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## Tartalmi kivonat

Absztrakt magyarul.

## Abstract

Abstract in English.

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# Introduction

[1]

# Chapter 1

## Light-sheet imaging of mammalian development

Unraveling the secrets of mammalian development has been a long standing challenge for developmental biologists and medical professionals alike. Understanding early embryonic development allows to shed light on questions such as human infertility and congenital diseases [todo]. This phase of early life is an incredibly complex and dynamic process spanning through large scales in space and time. Subcellular processes at the nanoscale are happening in the range of milliseconds or faster, while whole embryo reorganizations and tissue migration events take place over the course of hours [2]. Resolving these processes presents a true challenge, since to also understand the underlying mechanisms molecular specificity is just as crucial as high spatial and temporal resolution.

Fluorescence microscopy [3]

The most commonly used model organism for these kind of questions is the mouse embryo, that exhibits several common features to human development, and allows to study it. It has several advantages: full genome is sequenced [4], relatively fast reproduction rate facilitating genetic modifications, already extensively developed tools for genetics and handling [5, 6]. All of these make the mouse embryo the primary model organism when investigating biological processes in mammals.

fixed samples, histology, immunofluorescence -> no time lapse

ex vivo embryo culture

Hooke's Micrographia 1665 [7]

Marvin Minsky confocal microscope, no laser, moving sample [8] first laser scanning confocal: sample stationary, objective is moved Davidovits and Egger [9] first application of laser scanning confocal in "observation of endothelial cells lining the inside of the cornea" [10]

Imaging mouse embryonic development is an extremely challenging task, due to the

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intrauterine development of embryos. Although this prevents direct access to the embryos, for some developmental stages it is possible to culture them in an *ex vivo* environment using special media and carefully controlled environmental conditions [11, 12]. A second hurdle, aside from *in vitro* culturing, arises from the extreme light sensitivity [13] of these embryos, hindering the possibility of long term time-lapse experiments in standard confocal microscopes. Even a few hours of imaging every 15 mins can impair or arrest normal development [J1].

Despite these difficulties, numerous studies successfully used confocal microscopy to image live embryos in an *ex vivo* environment, although these studies typically only mostly wide-field, Oct4 - self renewal of pluripotent stem cells in ICM/epiblast [14]

Sox2 in ICM, also maternal proteins important, similar role as Oct4 Both Oct4 and Sox2 are necessary for ICM formation, without cells become trophoectoderm. Furthermore, for extraembryonic endoderm (ExEn) only Oct4 is required, while for extraembryonic ectoderm (ExE) only Sox2 is required. No time lapse, but confocal used to locate proteins in embryo [15]

inactivation of X chromosome and histone macroH2A1 mouse liver in confocal - fixed samples [16]

antibodies against amyloid  $\beta$ -peptide reduce Alzheimer in mouse, cryostat sections of mouse brain imaged in confocal[17]

aorta imaging in embryo slices, confocal, live stem cells from aorta endothelium? [18]  
fixed embryos, maintaining pluripotency in embryonic stem cells[19]

imaging thrombus formation in real time with intravital high-speed confocal microscopy[20]

turning blood into brain hematopoietic stem cells migrate to brain and express neuron-specific antigens [21]

papers from Takashi: apical domain for symmetry breaking [22]

Asymmetric division of contractile domains couples cell positioning and fate specification, Jean-Léon + Hervé [23]

specification of first embryonic lineage, venus trap [24]

surface tension and compaction [25]

self organization framework for symmetry breaking (review)

uj regi cikk Keller [26]

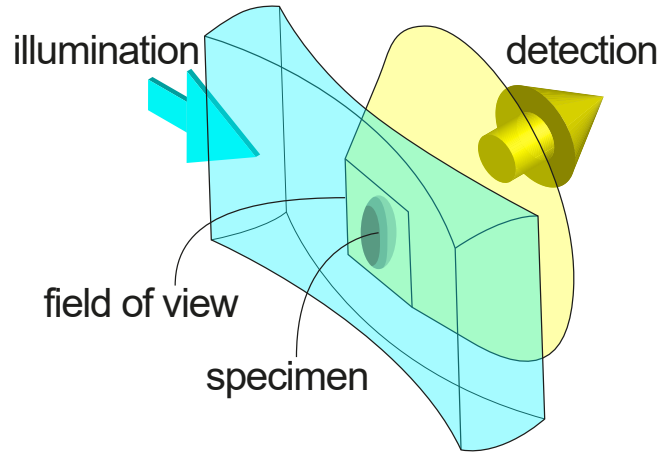
Light microscopy is one of the oldest methods that is still widely used today to investigate the inner workings of microscopic life. Light-sheet microscopy is a relatively new addition to the arsenal of tools that comprise light microscopy methods, and is especially suitable for live imaging of embryonic samples over extended periods of time [27, 28]. It is also easily adapted to the sample, allowing to image a large variety of specimens, from entire organs, such as cleared mouse brains [29], to the subcellular

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processes occurring inside cultured cells [30].

basic concept of spim

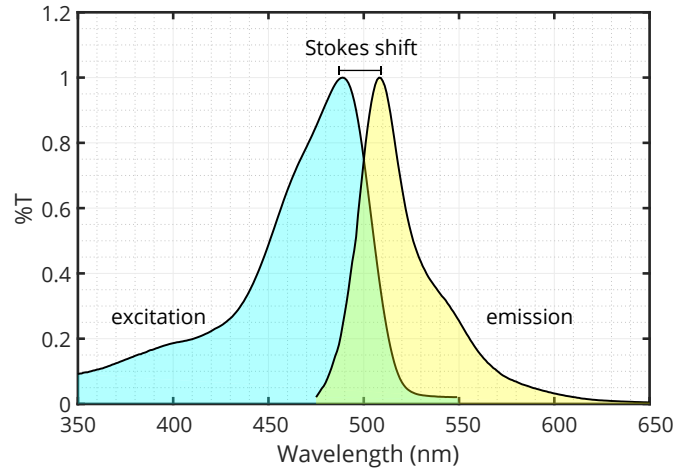


**Figure 1.1: Basic concept of single-plane illumination microscopy.** The sample is illuminated from the side by laser light shaped to a light-sheet (blue). This illuminates the focal plane of the detection lens, that collects light in a wide-field mode (yellow). The image is recorded, and the sample is translated through the light-sheet to acquire an entire 3D stack.

### 1.1 Wide-field fluorescence microscopy

Fluorescence microscopy [31], as a subset of light microscopy is one of the few methods that allow subcellular imaging of live specimens. As the name of the technique suggest, this method collects fluorescent light from the specimens which has numerous advantages, but also some drawbacks. Since biological tissue is usually not fluorescent, except for some autofluorescence at shorter wavelengths, fluorescent dyes or proteins have to be introduced to the system in order to be able to collect the necessary information.

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



**Figure 1.2: Excitation and emission spectrum of enhanced green fluorescent protein (EGFP).** Excitation spectrum in blue, emission spectrum in yellow. The separation between the two spectra is due to the Stokes shift, which is 19 nm for EGFP. Data from [32]. Emitted and excitation light can be separated by a long-pass filter at 500 nm.

### 1.1.1 Fluorescent proteins

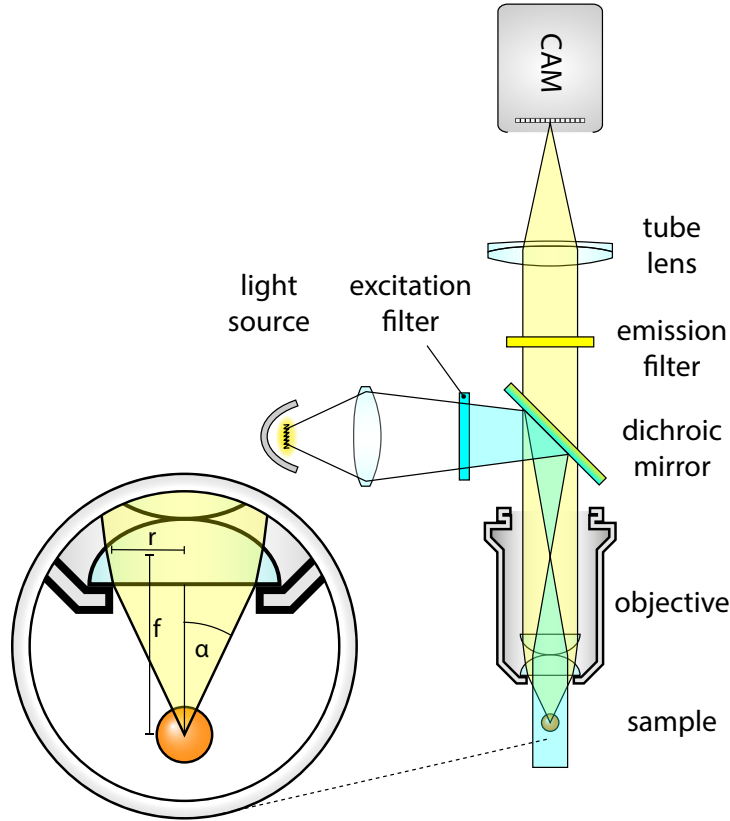
Traditionally synthetic fluorescent dyes were used to label certain structures in the specimens. Some of these directly bind to their target, such as DAPI to DNA, and others can be used when conjugated to an antibody specific to the structure of interest. The drawback of these methods is that the fluorescent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

The discovery of fluorescent proteins have revolutionized fluorescence microscopy. Since these molecules are proteins, they can be produced directly by the organism if the proper genetic modifications are performed. Even though this was a hurdle at the time of discovering the green fluorescent protein (GFP) [33], the first of its kind, genetic engineering techniques evolved since then [34], and not only has it been successfully integrated in the genome of nematodes [35], zebrafish [36], and mice [37], but many variants have been also engineered by introducing mutations to increase fluorescence intensity, and to change the fluorescent spectrum to allow multicolor imaging [37–40]. The usefulness and impact of these proteins are so profound, that in 2008 the Nobel Prize in chemistry was awarded to Osamu Shimomura, Martin Chalfie, and Roger Tsien “for the discovery and development of the green fluorescent protein, GFP” [41].

### 1.1.2 Wide-field image formation

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

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

in Figure 1.3.

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

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

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

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

The final field of view of the microscope will depend on the magnification, and also on the size of the imaging sensor:

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

where  $A$  is the area of the sensor.

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

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

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

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

### 1.1.3 Resolution of a wide-field microscope

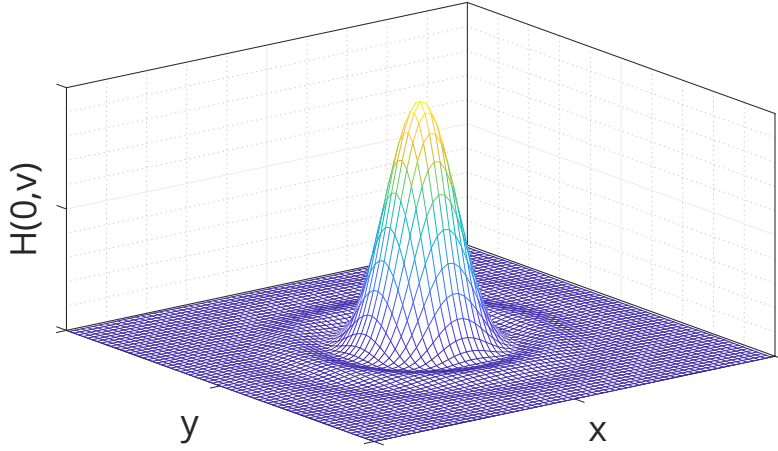
The resolution of an optical systems is defined by the size of the smallest distinguishable feature on the image. Practically this means the minimum distance between two point like objects so that the two objects can still be resolved. This mainly depends on two factors: the NA of the objective, and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

Even if the imaging sensor would have infinitely fine resolution, it is not possible to reach arbitrary high resolutions due to the wave nature of light and diffraction effects that occur at the aperture of the objective. This means that depending on the wavelength of the light, any point source will have a finite size on the image that will limit the resolution. The shape of this image is called the *point spread function*, or PSF, as this function describes the behavior of the optical system when imaging a point like source. This property of lenses was already discovered by Abbe in 1873 [42], when he constructed his famous formula for resolution:

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

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**Figure 1.4: Airy pattern.** Airy pattern calculated in Matlab based on Equation (1.8)

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

This equation can be also derived from the scalar theory of diffraction using a paraxial approximation (Fraunhofer diffraction, [43]) that describes the intensity of the electric field in the focus of a lens [44]:

$$H(u, v) = C_0 \left| \int_0^1 J_0(vr) e^{-i\frac{1}{2}ur^2} r dr \right|^2, \quad (1.6)$$

where  $C_0$  is a normalization constant, and  $J_0$  is the zero order Bessel function of the first kind. Furthermore, instead of the commonly used Cartesian coordinates  $x$ ,  $y$  and  $z$ , the following optical coordinates are defined:

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

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

To determine the lateral resolution of the system, let's substitute  $u = 0$  as the axial optical coordinate, and evaluate Eq. (1.6) which will give the intensity distribution in the focal plane:

$$H(0, v) = C_0 \left| \int_0^1 J_0(vr) r dr \right|^2 = \left( 2 \frac{J_1(v)}{v} \right)^2, \quad (1.8)$$

where  $J_1$  is the first order Bessel function of the first kind. This equation describes the famous Airy pattern (Fig. 1.4) which will be the shape of the PSF in the focal plane. The width of this pattern is the resolution, and although there are multiple definitions for this, the most commonly accepted is the Rayleigh criterion [rayleigh] which defines the resolution as the distance between the central peak and the first local minimum. As



this lies at  $v = 3.38$ , the resolution can be expressed by substituting this value to Eq. (1.7) and calculating the real distance ( $r$ ):

$$\delta_{xy} = \frac{3.83}{2\pi} \frac{\lambda_0}{n \cdot \sin \alpha} \approx 0.61 \frac{\lambda_0}{NA}, \quad (1.9)$$

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

Similarly, to calculate the intensity distribution along the axial axis, let's substitute  $v = 0$  to Eq. (1.6):

$$H(u, 0) = C_0 \left( \frac{\sin \frac{u}{4}}{\frac{u}{4}} \right)^2. \quad (1.10)$$

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

$$\delta_z = \frac{2n\lambda_0}{NA^2}. \quad (1.11)$$

Up till here, we only considered a single, point-like emitter. In a more realistic scenario, however the emitters are neither point-like, nor single. Effectively, however, for every emitter the PSF would be imaged on the sensor, and this creates the final image. In mathematical terms, this can be expressed as a convolution operation between the underlying fluorophore distribution of the object ( $O$ ) and the PSF ( $H$ ):

$$I(u, v) = O(u, v) * H(u, v). \quad (1.12)$$

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

### High NA imaging, and achieving isotropic resolution

In the previous paragraphs we defined optical resolution, and derived two formulas to calculate the lateral and axial resolutions. These formulas, however

but this only works for low angles, as the Fraunhofer diffraction equation used to derived these results is only an approximation of the Kirchoff diffraction equation. For high NA objectives, a more reliable way to calculate the resolution is the Stelzer-Grill-Heisenberg principle:

this result is base on the paraxial approximation of the Helmholtz equation, thus only apply for small NA. a more general method to calculate resolution for high NA imaging systems is SGH: A generally accepted method to calculate lateral ( $\sigma_{xy}$ ) and axial ( $\sigma_z$ ) resolution of an optical microscope is described by the Stelzer-Grill-Heisenberg, or

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SGH theory[45, 46]: A more general expression, works for large NA as well

$$\sigma_{xy} = \frac{\lambda}{\sqrt{3 - 2 \cos \alpha - \cos 2\alpha}} \quad (1.13)$$

$$\sigma_z = \frac{\lambda}{1 - \cos \alpha} \quad (1.14)$$

Generally, instead of  $\alpha$ , the numerical aperture (NA) is commonly used to characterize a lens' aperture.  $NA = n \cdot \sin \alpha$ , where  $n$  is the refractive index of the medium and  $\alpha$  stands for the angular aperture.

even for high NA axial/lateral ratio  $\sim 3 - 6$  (Figure 1.5) In principle it is possible to achieve isotropic resolution by increasing the acceptance angle to  $\alpha = 180^\circ$ , which means all light needs to be collected from the sample. Designing an objective that is capable of this feat, however, is currently beyond our technological capabilities, furthermore it would require an alternative method for sample positioning, since mechanical translation is not possible in such a configuration. One attempt at such an imaging system was the Multi-Imaging Axis Microscopy (MIAM) [47, 48] that consisted of 4 identical objectives arranged in a tetrahedral fashion to collect as much light as possible from multiple directions.

Another disadvantage of the wide-field microscope, is that it can not be used with thick specimens. Usually this type of microscopy is only used for a single layer of cells, because all the objects in the field of view will appear on the imaging plane, not just the plane in focus. These objects will appear blurred if close to the focus, or just evenly add to the background noise if they are further from the focus. This is why imaging specimens much thicker than  $10 \mu m$  will result in suboptimal image quality.

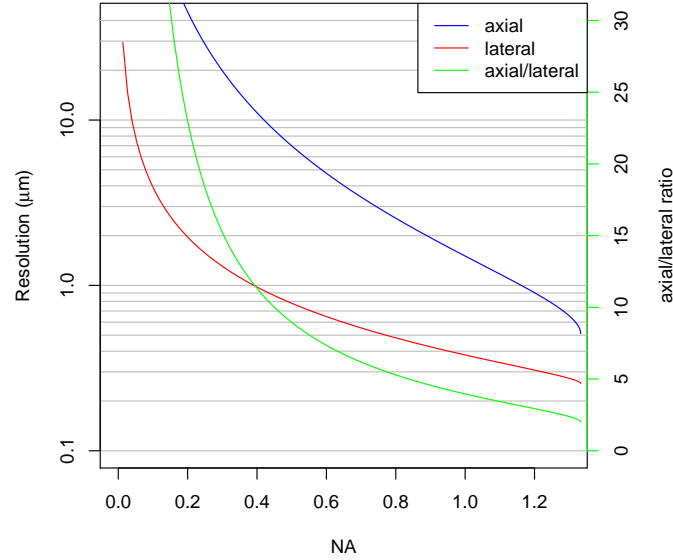
## 1.2 3D microscopy techniques

### 1.2.1 Laser scanning confocal microscopy

Laser scanning confocal microscopy [10] addresses the problem of noise originating from the out of focus planes. The principle for illumination and detection optics is very similar to a wide-field microscope, but for illumination a focused laser light is used.

The biggest change is in the detection method: the confocal microscope uses a pinhole, to exclude light coming from out of focus planes. Since only those rays are taking part in the imaging that originate from the focus, the image quality is highly improved. This microscopy technique is also capable of 3D imaging, with an axial resolution corresponding to a wide-field microscope.

However, the image can not be registered as simply as with the wide-field detection, since at any given time, only a very small portion of the sample will be used in the



**Figure 1.5: 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  $\lambda = 510nm$ , the emission maximum of GFP and  $n = 1.333$ , the refractive index of water, for water dipping objectives.

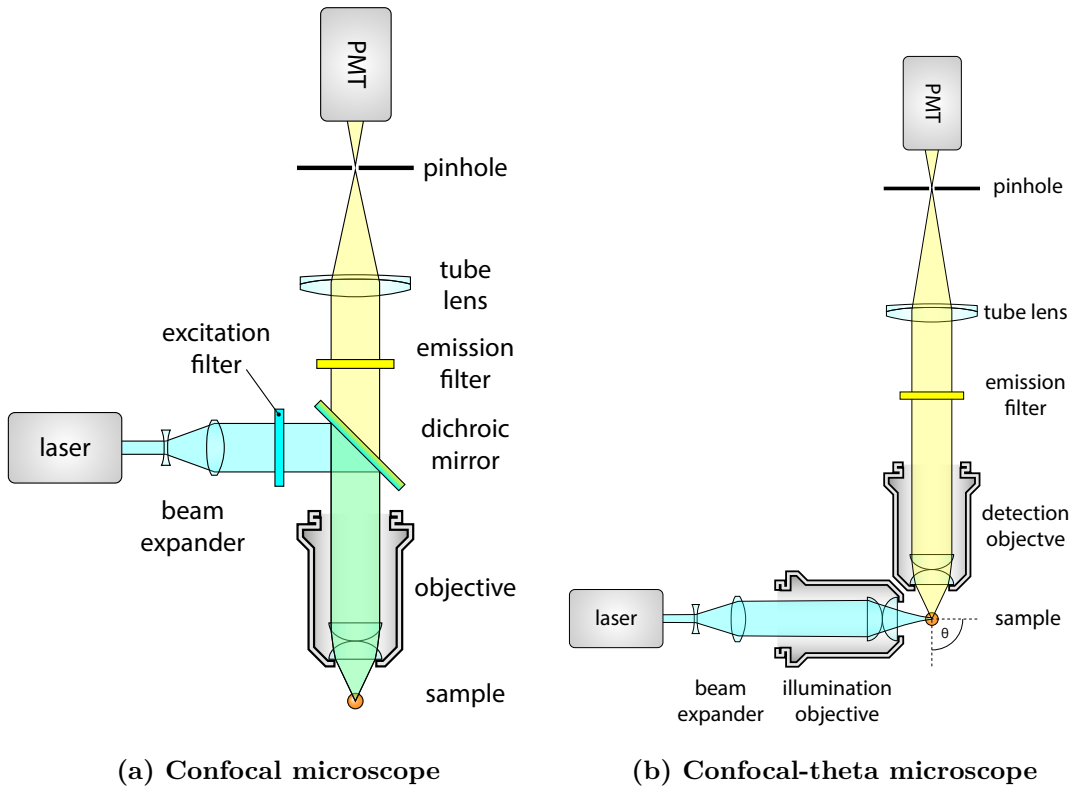
imaging. Because of this, a more sensitive detection is required, which generally means the use of a photomultiplier. To get the whole image, the focus is scanned through the whole sample (or rather, the sample is scanned through the focus), recording an intensity value for each position. The image is then generated using a computer based on the recorded position and intensity values.

Although this microscopy technique already has 3D capabilities, it's axial resolution is still limited by the SGH theory, since it uses only one objective. Imaging live specimens for an extended period of time with confocal microscopy although possible [49], is not ideal. Since for each voxel imaged, almost the entire specimen has to be illuminated, which results in a very high dose of radiation of the samples. This can be as much as 30–100 times bigger, than the dose used for the actual imaging [50]. High power of laser for an extended time frame can result in bleaching the fluorophores, thus resulting in a lower signal at later times, but the more significant issue is phototoxicity, that is when the cells themselves are damaged by the laser light.

### 1.2.2 Confocal-theta microscopy

Although confocal microscopy already provides a better resolution in all dimensions, the ratio of the axial and lateral resolution is still very high, due to the single objective illumination and detection. This seriously limits the microscope's 3D imaging capabilities,

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**Figure 1.6: Basic optical components of a laser scanning confocal and confocal-theta microscope.** Both type of microscopes use confocal images detection, which means that a pinhole is used to exclude light coming from out of focus points. Light intensity is measured by a photomultiplier for every voxel in the region of interest. The final image is generated on a computer using the positions and recorded intensity values. A regular confocal microscope (1.6a) uses the same objective for illumination and detection, while a confocal-theta microscope (1.6b) uses a second objective that is rotated by  $\vartheta$  around the focus. In this case,  $\vartheta = 90^\circ$ .

since in the  $z$  direction (i.e. along the imaging axis) the resolution would be significantly worse than in the other directions.

Confocal-theta microscopy [51] introduces a second objective to a regular confocal microscope, that is used to illuminate the sample (Figure 1.6b). Since this decouples the illumination and detection, using a filter cube is no longer necessary. The second objective is rotated by  $\vartheta$  around the focus, this is where the name of this setup originates.

Resolution is also improved compared to the regular confocal microscope, because the lateral resolution of the imaging objective now corresponds to the axial resolution of the detection objective. The combined resolution of the two-objective system can be calculated in the following manner [52]:

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

where  $\sigma_{ill} = \sigma_{xy}$  and  $\sigma_{det} = \sigma_z$  for the axial resolution of the system, and reversed for the lateral resolution of the system. This means, that the axial and lateral resolution would be the same (if the same objectives are used), and the resulting point spread function is almost isotropic.

Although this is a big improvement to confocal microscopy, the issue of photobleaching and phototoxicity is still not solved with a confocal theta microscope, which means that longer developmental processes are near impossible to follow with this type of microscopy.

### 1.3 Light-sheet microscopy

One main drawback of the point scanning methods introduced in the previous section is the inefficiency of the illumination scheme. Although almost the whole sample is illuminated, information is only collected from the focal volume. This results in two main limiting factors: acquisition speed depends on the point scanning speed, which in turn is inversely proportional to the signal to noise ratio. Furthermore, because of the inefficiency of the illumination scheme, photobleaching and phototoxic effects further reduce the usability of these techniques for live imaging, especially for long term experiments [50].

The main principle behind single plane illumination microscopy, that is illuminating the sample from the side by a very thin light-sheet, dates back to the early 20<sup>th</sup> century, when Siedentopf and Zsigmondy first described the ultramicroscope [53]. This microscope used sunlight as an illumination source, that was guided through a precision slit to generate a thin light-sheet. This allowed Zsigmondy to visualize gold nanoparti-

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cles floating in and out of the light-sheet. Since these particles are much smaller than the wavelength of the light, the device was called an ultramicroscope. His studies with colloids together with the development of the ultramicroscope led Zsigmondy to win the Nobel Prize in 1925.

previous to SPIM: Voie et al. fixed guinea pig cochlea, rotating and reconstruction in 3D [54], called Orthogonal-plane Fluorescent Optical Sectioning. Lateral resolution around  $10\mu\text{m}$  and axial resolution of  $26\mu\text{m}$ , with very large,  $1.5\text{mm}$  field of view. Since the specimens they imaged contained calcium rich bone tissue, optical imaging was only possible by using an optical clearing method. In this work, they used EDTA (ethylenediaminetetraacetic acid) dehydrated in ethyl alcohol, then it was cleared, finally immersed in a fluorescent dye bath. TO acquire 3D images, the specimen was rotated, and translated to compensate for the off-axis rotation.

follow-up to Voie and Spelman: [55] 3D reconstruction of the cochlea for the images

Similar to SPIM but not fluorescent: light scanning photomicrography [56]

Then, in 2002, Fuchs et al. introduces Thin Light-Sheet Microscopy [57] who use this technique to investigate the microbial life in seawater samples without disturbing their natural environment (by e.g. placing them on a coverslip). Using TSLM allowed the to image the bacteria directly in the staining solution containing SYBR Green I without having to deal with background illumination from the dye, which would have been an issue with confocal microscopy for example. Their light-sheet was similar to the one utilized in OPFOS, being  $23\mu\text{m}$  thin, and providing a  $1\text{mm} \times 1\text{mm}$  field of view.

The real breakthrough happened when light-sheet microscopy was combined with endogenous fluorescent labels: fluorescent proteins live imaging for long time and light-sheet these techniques were all combined in the 2004 Science paper from Huisken et al. that marks a landmark in light-sheet microscopy, and since then a widespread adoption started in biological research, with multiple groups implementing their own setups.

Since then however, light-sheet microscopy was seldom used, but in the last decade it was reinvented and combined with fluorescent microscopy. The first notable light-sheet fluorescent microscope (LSFM) was developed at EMBL in 2004 [1], that demonstrated the benefits of using a light-sheet in imaging developmental processes in three dimension.

Since then, light-sheet based imaging has gained more and more popularity, as it can be adapted and applied to a wide variety of problems. It was numerously proven to be a better choice than confocal microscopy [50, 58] especially in developmental biological applications [59]. It can also be used with a wide variety of specimens of different sizes, such as zebrafish embryo [60], mouse brain [61] or drosophila embryo [27]. It is also possible to use light-sheet microscopy in super-resolution, allowing for individual molecule localization [62].

### 1.3.1 Optics of light-sheet microscopy

A selective-plane illumination microscope (SPIM) uses a light-sheet to illuminate only a thin section of the sample (Figure 1.7). This illumination plane is perpendicular to the imaging axis of the detection objective and coincides with the focal plane. This way the image is taken of only that specific plane that is illuminated, thus providing much better signal to noise ratio. In case of conventional wide-field fluorescent microscopy, where the whole specimen is illuminated, light scattering from different regions contribute to a significant background noise. With selective-plane illumination, this problem is intrinsically solved, and it also provides a true sectioning capability. This makes SPIM especially suitable for 3D imaging.

### 1.3.2 Detection

The detection unit of a SPIM is basically equivalent to a detection unit of a wide-field microscope, without a dichroic mirror (Figure 1.7). Most important components are the objective together with the tube lens, filter wheel, and a sensor, typically a CCD or sCMOS camera.

One of the most important aspects that determine the resolution of the microscope is the detection objective. Since in developmental biology specimens require a water-based solution, these objectives are usually water dipping objectives directly submerged in the medium. Since the refraction index of water ( $n = 1.333$ ) is greater than the refraction index of air, these objectives tend to have a higher  $NA$ , which results in higher resolution. This, however, also depends on the sensor used, mainly on the pixel size ( $d_{sensor}$ ).

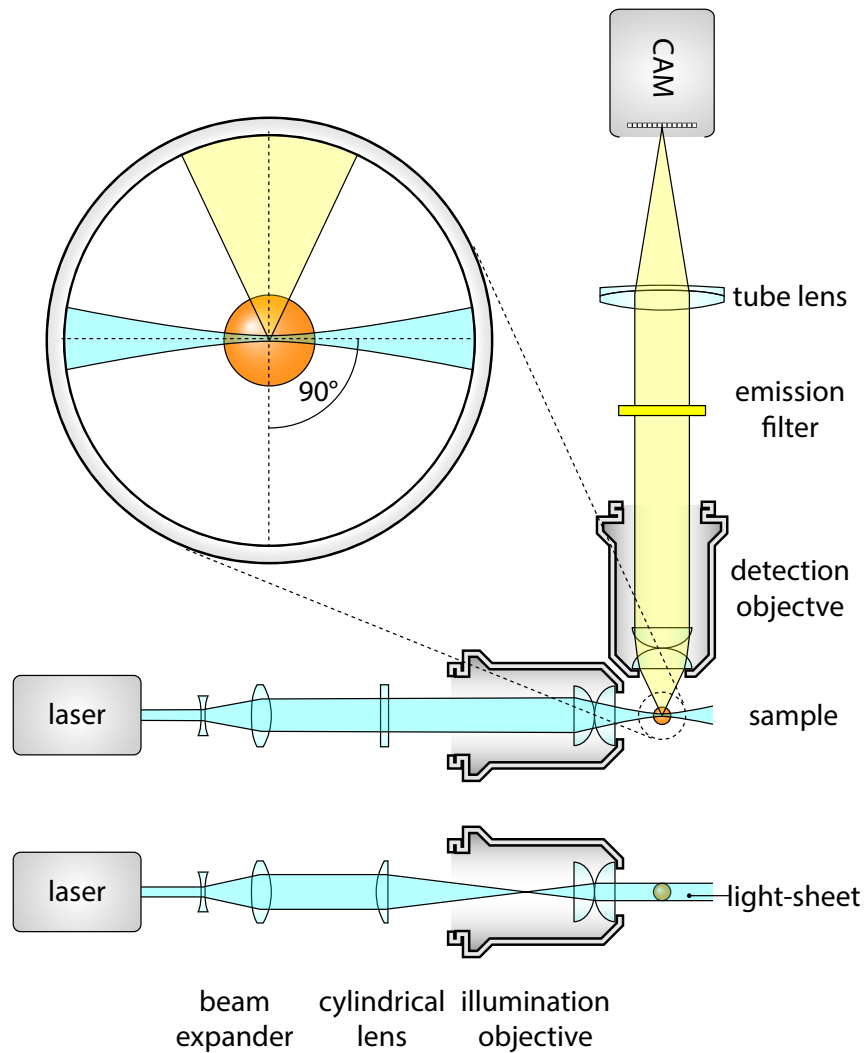
The magnification is typically  $10\times$ ,  $20\times$ ,  $40\times$  or  $100\times$  but these values are sound only when the objective is used together with the prescribed tube lens. These lenses are specially made to be used with the specific objectives, and are corrected for any aberrations. They typically have a focal length of 160–200mm.

### 1.3.3 Illumination

#### Using cylindrical lens

The light-sheet can be generated using a cylindrical lens, which focuses the laser beam in only one direction, and creating a thin sheet in the proximity of the focal point. However, to achieve light-sheets that are thin enough, one would need to use cylindrical lens with low focal lengths, but these are hardly accessible in well corrected formats. For this reason, its more common to use a longer focal length cylindrical lens in conjunction with a microscope objective, which is well corrected for chromatic and spherical aberrations [63]. This way, the light-sheet length, thickness and width can be adjusted for the specific imaging tasks.

## 1. LIGHT-SHEET IMAGING OF MAMMALIAN DEVELOPMENT



**Figure 1.7: Basic optical components of a SPIM.** A selective plane illumination microscope uses two objectives orthogonally aligned. One objective is used to generate a thin light-sheet that illuminates the sample from the side, while the other is used for detection. To generate an image of the specimen, a suitable tube lens is used to focus the light on the sensor of a detection unit (e.g. sCMOS camera). The light-sheet is generated by the illumination objective, using a beam that is previously shaped by a cylindrical lens.



For paraxial waves, i.e. waves with nearly parallel wave front normals, a general wave equation can be approximated with the paraxial Helmholtz equation [52, 64]

$$\nabla_T^2 + i2k \frac{\partial U}{\partial z} = 0 \quad (1.16)$$

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 we assume, that the light spreads in  $z$  direction.

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

$$U(r, z) = A_0 \cdot \frac{W_0}{W(z)} \cdot e^{-\frac{r^2}{W^2(z)}} \cdot e^{-i \cdot \phi(r, z)} \quad (1.17)$$

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

$$W(z) = W_0 \sqrt{1 + \left(\frac{z}{z_0}\right)^2} \quad (1.18)$$

where the parameter  $z_0$  is called the Rayleigh-range. This has the following connection with the beam waist:

$$z_0 = \frac{\pi W_0^2}{\lambda} \quad (1.19)$$

Which means, the thinner the beam waist, the shorter the Rayleigh-range, that is the beam divergence is faster for more focused beams.

Intensity of the emitted fluorescence is based on the intensity of the excitation light. In case of a Gaussian beam:

$$I(r, z) = U(r, z) \cdot U^*(r, z) = |A_0|^2 \cdot \left(\frac{W_0}{W(z)}\right)^2 \cdot e^{-\frac{2r^2}{W^2(z)}} \quad (1.20)$$

Apart from the circular Gaussian beam, the elliptical Gaussian beam is also an eigenfunction of Helmholtz equation (1.16):

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

This beam still has a Gaussian profile along the  $x$  and  $y$  axes, but the radii are uncoupled, which results in an elliptical beam. Since the beam waist is different along

## 1. LIGHT-SHEET IMAGING OF MAMMALIAN DEVELOPMENT

---

the two axes, the Rayleigh range is also different:

$$z_{x,0} = \frac{\pi W_{x,0}^2}{\lambda} \quad (1.22)$$

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

Intensity of the beam is the following:

$$I(x, y, z) = U(x, y, z) \cdot U^*(x, y, z) = |A_0|^2 \cdot \frac{W_{x,0}}{W_x(z)} \cdot \frac{W_{y,0}}{W_y(z)} \cdot e^{-\frac{2x^2}{W_x^2(z)}} \cdot e^{-\frac{2y^2}{W_y^2(z)}} \quad (1.24)$$

where

$$W_x(z) = W_{x,0} \sqrt{1 + \left(\frac{z}{z_{x,0}}\right)^2} \quad \text{and} \quad W_y(z) = W_{y,0} \sqrt{1 + \left(\frac{z}{z_{y,0}}\right)^2} \quad (1.25)$$

Since the illumination is uneven, the usable field of view is smaller than the actual illuminated region (Figure 1.8a). The width of the field of view  $w_{fov}$  is determined by the Rayleigh length, since this is in a direct relation with the beam divergence. To stay in the optimal region, the light-sheet should only be used in the range of 1 Rayleigh length on both sides of the beam waist (Figure 1.8b). In this range, the ratio between the thickest (at  $z = z_0$ ) and the thinnest (at  $z = 0$ ) part of the beam  $W(z)$  will be  $\sqrt{2} \approx 1.4142$  which is still acceptable.

Light-sheet height is determined by the profile of the beam along the vertical axis (Figure 1.8c). Since this is a Gaussian function (see Equation 1.17), only a small part in the middle can be used for imaging, because towards the sides the intensity dramatically drops. To allow a maximum 80% drop of intensity at the edges, the light-sheet height is  $h_{fov} = 2 \cdot 0.472 \cdot W_{x,0}$

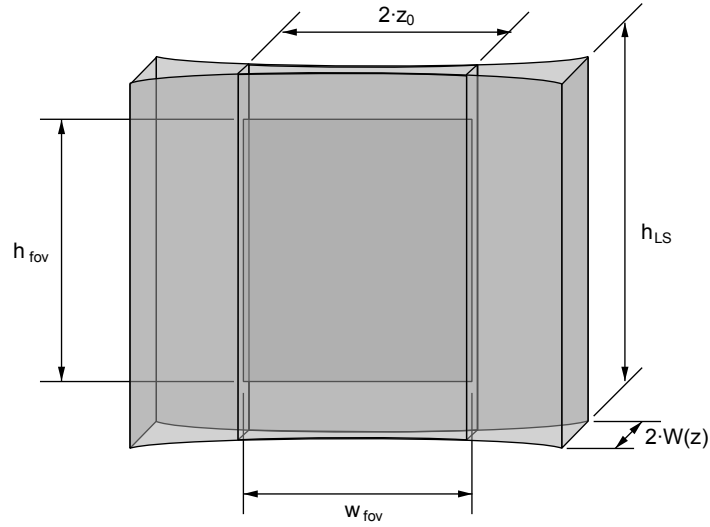
### Using focused beam scanning

Since using cylindrical lenses it's not possible to generate a homogeneous light-sheet, moreover at higher magnification the Rayleigh range would be too small, we also consider using focused beam scanning to generate the light-sheet (digital scanned light-sheet microscopy, DSLM). To generate a scanning beam, a galvanometer controlled mirror is used to alter the beam path. This can quickly turn around its axis which will result in an angular sweep with the laser beam. To change the angular movement to translation, a scan lens is used to generate an intermediate scanning plane. This plane is then imaged to the specimen by the tube lens and the illumination objective, resulting in a scanned focused beam.

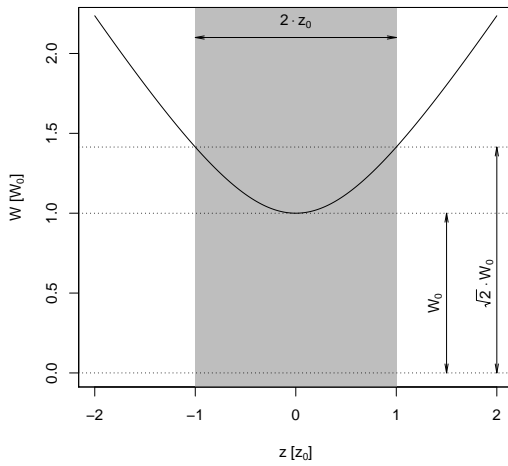
This method to generate the light-sheet has several advantages compared to a static

### 1.3 Light-sheet microscopy

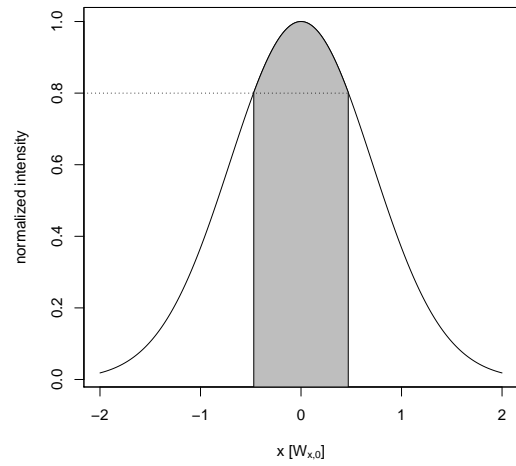
light-sheet. The height of this sheet is not determined by the cylindrical lens, but it can be dynamically modified. Also, the intensity is uniform through the whole height of the light-sheet.



(a)



(b)



(c)

**Figure 1.8: Light-sheet dimensions.** 1.8a shows a light sheet, with the field of view indicated. Since the light-sheet intensity is uneven, the field of view has to be confined to a smaller region. 1.8b The width and thickness of the field of view depends on the Rayleigh length of the beam ( $z_{y,0}$ ). 1.8c Height of the field of view is determined by the Gaussian profile of the elliptical beam.

### 1.4 Multi-view light-sheet microscopy

already in 1989 multi-view to increase axial resolution in conventional optical microscope. They imaged *Drosophila* metaphase plate in Zeiss Axiomat microscope using a 63x 1.2 NA water immersion objective lens. To increase the axial resolution, a special rotation stage was constructed, that allowed rotation around the object of interest to image it from a 90° tilted view. Using a fusion method in the Fourier space, the resolution increased to 0.25  $\mu\text{m}$  in lateral and 0.4  $\mu\text{m}$  in the axial direction for real samples.

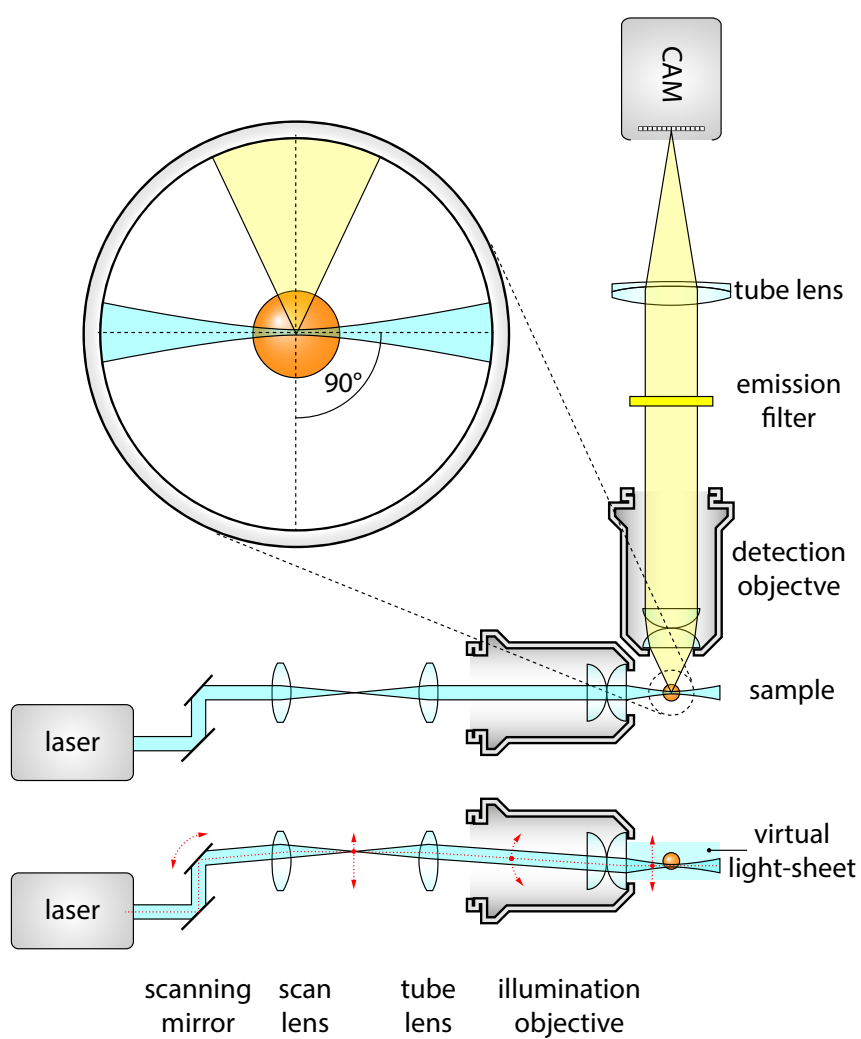
Multiple imaging axis microscopy (MIAM) [47] to image specimens from 4 directions in a tetrahedral objective configuration, could reach a 5.8 fold increase in axial resolution by combining the four views as a weighted average. Follow up for this: sample manipulation with optical tweezers / optical levitation in 3D [65](because of the lack of space for mechanical translation stage) positioning of a 20  $\mu\text{m}$  latex bead in a 100  $\mu\text{m}$  diameter volume by changing the intensity of 4 laser beams.

### 1.5 Imaging mouse preimplantation development

### 1.6 Imaging mouse postimplantation development

### 1.7 Imaging adult mice

things to improve: resolution axial resolution relative to lateral complete view reduce scattering



**Figure 1.9: DSLM illumination.** DSLM illuminates a specimen by a circularly-symmetric beam that is scanned over the field of view. This creates a virtual light-sheet, which illuminates a section of a specimen just like the SPIM. Light-sheet in DSLM is uniform over the whole field of view and its height can be dynamically altered by changing the beam scan range.

## Chapter 2

# Dual Mouse-SPIM

### 2.1 Previous Mouse-SPIM

mammalian development chromosome segregation cell specification symmetry breaking  
signaling pathways human infertility congenital diseases

current mouse-SPIM [J1] already is very good for answering many of these questions

advantages: immersion liquid and culture medium separated open-top sample holder  
allow standard microdrop *in vitro* embryo culture row of embryos - high throughput dual  
color imaging environmental chamber allows live imaging for 3 days, every

follow chromosomes by tracking kinetochores

from lattice paper: “because photodamage mechanisms that scale supralinearly with  
peak intensity have been identified for both visible [66] and two-photon [67] excitation.”

### 2.2 Light collection efficiency of an objective

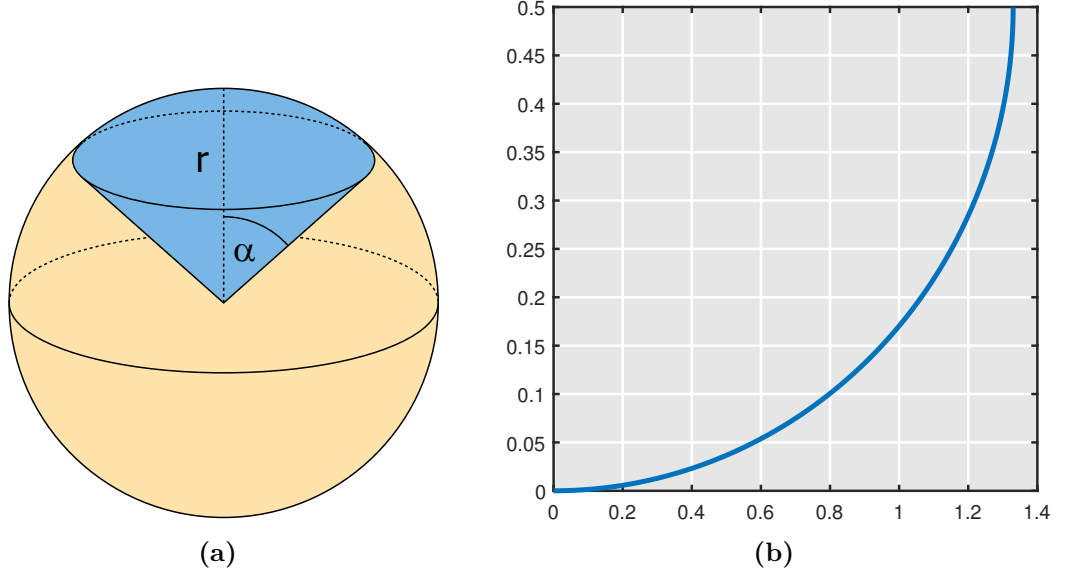
Resolution is not the only parameter of concern when designing a new microscope setup.  
We also have to consider light collection efficiency, since many application, especially  
live imaging applications require a tight photon budget. This means a single fluorophore  
can only emit so many times before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

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

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

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

## 2.2 Light collection efficiency of an objective



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

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

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

The surface area of the full sphere is calculated as:

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

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

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

Some more calculations: (Fig. 2.1b).

## 2. DUAL MOUSE-SPIM

### 2.3 Optical layout

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

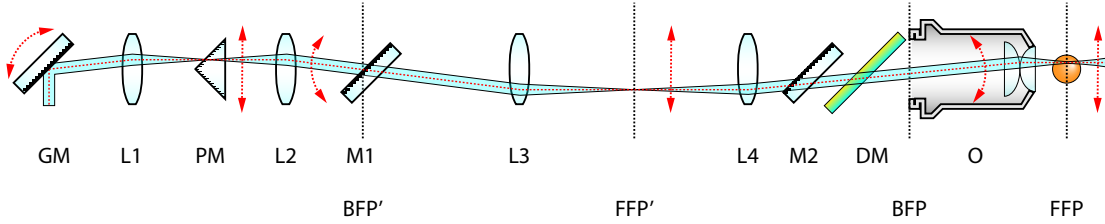


Figure 2.2: Simplified schematics for illumination.

When designing the optical layout of the setup, we had two principles in mind: simplicity and efficiency. Because of the symmetrical design it is possible that

### 2.4 Optical alignment

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

#### 2.4.1 Alignment of illuminaiton branches

The two illumination braches start with a common light source, a single mode fiber coupled to laser combiner, and they also share a galvanometric mirror that performs the beam scanning to generate the virtual light-sheet. Likewise shared is a scan lens focusing on the galvo mirror (GM), and the illumination splitter unit (PM, see Section 2.4.4).

Alignment for the illumination arms are done in three steps. First the laser beam is aligned on the first rail that holds the galvo, lens L1, and the splitter unit PM. This is performed by two kinematic mirrors placed between the fiber output and the galvo mirror. Using these two mirrors it's possible to freely align the beam along all 4 degrees of freedom: translation in two orthogonal directions, and rotation around two orthogonal axes. Beam alignment on the rail is tested by two irises at the two ends of the rail, if the beam passes through both of them we consider it centered and straight on the optical axis.



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

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

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

**Adjusting beam axial position.**

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

### 2.4.2 Alignment of detection branches

Since the detection path is equivalent to a wide-field detection scheme, its alignment is much simpler than that of the illumination branches. The only difference is the detection branch merging unit (see Sec. 2.4.5.) that features two moving mirrors. This, however

## 2. DUAL MOUSE-SPIM

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

The final alignment procedure

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

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

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

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

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

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

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

### 2.4.3 3D figures

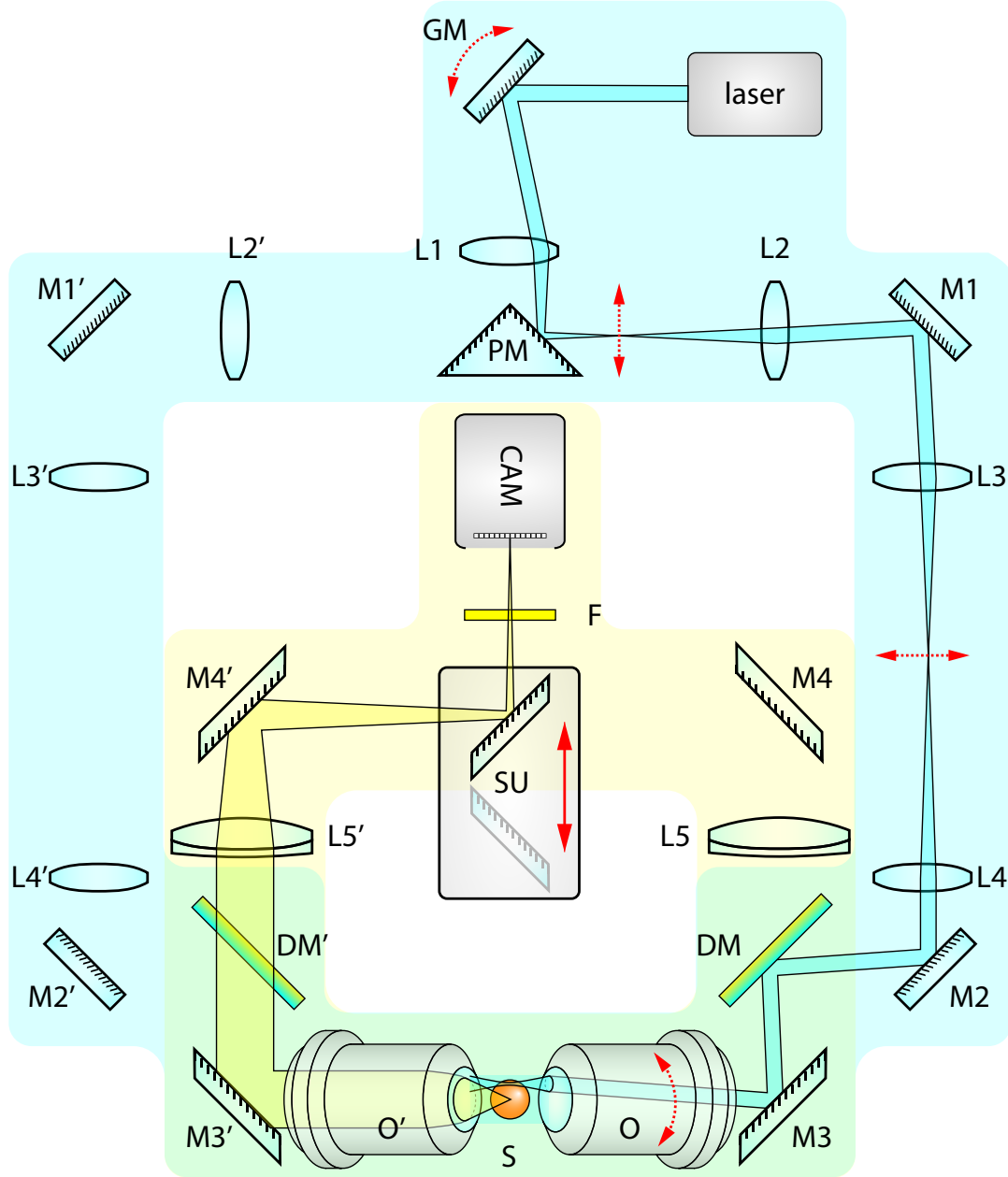
### 2.4.4 Illuminaiton splitter unit

### 2.4.5 Detection merging unit

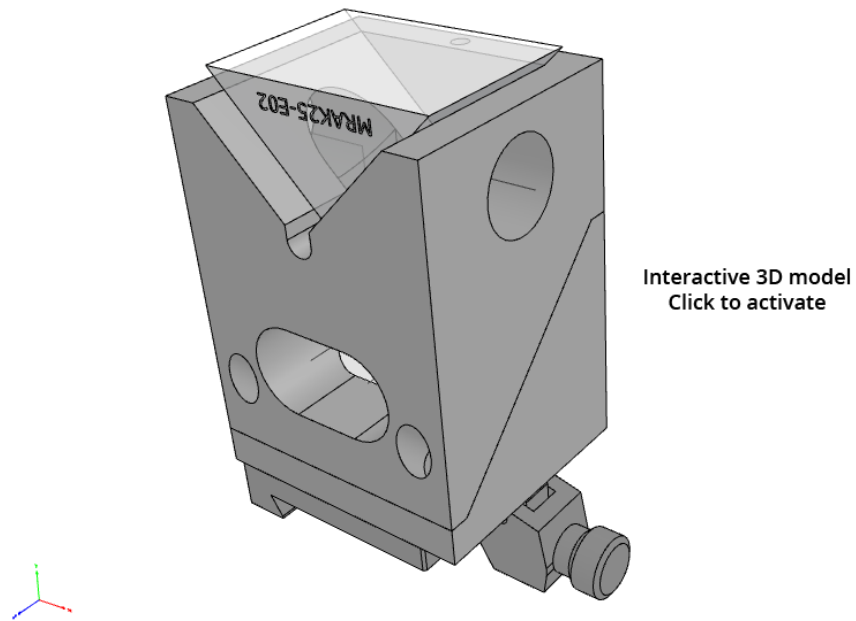
## 2.5 Results

Resolution for Lattice light-sheet [68]:  $230\text{ nm}$  in  $x$  and  $370\text{ nm}$  in  $z$  [69]

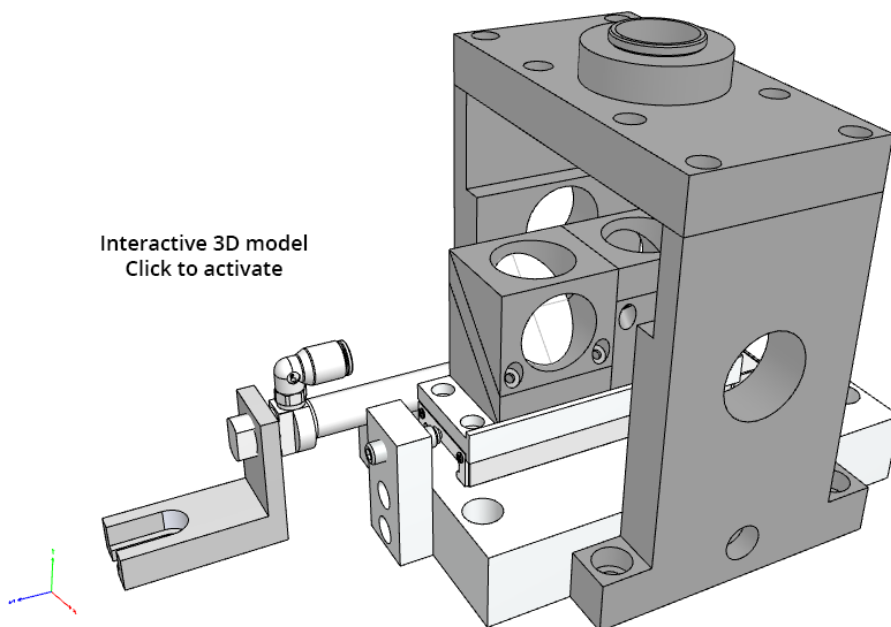
## 2. DUAL MOUSE-SPIM



**Figure 2.3: 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, SU – switcher unit



**Figure 2.4: Illumination branch splitting block.** 3D model of the custom designed splitter block used in the illumination branch. Depending on the horizontal beam position at the lower port, the beam will be reflected either to the right or to the left. Because of the mirror alignment, the beam is rotated 90 degrees.



**Figure 2.5:** 3D model of the custom designed unit to enable quick switching between the two detection paths. A high precision stainless steel stage holds two mirror blocks facing opposite directions, and bouncing the light upwards, towards the camera. The units are translated by pneumatic cylinder to ensure high speed and reproducibility.

## 2. DUAL MOUSE-SPIM

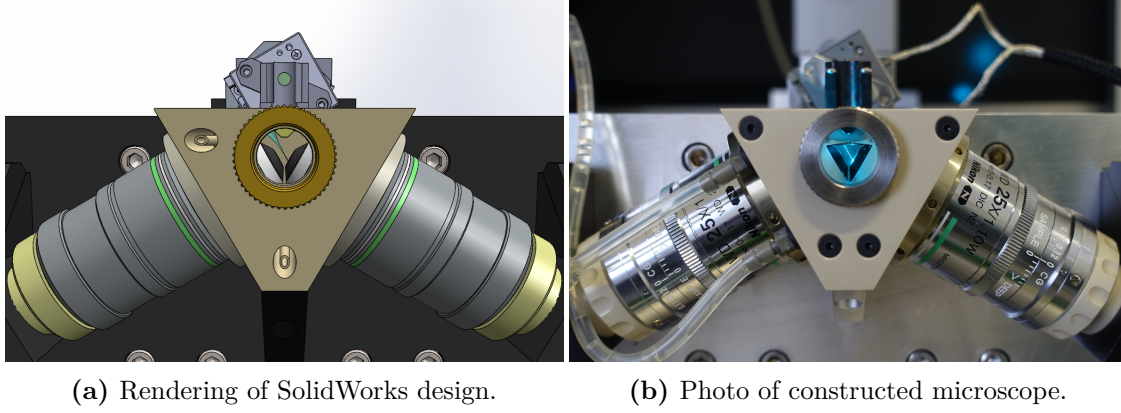


Figure 2.6: Front view of the Dual Mouse-SPIM.

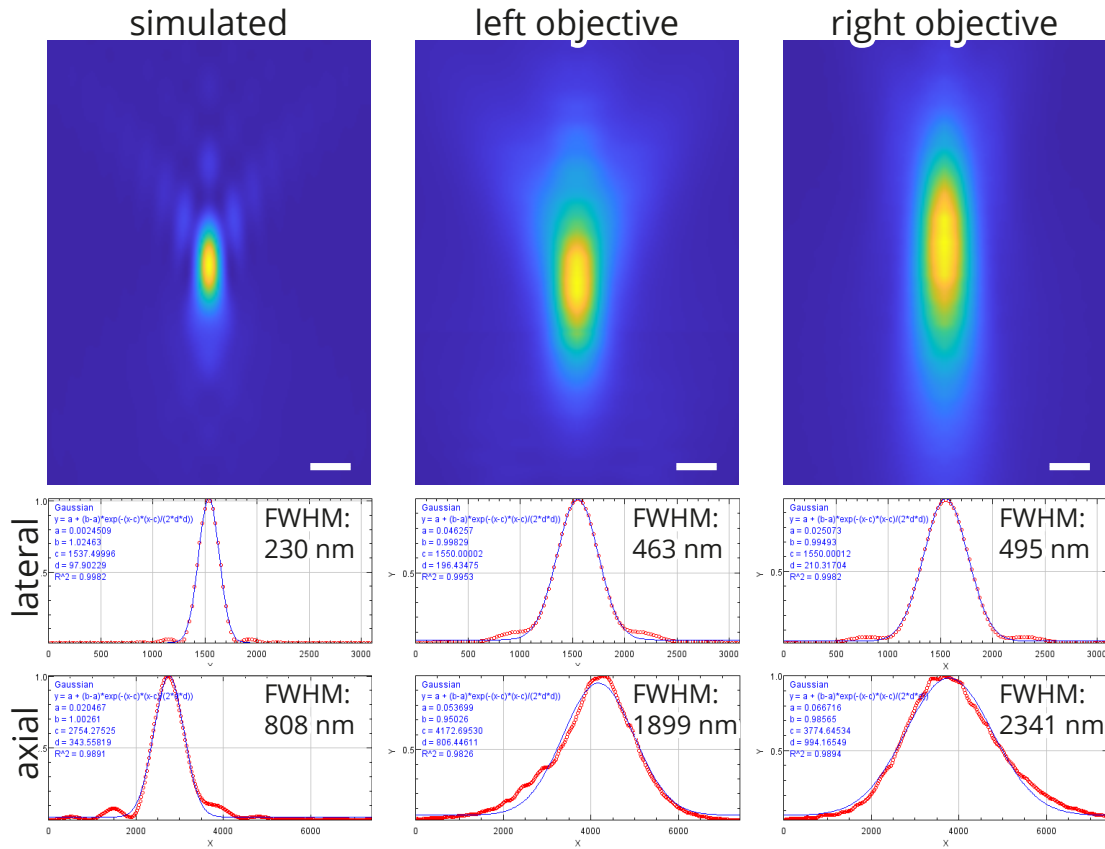


Figure 2.7: Simulated and measured PSF of Dual Mouse-SPIM. Top row: axial sections of simulated and measured point spread functions. Middle row: lateral intensity profile and Gaussian fit. Bottom row: Axial intensity profile and Gaussian fit. Simulations were performed based on the Gibson-Lanni model. Immersion medium and sample refractive index: 1.330, coverslip (FEP foil) refractive index: 1.344, coverslip distance: 1900  $\mu\text{m}$ , coverslip thickness: 50  $\mu\text{m}$ . Scale bar: 500 nm.

## Chapter 3

# Image processing

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

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

As it is apparent from this table,

proportional to the symbol frequencies. A string of symbols can be encoded by applying this process recursively: The sub-interval from the previous step is subdivided again using the same proportions. Figure4.2. shows an example of arithmetic coding using

## 3. IMAGE PROCESSING

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### 3.1 Multi-view fusion techniques

#### 3.1.1 Why multi view

#### 3.1.2 registration

Image based registration

Bead based registration

Affine transformation

#### 3.1.3 fusion

Average

Sigmoidal weighted average

Fourier mixing

Huisken had something like this

Wavelet-based fusion

Multi-view deconvolution

[52] Uros thesis [70], [71] Spatially variant deconvolution

### 3.2 Image compression

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

In this paper I will review the basics of image compression based on Sayood's textbook, Introduction to Data Compression [72]. I will introduce some basics of information theory and entropy, followed by discussing two widely used entropy coding algorithms, Huffman coding and arithmetic coding. Section 3 will be about transform coding, specifically Discrete Cosine Transform (DCT) and wavelet transform while also touching upon techniques based on differential pulse code modulation. Finally I will show how some of



the most widely used image compression standards use these methods to achieve effective image compression.

## 3.3 Entropy coding

### 3.3.1 Information and Entropy

For the purpose of data compression it is useful to quantify the amount of *information* contained within a piece of data. The first rigorous definition of information was presented in an extremely influential paper by Shannon, published in two parts in 1948 [73, 74].

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

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

2 independent events:

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

self-information is additive:

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

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

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

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

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

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

### 3.3.2 Huffman coding

Huffman coding is a prefix-free, optimal code that is widely used in data compression. It was developed by David A. Huffman as a course assignment on the first ever course on information theory at MIT, and was published shortly afterwards [75]. It is a variable length binary code which assigns different length codewords to letters of different probabilities. It is able to achieve optimal compression, which means the total length of the coded sequence will be minimal.

Although it produces a variable length code which can introduce some issues with decoding, it is still uniquely decodable. It achieves this property by using prefix-free

### 3. IMAGE PROCESSING

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**Table 3.1: Examples of a random binary code (#1) and a prefix-free binary code (#2).** Code #2 is uniquely decodable, while for code #1 it's necessary to introduce boundaries between codewords to be able to distinguish them.

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

codewords, meaning that none of the codewords are prefixes of any other codewords. This property can be exploited when decoding the codeword, since during this procedure the number of bits for the next codeword can not be determined in advance. However if no codeword is a prefix of another codeword, by simply reading the successive bits one by one until we reach a valid codeword, it's possible to uniquely decode the message.

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

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

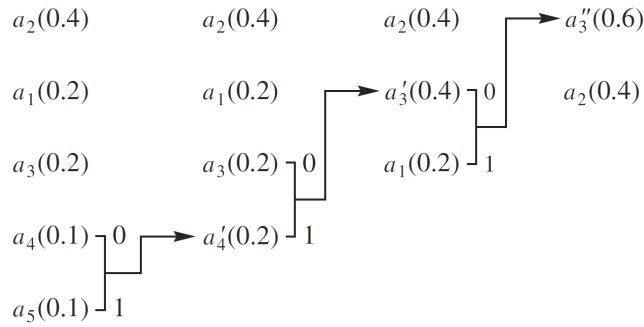
1. For a letter with higher frequency the code should produce shorter codewords, and for letters with lower frequency it should produce longer codewords.
2. In an optimum code, the two least frequent codewords should have the same lengths.

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

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

**Table 3.2: Huffman code table.**

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



**Figure 3.1: Building the binary Huffman tree.** The letters are ordered by probability, these will be the final leaf of the tree. To join the to branches at every iteration we join the to nodes with the smallest probability, and create a new common node with the sum of the probabilities. This process is continued until all nodes are joined in a root node with probability of 1. Now, if we traverse down the tree to each leaf, the codeword will be defined by their position.

To construct such a code, the following iterative procedure can be used. Let's consider an alphabet with five letters  $A = [a_1, a_2, a_3, a_4, a_5]$  with  $P(a_1) = P(a_3) = 0.2$ ,  $P(a_2) = 0.4$  and  $P(a_4) = P(a_5) = 0.1$  (Table 3.2). The entropy for this source is 2.122 bits/symbol. Let's order the letters by probability, and consider the two least frequent. Since the codewords assigned to these should have the same lengths, we can assign their codewords as

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

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

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 letter, its probability is 1. We can represent the end result in a binary tree (see Figure 3.1), where the leaves are the letter of the alphabet, nodes are the merged letters, and the codewords are represented by the path from the root node to each leaf (compare with Table 3.3). The average length of this

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Table 3.3: Huffman code table.

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

code is

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

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

#### 3.3.3 Arithmetic coding

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

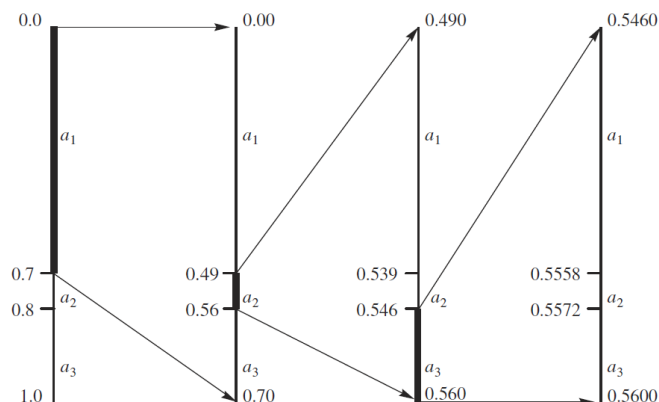
Let's consider the following example: take alphabet  $A = [a_1, a_2]$  with  $P(a_1) = 0.95$  and  $P(a_2) = 0.05$ . The first order entropy for this source is  $-0.95 \log 0.95 - 0.05 \log 0.05 = 0.2864$  bits/symbol, however if we assign the Huffman code we will have to use  $c(a_1) = 0$  and  $c(a_2)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

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

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

Table 3.4: Example alphabet for arithmetic coding.

Letter	Probability
$a_1$	0.7
$a_2$	0.1
$a_3$	0.2

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

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

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

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

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

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#### 3.4 Transform coding

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

##### 3.4.1 Discrete Cosine Transform

Discrete Cosine Transform [76] is closely related to the well known Fourier transform. The main idea behind this is to represent a function by a weighted sum of different sine and cosine functions. This provides a different view, instead of looking at the data in the time domain, we gain information about the frequencies that compose the signal.

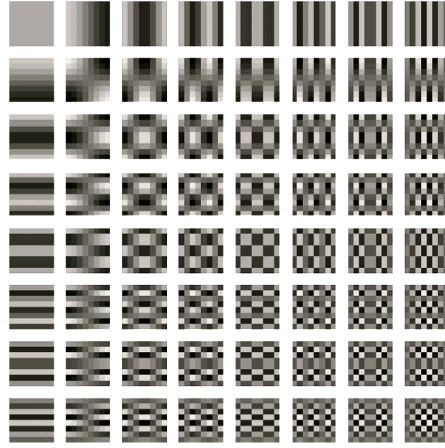
Discrete Cosine Transform is a variant of the discrete Fourier transformation, however instead of making the signal periodic which can introduce large jumps at the edges, the signal is extended in a symmetric way. Since this will give a smooth transition even at the boundaries, the transform does not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

The DCT base functions are defined in the following way:

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

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

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



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

each dimension.

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

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

### 3.4.2 Discrete Wavelet Transform

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

A solution for this is to use different base functions, namely ones with finite support. This way we will not only be able to get information about the frequency, but also about

### 3. IMAGE PROCESSING

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the localization of that frequency in some extent.

One option for such a set of local basis functions, and certainly the most popular one, is the multi-resolution analysis based on wavelets. The term “multi-resolution analysis” in the context of wavelets was introduced in the late 1980s by Stéphane Mallat [77], though research on wavelets had been ongoing for several years before that.

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

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

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

The oldest wavelets are the Haar wavelets, and because of their simplicity, they provide a good example on how wavelet transformation works. The Haar wavelet scaling  $\phi$  and wavelet  $\psi$  functions are the following:

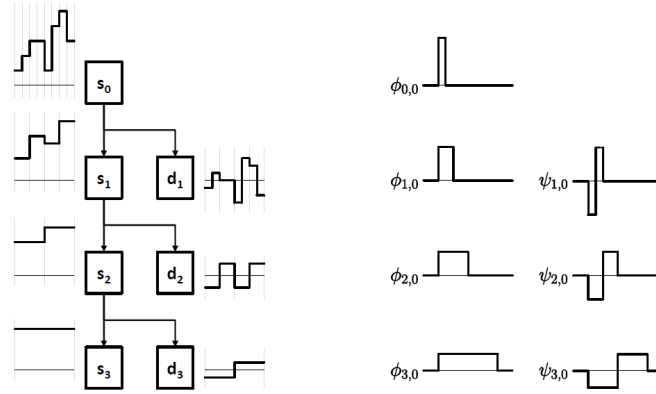
$$\phi(t) = \begin{cases} 1 & \text{if } 0 \leq t < 1 \\ 0 & \text{otherwise} \end{cases} \quad (3.10)$$

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

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

Figure 3.4. (left) schematically shows the decomposition of a signal into low-pass com-





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

ponents corresponding to the scaling function, and high-pass components corresponding to the wavelet. The signal  $s_0$  can be represented by the translated scaling function at a finer scale  $l_0$ , which can be decomposed to the sum of a coarser approximation  $s_1$  corresponding to the scaling function and the detail part  $d_1$  corresponding to the wavelet functions. This decomposition to a sum of coarser approximation and detail can be continued multiple times, thus resulting in the previously mentioned multi-resolution analysis.

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

### 3.4.3 Pixel prediction

## 3.5 Conclusion

In this paper I have introduced some of the most important techniques for image compression. Many of these are used in different image compression algorithms, such as JPEG [78], JPEG-LS [79] and JPEG2000. The common point for each of these formats is that they first transform the images to effectively reduce first order entropy, either by predicting the pixel values and coding only the differences (JPEG-LS), or by using either Discrete Cosine Transform (JPEG) or Discrete Wavelet Transform (JPEG2000). In the case of lossy standards after this transformation step a quantization is also performed depending on the desired quality setting for the coder. Finally as the last step

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the transformed and quantized coefficients are compressed by an arithmetic coder, such as Huffman coding in JPEG or arithmetic coding in JPEG2000. The end result in each case is a highly optimized compression that greatly reduces the file size.

## Chapter 4

# Real-time, GPU accelerated image processing pipeline

### 4.1 GPU architecture

### 4.2 Live fusion

#### 4.2.1 MuVi-SPIM

#### 4.2.2 Bead based registration

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

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

#### 4.3.1 Data sizes in microscopy

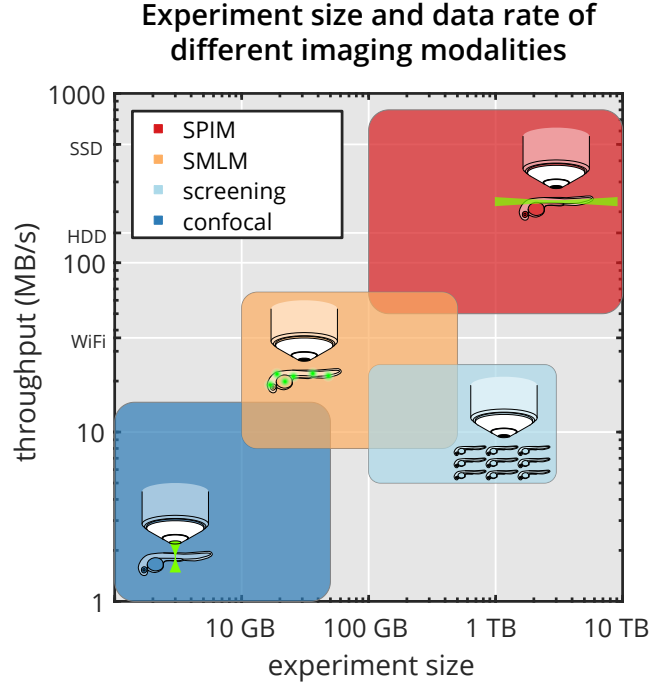
#### 4.3.2 Lossless compression performance

#### 4.3.3 Benchmarking

### 4.4 Noise dependent lossy compression

### 4.5 Methods

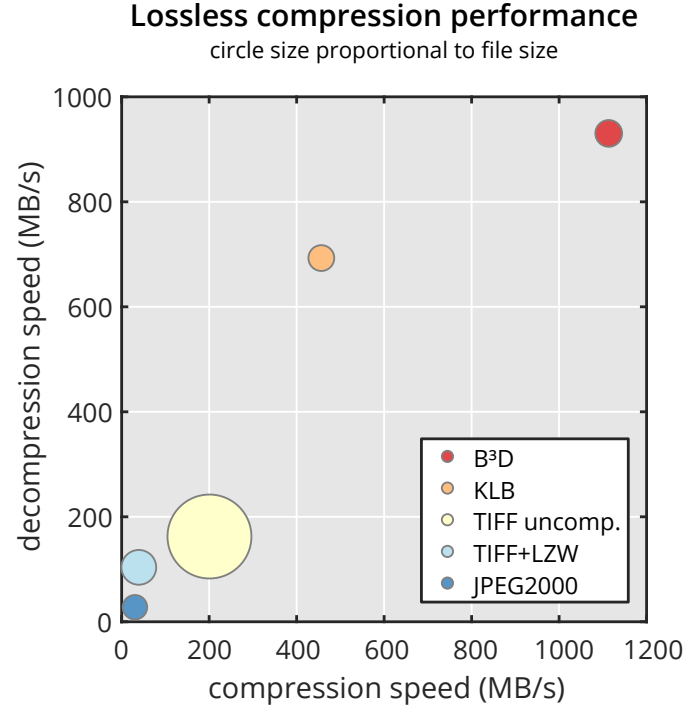
#### 4. REAL-TIME, GPU ACCELERATED IMAGE PROCESSING PIPELINE



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



**Figure 4.2: Lossless compression performance.** Performance comparison of our B<sup>3</sup>D 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 4.2).

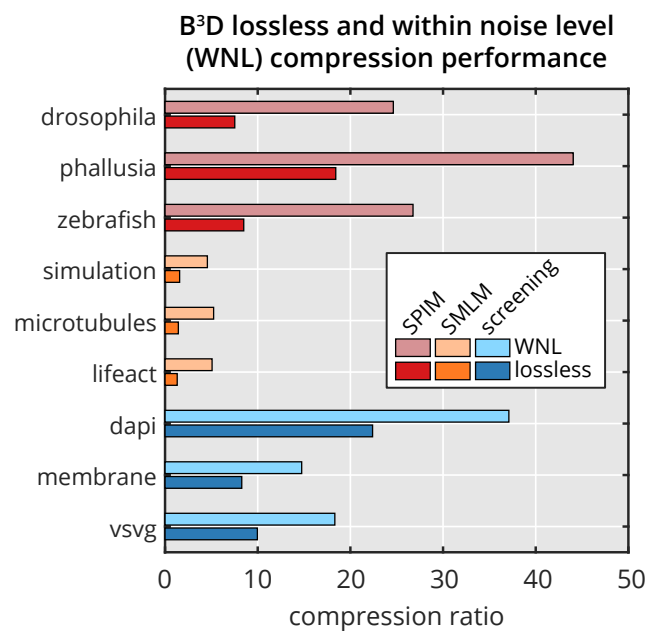
	write speed	read speed	CR	file size
<b>B<sup>3</sup>D</b>	1,115.08 MB/s	928.97 MB/s	9.861	100%
<b>KLB</b>	283.19 MB/s	619.95 MB/s	10.571	93.28%
<b>JPEG2000</b>	31.94 MB/s	26.38 MB/s	11.782	83.69%
<b>JPEG2000</b>	202.32 MB/s	161.08 MB/s	1.00	986.1%
<b>TIFF + LZW</b>	40.85 MB/s	102.37 MB/s	5.822	169.37%

**Table 4.2: 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 4.2.

#### 4. REAL-TIME, GPU ACCELERATED IMAGE PROCESSING PIPELINE

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 [80]	1,005.38
<b>vsvg</b>	screening	wide field fluorescence images of CFP-tsO45G proteins in HeLa Kyoto cells [80]	1,005.38
<b>membrane</b>	screening	wide field fluorescence images of membrane localized CFP-tsO45G proteins labeled with AlexaFluor647 in HeLa Kyoto cells [80]	1,005.38

Table 4.3: Datasets used for benchmarking compression performance.



**Figure 4.3: Lossless compression performance..** For description of datasets see Table 4.3.

## Chapter 5

# Discussion



## 5.1 New scientific results

**Thesis I.** *I have designed and constructed a new light-sheet microscope suitable for high sensitivity imaging of delicate samples. A novel arrangement of two high numerical aperture objectives in 120 degrees allows for near isotropic resolution while increasing light collection efficiency by a factor of two.*

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

Live imaging of light sensitive specimens, such as a developing mouse embryo is a challenging task

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

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

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

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

All microscopy images contain inherent noise, that is

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

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

$B^3D$  is an efficient, GPU-based image compression library allowing lossless and noise dependent lossy compression of microscopy images. Since many high-speed microscopy methods generate immense amounts of data, easily reaching terabytes per experiment, image compression is especially important to efficiently deal with such datasets.

## 5. DISCUSSION

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Finally, I would like to thank Gustavo Quintas Glasner de Medeiros and Nils Norlin, as they both participated in the microscope design and program development, and both of them inspired me greatly. Without their help, I couldn't have written this paper.

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