use. 50 µl of the diluted bead solution was added with a heated pipette tip to  
<sup>13</sup> 450 µl of gel solution at 70° to prevent polymerization. The gel was thoroughly vortexed,  
<sup>14</sup> and loaded to glass micropipettes (Brand 100 µl). The gel was allowed to cool to room  
<sup>15</sup> temperature and stored in a petri dish under dH<sub>2</sub>O at 4° until use. For imaging, a small  
<sup>1</sup> piece of gel was extruded from the capillary, cut off, and placed in the sample holder.  
<sup>2</sup> After positioning the gel to the bottom of the sample holder, the holder was filled with  
<sup>3</sup> 200 µl dH<sub>2</sub>O.

**4    3.6.2 Preparing the sample holder**

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

**17    3.6.3 Drosophila embryo imaging**

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

**24    3.6.4 Mouse zygote imaging**

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

## **4 3.7 Conclusions**

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

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

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

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

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

<sup>4</sup> Chapter 4

<sup>5</sup> GPU-accelerated image  
<sup>6</sup> processing and compression

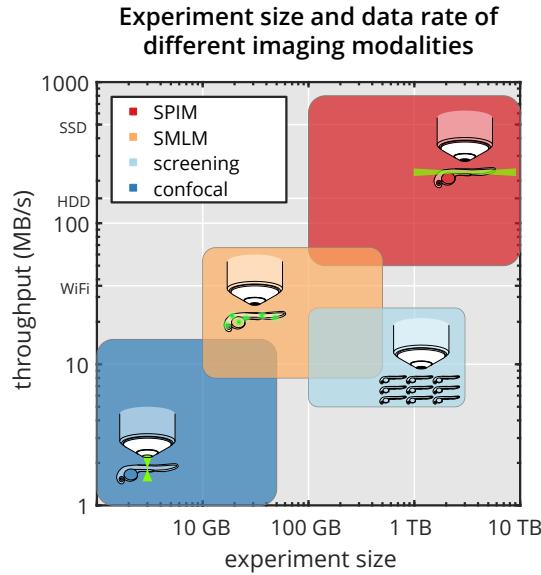
<sup>7</sup> 4.1 Challenges in data handling for light-sheet microscopy

<sup>8</sup> When using any kind of microscopy in research, image processing is a crucial part of  
<sup>9</sup> the workflow. This is especially true for light-sheet microscopy, since it is capable of  
<sup>10</sup> imaging the same specimen for multiple days, producing immense amounts of data. A  
<sup>11</sup> single overnight experiment of *Drosophila* development (which is a very typical use-case  
<sup>12</sup> for light-sheet microscopy) can produce multiple terabytes of data.

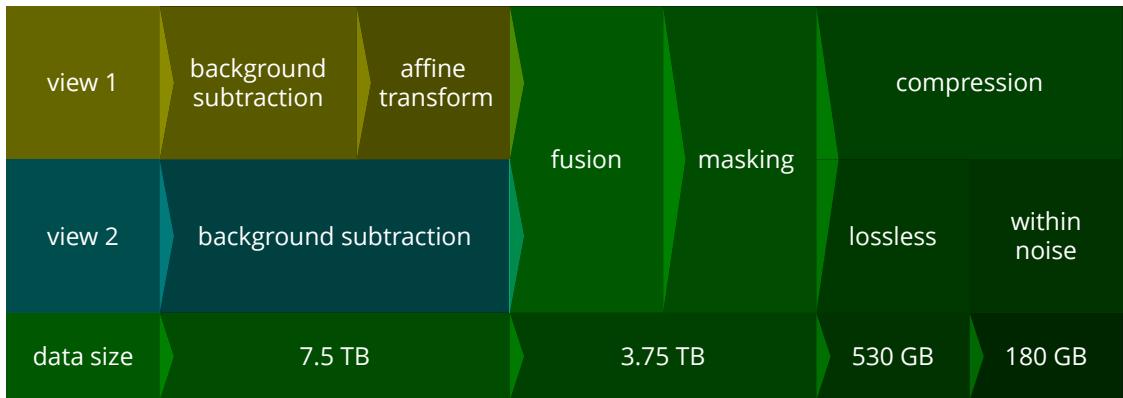
<sup>13</sup> Apart from light-sheet microscopy, many other microscopy modalities also suffer from  
<sup>14</sup> this problem. Methods, such as high content screening [124–126], where tens of thousands  
<sup>15</sup> of different genotypes are imaged generating millions of images; and single molecule  
<sup>16</sup> localization microscopy (SMLM) [127–129], where just a single plane of a sample is  
<sup>17</sup> imaged hundreds of thousands of times to acquire super-resolved images.

<sup>18</sup> Not only these methods are capable of generating data extremely fast, but with  
<sup>19</sup> the sustained high data rate a single experiment can easily reach multiples of terabytes  
<sup>20</sup> (Figure 4.1). Handling this amount of data can quickly become the bottleneck for many  
<sup>21</sup> discoveries, which is a more and more common issue in biological research [130–132].

<sup>22</sup> This chapter will focus on addressing these challenges by presenting a real-time,  
<sup>23</sup> GPU-based image preprocessing pipeline developed in CUDA [133] consisting of two  
<sup>24</sup> parts (Figure 4.2). The first part is a fast image fusion method for our workhorse light-  
<sup>25</sup> sheet microscope, the MuVi-SPIM [45], that enables live fusion of the images arriving  
<sup>26</sup> from two opposing cameras. The second part of the pipeline, which can also be used  
<sup>1</sup> in a standalone way, is a real-time image compression library that allows for lossless  
<sup>2</sup> and noise-dependent lossy compression of the data directly during acquisition even for  
<sup>3</sup> high-speed sCMOS cameras.



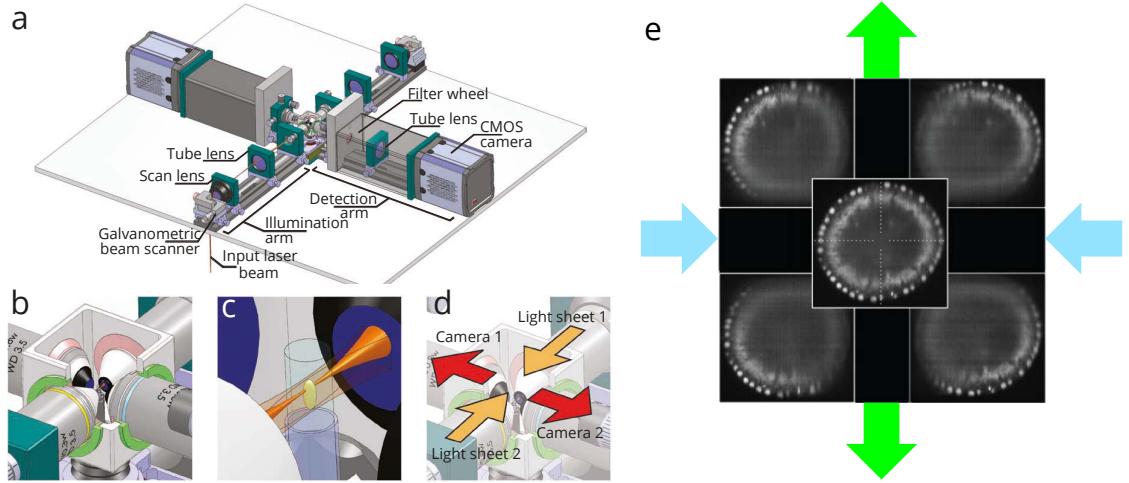
**Figure 4.1: Experiment sizes and data rate of different imaging modalities.** Comparison of single-plane illumination microscopy (SPIM, red), high-content screening (light blue), single molecule localization microscopy (SMLM, orange) and confocal microscopy (blue) by typical experiment size and data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



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

## 4.2 Real-time preprocessing pipeline

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



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

#### 4.2.1 Multiview SPIM for *in toto* imaging

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

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

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

4    **4.2.2 Image registration**

5    To perform image fusion of multiple views, first image registration is necessary: the  
 6    coordinate systems of the views have to be properly overlapped. Ideally a single mirroring  
 7    transformation would be enough to superpose the two camera images, however in practice  
 8    the microscope can never be aligned with such precision. Other types of transformations  
 9    are also necessary: translation to account for offsets in the field of view; scaling in case of  
 10   slightly different magnifications; and also shearing if the detection plane is not perfectly  
 11   perpendicular to the sample movement direction [136]. To combine all of these effects,  
 12   a full, 3D affine transformation is necessary to properly align the two camera images  
 13   (Figure 4.4 a). This transformation can be represented by a matrix multiplication with  
 14   12 different parameters:

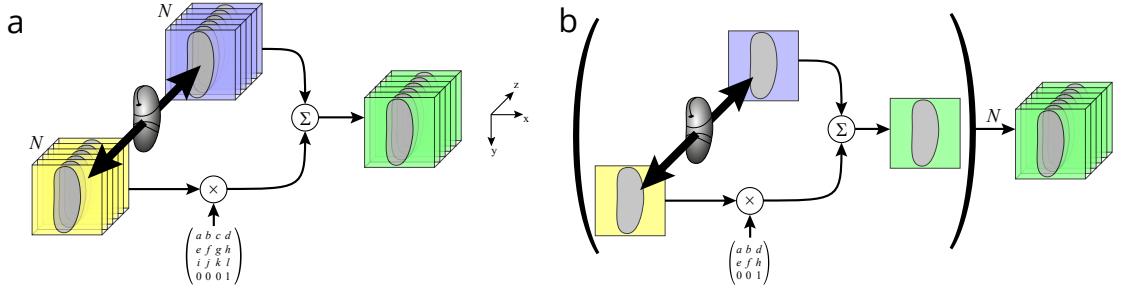
$$\begin{pmatrix} x, \\ y, \\ z, \\ 1 \end{pmatrix} = \begin{pmatrix} a & b & c & d \\ e & f & g & h \\ i & j & k & l \\ 0 & 0 & 0 & 1 \end{pmatrix} \times \begin{pmatrix} x \\ y \\ z \\ 1 \end{pmatrix} = \begin{pmatrix} ax + by + cz + d \\ ex + fy + gz + h \\ ix + jy + kz + l \\ 1 \end{pmatrix}$$

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

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

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

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



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

4 the sample [75].

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

### 12 4.2.3 2D fusion of opposing views

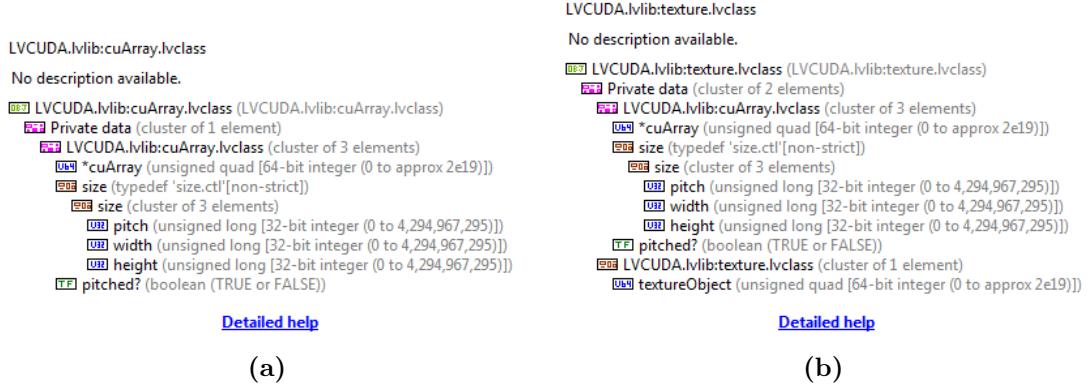
13 To speed up the image processing, we take a new approach to fusing the images. As the  
14 two objectives of MuVi-SPIM ideally image the same \$z\$ plane, it should be possible to  
15 reduce the alignment problem to a 2D affine transformation:

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

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

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

## 4.2 Real-time preprocessing pipeline



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

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

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

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

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

4 where  $\sigma_{xy}$  is the lateral resolution, and  $\sigma_z$  is the axial resolution of the microscope, which  
 5 are 277 nm and 1099 nm respectively.

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

### 9 4.2.4 CUDA implementation of direct fusion

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

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

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

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

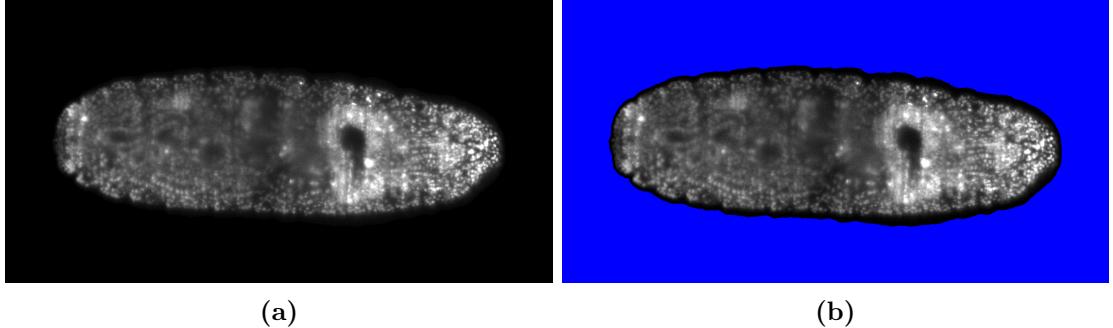
### 12 4.2.5 Background subtraction and masking

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

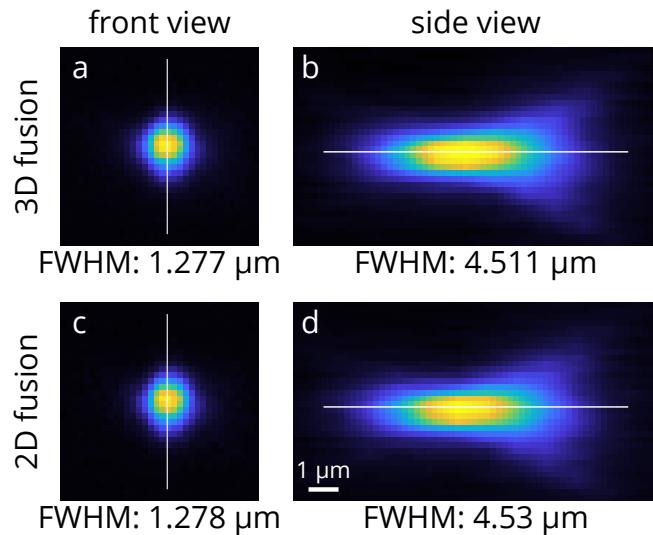
17 To perform the background subtraction, dark images are recorder without the laser  
18 turned on. Typically 500–2000 images are taken which are averaged, to capture the pixel  
19 non-uniformities of the camera sensors. As this is camera specific, the step is repeated  
20 for all cameras. If necessary,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

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

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



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



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

#### 4.2.6 Results

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

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

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

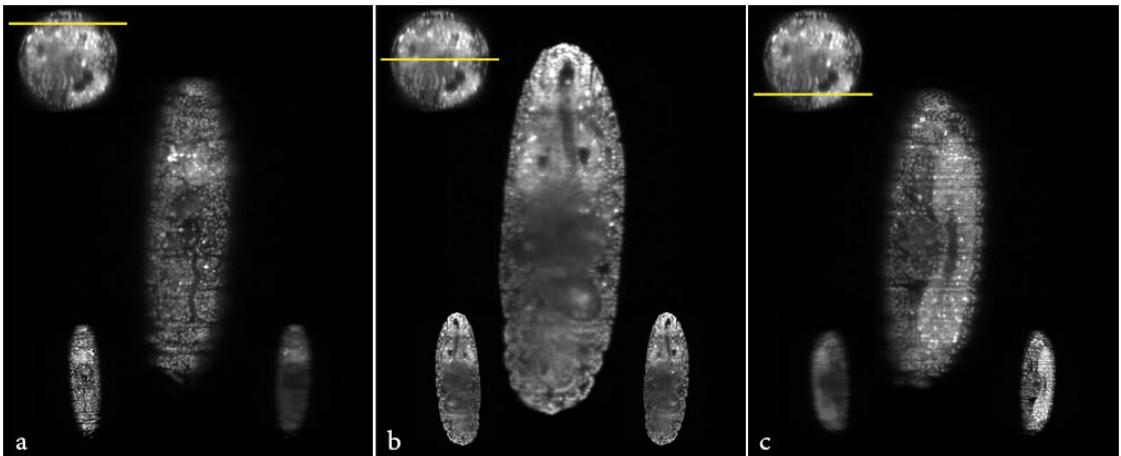
Substituting these values to Eqs. 4.1 – 4.3 and evaluating for the full range of  $x, y, z$ , the maximum contribution of the discarded coefficients for each coordinate will be:

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

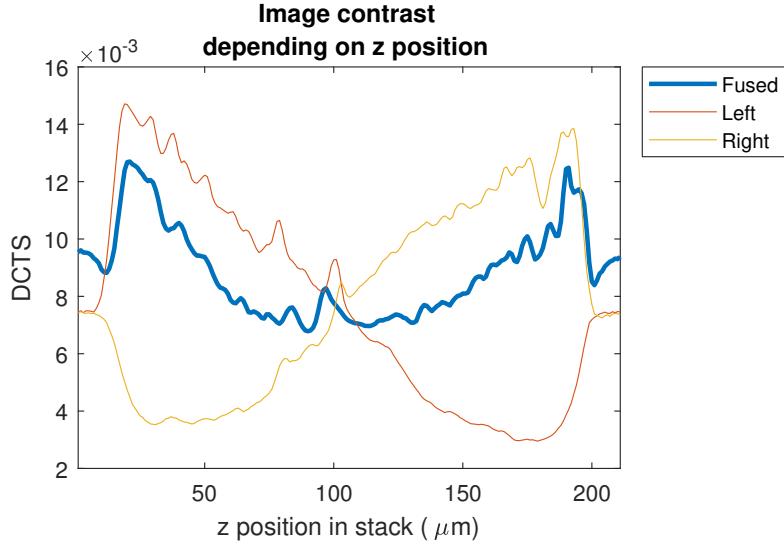
- 4 As these are below the resolution of the microscope, discarding the coefficients and
- 5 reducing the 3D transformation to a 2D transformation will not have any negative effects
- 6 on the image quality.

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

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



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



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

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

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

14 **4.2.7 Methods**

15 **Speed measurements**

16 Processing speed of the CPU and GPU implementation of the processing pipeline was  
 17 measured with the profiling tool of LabVIEW, and are an average of 10 runs. The bench-  
 1 marking computer had 2 Intel Xeon E5620 processors with 8 processing cores running at  
 2 2.4 GHz, 24 GB RAM and an Nvidia GeForce GTX 750 graphics card. Test images were  
 3 2048×2048 pixels at 16 bit depth.

#### 4.3 B<sup>3</sup>D image compression

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

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

26 To address these issues, we developed B<sup>3</sup>D, a GPU based image compression library  
27 capable of high-speed compression of microscopy images during the image acquisition  
28 process. By utilizing the massively parallel processing architecture of a GPU, we were not  
29 only able to reach a compression and decompression speed of 1 GB/s, but our algorithm  
30 also keeps the load off the CPU, making it available for other computing tasks related  
31 to operating the microscope itself.

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

4   **4.3.1 Compression algorithm**

5   **Lossless compression**

6   The algorithm design initially had three main requirements to ensure suitability for a  
 7   high-speed microscopy environment: 1) low complexity for fast execution, 2) no data  
 8   dependencies to enable parallel execution, and 3) full reversibility to ensure lossless com-  
 9   pression. Since existing image compression standards such as JPEG2000 [142] or JPEG-  
 10   LS [105] were designed to maximize compression ratio even at the expense of compression  
 11   speed, these were not suitable for our needs. Furthermore, most steps inherently require  
 12   sequential execution, thus preventing efficient parallelization. Promising efforts have been  
 13   made to enable parallel compression by simplifying JPEG-LS, by removing the limiting  
 14   steps from the algorithm (such as Golomb parameter adaptation or context modeling)  
 15   [107, 108]. Although some of these methods could reach a significant speedup with min-  
 16   imal expense in compression ratio, their performance was still not enough for real-time  
 17   compression of high-speed microscopy data.

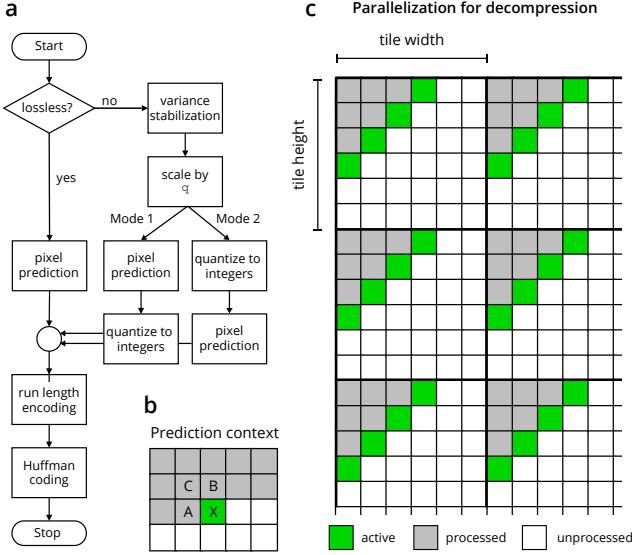
18   As in the case of other image compression methods, our algorithm has two main  
 19   components: decorrelation and entropy coding (Figure 4.10a). In this work we focused  
 20   on implementing a parallel encoder and decoder for the decorrelation part that would  
 21   also be compatible with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

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

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

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

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



**Figure 4.10: B<sup>3</sup>D algorithm schematics.**

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

4 where  $N_{th}$  is the number of threads,  $N_t$  is the number of tiles,  $a_t$  is the tile width and  
 5 height for a square tile. Although the threads can be maximized by making smaller tiles,  
 6 the compression ratio will be affected, since the corner pixel can't be predicted, and for  
 7 the edges only one neighbor can be used instead of 2. We find square tile between 16 and  
 8 64 pixels a good choice between decompression speed and compression ratio.

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

### 18 Within-noise-level compression

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

4 are smaller than the standard deviation of the noise ( $\sigma$ ), which we call within-noise-level  
5 (WNL) compression.

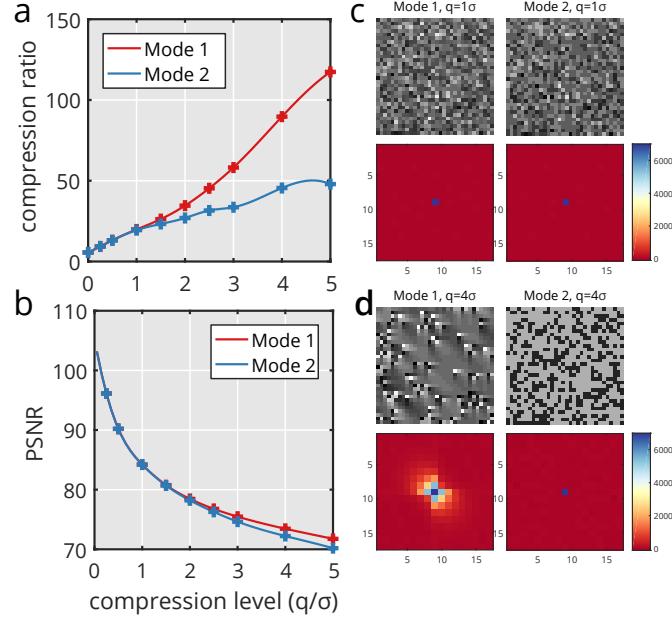
6 Our algorithm is inspired by both the near-lossless mode of JPEG-LS [105], where  
7 the maximum compression error can be set to a predefined value, and the square root  
8 compression scheme [147, 148], that quantizes its input relative to its square. We combine  
9 the two methods to achieve noise-dependent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

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

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

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

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



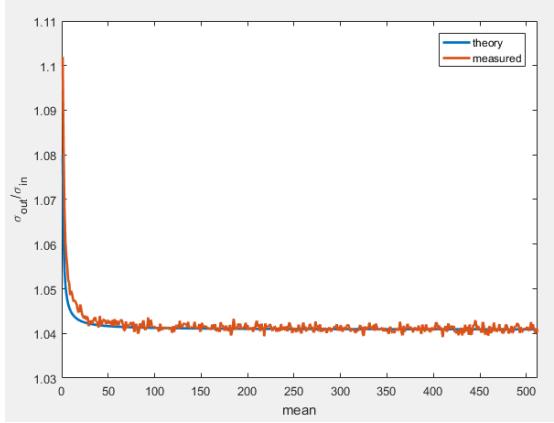
**Figure 4.11: Options for noise-dependent lossy compression.** Comparing Mode 1 (prediction then quantization) and Mode 2 (quantization then prediction) of noise-dependent lossy compression in terms of compression ratio, peak signal-to-noise ratio (PSNR) and spatial correlations introduced to random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

#### 4 Additional noise introduced by compression

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

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

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



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

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

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

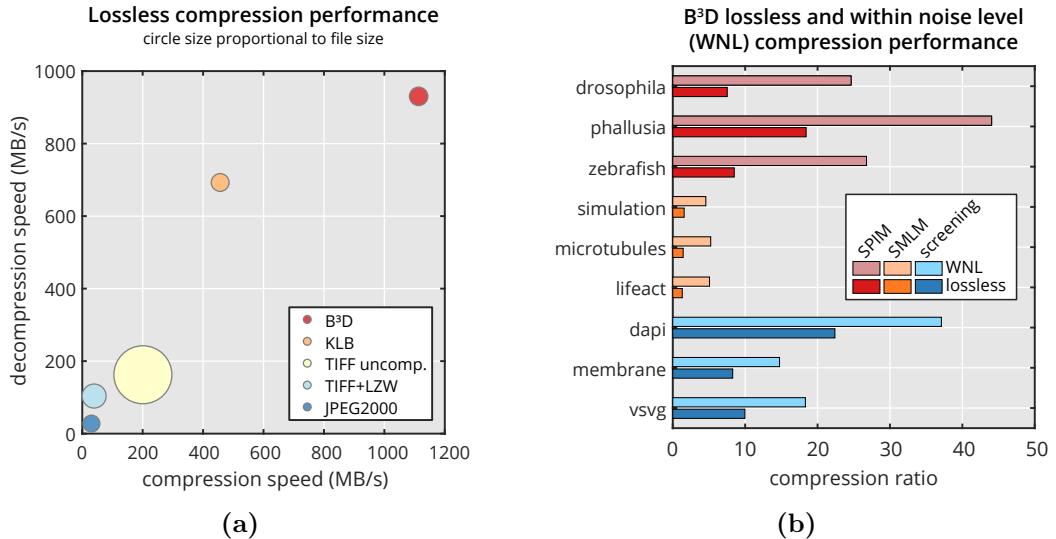
5 We verified this by compressing 512 images ( $1024 \times 1024$  pixels) of random Poisson noise  
6 with different means (1..512), and calculated the standard deviation for each frame after  
7 the decompression step. Finally, we plotted the ratio  $\sigma_{out}/\sigma_{in}$  as a function of the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

#### 10 4.3.2 Evaluation of the compression algorithm

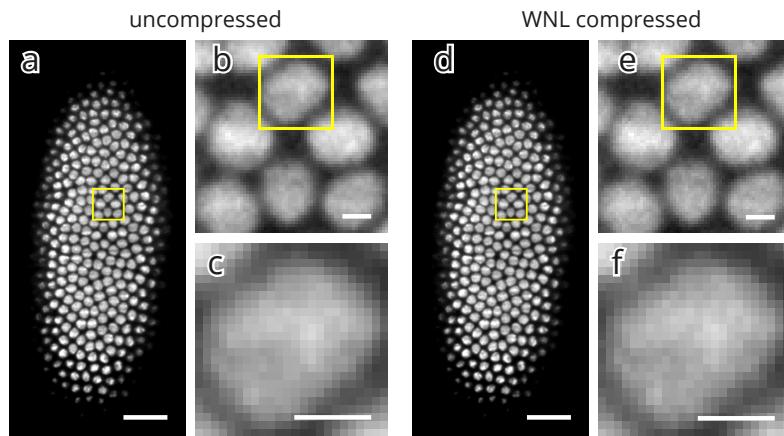
11 We compared our algorithm's performance with some of the most commonly used image  
12 formats in the scientific field: TIFF (LZW) and JPEG2000. Furthermore, we also in-  
13 cluded the state of the art KLB compression [139], which was especially designed for fast  
14 compression of large light-sheet datasets. We measured compression speed, decompres-  
15 sion speed and resulting file size for all algorithms (Figure 4.13a). Only B<sup>3</sup>D is capable  
16 of handling the sustained high data rate of modern sCMOS cameras typically used in  
17 light-sheet microscopy, while still maintaining compression ratios comparable to more  
18 complex, but much slower algorithms (Table B2 in Appendix B).

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

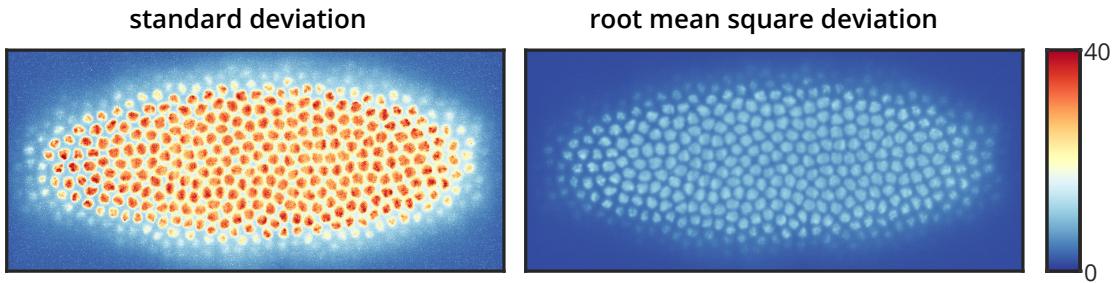
### 4.3 B<sup>3</sup>D image compression



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



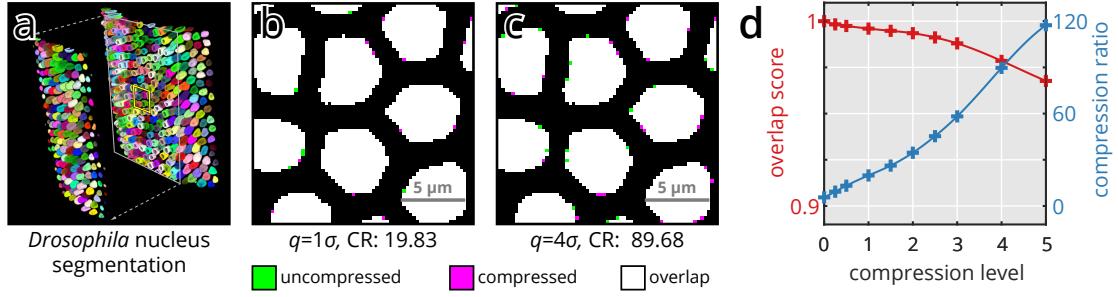
**Figure 4.14: Image quality of a WNL compressed dataset.** WNL compression of a *Drosophila* recording taken in the MuVi-SPIM setup. Compression ratio: 19.83. (a–c) Uncompressed image of the whole field of view (a), and zoomed in smaller regions (b, c). (d–f) WNL compressed image of the whole field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



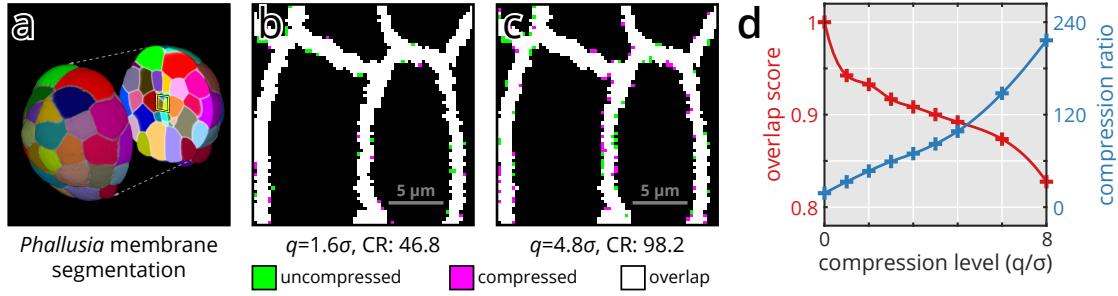
**Figure 4.15: Compression error compared to image noise.** To compare the difference arising from WNL compression to image noise, we imaged a single plane 100 times in a *Drosophila melanogaster* embryo expressing H2Av-mCherry nuclear marker at 38 ms intervals. The whole acquisition took 3.8 s, for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

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

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



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



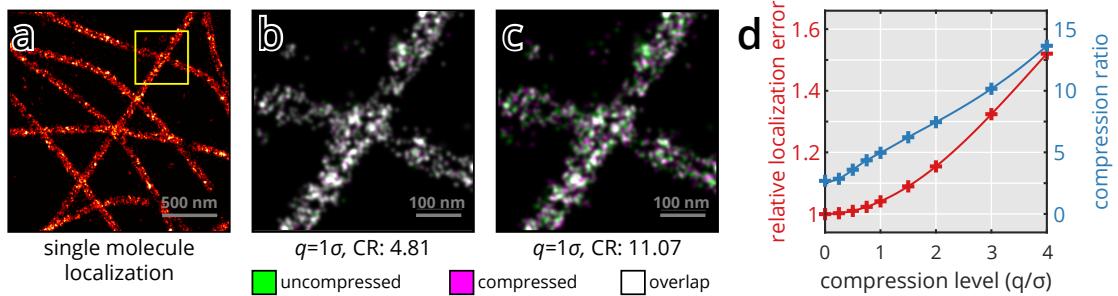
**Figure 4.17: Influence of noise-dependent lossy compression on 3D membrane segmentation.** A *Phallusia mammillata* embryo expressing PH-citrine membrane marker was imaged in MuVi-SPIM [45], and 3D membrane segmentation was performed (Section 4.3.4) (a). The raw dataset was subsequently compressed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

4 noise ratio (Figure 4.19).

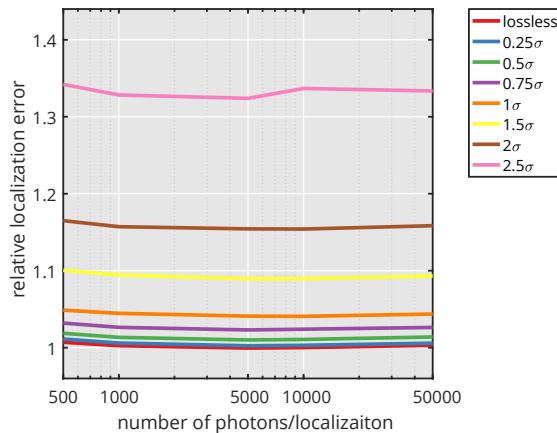
#### 5 4.3.3 HDF5 integration

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

### 4.3 B<sup>3</sup>D image compression



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



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

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

11 Writing compressed HDF5 files is possible if the user has direct control over the  
12 file writing routine, as an additional command is required to set up compression. This  
13 functionality has been tested in the following environments: C++, Python 2.7.10 and  
14 3.5.3, LabVIEW 2015.

#### 15 4.3.4 Methods

##### 16 Compression benchmarking

17 For all presented benchmarks, TIFF and JPEG2000 performance was measured through  
18 MATLAB's imwrite and imread functions, while KLB and B<sup>3</sup>D performance was mea-  
19 sured in C++. All benchmarks were run on a computer featuring 32 processing cores  
20 (2×Intel Xeon E5-2620 v4), 128 GB RAM and an NVIDIA GeForce GTX 970 graph-  
21 ics processing unit. Read and write measurements were performed in RAM to minimize  
22 I/O overhead, and are an average of 5 runs. SMLM datasets were provided by Joran De-  
23 schamps (EMBL, Heidelberg), and were originally published in [152] and [153]. Screening  
24 datasets were provided by Jean-Karim Hériché (EMBL, Heidelberg), and were originally  
25 published in [154]

##### 26 Light-sheet imaging

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

##### 32 3D nucleus segmentation

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

4 Sørensen–Dice index [155, 156]:

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

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

6 **3D membrane segmentation**

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

15 **Single-molecule localization imaging**

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

21 **Single-molecule localization data analysis**

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

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

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

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

9 **Code availability**

10 Code used for analyzing data, B<sup>3</sup>D source code and compiled binaries, including a filter  
11 plugin for HDF5, is available for download at <https://git.embl.de/balazs/B3D>.

12 **4.4 Conclusions**

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

<sup>4</sup> **Chapter 5**

<sup>5</sup> **Discussion**

<sup>6</sup> **5.1 New scientific results**

<sup>7</sup> **Thesis I.** *I have designed and constructed a new light-sheet microscope suitable for*  
<sup>8</sup> *high resolution imaging of delicate samples. A novel arrangement of two high numeri-*  
<sup>9</sup> *cal aperture objectives in 120 degrees combined with a tilted light-sheet allows for near*  
<sup>10</sup> *isotropic resolution while increasing light collection efficiency by a factor of two.*

<sup>11</sup> Corresponding publications: [J3],[J2], [J1]

<sup>12</sup> Dual Mouse-SPIM is a novel design for symmetric light-sheet microscopy. The use  
<sup>13</sup> of high NA objectives in 120° not only increases volumetric resolution compared to the  
<sup>14</sup> conventional 90° setup, but due to the larger detection angle, light collection efficiency  
<sup>15</sup> is doubled. This is especially beneficial for delicate, light-sensitive specimens, such as  
<sup>16</sup> mouse embryos, since phototoxic effects are reduced while the contrast is preserved.

<sup>17</sup> As part of the microscope I have designed a custom beam splitter unit that allows  
<sup>18</sup> the use of a single galvo scanner to generate the light-sheet for both objectives. I have  
<sup>19</sup> also designed a custom detection merging unit that allows the use of a single camera for  
<sup>20</sup> both detection views.

<sup>21</sup> I have characterized the optical properties of the microscope, measured the illumina-  
<sup>22</sup> tion profile and point spread function. With a 3.6 µm thick light-sheet, a 95 µm field of  
<sup>23</sup> view is evenly illuminated. Dual view imaging of bead samples revealed a lateral resolu-  
<sup>24</sup> tion of 314 nm, and axial resolution of 496 nm. This is a 2.67× improvement compared  
<sup>25</sup> to the axial resolution of a single lens. I have also demonstrated the imaging capabilities  
<sup>26</sup> of the microscope on *Drosophila* embryos and mouse zygotes.

<sup>1</sup> **Thesis II.** *I have developed a GPU-based image processing pipeline for multi-view*  
<sup>2</sup> *light-sheet microscopy that enables real time fusion of opposing views.*

<sup>3</sup> Corresponding publications: [C1], [C2], [C3]

4 I have developed a GPU-based image preprocessing pipeline, which integrates di-  
5 rectly to our universal microscope control software in LabVIEW. The pipeline currently  
6 supports background subtraction and background masking, furthermore it is capable of  
7 fusing opposing views of the same plane faster than real-time. I have shown that it is  
8 possible to reduce the registration of opposing camera views from a 3D alignment to a 2D  
9 alignment without any negative effects in image quality and resolution. This massively  
10 reduces the necessary computing resources, and allows the use of CUDA textures for  
11 faster than real-time image fusion. Processing speed of this implementation is 138 fps, a  
12  $18.3\times$  increase compared to a single threaded CPU implementation.

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

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

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

24 I developed a GPU-based parallel image compression algorithm called B<sup>3</sup>D, capable  
25 of over 1 GB/s throughput, allowing live image compression. To further reduce the data  
26 size, I developed a noise dependent lossy compression that only modifies the data in  
27 a deterministic manner. The allowed differences for each pixel can be specified as a  
28 proportion of the inherent image noise, accounting for photon shot noise and camera  
29 readout noise. Due to the use of pixel prediction, the subjective image quality is higher  
30 than for other methods that simply quantize the square root of the images.

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

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

35 As data integrity in microscopy is paramount for drawing the right conclusions from  
36 the experiments, using a lossy compression algorithm might be controversial. I have  
37 shown that the within noise level (WNL) mode of B<sup>3</sup>D does not significantly affect the  
1 results of several commonly used image analysis tasks. For light-sheet microscopy data I  
2 have shown that WNL compression introduces less variation to the image than the photon  
3 shot noise. When segmenting nuclei of *Drosophila* embryos and membranes of *Phallusia*

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

### 11 5.2 Application of the results

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

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

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

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

## **5.2 Application of the results**

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<sup>3</sup> (camera and sensor manufacturing).

# <sup>4</sup> Appendix

## <sup>5</sup> A Bill of materials

### <sup>6</sup> Optical components

- <sup>7</sup> • Lenses

- <sup>8</sup> – 2×Nikon CFI75 Apo LWD 25x/1.10w water dipping objectives
- <sup>9</sup> – 2×Nikon ITL200 200 mm tube lens
- <sup>10</sup> – 2×200 mm x 25 mm Dia. achromatic lens (Edmunds Optics, #47-645)
- <sup>11</sup> – 2×400 mm x 40 mm Dia. achromatic lens (Edmunds Optics, #49-281)
- <sup>12</sup> – 3×75 mm x 25 mm Dia. achromatic lens (Edmunds Optics, #47-639)
- <sup>13</sup> – SILL 112751 1:2 beam expander

- <sup>14</sup> • Filters

- <sup>15</sup> – 2×BrightLine quad-edge dichroic beam splitter (Semrock, Di03-R405/488/561/635-t3-25x36)
- <sup>16</sup> – EdgeBasic 488 nm long pass filter (Semrock, BLP01-488R-25)
- <sup>17</sup> – BrightLine 525/50 band pass filter (Semrock, FF03-525/50-25)
- <sup>18</sup> – EdgeBasic 561 nm long pass filter (Semrock, BLP02-561R-25)
- <sup>19</sup> – RazorEdge 647 nm long pass filter (Semrock, LP02-647RU-25)

- <sup>21</sup> • Mirrors

- <sup>22</sup> – 6×1” Broadband Dielectric Elliptical Mirror (Thorlabs, BBE1-E03)
- <sup>23</sup> – 2×30 mm Broadband 1/10λ Mirror (OptoSigma, TFMS-30C05-4/11)
- <sup>24</sup> – 10 Pack of 1” Protected Silver Mirrors (Thorlabs, PF10-03-P01-10)
- <sup>25</sup> – Knife-Edge Right-Angle Prism Dielectric Mirror (Thorlabs, MRAK25-E02)

### <sup>26</sup> Mechanical components

- <sup>27</sup> • 40 mm travel range pneumatic cylinder (Airtac, HM-10-040)
- <sup>28</sup> • 5/2-way electric valve (Airtac, M-20-510-HN)
- <sup>29</sup> • 2×25 mm extended contact bearing steel stage (OptoSigma, TSDH-251C)
- <sup>30</sup> • 30x90 mm stainless steel slide (OptoSigma, IPWS-F3090)
- <sup>31</sup> • close proximity gimbal mirror mount (Thorlabs, GMB1/M)
- <sup>1</sup> • 30 mm cage elliptical mirror mount (Thorlabs, KCB1E)
- <sup>2</sup> • Melles Griot Performance Plus Optical Breadboard
- <sup>3</sup> • 4×Newport Stabilizer, High Performance laminar Flow Isolator, I-2000 Series

**4 Custom parts**

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

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

**13 Electronics**

- 14 • Embedded system (National Instruments, cRIO-9068) equipped with:**
  - 15 – 2×C series digital I/O card (NI 9401)**
  - 16 – 1×C Series 100 kS/s 4-channel Voltage Output Module (NI 9263)**
  - 17 – 1×C Series 25 kS/s 16-channel Voltage Output Module (NI 9264)**
- 18 • Omicron SOLE-3 laser combiner, with 488, 561 and 638 nm laser lines**
- 19 • Andor Zyla 4.2 sCMOS Camera**
- 20 • Galvanometric scanner mirror (Cambridge Technology, 6210B)**
- 21 • 6 position filter wheel (Ludl Electronic Products, 96A361)**
- 1 • Filter wheel controller unit (Ludl Electronic Products, MAC5000)**
- 1 • 2 piezoelectric stages (Nanos Instruments, LPS-30-30-1-V2\_61-S-N)**
- 2 • 2 stage controller boards (Nanos Instruments, BMC101)**

## B Supplementary Tables

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### <sup>3</sup> B Supplementary Tables

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

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

**Table B2: Lossless compression performance.** B<sup>3</sup>D is compared with various popular lossless image compression methods regarding write speed, read speed and compression ratio (original size / compressed size). Data visualized on Figure 4.13.

	write speed	read speed	CR	file size
<b>B<sup>3</sup>D</b>	1,115.08 MB/s	928.97 MB/s	9.861	100%
<b>KLB</b>	283.19 MB/s	619.95 MB/s	10.571	93.28%
<b>JPEG2000</b>	31.94 MB/s	26.38 MB/s	11.782	83.69%
<b>TIFF uncompressed</b>	202.32 MB/s	161.08 MB/s	1.00	986.1%
<b>TIFF + LZW</b>	40.85 MB/s	102.37 MB/s	5.822	169.37%

## B Supplementary Tables

**Table B3:** Datasets used for benchmarking compression performance.

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

## 4 C Light collection efficiency of an objective

5 Let's define light collection efficiency  $\eta$  as the ratio of collected photons and all emitted  
6 photons:

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

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

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

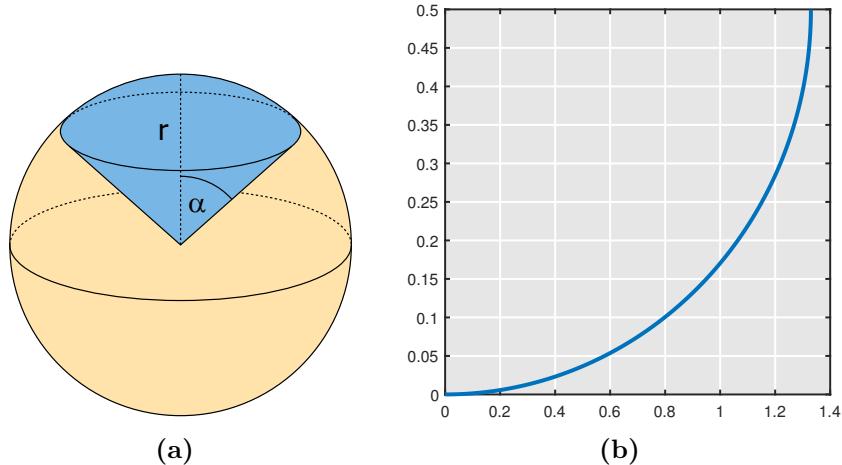
13 The surface area of the full sphere is calculated as:

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

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

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

2 As most objectives are characterized by the numerical aperture, we also plot  $\eta$  as a  
3 function of the NA on Figure C1b.



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

**4 D 3D model of Dual Mouse-SPIM**

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

7 Navigation:

- 1 • zoom: mouse wheel / right click and drag vertically
- 2 • rotate: left click and drag
- 3 • pan: left + right click and drag / Ctrl + click and drag

Interactive 3D figure  
Click to activate

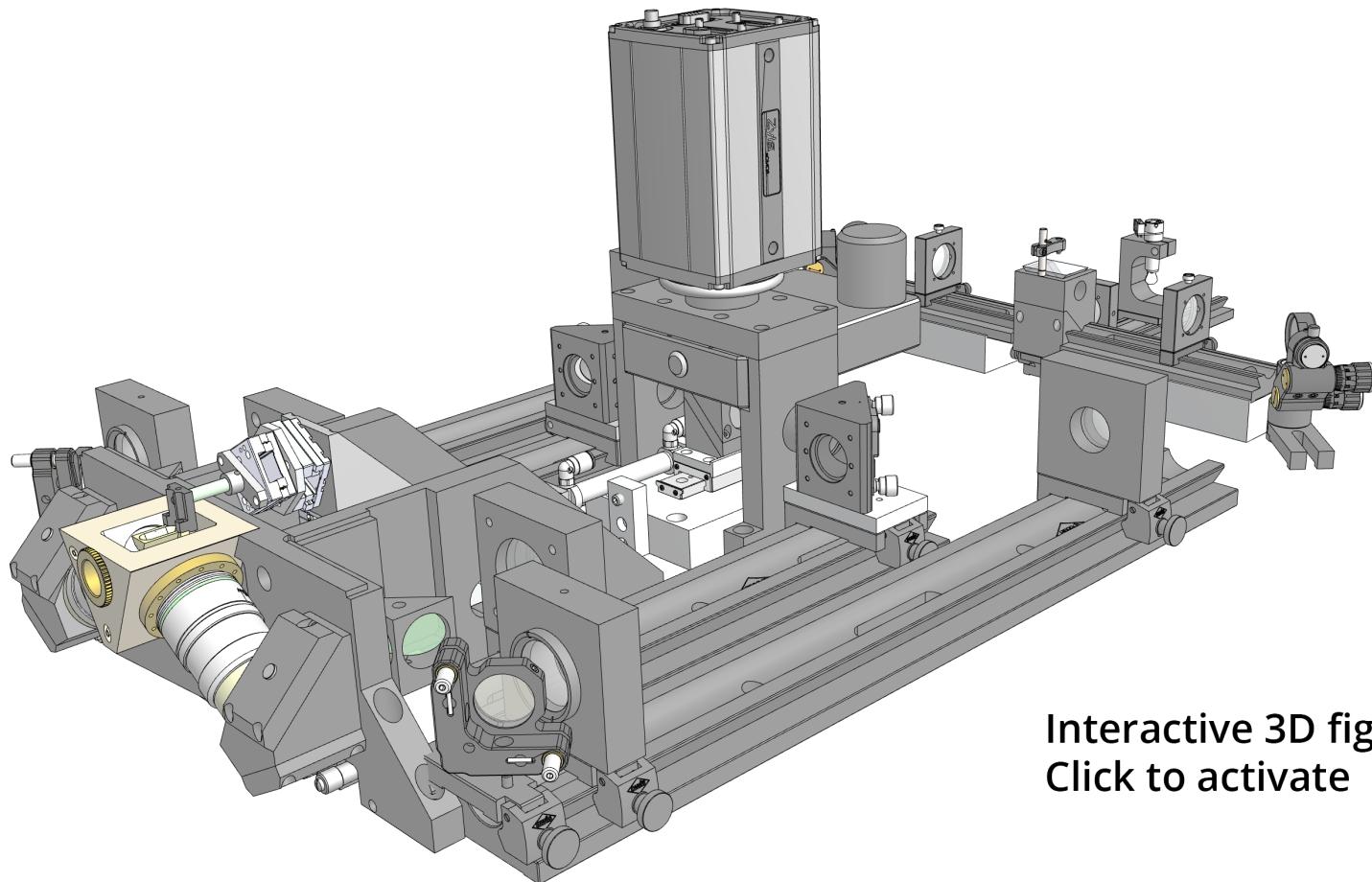


Figure D1: 3D model of DualMouse-SPIM.

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## <sup>25</sup> Acknowledgements

<sup>26</sup> The work presented in this thesis has been carried out in the Hufnagel group at the  
<sup>27</sup> European Molecular Biology Laboratory (EMBL) in Heidelberg, Germany. I would like  
<sup>28</sup> to thank Lars Hufnagel for this unique opportunity to work in the field of microscopy,  
3255 and for his continuous support through the project.

<sup>3256</sup> I am very grateful to the members of my thesis advisory committee: Jonas Ries, Takashi  
3257 Hiiragi and Balázs Rózsa; thank you for the support and the fruitful discussions.

<sup>3258</sup> I would also like to thank Prof. Péter Szolgay for the opportunity to be part of the  
3259 Roska Tamás Doctoral School of Sciences and Technology at Pázmány Péter Catholic  
3260 University.

<sup>3261</sup> I would like to thank Gustavo Quintas Glasner de Medeiros and Nils Norlin for the  
3262 microscope building and debugging sessions, and for sharing all their experience and  
3263 knowledge. A big thank you to Marvin Albert and Joran Deschamps for their help with  
3264 the compression algorithm, and also for sharing the ups and downs of EMBL PhD life.

<sup>3265</sup> Many thanks to all friends and colleagues who helped me throughout my four years:  
3266 Dimitri Kromm, Ulla-Maj Fiuzza, Li-Ling Yang, Stefan Günther, Lucía Durrieu, Patrick  
3267 Hoyer, Laura Panavaite, Uroš Kržič, Petr Strnad, Sebastian Streichan, Aldona Nowicka,  
3268 Christina Besir, Tatjana Schneidt, Judith Reichmann, Yu Lin, Nils Florian Wagner,  
3269 Christian Tischer; it has been a truly memorable time.

<sup>3270</sup> Development of the microscope would not have been possible without the expert help of  
3271 Alfons Riedinger, Alejandro Gil Ortiz, Leo Burger, Sascha Blättel, Tim Hettinger and  
3272 all other members of the EMBL electronic and mechanical workshops.

<sup>3273</sup> Special thanks goes to Judit Tóth, István Gärtner and Attila Mócsai for introducing me  
3274 to the world of natural sciences, and Prof. Szabad János for showing me EMBL for the  
3275 first time.

<sup>3276</sup> I am eternally grateful to my parents and my first scientific advisors, Béla Balázs and  
3277 Ilona Fige, and my sister, Eszter for their unconditional support.

<sup>3278</sup> Finally, Melánia: Thank you for your infinite patience, support and love.