

Grade: 1,3

Protocol for the experiment
Confocal Laser Scanning Microscopy

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1 Introduction

Widefield fluorescence microscopy (WFFM) was developed in 1904 and has since offered the possibility of examining fluorescent-labelled parts of biological samples [1]. For this purpose, the sample is irradiated with excitation light, which leads to the excitation of the fluorochromes and consequently to the emission of fluorescence. [2]. However, images of thick samples in particular often appear blurred due to out-of-focus light from excited layers above and below the focal plane. The confocal laser scanning microscopy (CLSM) developed by Minsky in 1954 solved this problem by placing a pinhole in front of the detector so that only focused light rays from a point on the focal plane reach the detector. By scanning the entire sample, sharp images can be obtained with this method [1].

In the scope of this labwork, first the refraction and interference properties of light were investigated. For this purpose, single and double slit experiments, in which the slit width and separation were changed, as well as a pinhole experiment were performed. Photographs of the respective diffraction pattern were taken, distances measured during the experiment and the final data analysed afterwards.

In the second and third part the PSFs (Point Spread Function) and cross sections of microspheres and neurons were recorded using different objectives and microscope configurations. The FWHM (Full width at half maximum) of each measurement was determined from the recorded graphs and images, and differences between the images were analysed [3].

2 Technical background

2.1 Basic wave optics

2.1.1 Interference

Interference is the superposition of two (electromagnetic) waves to a resultant one. If the superimposed waves have the same wavelength, constructive or destructive interference occur. In constructive interference, the waves have a phase difference of 2π and its integer multiples, resulting in a higher amplitude. While in deconstructive interference, where the waves cancel each other out, there is a phase difference of π and its uneven integer multiples [4].

2.1.2 Refraction

Refraction of light waves occurs at interfaces of media with different refraction indices, where the propagation direction of the waves changes due to the change of light [3]. This phenomenon can be explained by the Huygens-Fresnel principle. In which each point of a wave front is the origin of a secondary wave, which forms a new wave front with other secondary waves [5].

The propagation of the wave at the transition from medium 1 to medium 2 can be described using Snell's Law (see formula 1):

$$n_1 \cdot \sin(\alpha_1) = n_2 \cdot \sin(\alpha_2) \quad (1)$$

Where n_1 and n_2 are the refraction indices of the first and second medium and α_1 and α_2 are the angles to the normal of the interface in the first and second medium.

Rearranging the above formula 1 and taking into account the changed wavelength and speed of light, the following holds (see formula 2):

$$\frac{n_2}{n_1} = \frac{\sin(\alpha_1)}{\sin(\alpha_2)} = \frac{\lambda_1}{\lambda_2} = \frac{c_1/f}{c_2/f} \quad (2)$$

Where λ_1 and λ_2 are the wavelengths and c_1 and c_2 are the velocities of light in the first and second media, respectively. f is the frequency, which is constant [3].

2.1.3 Diffraction

Diffraction is a phenomenon that occurs when light waves interact with objects or structures whose size is approximately equal to the wavelength of the light.

An example of this is the single-slit experiment, in which light waves are diffracted at a narrow slit. Due to this, an elongated diffraction pattern forms on a screen behind it, in which bright maxima and dark minima alternate periodically (see figure 1a) and 1b)). The intensity of the maxima is highest in the centre of the pattern and decreases towards the edges (see figure 1c)).

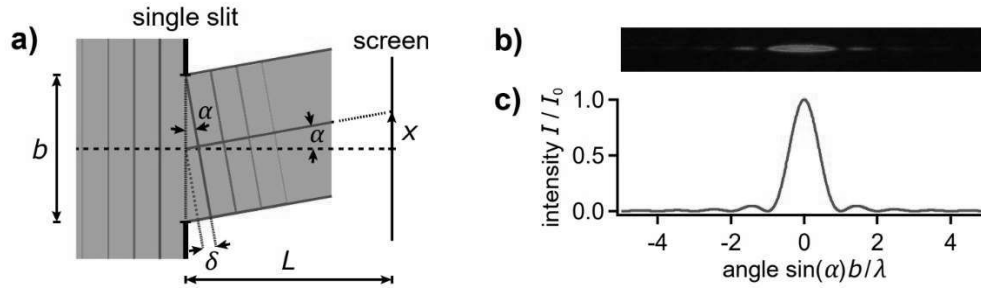


Figure 1: a) Diffraction at a single slit of width b . α is the angle of the n^{th} -order minimum, δ the path difference, x the distance between the central maximum and the n^{th} -order minimum, and L is the slit-screen distance b) Single slit diffraction pattern, c) Intensity profile of the single slit diffraction pattern [3]

By means of the following formula the theoretical angle $\alpha_{th,n}$ of the n^{th} -order minimum can be determined (see formula 3):

$$\alpha_{th,n} = \arcsin\left(\frac{n \cdot \lambda}{b}\right) \quad n = 1, 2, 3 \dots \quad (3)$$

Where n is the order of the minimum, λ the light's wavelength and b is the slit width [3].

In a double slit experiment with slit spacing g , an intensity profile is obtained which has an envelope similar to the pattern of the single slit experiment (see figure 2) [6]. In addition, periodic intensity oscillations occur in the maxima of the pattern, the frequency of which depends on the slit separation [7].

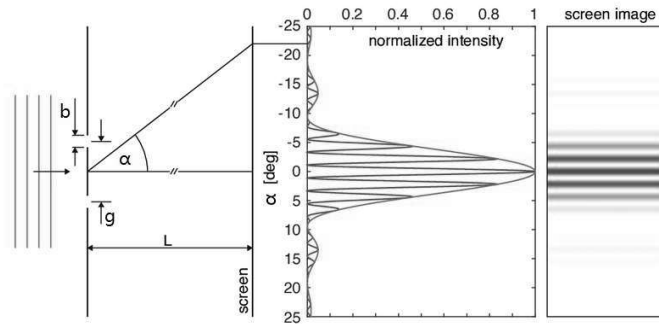


Figure 2: Diffraction at a double slit of width b . g is the slit separation, α the angle of the n^{th} -order minimum, x the distance between the central maximum and the n^{th} -order minimum, and L is the slit-screen distance. At the right side the resulting intensity profile and screen image can be seen [6]

The angle of the n^{th} order minimum of a double slit with slit spacing g is calculated as follows (see formula 4):

$$\alpha_n = \arcsin\left(\frac{n \cdot \lambda}{2g}\right) \quad n = 1, 2, 3 \dots \quad (4)$$

If a circular aperture is used instead of a slit, a two-dimensional airy disk is created on the screen, which consists of minima and maxima arranged circularly around each other. The intensity has its maximum in the centre and decreases towards the outside [3].

2.2 Widefield fluorescence microscopy

2.2.1 Setup and working principle

The principle of the WFFM is based on the excitation of fluorochromes contained in the sample using excitation light of a narrow wavelength range. The subsequent fluorescence signals emitted from these, which have a longer wavelength than the excitation light, are recorded by a detector or camera.

This microscopy technique allows imaging of individual structures of biological samples that are labelled with fluorescent dyes before [2]. The problem with this technique is that the responses of all layers to the excitation light are recorded, causing images of thicker samples to appear blurred due to the out-of-focus light [1].

2.2.2 Resolution and FWHM

During imaging with WFFM, diffraction effects occur due to the use of objectives that resemble a circular aperture. This causes small round structures of the sample to appear as hourglass shaped PSFs on the final image, which correspond to the airy pattern.

In order to distinguish two points or objects with the microscope as two individual ones, the Rayleigh criterion must be fulfilled. This states that the minimum resolvable distance d is the one at which an object is located in the first minimum of the airy pattern of another object. If the two objects are closer to each other, they cannot be perceived as two separate ones. Theoretically, d is calculated according to formula 5:

$$d = \frac{0.61\lambda_{em}}{NA} \quad (5)$$

Where λ_{em} is the emitted wavelength of the fluorescence and NA is the numerical aperture of the microscope. This is calculated from the refractive index n of the medium between objective and sample and the aperture angle α according to the formula $NA = n \cdot \sin(\alpha)$.

Since it is complicated to determine the resolution of the microscope based on the Rayleigh criterion in a practical experiment, the FWHM of the PSF of a small object like a microsphere is used instead. The lateral theoretical $FWHM_{x,y}$ is thus calculated as follows (see formula 6):

$$FWHM_{x,y} = \frac{0.51\lambda_{em}}{NA} \quad (6)$$

The axial resolution $FWHM_z$ is calculated using following formula 7:

$$FWHM_z \cong \frac{1.77n\lambda_{em}}{NA^2} \quad (7)$$

The use of an oil objective can improve resolution by increasing the NA due to the higher refractive index of oil compared to air and the larger effective opening angle. Therefore, more emitted light waves enter the objective.

Using a larger aperture angle also contributes to better resolution, but this is limited by the critical angle for internal reflection [3].

2.3 Confocal laser scanning microscopy

2.3.1 Setup and working principle

In CLSM, as in WFFM, the sample labelled with fluorescence dyes is irradiated with excitation light of a narrow wavelength range and the emitted fluorescence of the sample is detected. In addition, CLSM uses a disk with a pinhole in front of the detector, which allows only focused light from one point of the focal plane to pass through and reach the detector. The out-of-focus light of the remaining layer is repelled by the disk and thus does not contribute to the image. In this way, the so-called "optical sectioning" leads to a sharp image and the possibility to record individual slices of the sample [1].

Since each point has to be scanned one after another, a setup of two mirrors is often used in the microscope, one for the movement of the excitation and emission light in the x-direction and one for the movement of the light in the y-direction. If a z-stack of the sample needs to be imaged, the objective is moved in the z-direction after each fully scanned slice [3].

2.3.2 Resolution and FWHM

By using the pinhole, the PSFs of illumination and detection are diffraction-limited. Their multiplication gives the resulting effective PSF. The lateral resolution $FWHM_{x,y}$ of a CLSM with a

pinhole of the diameter of 1 Airy Unit (AU) is approximately the same as that of the WFFM (see formula 6 and 8):

$$FWHM_{x,y} = \frac{0.51\lambda_{ex}}{NA} \quad (8)$$

Here λ_{ex} is the wavelength of the excitation light.

The lateral resolution $FWHM_z$ is better than that of the WFFM and is calculated as follows (see formula 9):

$$FWHM_z = \frac{0.88\lambda_{ex}}{n - \sqrt{n^2 - NA^2}} \quad (9)$$

Using smaller pinholes theoretically improves resolution, but it also blocks more of the emitted light at the pinhole, so more signal is lost [3].

2.4 Nyquist-Shannon sampling theorem

When recording images with the WFFM and the CLSM, it is important to ensure that the Nyquist-Shannon criterion is met. This states that when converting analog to digital signals, the sampling frequency must be at least more than twice the frequency of the analog signal so that the information of the digital signal corresponds to that of the analog signal without loss of information.

For the microscopes it applies equivalently that the selected pixel size of the detection system must be at least smaller than half of the structure size of the sample. Thus, the following applies to the pixel size $PS_{Nyquist}$ (see formula 10):

$$PS_{Nyquist} < \frac{\Delta x}{2} \quad (10)$$

Where Δx is the structural size in the sample.

A further reduction in signal loss during conversion occurs when choosing a pixel size of one third of the structural size. Smaller pixel sizes lead to an "empty magnification", where no further improvement of the information content occurs [3].

3 Execution of the experiment

In the first part of the practical work, single slit, double slit and pinhole diffraction experiments were performed. For this purpose, the optical bench was set up by attaching a laser with a wavelength of 633 nm.

First a single slit having a width of 0.8 mm was fixed in front of the laser. Since this slit was wide enough for the laser to pass through without diffraction effects, a beam-widening lens system was used. The laser was adjusted so that it passed through the centre of the slit and the diffraction pattern formed on the wall behind it. For later analysis, the distance between the centre maximum and the 10th-order minimum in the pattern was measured and photographs of the pattern were taken. In addition, the distance from slit to screen was measured.

The single slit experiment was repeated using slits of 0.4 mm and 0.1 mm width. As before, the resulting diffraction patterns were measured for later analysis and images were taken for comparison.

Subsequently, double slit experiments were performed by attaching double slits with different slit widths and slit separation in front of the laser. One after another, slits of the following width b and separation g were illuminated:

1. $b = 0.1 \text{ mm}$, $g = 0.3 \text{ mm}$,
2. $b = 0.2 \text{ mm}$, $g = 0.3 \text{ mm}$
3. $b = 0.15 \text{ mm}$, $g = 0.25 \text{ mm}$
4. $b = 0.15 \text{ mm}$, $g = 1 \text{ mm}$

Photographs were taken of the resulting diffraction patterns, which were used for later qualitative evaluation.

Finally, the double slits were replaced by a pinhole of unknown diameter. The distance between centre maximum and 2nd-order minimum in the resulting diffraction pattern was measured and images of the pattern were taken for evaluation.

At each measurement during the diffraction experiment accuracy was estimated, which was used for later calculations during the evaluation.

In the next part of the laboratory work, investigations with a microscope in the widefield configuration were examined. For this purpose, a low magnification air objective with a magnification of 20 and a NA of 0.45 was used, and sample 4 with 0.2 μm fluorescent microspheres was mounted in the microscope. The examination was performed with an excitation light of wavelength 480 nm,

resulting in an emission light with wavelength 540 nm. The pixel size was set to 0.37 $\mu\text{m}/\text{px}$. During the experiment exposure time, brightness and gain were adjusted by eye using the microscope software. With these settings a single bead was searched for. A PSF and its intensity profile in the xy-plane were recorded, and a z-stack was taken, in which the z-layers of the bead were traversed in 15 steps and cross sections in the xz- and yz-plane were generated.

The same steps were repeated again using an oil objective with a magnification of 100 and a NA of 1.45, for which a drop of oil was placed between the sample and the objective. In addition, sample 4 was replaced by sample 5 with beads of size 0.1 μm and the pixel size was reduced to 0.07 $\mu\text{m}/\text{px}$. The wavelength remained identical.

As before, a fluorescent microsphere was selected and a PSF and intensity profile were recorded from it. In 28 steps, z-stacks were created and the xz- and yz-planes of the PSF were generated.

In the third part of the practical work, samples were examined with the microscope in confocal configuration. For the experiments in this configuration the oil objective with magnification of 100 and NA of 1.45 and sample 5 with micro beads of size 0.1 μm were used. The wavelength of the excitation light was set to 488 nm, resulting in an emission light wavelength of 520 nm. In addition, a pinhole with a size of approximately 1 AU was used. The pixel size was set to 0.07 $\mu\text{m}/\text{px}$. During the experiment, laser power and voltage were adjusted for optimal image quality.

As before, the xy-PSF of a bead and its intensity profile were recorded and a z-stack of the bead was taken in 26 steps. From this, an xz- and yz-cross section was generated using the microscope software. With the identical settings this procedure was repeated with a pinhole having a diameter of 3.8 AU.

In the last part of the laboratory work, mouse neurons were imaged. Different components of the cells were labelled in advance with sundry fluorophores:

- DAPI (channel 1): Emission colour: blue (nuclei), Wavelength of excitation light: 405 nm
- Cy2 (channel 2): Emission colour: green (tubulin), Wavelength of excitation light: 488 nm
- Cy3 (channel 3): Emission colour: orange (nestin), Wavelength of excitation light: 561 nm

The oil lens with magnification of 100 and NA of 1.45 was used for the investigation.

First a single channel xy-image was taken with channel 2 in widefield configuration. Subsequently, this procedure was repeated using the same channel with the microscope in confocal configuration, where the pinhole had a diameter of 1 AU.

Then, multicolour imaging was performed with all three channels, for which the microscope remained in the confocal configuration. First, all three channels were tested in sequence. Subsequently, an image of the xy-plane was acquired, for which all three channels were addressed quickly one after another, so that "cross reactions" were minimized and finally a three-color image was obtained. As before, a z-stack was recorded in 6 steps, from which xz- and yz-cross sections were created using the microscope software. In addition, a two- and three-dimensional maximum intensity projection was acquired, using the software selecting the xy-point with the highest intensity from all z-sections for the final image.

Finally, to measure the laser power for later calculations, the sensor of a laser power meter was placed near the sample and the power was measured using the previously used settings and a light of wavelength 488 nm.

4 Results

The diffraction patterns of the single slit experiments are shown in Figure 3. The slit width was reduced from 0.8 mm to 0.4 mm and 0.1 mm for the different runs.

All three diffraction patterns are elongated, horizontally oriented patterns with minima and maxima alternating periodically. All patterns have a bright circular centre. As the distance from this increases, the brightness of the maxima becomes lower until no maxima are visible at the outermost edge.

Figure 3A shows the pattern of the 0.8 mm slit. The bright maxima are narrow and have sharp edges. In addition, the centre maximum has a relatively circular shape.

In comparison, the pattern of the 0.4 mm slit width has wider maxima with fuzzier edges, as well as a slightly oval-shaped centre maximum (see figure 3B). Moreover, the pattern appears slightly wider in the vertical direction than the previous one.

The pattern of the 0.1 mm slit can be seen in Figure 3C. Compared to the two previous patterns, the centre maximum appears significantly wider and more oval-shaped. In addition, the maxima are also significantly wider and more blurred than in the previous patterns. The pattern also appears wider in the vertical direction.

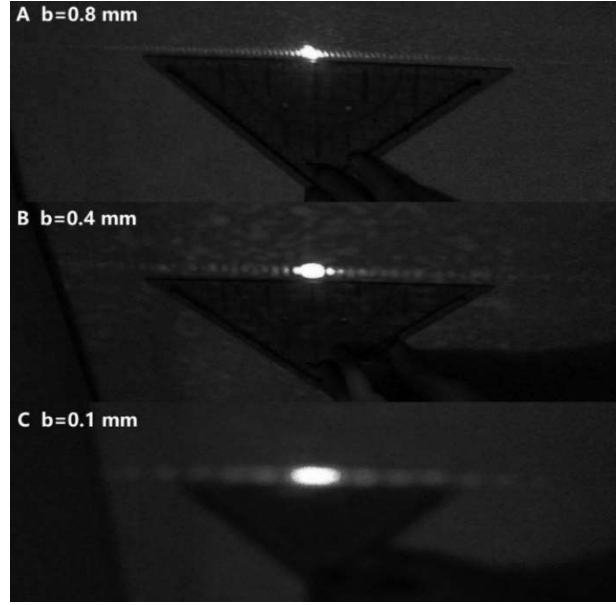


Figure 3: Diffraction pattern of the single slits with different slit width b

In the measurements of the diffraction pattern, the measured distance between slit and screen amounted to $4396 \text{ mm} \pm 10 \text{ mm}$. When measuring the slit-screen distance, an accuracy of 10 mm was estimated due to measurement and application errors, which was taken into account in further calculations.

For all samples, the distance x_n from an n^{th} -order minimum to the centre maximum was measured. Using this measured distance, the slit-screen length L and the current order n , the angle of the first-order minimum α_1 was calculated according to following formula 11:

$$\alpha_1 = \frac{\arctan\left(\frac{x_n}{L}\right)}{n} \quad (11)$$

As measurement errors occurred during the measurements, the accuracy of the calculated angle $\Delta\alpha_1$ was determined by means of these using the error propagation formula (see formula 12):

$$\Delta\alpha_1 = \left| \frac{\partial\alpha_1}{\partial x} \right| \cdot \Delta x + \left| \frac{\partial\alpha_1}{\partial L} \right| \cdot \Delta L \quad (12)$$

Where Δx is the estimated accuracy of the measured distance x_n and ΔL is that of the slit-screen distance.

In formula 12, formula 11 was inserted for α_1 and the derivatives were calculated, resulting in the following formula (see formula 13):

$$\Delta\alpha_1 = \left| \frac{L}{n \cdot (x_n^2 + L^2)} \right| \cdot \Delta x + \left| -\frac{x_n}{n \cdot (x_n^2 + L^2)} \right| \cdot \Delta L \quad (13)$$

In addition to the calculated angle from the measured data, the theoretical angle of the first order minimum $\alpha_{th,1}$ was calculated using the laser wavelength λ of 633 nm and the currently used slit width b (see formula 3 and 14):

$$\alpha_{th,1} = \arcsin\left(\frac{1 \cdot \lambda}{b}\right) \quad (14)$$

The calculated angles of the three slits are plotted in Table 1.

Table 1: Calculated angles of the first order minimum

Slit width b [mm]	Order n	Measured distance x_n [mm]	Experimental angle α_1 [°]	Theoretical angle $\alpha_{th,1}$ [°]
0.8	10	32 ± 1.5	0.04171 ± 0.00004	0.04534
0.4	10	70 ± 2	0.09123 ± 0.00005	0.09067
0.1	5	133.5 ± 4	0.34789 ± 0.00020	0.36268

In addition to the single slit experiments, double slit experiments were performed. The resulting patterns of the four runs are shown in Figure 4. All patterns are elongated, horizontally oriented and have a bright centre maximum. Starting from this, minima and maxima can be seen in periodic alternation, fading towards the edge.

The first two images 4A and 4B were taken with the same slit separation of 0.3 mm, but with a different slit width of 0.1 mm and 0.2 mm. It can be seen in both images that there are additional periodic intensity oscillations in the maxima. Differences between the two patterns can be seen in the width of the maxima, as those in image 4 B are significantly wider than in 4A, and the edges blur slightly with each other in 4B.

Images 4C and 4D were taken with an identical slit width of 0.15 mm. This value is midway between the slit widths of the first two images. The slit separation was changed from 0.25 mm in figure 4C to 1 mm in 4D. Despite the difference in slit separation, no significant difference can be seen between the two patterns when viewed. Both appear more blurred than the two before and look similar to those from the single slit experiment as there is no discernible intensity oscillation

in the maxima. The centre maximum and the maxima extending from the centre have an oval shape and appear broader than those of the first two patterns.

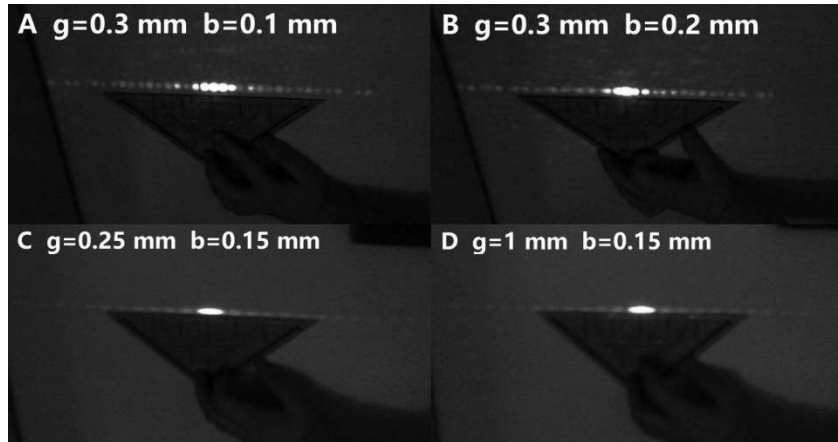


Figure 4: Diffraction pattern of the double slits with different separation g and width b

The pattern that emerged when the pinhole was illuminated is shown in Figure 5. A circular pattern can be seen, which has a bright, round and slightly oval centre. Around this are oriented ring-shaped periodically changing minima and maxima. With increasing distance from the centre, the maxima become ring segments and decrease in brightness.

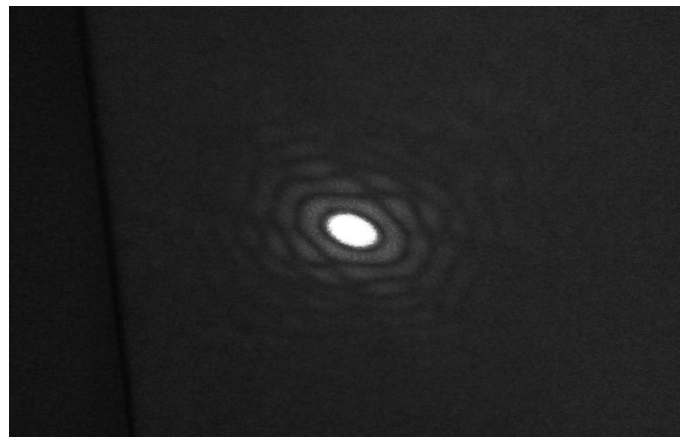


Figure 5: Diffraction pattern of the pinhole

To determine the pinhole, formula 14 was converted according to the hole width b and formula 11 was inserted for $\alpha_{th,1}$, so that the diameter of the hole could be calculated with resulting formula 15:

$$b = \frac{n \cdot \lambda}{\sin\left(\arctan\left(\frac{x}{L}\right)\right)} \quad (15)$$

The measured distance between the 2nd-order minimum and the centre maximum was (23 ± 2) mm. This resulted in (see formula 15):

$$d = b = \frac{2 \cdot 633 \cdot 10^{-9} \text{ m}}{\sin\left(\arctan\left(\frac{23 \cdot 10^{-3} \text{ m}}{4396 \cdot 10^{-3} \text{ m}}\right)\right)} = 2.42 \cdot 10^{-4} \text{ m}$$

The accuracy of the hole diameter Δd was calculated according to the formula of error propagation (see formula 16):

$$\Delta d = \left| \frac{\partial d}{\partial x} \right| \cdot \Delta x + \left| \frac{\partial d}{\partial L} \right| \cdot \Delta L \quad (16)$$

Inserting formula 15 for d resulted in (see formula 17):

$$\begin{aligned} \Delta d &= \left| \frac{Ln\lambda}{x^2 \sqrt{\frac{x^2}{L^2} + 1}} \right| \cdot \Delta x + \left| \frac{n\lambda}{x \sqrt{\frac{x^2}{L^2} + 1}} \right| \cdot \Delta L \\ &= \left| \frac{4396 \cdot 10^{-3} \text{ m} \cdot 2 \cdot 633 \cdot 10^{-9} \text{ m}}{(23 \cdot 10^{-3} \text{ m})^2 \sqrt{\frac{(23 \cdot 10^{-3} \text{ m})^2}{(4396 \cdot 10^{-3} \text{ m})^2} + 1}} \right| \cdot 2 \cdot 10^{-3} \text{ m} \\ &\quad + \left| \frac{2 \cdot 633 \cdot 10^{-9} \text{ m}}{23 \cdot 10^{-3} \text{ m} \sqrt{\frac{(23 \cdot 10^{-3} \text{ m})^2}{(4396 \cdot 10^{-3} \text{ m})^2} + 1}} \right| \cdot 10 \cdot 10^{-3} \text{ m} \\ &= 2.2 \cdot 10^{-5} \text{ m} \end{aligned} \quad (17)$$

The determined result of the pinhole diameter was therefore $(242 \pm 22) \mu\text{m}$.

In the next part of the lab work, images of the PSF in the xy-plane were taken with different microscope settings and the corresponding intensity profile and the xz- and yz-cross section were generated.

The images of the first run were recorded in widefield configuration, with a bead diameter of 0.2 μm and an air objective with a NA of 0.45 and a magnification of 20. In figure 6B, the PSF in all planes can be seen. The xy-PSF looks circular, pale and blurred at the edges, whereas the faded and blurred cross sections have an elongated and slightly hourglass shape.

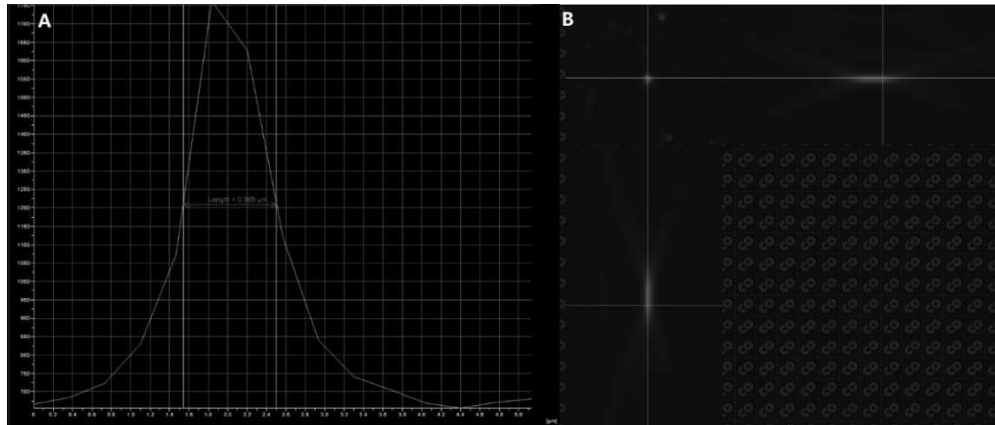


Figure 6: Intensity profile of the PSF (A) and PSF of all planes (top left: xy-plane, bottom left: xz-plane, top right: yz-plane) (B) taken in widefield configuration and with an air lens

The intensity of the PSF have a symmetrical course, reaching its maximum intensity of 1750 in the middle (see figure 6A). Based on the curve, a FWHM of 965 nm could be determined.

By using the emitted wavelength of the bead λ_{em} of 540 nm and the NA of 0.45, the following result for the theoretical lateral resolution was obtained with formula 6:

$$FWHM_{x,y} = \frac{0.51 \cdot 540 \text{ nm}}{0.45} = 612 \text{ nm}$$

The second run in the widefield configuration of the optical microscope was performed using an oil objective with magnification of 100 and NA of 1.45, as well as a bead diameter of 0.1 μm .

In figure 7B, it can be seen that the PSF resembles the PSF of the previous image 6B in its hourglass shape, but is now much brighter and sharper in all planes.

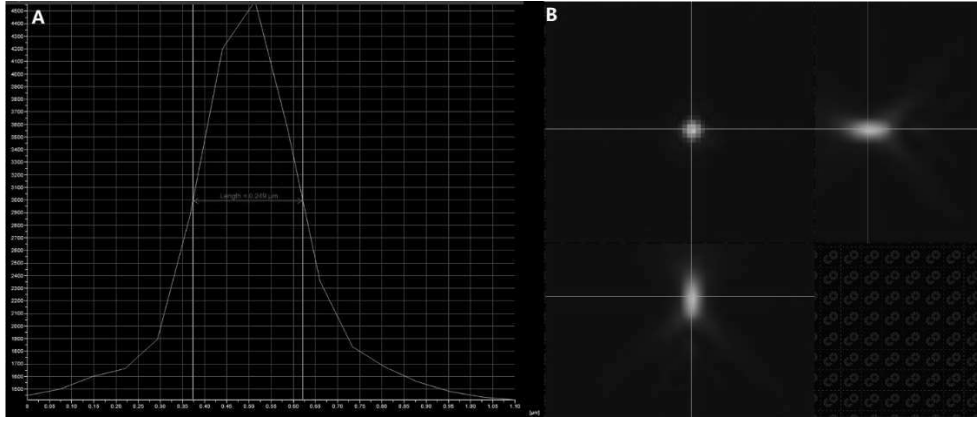


Figure 7: Intensity profile of the PSF (A) and PSF of all planes (top left: xy-plane, bottom left: xz-plane, top right: yz-plane) (B) taken in widefield configuration and with an oil lens

The intensity profile of the PSF looks similar in shape to the previous one, but has the difference of being narrower with a FWHM of 249 nm and having a higher intensity of about 4500 (see figure 7A). With an emission wavelength of 540 nm and the NA of 1.45, the theoretical value of the lateral FWHM amounted to (see formula 6):

$$FWHM_{x,y} = \frac{0.51 \cdot 540 \text{ nm}}{1.45} = 189.93 \text{ nm}$$

In the third run, images of a bead having a diameter of 0.1 μm were recorded with the microscope in the confocal configuration. The pinhole had a diameter of 1 AU and the oil objective had a magnification of 100 and a NA of 1.45.

The xy-PSF has a circular shape and in contrast to the PSF of the widefield images it shows fluctuations of the brightness values (see figure 8B). The cross sections of the PSF are elongated like the widefield images, but are now more oval shaped instead of hourglass shaped. As in the xy-plane, the pixel values are subject to fluctuations.

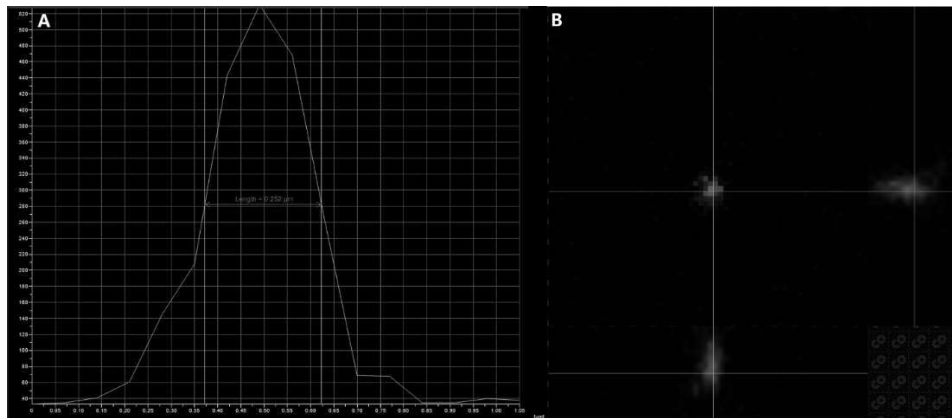


Figure 8: Intensity profile of the PSF (A) and PSF of all planes (top left: xy-plane, bottom left: xz-plane, top right: yz-plane) (B) taken in confocal configuration with a pinhole of 1 AU

The intensity profile has a similar shape and, with 252 nm, a similar FWHM to that recorded in the widefield configuration with the oil objective (see figure 8A). However, the intensity is significantly lower at about 520. Slightly angular gradations occur at the edges of the profile. With an irradiated wavelength of 488 nm and a NA of 1.45, the lateral FWHM amounted to (see formula 8):

$$FWHM_{x,y} = \frac{0.51 \cdot 488 \text{ nm}}{1.45} = 171.64 \text{ nm}$$

Figure 9B shows the PSF and its xz- and yz-cross section recorded with an extended pinhole of 3.8 AU using the microscope in the confocal configuration. The other settings were identical to the previous confocal examination.

The PSF resembles that of the previous confocal image, but appears slightly paler and blurrier than in the previous image. There are slight fluctuations in the pixel values, however, in the xy-plane as well as in the cross sections a bright round circle can be seen in the centre.

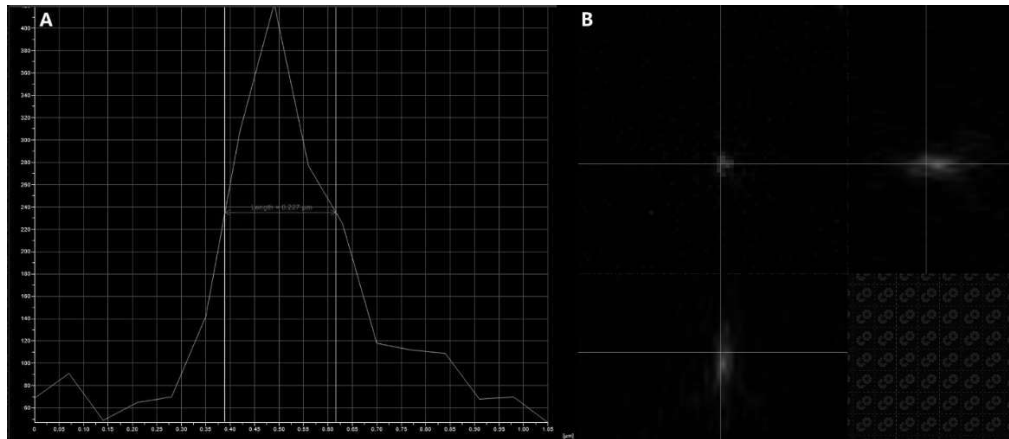


Figure 9: Intensity profile of the PSF (A) and PSF of all planes (top left: xy-plane, bottom left: xz-plane, top right: yz-plane) (B) taken in confocal configuration with a pinhole of 3.8 AU

The PSF has a slightly smaller FWHM of 227 nm than the previous confocal image and has a lower intensity of 420 (see figure 9A). In addition, there are strong steps in the edge regions of the PSF.

In both, widefield and confocal mode, additional recordings of the xy-plane of mouse neurons with channel 2 were taken. For the images, an oil objective with a magnification of 100 and a NA of 1.45 was used. The confocal pinhole had a size of 1AU.

The widefield image in Figure 10A appears blurred greenish, especially in the upper part of the image. In the lower part of the image, blurred elongated and round structures are visible. In comparison, the confocal image in Figure 10B has predominantly dark to black areas. In the central and lower image area, small elongated structures can be seen that stand out sharply from the background.

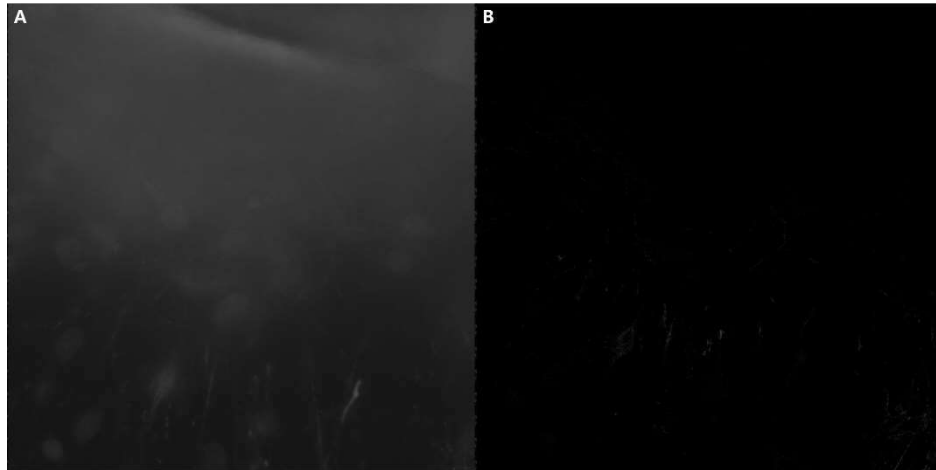


Figure 10: Channel 2 recording of mouse neurons in widefield (A) and confocal (B) configuration

At the end of the laboratory work, images of the cells were taken with all three colour channels in confocal mode with the same oil objective as before (see figure 11). There are predominantly blue roundish structures, which are probably cell nuclei, and orange elongated structures on the image. In addition, small dot-like greenish structures can be seen in the central area and in the lower right edge of the image.

The structures in the cross sections of the xz- and yz-plane in image 11B have a similar shape as in image 11A. Thus, the blue structures appear roundish, the orange ones elongated but somewhat more point-like, and the green structures dot-like.

In the maximum intensity projection in image 11C, in contrast to image 11A, bright structures are visible throughout the image. In the upper area, there are a large number of blue roundish structures and a few green and orange areas can be seen. In the lower part of the image, there are more of the green and orange elongated structures and a few blue ones. Figure 11D shows the image of the maximum intensity projection in three dimensions.

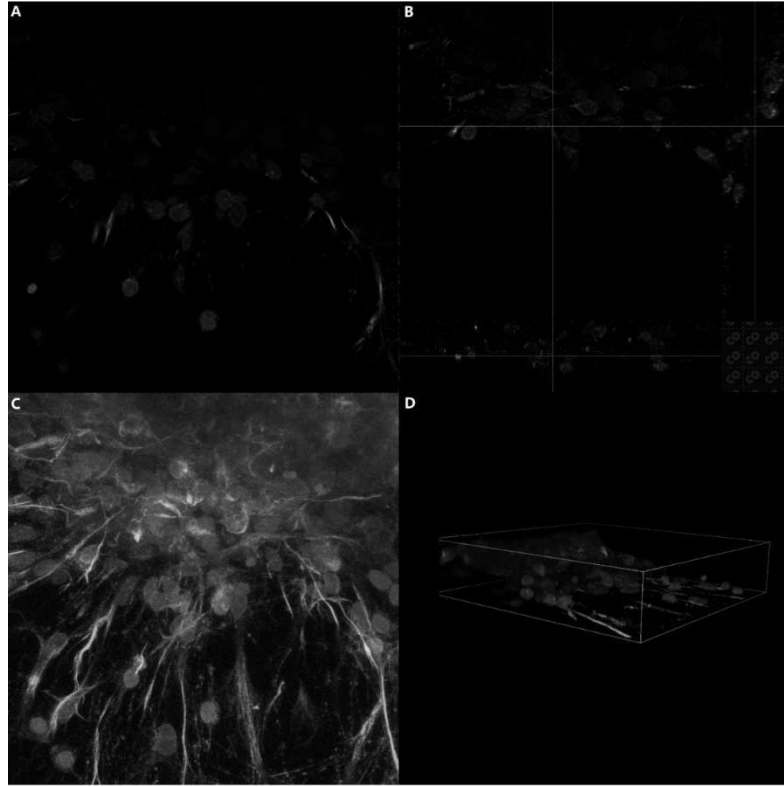


Figure 11: Three channel recording of mouse neurons in confocal configuration (A), PSF of all planes (top left: xy-plane, bottom left: xz-plane, top right: yz-plane) (B), maximum intensity projection (C) and three-dimensional image of the maximum intensity projection (D)

The irradiance of the light of wavelength 488 nm using the same microscope settings as before was measured with a laser power metre in the area of the sample. With a measured power P of about 14 μW and the lateral $FWHM_{x,y}$ of 252 nm from figure 8A, this resulted in an irradiance I of (see formula 18):

$$I = \frac{P}{\pi \cdot \left(\frac{FWHM_{x,y}}{2}\right)^2} = \frac{14 \cdot 10^{-6} \text{ W}}{\pi \cdot \left(\frac{252 \cdot 10^{-9}}{2}\right)^2} = 28.07 \cdot 10^7 \text{ W/m}^2 \quad (18)$$

5 Discussion

The diffraction patterns of the single slit experiments showed the typical periodic alternation of minima and maxima and the intensity decreased outward. With decreasing width of the slit, the alternation of maxima and minima became wider and the associated angle of the n^{th} -order minimum larger, which according to formula 1 complies with the expectations. In addition, it was

found that as the columns became narrower, the pattern appeared more blurred. This could be caused by a stronger diffraction effect of the light at a narrower slit, which resulted in significantly more interference of the light waves.

The calculated angle values for the 0.8 mm, 0.4 mm and 0.1 mm slit showed the differences between the experimental and the theoretical angle of 0.00363° , 0.00056° and 0.01479° . Thus, these values were above the accuracies of 0.00004° , 0.00005° and 0.00010° for each measurement. It can be assumed that these deviations occurred due to underestimated measurement errors. In addition, the large difference between the theoretical and experimental angle value for the 0.1 mm slit can be explained by the blurred edges of the diffraction pattern, which made it difficult to measure.

In the double slit experiment, the two patterns of the 0.3 mm slit separation showed periodic intensity oscillations in the main maxima. This is in accordance with expectations, since depending on the separation width of the two slits, interference between the diffraction patterns of the two slits occurred. Increasing the slit width while keeping the same slit separation showed a broadening of the main minima and maxima as before in the single slit experiments.

The two patterns of the 0.15 mm slit width resembled the single slit image at a slit width of 0.1 mm, as no intensity variations were seen in the main maxim. Despite a variation of the slit separation, they looked almost identical. The reason for this could be the poor quality of the images, as the camera had difficulties in focusing the pattern. However, it is likely that the width of the undetectable periodic intensity oscillations in the main maxima changed as the slit separation increased.

The pinhole pattern was similar in shape to the Airy pattern, as expected, but it was oval-shaped and the maxima at the edge were not ring-shaped but smaller segments. It is suspected that this occurred due to an uneven, not optimally circular pinhole, resulting in unwanted interference and diffraction effects.

The widefield fluorescence microscope images showed that the PFS had the typical hourglass shape. It was also found that the PSF was significantly brighter and sharper when using the oil objective with the higher NA of 1.45 than when using the air objective with an NA of 0.45. The measured lateral FWHM confirmed this quantitatively, as this was significantly better at 249 nm when using the oil objective than that of 965 nm when using the air objective. The reason for this could be that due to the higher refraction index of the oil, less refraction effects occurred at the objective and more light was coupled in.

When using the NA of the air objective the calculated theoretical lateral FWHM was 612 nm, which was significantly lower than the measured one. The reason for this could be that the theoretical formula only considers the wavelength and NA, but in reality, much higher values can occur due to unforeseen refraction effects and contamination on the lens.

The theoretical PSF using the NA of the oil objective was 189.93 nm, and thus slightly lower than the measured PSF. The difference was not as large as with the air objective, so it can be assumed that the oil objective worked more accurate. The deviation here can again be attributed to deviations of the objective.

The pixel size selected when imaging with the air objective was 370 nm, which was 2.6 times smaller than the lateral FWHM at 965 nm. Hence, the Nyquist-Shannon criterion was met, so there was no loss of information during conversion to the digital image.

The chosen pixel size with 70 nm using the oil lens was about 3.5 times smaller than the determined PSF, so the Nyquist-Shannon criterion was fulfilled, but one can also speak of "empty magnification", because the value of the pixel size was more than three times smaller. Due to this, no improvement of the information content occurred during the transfer to the digital signal.

The PSFs recorded in confocal configuration showed fluctuation. On the one hand, this occurred due to the lower signal intensity, since information in the form of photons was lost at the pinhole, resulting in a higher statistical noise of the pixels. On the other hand, due to the scanning process, each point was scanned individually, so they were each taken at a slightly different time point.

The measured lateral FWHM of the pinhole with the diameter of 1 AU had the value of 252 nm and thus corresponded approximately to that of the lateral FWHM, which was taken in widefield configuration and with the same oil objective. It would have been expected that possibly the image in confocal configuration would be slightly better, since out-of-focus light was reduced.

The pixel size was set to 70 nm for the image in confocal configuration, which was 3.6 times smaller than the lateral FWHM and thus the Nyquist-Shannon criterion was met, but at the same time "empty magnification" was present.

The theoretical lateral FWHM of 171.64 nm was slightly lower than the measured one, although this could be explained as before by deviations of the objective. Since the higher wavelength of the excitation light and not the lower wavelength of the emission light was taken for calculations this time, the theoretical resolution of the confocal experiment was better than that of the widefield experiment.

When the pinhole was enlarged to 3.8 AU, the PSF looked paler in all planes and had a circular bright spot in the centre representing the bead. Additionally, the measured lateral FWHM amounted to 227 nm, which was smaller than that at a pinhole of 1 AU. Theoretically, resolution

should increase and brightness should decrease with a smaller pinhole as the PSF becomes more diffraction limited. It is assumed that the better resolution when using a pinhole with a diameter of 3.8 AU occurred due to statistical reasons.

The examination of the neurons with one channel showed that the image taken in widefield configuration appeared greenish and blurred. The reason for this was probably the out-of-focus light, as the sample was thicker than the previously examined beads. Therefore, emission light from excited layers above and below the focal plane also contributed to the image. In the image taken in confocal configuration, the information from the unfocused layers was blocked by the pinhole, so that only a few sharp structures were visible against a black background.

The calculated irradiance of the light of wavelength 488 nm was 2.81 MW/m^2 , this value was almost 3000 times higher than the solar irradiance of 1000 W/m^2 in central Europe [3]. For this reason, care had to be taken during imaging not to irradiate the sample for too long in order to avoid photo bleaching and damage.

6 Conclusion

In this experiment, the CLSM proved to be an effective tool for high-resolution imaging of thicker samples such as neurons, since out-of-focus light did not interfere with the image.

When microspheres were examined using the confocal setting of the microscope, no significant difference in resolution was obtained compared to the widefield configuration. However, using an oil objective with high NA in the microscope experiments proved to be effective in increasing resolution and brightness as more light was coupled in the objective.

Based on the refraction experiments at the slit and pinhole, the PSF occurring in the microscopy experiments could be explained.

Since only a few recordings were performed in the widefield and confocal configuration, no precise statement could be made about the difference of the two modes due to suspected statistical fluctuations. In order to quantify the influence of the confocal pinhole on the image resolution, further recordings would have to be taken in widefield and confocal configuration.

7 References

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