Linear and nonlinear optical imaging of moss chloroplasts
Over the last three decades, two-photon microscopy (2PM) has gained considerable attention for bio-medical imaging as a result of depth resolved imaging in highly-scattering tissues by nonlinear two-photon (2P) effects. 2P effects include second harmonic generation (SHG) and two-photon excited fluorescence (2PEF). This thesis evaluates the feasibility of using an in-house 2PM setup for imaging the mouse retina. For this purpose, moss leaf chloroplasts (Plagiomnium affine) were imaged by linear and nonlinear optical imaging techniques using two objectives, a dry and a water immersion objective. The results show that use of a water immersion objective in the in-house setup yields depth-resolved 2P images of chloroplasts in the moss leaf. This result agrees well with previous work, indicating that the 2PM would be capable of imaging the retina.

The thesis also studies the need for dark-adapting the moss samples in order to reduce starch signal uncertainty adversely affecting chloroplast SHG imaging. Eliminating the dark-adapting process should shorten the SHG measurement process for future work. This uncertainty is studied by comparing 2P images of both dark-adapted and normal types of moss. The results showed no discernible differences between the two chloroplast sample types, suggesting that starch granules have no effect on SHG imaging of chloroplasts. Future work could use filtering before the detector in order to discriminate SHG from 2PEF, as this would reveal starch granules as bright spots inside the chloroplasts.
Under de tre senaste årtiondena har intresset för tvåfoton mikroskopi (2FM) ökat avsevärt i de biologiska och medicinska områden på grund av dess förmåga att avbilda celler djupt i levande sampel som följd av dess icke-linjära tvåfotonegenskaper. Tvåfotoneffekter omfattar tvåfoton excitert fluorescens (2FEF) och frekvensfördubbling (FD). Detta slutarbete utvärderar 2FM anordningens förmåga att avbilda näthinnan av möss. Utvärderingen genomförs genom att avbilda simplare biologiska sampel, i detta fall celler av ett mossblad (Plagionnium affine), vilket innehåller kloroplaster. 2FM anordningen avbildar tvåfoton signaler från sampen med luft och vatten baserade mikroskop immersionsoobjektiv. Resultaten visar att användning av ett vatten immersionsoobjektiv kan producera bilder av kloroplaster på ett 200 ums djup. Detta resultat matchar med tidigare forskning, vilket också tyder på att 2FM anordningen har möjligheten av att avbilda retinan. Framtida arbete kunde undersöka hur trefoton mikroskopi tillämpas på biologiska sampel, då denna använder längre vånglängder för att nå celler på djupare nivå, samt att trefotoneffekter ökar både bildens resolution och mikroskopets förmåga att avbilda mindre celler.


**Nyckelord:** Tvåfoton mikroskopi, Tvåfoton excitert fluorescens, Frekvensfördubbling

**Språk:** Engelska
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Along the journey several people have supported me including Hanne, Susobhan, Yadong, Ken and Camilla, who assisted me in various tasks. Finally, I would like to thank my family and friends, especially my fiancee, for giving me mental and emotional support for the duration of the thesis.

Espoo, July 29, 2019

Robin Ångerman
## Abbreviations

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<th>Description</th>
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<tbody>
<tr>
<td>1P</td>
<td>One-photon</td>
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<td>2P</td>
<td>Two-photon</td>
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<td>3P</td>
<td>Three-photon</td>
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<td>1PEF</td>
<td>One-photon excitation fluorescence</td>
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<td>2PEF</td>
<td>Two-photon excitation fluorescence</td>
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<tr>
<td>3PEF</td>
<td>Three-photon excitation fluorescence</td>
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<tr>
<td>1PM</td>
<td>One-photon microscopy</td>
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<td>2PM</td>
<td>Two-photon microscopy</td>
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<td>3PM</td>
<td>Three-photon microscopy</td>
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<tr>
<td>1PCLSM</td>
<td>One-photon confocal microscopy</td>
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<tr>
<td>2PLSM</td>
<td>Two-photon laser scanning microscopy</td>
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<tr>
<td>CARS</td>
<td>Coherent anti-Stokes Raman scattering</td>
</tr>
<tr>
<td>CW</td>
<td>Continuous wave</td>
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<tr>
<td>FLIM</td>
<td>Fluorescence lifetime imaging</td>
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<td>FRET</td>
<td>Förster resonance energy transfer</td>
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<td>GFP</td>
<td>Green fluorescent protein</td>
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<tr>
<td>LSM</td>
<td>Laser scanning microscopy</td>
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<tr>
<td>PL</td>
<td>Photoluminescence</td>
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<td>SHG</td>
<td>Second harmonic generation</td>
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<td>THG</td>
<td>Third harmonic generation</td>
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<tr>
<td>MPI</td>
<td>Multiphoton imaging</td>
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<tr>
<td>MPM</td>
<td>Multiphoton microscopy</td>
</tr>
<tr>
<td>NA</td>
<td>Numerical aperture</td>
</tr>
<tr>
<td>NIR</td>
<td>Near-infrared</td>
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<tr>
<td>OCT</td>
<td>Optical coherence tomography</td>
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<tr>
<td>PMT</td>
<td>Photomultiplier tube</td>
</tr>
<tr>
<td>QD</td>
<td>Quantum dot</td>
</tr>
<tr>
<td>TIR</td>
<td>Total internal reflection</td>
</tr>
<tr>
<td>SBR</td>
<td>Signal to background ratio</td>
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<tr>
<td>WA</td>
<td>Water immersion</td>
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Chapter 1

Introduction

Study of synaptic networks and monitoring of intracellular transport dynamics at the micrometer level have become important research topics in neuroscience and medicine. Traditionally, synaptic networks have been studied using one-photon imaging (1PI) techniques, such as linear confocal imaging and optical coherence tomography. However, 1PI methods are limited by either the effective resolution or imaging depth, since the image contrast degrades when UV-light is absorbed by fluorescent molecules in tissue, such as the retina of the eye [23]. Moreover, UV-light is known to scatter in opaque tissue, thereby reducing the imaging depth. One solution to improve depth resolved and high-resolution imaging is to use multiphoton imaging (MPI). Over the last decades, MPI has attracted considerable attention in the biosciences [20, 66, 85] due to its depth resolving capabilities, and intrinsic 3D imaging.

Nonlinear two-photon effects can be divided into two types: two-photon excitation fluorescence (2PEF) and second harmonic generation (SHG). Unlike conventional 1PI that excites samples at a shorter wavelength than the absorption maxima of fluorophores, 2PEF is typically generated using a femtosecond laser centered at a longer wavelength as a result of the simultaneous absorption of two photons into an atom. Both nonlinear effects are efficiently excited using a femtosecond pulsed laser in the red to IR wavelength range, generating blue-shifted light in the samples. Because of the longer wavelength, the photons penetrate deeper into biological tissue. This causes 2PEF only in those molecules within the microscope focal volume, leading to increased resolution due to suppressed background noise. SHG is another two-photon effect which was found to occur in non-centrosymmetric media [49], such as cell boundaries or extracellular matrices. SHG is a sum-frequency generation process that also requires a phase matching condition in order to be generated. Unlike 2PEF, SHG does not insert energy into the system as a result of almost perfectly frequency-doubled photon generation. Thus, the wavelength used for SHG imaging differs from that used in 2PEF
imaging.

Considerable research has been devoted to using MPI for retinal and plant-based imaging [13, 20, 66, 76, 85]. However, only one study has attempted to image chloroplast using SHG imaging in the moss species *Plagiomnium affine* [76]. Moreover, SHG imaging of chloroplast is hindered by starch granules, since starch is another source of SHG, thus introducing uncertainty into measurements of SHG in chloroplasts [13]. Nevertheless, the production of starch can be halted by withdrawing light from the growing conditions.

Therefore, the thesis focuses on evaluating the feasibility of using the proposed two-photon setup for imaging complex bio-samples, such as the mouse retina. The feasibility will be determined by comparing chloroplast images from the moss plant *Plagiomnium affine* generated using the proposed setup against the one-photon images (Sec. 5.1) as well as images from previous work [76]. For this purpose, the images are evaluated in terms of three imaging parameters: 2P collection efficiency (SHG and 2PEF), resolution and brightness. The microscope images are generated using both a standard and a water immersion (WA) microscope objective.

Since starch introduces uncertainty into second harmonic generation (SHG) imaging of chloroplasts, the thesis also determines the need for dark-adapting moss for SHG imaging of chloroplast. This is accomplished by using the proposed setup for imaging two types of the moss, dark-adapted and moss maintained under normal light conditions. 2P images of both moss types are compared, in order to determine the effect of starch granules on chloroplast SHG imaging.

The thesis is limited to optical imaging of moss. The literature review focuses on only multiphoton imaging of the retina, which will form a basis for further research using the optimized multiphoton setup.

## 1.1 Structure of the thesis

The thesis will be structured as follows. Chapter 2 describes the fundamentals and advancements in linear and nonlinear optical imaging, as well as their application for biological samples. The chapter also considers the challenges of using multiphoton imaging in highly scattering samples. Chapter 3 reviews and discusses the optical properties of bio-samples and the fluorescent probes used for labeling them. Chapter 4 describe the experimental setup used for imaging the biological samples. The chapter also presents the typical setup elements used for bio-imaging. The chapter additionally lists the samples and describes their preparation. Chapter 5 presents the measurement results and discusses the effect of starch granules on chloroplast 2PEF and SHG imaging. Finally, Chapter 6 summarizes the work by discussing the results, as well as describes future work for improving the setup and further research.
Chapter 2

Fundamentals of optical imaging techniques

Optical imaging reveals information about biological structures at a micrometer scale, which is necessary in several fields of science and medicine. Because this thesis focuses on optical imaging techniques, it is important to understand the fundamental phenomena of optical microscopy, such as molecular photoluminescence (PL) and harmonic generation. PL, or in special cases harmonic generation, is generated in biological molecules when excited by a light source, such as a laser. Optical microscopes measure PL, which generate contrast in order to study material properties, including morphology, transport dynamics, and optical parameters.

This chapter reviews the principles of linear and nonlinear optical imaging techniques in neuroscience and medicine. Section 2.1 introduces linear one-photon optical imaging techniques, such as one-photon confocal imaging, the ancestor of confocal imaging techniques, and optical coherence tomography (OCT). Section 2.2 discusses nonlinear multiphoton imaging techniques using nonlinear optics, including two and three-photon imaging techniques. Section 2.3 further discusses advanced imaging techniques, such as Coherent anti-Stokes Raman scattering (CARS) imaging. Multiple imaging techniques permit multimodal imaging, which cover a wider range of tissue, i.e. bands of wavelengths. Section 2.4 describes typical confocal imaging setup parameters and elements. Section 2.5 presents the challenges of multiphoton imaging, and their effect on the measurement setup. Finally, in Sec. 2.6, a complete review of current and advanced imaging techniques is shown and their application in life-science.

Optical imaging evolved in 1961 when Kaiser and Garrett excited two-photon fluorescence [47], and when Minsky [58] invented confocal laser scanning microscopy. Both techniques improve radial and axial resolution compared with conventional light microscopes. Both imaging methods also intrinsically image samples in 3D, by employing different methods, 2PEF even penetrated deeper into scattering
tissue. Maria Göppert-Mayer [27] predicted two-photon excited fluorescence in her doctoral thesis in 1931, K&G observed it 30 years later and with pioneering work conducted by Denk et al. in 1990 [19], the two-photon laser scanning microscope, imaging retinal neurons, revolutionized bio- and neuroscience.

The basic principles of optical imaging include PL and harmonic generation. PL arises from a three stage process [66] where: 1) a fluorescent molecule, a fluorophore, is excited by an excitation source photon. The photon excites the electron of a molecule from the ground state to an upper energy excited state, 2) excess energy is transferred into heat, and finally, 3) a photon is emitted by an electron relaxation, which is usually red-shifted [91]. Reflected and emitted photons are generated and collected by a laser scanning microscope (LSM) and detector, respectively. Images are constructed by raster scanning a diffraction limited focal volume, pixel by pixel, across the sample and then processing the data in a computer after the detector to reveals internal structures.

Two-photon and multiphoton processes occur in a fluorophore by simultaneous absorption of (two) photons with equal wavelength, called a nonlinear process. Step 1 differs for one- and multiphoton processes [38]. In practice, a fluorophore can be excited by two photons (in the infrared range), which are up converted into a blue-shifted photon, which is nearly double in the frequency and half the wavelength [91]. Two photons excite the molecule, the first photon excites the electron to a virtual state and the second photon excites the electron to an upper energy- or vibrational state. Finally, the electron relaxes to the ground state emitting isotropic, frequency-doubled emission fluorescence in form of a photon[66]. It allows for imaging of several molecules in the visible range, endogenous fluorophores, such as serotonin and nicotinamides [48, 57].

Another method for contrast imaging is harmonic generation. Second harmonic generation (SHG) require non-centrosymmetric media and phase-matching to be excited in tissue [38]. SHG is unrelated to electronic states and perfectly converts into frequency-doubled photons when degenerated in media. Also, due to the lack of energy transfer, SHG avoids photodamage in the specimen. Symmetrically distributed dipoles disables the second-order nonlinear susceptibility, thus SHG is nonexistence in cube-lattice media [8]. Alternate imaging techniques parallel to fluorescence imaging include fluorescence life-time imaging (FLIM) [28], Förster resonant energy transfer (FRET) [38], and CARS [59].
2.1 Linear imaging techniques based on one-photon optics

This section introduces one-photon imaging techniques, including one-photon confocal microscopy and optical coherence tomography. Linear imaging is a well-developed technique for monitoring and detection of molecular concentrations and tissue-specific neurons, respectively. However, one-photon imaging techniques are limited either by depth or resolution, which can be solved by methods employed in the next section, Sec. 2.2. Nevertheless, one-photon confocal imaging is the ancestor of modern multiphoton imaging and it explains the working principle of the imaging technique.

M. Minsky coined the term confocal laser scanning microscope (CLSM) in 1961, improving the axial and radial resolution compared to conventional optical microscopes [58]. A CLSM enhances resolution [99] both axially and transversely by scanning a point source through a collector lens and directing the PL from the sample to the detector using a magnifying objective. Łukosz [56] describes how a double-focused lens system improves the resolution at a cost of field of view. Nevertheless, field of view is regained by scanning the focal spot pixel by pixel in a raster-scan, thus recreating the image plane. A point like focal spot collects PL from the sample, but the excitation source illuminates the whole beam path which generates noise in form of scattered photons [99]. The noise, i.e. the out-of-focus photons, is rejected by the introduction of a pinhole before the detector. The pinhole rejects scattered photons from both the bulk and from the focal spot. Mainly ballistic photons cross the pinhole [20], thus much of the signal is lost in highly scattering specimen, such as twice-scattered photons inside the focal spot [75]. As a result, CLSM can obtain 3D resolution of the sample by scanning the laser layer-by-layer along the z-axis, the objective focuses light through the pinhole, thus out-of-focus photons outside the focal volume are filter by the pinhole [36, 38].

Illumination of the entire beam path limits applications of CLSM, such as mouse brain imaging, due to out-of-focus fluorophore excitation. As a consequence, electrophysiological recordings or spatial separation of specific neurons in brain samples is hindered by out-of-focus excitation generating background noise [108]. Nevertheless, LSM is useful for fluorescence imaging applications, such as monitoring neuron activity, which requires high speed optics. The laser source provide high intensity coherent light that outputs all power at the scanned point.

3D resolution is achieved by scanning a diffraction-limited focal spot across a translucent sample of the thickness of about 100 μm. Only PL originating from the focal volume reaches the detector through a pinhole. However, later we will see that the axial intensity, outward from the focal volume stays constant, thus reducing the image contrast. Resolution is ultimately limited by the excitation
beam properties [99].

Confocal microscopes best utilize laser light sources, unlike optical microscopes where any source can be applicable in the UV- to IR-range [99]. A laser provides a larger range of wavelengths, useful for exiting a variety of fluorescent molecules. The wavelength of an excitation laser source used by a confocal microscope, usually in the blue range, is lower than the emission peak of the fluorophore, because typically the molecule absorbs at a lower wavelength and emits at a higher wavelength, caused by relaxation effects causing red-shifting of the re-emitted photon. Multiphoton microscope laser sources excite the fluorophores at higher wavelengths in the near infrared (NIR) to infrared region [85]. Interference effects caused by coherent laser light are avoided due to a moving beam by scanning. A diffraction limited confocal spot is optimally utilized by a low power monochromatic laser beam compared to conventional light sources, such as xenon arc lamp [96].

Hattar et al. [32] demonstrated an opsins-like protein, melanopsin, using linear CLSM in the retinal ganglion cell (RGC) responsible for photosensitivity and triggering activity of parallel visual systems. Melanopsin, rather than the typical photoreceptor rods and cones, is the intrinsically photosensitive photopigment projecting information about photic response and environmental illumination through the RGC to the brain. Hattar imaged retinal neurons tagged with antibodies using immunocytochemistry or labeled using intracellular dye. By labeling the RGC using immunocytochemistry, several neuron parameters are shown, such as size, morphology and location of melanopsin-expressing RGCs. The work concludes the RGC as the photoentreating neuron of the circadian pacemaker, activated by photosensitive melanopsin.

Another one-photon imaging technique, based on optical coherence domain reflectometry, reveals structural details of the retinal cross-section due to inhomogeneities of the refractive index. Cross-sectional views of the tissue can be observed to determine diseases which helps evaluate the health of the tissue [90]. This interferometry based imaging technique measures reflected infrared light, in the range of 1200-1800 nm (but also visible). Additionally, the coherence length of the infrared laser determines depth resolution and coherence time of the point-spread function, i.e. the size of the focal volume. OCT images deeper, but with lower resolution compared to linear confocal imaging techniques, up to a depth of 2 cm and 1.5 mm in transparent and scattering tissue, respectively [79]. OCT interference signals measure several factors that generates contrast in the tissue, including tissue absorption, location, reflectance and birefringence.

Light generation for OCT is achieved by various light sources, including edge-emitting LEDs (ELED), mode-locked lasers or light sources based on supercontinuum generation (SCG). Mode-locked high-frequency lasers, in contrast to ELEDs, provide stable and high-power output. However, bulky tabletop lasers are highly
immobile and become unpractical for portable applications. Yuan et al. [107] attempted using OCT based on SCG as an alternative to NIR light and they discovered SC-OCT provide comparable contrast compared with a Titanium:sapphire (Ti:sapphire) laser. Supercontinuum generation based OCT is desirable for tunability in the infrared, short coherence times, large spatial coherence and bright illumination.

Li et al. [54] imaged the mouse retina in vivo using a 1280-nm OCT. The results showed good correlation with ERG measurements in determining retinal thinning by retinal degradation related diseases. The study showed several advantages of OCT, including high-resolution high-depth imaging in a rapid and reproducible manner. OCT shows structural changes in the retina on a scale of 20 μm, it also shows the displacement of the retina as a result of surgical operations. In vivo follow up on diseases would require less animals, thus giving higher quality data that can be cross-correlated with ERG measurements. OCT images reflective regions in order to determine the retinal boundaries, which can be used for characterization of the retina. However, the paper determined resolution is still limited and resolving structural details were not possible, while resolving structural changes was possible. Nevertheless, new research presents OCT with an axial resolution of 3 μm [21].

2.2 Nonlinear imaging based on multiphoton processes

The previous section described one-photon optical imaging technologies used for retinal imaging. One-photon techniques lack either resolution or depth, which can be solved using multiphoton effects. The confocal microscope can easily be modified for two-photon processes, which use high-frequency laser sources and detection without a pinhole. Two-photon processes excite atoms by the simultaneous absorption of two NIR photons and subsequently emitting one frequency doubled photon, which results in quadratic intensity dependence. As a result, the objective focal volume is spatially limited, which enables 3D imaging and lowers the background noise. This section reviews multiphoton techniques, including imaging based on two- and three-photon excitation and respective harmonic generations, based on nonlinear imaging of biological samples, such as the mouse retina.

2.2.1 Two-photon excitation fluorescence imaging

Two-photon excited fluorescence (2PEF) is an extremely rare phenomenon in nature, occurring under normal conditions in rhodamine B, a fluorescent probe, every 10 million years [20]. In contrast, a one-photon absorption event of the same
probe occurs once every second. Two-photon microscopy (2PM) has generated much interest for biological applications [23] as the technique of choice for imaging thick samples [52]. The probability of 2PEF event increases by a high temporal and spatial confinement of photons, i.e. a large photon flux focused into a tight focal volume in the sample. Temporal confinement in 2PM is realized using a high-frequency pulsed laser, generating a sufficient photon flux for 2P events (a 2P absorption event occurs in the span of ca 15 femtoseconds); Spatial confinement is achieved using a large numerical aperture (NA) objective, which focuses photons into a minimal focal volume. The difference in the focal volume between the two-photon (2P) and confocal one-photon microscope is compared in Fig. 2.1. As shown in the figure [110], the tight focus of a 2PM setup is generated by a large photon flux in the focal plane that results in the excitation of fluorescent molecule only in the focus by the absorption of two NIR photons.

For generating a sufficient photon flux for 2P events, 2P PL is most optimally excited using a high-frequency or femtosecond (fs) laser. Femtosecond lasers and temporal confinement are commonly realized using Kerr lens mode-locked Ti:sapphire lasers [66]. Kerr lens mode-locking is a phenomenon that relies on the intensity dependent refractive index in a Ti:sapphire crystal, which gradually changes along the radial direction. Mode-locking leads to self-focusing of laser modes, similar to a convex lens, in the crystal center. As a result, longitudinal modes interfere constructively (i.e. produce noise) only in the center and become locked in phase with the laser cavity. Fluctuations in the noise produce short pulses at the femtosecond scale [40]. For 2PM applications, Ti:sapphire lasers in the infrared range are a common excitation source in terms of its flexible parameters, which are further explained in Section 2.4. 2PM utilizes the infrared range of 700-1100 nm in order to match the wavelength halved one photon (1P) absorption maxima of several fluorescent molecules [103]. This infrared light enables deeper penetration and less absorption in the tissue. 2PEF may also be excited by a continuous-wave laser, although a much larger excitation power is required in this case to create an equal amount of 2PEF photons [20].

In order to obtain a tight focal volume, spatial confinement of the excitation beam is conducted using a high numerical aperture (NA) objective. Since the 2PEF depends on the fourth power of the NA, high-NA objectives are highly desirable for increasing the axial and spatial resolution of the point-spread function, further discussed in Section 2.4 [18]. The number of photons absorbed by a fluorophore per pulse, with duration \( t_p \), may be calculated with

\[
\eta_a = \frac{p_0^2 \sigma}{\tau_p f_p} \left( \frac{\pi (NA)^2}{hc\lambda} \right)^2, \tag{2.1}
\]

where \( p_0^2 \) is the average laser power, \( \tau_p \) is the laser pulse duration, \( f_p \) is the repe-
tition rate and NA is the numerical aperture of the focusing objective. Equation 2.1 shows that the absorption rate is determined by a square dependence of the laser power, or the electric field, and a quadratic proportionality of the photon flux at the focal plane, i.e. the intensity. A short pulse duration, a high repetition rate and a large numerical aperture objective defines an efficient excitation of a multiphoton process.

The two-photon absorption probability quadratically depends on the intensity, thus fluorophores are only excited in and near the focal volume of about 1 femtoliter (fl). This means optical sectioning is possible in the axial direction, while the intensity falls off as the fourth power of the distance. As a result, 3D images can be reconstructed from the contribution of useful photons, both scattered and ballistic, and stack imaging of focal slices provides cross-sectional views of samples [19]. Photons outside the focal volume are either too diluted or lack the energy to excite the molecules bandgap [20]. Therefore, there is less photodamage in the specimen and unlike linear confocal microscopy where the whole beam path is illuminated, there is no requirement for a pinhole before the collecting optics, which reduce the signal. Matters concerning absorption and scattering are explained in Section 3.2.

The advantages of 2PM lay in its intrinsic sectioning capability; 1PCLSM linearly depends on the intensity in the axial direction. Therefore, despite of capabilities for 3D sectioning by a screening pinhole, illumination of the whole beam path leads to photodamage in the whole volume [75]. However, Section
2.5 discusses the possibility of larger photodamage in the focal volume of a 2PM compared with imaging based on linear optics. As a result of illuminating the entire beam path using CLSM, electrophysiological measurements combined with visual stimulation is disturbed. In contrast, longer-wavelength fs lasers solely excite the focal volume due to a lowered probability of 2P events outside the focal plane [18]. 2PM also collects scattered photons, unlike 1PM, because photons only generate in the focal point, off-focus infrared scattered photons do not excite the fluorophores as efficiently. By also collecting scattered photons for 2PM, it efficiently increase the resolution since more signal photons are collected. In addition, the resolution can be improved using a pinhole, however, at a loss of signal [85]. CLSM setups rejects most of the out-of-focus photons scattering inside the sample due to a pinhole, as a result useful scattered photons also gets filtered, since all scattered photons hit the pinhole [66].

Section 2.4 discusses the confocal microscope resolution, and the linear CLSM resolution looks to be two times higher than that of 2PM on paper. However, due to various factors decreasing the effective resolution of CLSM, 2PM improve depth resolved imaging of bio-samples. Contributing factors include photons lost by pinhole rejection; a lower laser intensity is used for optimal images, and a larger than necessary objective is used. In contrast, the 2PM compensates the lower resolution by collecting all photons, ballistic and scattered, to obtain a high signal-to-noise ratio [110]. Ultimately, 2PM is limited by imaging depth, the issue can be solved by higher order multiphoton imaging, such as 3PM, or optimization of the pulse characteristics, discussed in Section 2.2.3 and Section 3.2, respectively.

Quadratic intensity dependence allows multiphoton microscopy (MPM) to manipulate fl volumes in tissue. A method for analyzing time-resolved processes, such as transport properties in neurobiology, is monitoring the release of caged compounds by photolysis [53]. Molecules release a caged compound, such as calcium, by the absorption of a UV photon or a multiphoton excitation process [9]. The intrinsic 3D resolution of multiphoton microscopes can, with pinpoint control, release caged compounds, such as calcium ions (Ca^{2+}), to monitor the activity of receptors [19]. For specific molecules, total photolysis is achievable within the 2PE focal volume. Thus, in combination with calcium sensitive dyes, 2PM setups monitor jumps in calcium transport [9]. Advantages of uncaging include the ability to label cells beforehand, thus avoiding measuring uncertainties due to physical damage to the tissue. The caged molecules remain inactive until they are illuminated [53]. Linear confocal microscopy will release the cage compound through the illumination cone, thus making it unpractical for measurements of single neurons.

As earlier stated, multiphoton imaging (MPI) has become an important tool for studying tissue in neurosciences. Imanishi et al. [43] monitored the distribution of
the intrinsically fluorescent compound vitamin A in the mouse retina by \textit{in} and \textit{ex vivo} two-photon imaging. The paper demonstrated retinyl ester storage particles (RESTs) another intrinsically fluorescent compound essential for recycling 11-cis-retinal, which is a chromophore enabling regeneration of rod and cone pigments. Inherited blinding disorders can be detected at an early stage, thus monitoring REST function allows for evaluation of diseases, such as the disease group Leber congenital amaurosis [72]. The usefulness of two-photon imaging shows in \textit{in vivo} applications, such as the eye appearing transparent for the NIR 2PM wavelengths, which efficiently excites molecules deep within the mouse retina. Also, photo-bleaching in the illumination cone is avoided due to a quadratic dependency on the intensity, making two-photon imaging suitable for \textit{in vivo} imaging of living tissue. [44]

2.2.2 Imaging based on second harmonic generation

Second harmonic generation (SHG) microscopy is another nonlinear 2P technique for generating contrast in scattering tissue parallel to 2PEF. Like 2PEF, SHG generates a frequency-doubled photon using two NIR excitation photons. However, the SHG photon does not concern electronics states, thus no energy is transferred or lost between incident radiation and the molecule [44]. Additionally, the generated photon will almost perfectly up-convert to half the wavelength and double the energy, thus SHG emits narrowband emission. As a result, narrowband SHG provides an additional imaging channel besides 2PEF, which is usually red-shifted and broadband. Further, since SHG does not involve electron states, it enables longer imaging session due to less power induced photodamage.

SHG up-converted photons are based on sum-frequency generation under the condition of phase matching [38]. Sum-frequency generation combines the energy and wavenumber of two input photons, which are annihilated in the process, and emits anisotropic radiation at half the wavelength and double the frequency. Thus, sum-frequency generation can weaken due to out of phase oscillation, i.e. a perfect phase matching condition $\Delta k = 0$ should be met. Coherent dipole oscillations interfere constructively to create perfectly up-converted radiation of the input photo pairs, unlike 2PEF which loses energy to internal conversion. In other words, frequency matching of the input waves and a nonlinear polarization (fixed phase relation) produces SHG. Additionally, during the length of the tissue, incident photon energy is periodically converted into the second harmonic generated photons [8].

SHG is a second order nonlinear process that also attenuates radially from the focal volume as $1/r^2$. Due to an inverse proportionality to the square of the radius, SHG enables 3D imaging of samples. A strong incident electric field induce nonlinear polarization in the atoms of a molecule. Second order nonlinear processes,
such as SHG, are induced by nonlinear polarization in media. Atom dipole moments oscillate in unison, like a phased array, using the excitation frequency all the oscillating atoms add coherently to the forward propagating second harmonic beam [8]. The overtone oscillations of $2\omega$, a sum of the input photons, interfere constructively, thus the induced dipole moment is proportional to the square of the input field and it will create a beam proportional to the fourth power of the intensity [8].

SHG require non-centrosymmetric media [38], or crystals lacking inversion symmetry to occur. The nonlinear susceptibility disappears inside medium lacking inversion symmetry, such as cube lattice, although the symmetry will be broken at a medium surface or a change of refractive index [82]. Therefore, tissue membranes such as chloroplast membranes generate SHG, and extra cellular matrices such as microtubules in the sclera of the eye generate SHG due to polarization anisotropy. For instance, SHG can determine structural orientation through polarization anisotropy, such as changing excitation laser polarity affecting the intensity.

Imanishi et al. [44] studied the spatial orientation of collagen in the sclera of the eye. Collagen is a structural part of connective tissue, ubiquitously found in the human body. Moreover, in addition to the structural organization of the extracellular matrix collagen, the incident polarization of the light affects SHG strength, aiding in the analysis of unknown tissue [88]. Another interest for SHG imaging in living tissue is microtubules (MTs), which also generate second harmonics in the mouse retina [55].

Lim et al. measured the forward transmitted MT-SHG in the ex vivo mouse retina. Neuronal axons in the retina are mechanically supported by MTs, which also functions as origin for birefringence [42] and reflection coefficient in the axons. SHG arise due to structural orientation and the birefringent nature of the MT cytoskeleton [4]. Several distortions of vision, including retinal nerve fiber layer thinning, glaucoma and neurodegradation, begin by a reorganization of the MT structure causing various medical conditions, such as intraocular pressure elevations in the eye [5]. As a result of combining 2PEF and SHG, it generates two sources of contrast, and by distinguishing the source of SHG by co-localization of emission and following allows for monitoring of MT health.

Introducing higher order harmonics, such as third harmonic generation (THG), can provide another source of contrast due to THG not requiring inversion symmetry and functioning using longer wavelengths [38]. Section 2.2.3 describes principles and application of 3PEF and THG in bio-sciences, in the latter case refractive index change generate contrast, even in symmetric lattice media.
2.2.3 Three-photon microscopy techniques

Three-photon microscopy (3PM), including three-photon excitation fluorescence (3PEF) and third harmonic generation (THG), provides a new window of wavelengths for nonlinear imaging [50]. Due to a longer wavelength, the attenuation of the pump signal meets a minimum between the wavelengths 1300-1700 nm because of minimizing the Rayleigh scattering, as well as UV and infrared absorption. 3PM extends the available wavelengths by using three NIR photons as an excitation signal. Longer wavelengths result in deeper penetration in scattering tissue, and a larger variety of available fluorescent probes (as the 3PM one-photon equivalent wavelength is 400-560 nm, 2PM will struggle finding dyes at an equivalent wavelength range of 1200-1700 nm). The 3PM scales more efficiently based on higher order nonlinear effects compared to 2PM, the 3PM signal attenuates as the fourth power of the radius from the focal volume. As a result, the 3PM signal to background ratio (SBR) is a magnitude larger than the 2PM, since it has a square dependence on the signal attenuation [69].

Hell et al. [35] pioneered in 3PM in the early 1990's using a 900 nm Ti:sapphire laser. Later work [41, 69] used longer wavelengths in the range 1200-1700 nm, termed the "optical window" of bio-imaging. The window is optimized for retinal imaging based on its nonlinear optical properties, causing deeper penetration (longer attenuation length), and enabling more variety of excitable fluorophores. Palczewska et al. [70] imaged intrinsic fluorescence in the mouse retinal pigment epithelium. The excitation was centered at 910 nm, which provided a 0.3 ratio of 3PEF to 2PEF. The ratio was calculated using \( \sigma_3 / \sigma_2 \), where \( \sigma \) is the three- and two-photon absorption cross-section, respectively [104]. Thus, suitable three-photon cross-sections belong to intrinsic fluorophores, such as condensation products of all-trans-retinal, including retinal dimers (A2E, A2DHP-PE) and retinosomes. However, a 0.3 ratio of 3PEF to 2PEF indicate that longer wavelengths can increase the ratio. Nevertheless, 3PM was demonstrated by Hell et al. [35] to achieve a greater resolution than 2PM by measuring the lateral- and axial resolution of BBO-stained polystyrene micro beads and determining the power-dependence of the fluorescence signal.

As laser sources develop to use longer wavelengths at a higher frequency and power, improved depth resolved imaging is achieved. Ouzounov et al. [69] utilized 3PM for brain cell imaging and the paper achieved deeper imaging depth with a significant improvement in SBR. The paper demonstrated an improved spatial discrimination due to improved SBR of a 3PLSM compared to a 2PLSM at a ratio of 40-1, which also lead to a greater contrast. 3PLSM is cable of imaging at depths of 1 mm, whereas 2PLSM fall of at 250 \( \mu \)m. SBR is the limiting factor of 2PM [93] and 3PM reduce background noise by higher spatial confinement of the signal in the focal volume due to higher order nonlinear excitation. The earlier mentioned limit
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of 2PM, SBR, scales as $1/z^4$ for 3PM compared with 2PM fluorescence degradation of $1/z^2$. This allows for higher contrast, thus improved spatial localization of cells by suppressing background noise.

3PM enables non-invasive imaging due to a longer wavelength excitation. Horton et al. [41] utilized a 1700 nm laser, compared with Ouzounov’s 1300 nm laser, because of a more optimized wavelength for reducing attenuation in a mix of brain matter and water. A three-photon laser tuned to 1700 nm has an effective attenuation length of an order of magnitude larger than that of a 2PM tuned to ca 780 nm. Fig. 2.2 shows a three-photon image of the mouse brain up to a depth of 1000 μm. As shown in the figure, several brain regions are shown in the image, such as axons in the external campus in the CA1 region of the hippocampus at a depth of 840 μm. The image consists of both 3PEF elements, such as endogenous blood vessel fluorescence and exogenous RFP-labeled pyramidal neurons, and THG signals.

In addition to 3PEF, third harmonic generation (THG) can be generated in parallel to gain additional information about the observable specimen. Squier et al. [87] applied THG microscopy to bio-applications and suggested THG as an alternative method for contrast imaging. As in the case of SHG, THG does not transfer energy to the sample, thus resulting in longer imaging sessions of the specimen, and a narrowband channel for imaging. Contrast is achieved mainly from change in refractive index between boundaries or in change of the third-order nonlinear susceptibility, such as at the boundaries of cell walls. Unlike SHG, THG does not require samples lacking inversion symmetry. However, homogeneous refractive index in media induces a Gouy phase shift which destructively interfere around the center of generation [38], causing the generation to annihilate. Multiple imaging channels enables 2 to 3 data channels for tissue morphology imaging, termed multimodal imaging. Multimodal imaging can observe co-localization of signals in order to demonstrate the separate effects and simultaneously spatially locate signal components or tissue. THG intensity is dependent on several contributing factors, including the cubic of the excitation power, wavelength range, pulse duration, and polarization dependence.

Aptel et al. [3] utilized up to three imaging channels, such as 2PEF, SHG and THG, for label-free nonlinear imaging of the human cornea in order to reconstruct 3D images down to a depth of 500 μm. The cornea contains NAD(P)H, which act as a metabolic reporter that generates intrinsic 2PEF [74]. SHG arise in structurally organized collagen and accompanying fibrils [98]. THG resolve varying tissue inhomogeneities and interfaces, including stacked membranes, intracellular medium and nuclei composition at a scale of 100 nm. Harmonic generation anisotropy, polarization [68] and coherence length [17] dependence provide different methods for detecting various tissues, such as stratum interfaces, nuclei composition and
Figure 2.2: 3D z-stack image based on 4-μm-steps in the mouse brain. Both 3PEF and THG is expressed in the RFP-labelled brain matter. (Courtesy of ref. [41])
2.3 Advanced imaging techniques - Multimodal imaging

The previous sections reviewed multiphoton imaging techniques based on recorded intensity from two and three-photon effects. By combining multiple imaging techniques, several sample characteristics generate multimodal data, such as structural, spatial and optical properties, which are not intensity dependent. These properties can be measured by advanced techniques, including single-photon counting, resonant energy transfer and coherent anti-Stokes Raman scattering. This section reviews multimodal imaging techniques, which can be utilized parallel to confocal imaging.

The PMT is a single-element detector that has also found use in photon-counting, or fluorescence lifetime imaging microscopy (FLIM), due to its narrow transient time spread and short output pulses [39]. PMTs are further discussed in Section 2.4. FLIM can be divided into two methods, time- and frequency-domain FLIM, which both measure fluorophore lifetimes. Time-domain FLIM, or time-correlated single-photon counting (TCSPC) rebuild a histogram based on measured photons delays, and the frequency-domain measure fluorescent system harmonic response to modulated excitation light [28]. FLIM generates contrast in the case of two dyes emitting the same wavelength fluorescence. Now, a high-speed photon-counting circuit correlates emitted and collected photons, which discriminates fluorophore lifetimes and identifies the two fluorescent molecules. In time domain, a histogram shows the fluorescence lifetimes collected from each pixel during the raster scan, thus lifetimes in addition to intensity is also generated. In \textit{vivo} applications utilize PMTs that require high sensitivity, thus low power can be applied to the specimen and as a result photo-induced damage is reduced. In addition, 3D imaging will utilize FLIM due to scattering tissue having low fluorescent yield.

Gratton et al. [28] compared the FLIM methods TCSPC and frequency-domain FLIM with the focus on circuitry speed and signal to noise ratio (SNR). Time-domain FLIM, TCSPC, showed greater SNR in low fluorescence conditions, while the frequency-domain method adapted better to high-frequency data acquisition in bright conditions due to less distortions of the image. Also, instrumentation affect measurements in sense of noise cancellation, and faster circuitry intrinsically increases SNR.

Miura et al. [63] studied porcine RPE-choroid samples using 2PM and FLIM. The work investigated the effect of oxidative stress on RPE structures. Melanosomes
in the RPE revealed stress induced granules, which emitted bright 2PEF. Melanosomes, oxidized melanosomes and granules were discriminated using FLIM. The paper demonstrated the uncertainty of instrumentation on recordings, such as varying lifetimes were recorded, which Gratton also concluded to exist. The groups work resulted in references of melanosome and granule FLIM and 2PEF images that can be used for further research and clinical reference. The work identified lipofuscin related compounds or the signals of its byproducts and eliminated the possibility of them being RESTs as discovered by Imanishi et al [43].

FLIM also applies to distance measurements based on resonant energy transfer between fluorescent protein, i.e. Förster resonance energy transfer (FRET) [38, 46]. FRET is a quenching process of a donor-acceptor protein pair tagged with matching emission wavelength probes, such as green fluorescent protein (GFP). The quenching is a non-radial process that reduce the donor fluorescent lifetime, thus fluorophores emitting similar wavelength PL become discernable. As a result, FLIM is a perfect match for FRET measurements due to the ability of separating quenching from low concentration of fluorophores. The donor concentration fluorescence lifetime remains the same and no FRET process occurred. FRET applies to proteins in a vicinity of 10 nm due to a \( r^{-6} \) relationship with distance and FRET efficiency. As a result, FRET resolution outclasses optical microscopes by a factor of 10, i.e. 2PM resolution detects focal volumes of 1 fl or 100 nm axially while FRET measure 10 nm molecule spacing. The quenching process is a dipole-dipole interaction, thus dipole moments must be perpendicular for FRET to occur. Additionally, other factors affecting process include multiplicity preservation and emission spectra matching. FRET reduce lifetimes, thus contrast is generated by comparing PL lifetimes before and after acceptor-donor interactions, e.g. a low concentration of fluorophores emit a low fluorescence either due to quenching or FRET, in the latter case lifetimes are shortened.

Another third order nonlinear imaging technique includes Coherent anti-Stokes Raman scattering (CARS), which is a nonlinear four-wave mixing process based on spontaneous Raman scattering (SRS) [77]. Similar to 3PM, CARS excite third order nonlinear processes in the sample, and as a result CARS quadratically depends on the pump intensity. Mixing of overlapping pump and Stokes beams probe the inherent vibrational frequency of fluorophores. Section 2.2.3 discussed the attenuation of radiation, including losses due to oscillations in the infrared. Thus, using a photon pair of NIR wavelengths excite Raman-active molecules, generating perturbations in the form of anti-Stokes photons. As a result, the pump probe perturbations are measurable as coherent vibrations in the molecule atom covalent bonds, such as carbon-hydrogen bonds. CARS applies to several biological structures, including lipids, protein and volumes of water [38]. Multimodal nonlinear imaging using CARS and 2PEF visualize the retina without
the aid of extrinsic fluorescent probes. Retinal tissue emit photons of different energy due to the coupling of electronic or vibrational states by 2PEF and CARS, respectively. Unfortunately, the CARS signal excite non-resonant background in the focal volume [77] which reduce image quality by contrast degradation. Nevertheless, several methods can reduce the background noise, such as epi-detection and SRS.

Masihzadeh et al. [59] showed multimodal images of retinyl ester storages (RESTs) by 2PEF and CARS. As the work demonstrated, CARS can image the rod outer segments, and 2PEF can image collagen and fibroblasts by absence of signal in the mouse sclera-RPE region. The results showed CARS intensity as a function of molecule size and shape. CARS is anisotropic, thus a larger portion of the signal is emitted in the forward direction, and for biomolecules with the size of RESTs the signal is equal. Resonant CARS signals and non-resonant background show internal structures by matching the difference frequency with fluorophore vibrational properties, thus CARS target a narrow section of the Raman spectrum and several molecules can be spatially targeted.

2.4 Microscope characteristics

This section reviews microscope characteristics, such as resolution, imaging depth and spatial distribution of the focal volume. In order to interpret the quality of the MPM images, it is important to understand how the light is collected. The section also reviews basic components of a confocal laser scanning microscope, including illumination, scanning optics, and detection.

The microscope resolution determines the tiniest discernible feature of a sample. Typically, resolution (R) is defined as

$$ R = \frac{\lambda}{2 NA} $$  \hspace{1cm} (2.2)

where $\lambda$ is the excitation source wavelength and NA is the objective numerical aperture. As the equation indicates, the resolution of a 2PM setup, compared to a 1PM setup, would be worse by a factor of two. However, ultimately, the resolution is limited by the signal-to-background ratio (SBR), as out-of-focus photons will degrade the contrast of a 1PCLSM, thus it would require an infinitely small pinhole [30, 110]. As a result, the effective resolution of a 2PLSM is greater compared to a 1PM setup caused by a higher SBR, and consequently the spatial profile of the 2PM setup focal volume will be smaller as seen in the next paragraph [85].

Equation 2.2 can be extended to 3D, which models the illumination point spread function (IPSF), i.e. the focal volume of the 2PLSM. The 2PM resolution is effectively proportional to the axial 1/e radii (IPSF$^2$) [83]. Calculating the
diffraction-limited 1/e radii of the IPSF can be conducted by a Gaussian approximation for a non-saturated fluorophore excitation. High-σ probes can saturate the fluorophore, such as QDs [110], reviewed in Chapter 3. The transverse radii depends on the NA in different regions (NA = 0.7), and on the fluorophore action cross-section. For NA values over 0.7, the resolutions depends inversely on the power instead [110]. The approximation calculates the central lobe as an averaging of the 2PE potential, which gives an effective volume approximation [83]. The following equation models the IPSF for a confocal one-photon microscope [99]:

\[ I(v)_c = \left( \frac{2J_1(v)}{v} \right)^4. \quad (2.3) \]

Equation 2.3 describes the spatial distribution of the focal volume where \( J_1(v) \) is the first order Bessel function and \( v \) is the normalized radial distance defined as

\[ v = kr \sin(\alpha), \quad (2.4) \]

where \( k \) is the wave number, \( r \) is the radial distance from the optical axis and \( \sin(\alpha) \) is the NA of the objective. Similarly, the IPSF of a 2PM setup is represented by the following equation:

\[ I(v/2)_{2p} = \left( \frac{2J_1(v/2)}{(v/2)} \right)^4. \quad (2.5) \]

Comparing 2.3 and 2.5 we notice that the 2PM IPSF will be wider due to a longer excitation wavelength used in regard of both axial and transverse resolution. Confocal one-photon microscopy can have a larger resolution due to the pinhole, but the strength of 2PM lies in the optical discrimination capabilities, which enhance contrast by collecting useful photons only [85]. However, several factors limit confocal microscopy, including an imperfect pinhole, contrast, and collected photons per pixel [110]. Nonlinear excitation fluorescence only arise in the volume, thus only useful photons increase the resolution. Another advantage of 2PM is the spectral separation of the signal and excitation light. Molecules are commonly excited using infrared light and they emit nearly frequency doubled light in the visible range. Thus, the excitation beam can be filtered out by dichroic mirrors and in the process separating the excitation beam and directing the signal to the detector [85]. Also, comparing the intensity as a function of axial (z) distance from the focal volume, it shows that the intensity remains constant for confocal imaging and it falls of inversely to the square of the intensity for two photon imaging (Fig. 2.1).

Several factors affect the microscope resolution, which can be modelled using an effective resolution instead. The effective resolution includes contributing factors, such as contrast and photons collected from the focal volume. The two-photon
microscope excels in this regard compared to linear CLSM, which requires a pinhole before the detector for filtering out-of-focus scattered photons. The loss due to scattering is modelled by the Mie (Rayleigh) theory, where scattering scales inversely proportional to \( \lambda^4 \), thus decreasing resolution as the wavelength increases [61]. Ultimately, 2PM sacrifices resolution for intrinsic 3D imaging. Additional image contrast is gained using an immersion medium between the objective front lens and the cover slip. The reduction in refractive index difference between the objective medium-cover-glass interface results in a greater collection of higher diffraction orders. Immersion mediums, including oils or water, approach the refractive index of glass \( n_{\text{glass}} = 1.5 \) by 1.518 (\( n_{\text{oil}} \)) and 1.33 (\( n_{\text{water}} \)), respectively.

Exciting fluorescence efficiently in biological samples require the correct illumination source. One-photon confocal microscopy elements provide a basis for multiphoton imaging setups. The linear setup can be modified to support multiphoton imaging by introducing a pulsed mode-locked laser, which replaces the continuous wave (CW) laser. The CLSM consist of three main components: an illumination source, scanning optics, and detection. Thus, beam scanning and data acquisition of MPM is similar to linear CLSM. The excitation source differs in regards of the output, i.e. pulsed versus CW excitation.

Unlike a linear 1PCLSM, a multiphoton setup requires a pulsed laser in order to excite two-photon effects efficiently. Section 3.4 discussed the two-photon absorption cross-section, which naturally requires a high-intensity mode-locked excitation source for generating a large photon flux in order to excite a two-photon process. Therefore, the passively mode-locked Titanium:sapphire (Ti:sapphire) femtosecond laser is the most common amplifier laser for MPM applications [85]. The Ti:sapphire laser is the application of choice due to several factors, including wavelength tunability, broadband amplification and short high-intensity pulses [66].

The sapphire gain medium generates short pulses at a scale of nano- to picoseconds, and at a rate of several KHz up to 100 MHz. Pulse generation occurs in the titanium-doped sapphire crystal due to a phenomenon known as Kerr-lens mode-locking, which is already reviewed in Section 2.2.1 [66]. Adding to this, each pulse consist of several modes eqidistanted by the laser repetition rate and it can be utilized other applications, such as optical frequency combs [31]. A broadband gain medium results in wide tunability in the range of 700-1100 nm, which is optimal for tissue imaging, such as imaging of the mouse retina [85]. Mode-locking is possible by several methods, including passive Kerr-lens mode-locking, synchronous pumping and acousto-optic mode-locking [86]. However, the mode-locking method in use limits the tunability by several factors, such as reflectance of the optics, cavity length, or dispersion characteristics of the lasing cavity [40, 86]. Tunability also inherently amplifies the pump in a wide spectral
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range. Furthermore, returning to the Kerr effect, the pump is further broadened due to the generation of additional modes as a result of self-amplification, thus a wide range of frequencies are amplified by the gain medium and effectively increasing the tuning range [40]. As a summary, amplification of the pump laser power is initiated by the mode-locking exceeding background noise resulting in population inversion, which broadens a wide range of frequencies.

Pulsed lasers started out as dye lasers that required high maintenance. Later, Ti:sapphire amplifiers provided robust and solid state means of pulse generation, and other possible high-frequency lasers are based on Cr, Yb and Nd-doping, which lase in different wavelength regimes [85]. Various Ti:sapphire lasers components evolve continuously, such as saturable mirrors initiate mode-locking by suppression of CW-operation, and as a result improve laser operator convenience. The Ti:sapphire laser is robust but bulky and the invention of high-intensity diodes would replace the intermittent seed CW laser used for exciting modes in the gain medium [40].

In order to generate an image based on the re-emitted laser intensity, several microscope elements are required, including filtering, beam expanding, and scanning elements. The scanning microscope comb a sample in raster scanning pattern, pixel by pixel. As such, it requires synchronized scanning and data acquisition. Scanning optics can include a galvanometer scanning (GS) mirrors, which deflect the collimated excitation beam through the telescope path, which overfills the objective for a diffraction-limited focus. The experimental setup beam path is focused using a telescope path, which includes a beam expander that adjusts the beam radius at the back of the objective. The beam expander consist of a scan (SL) and tube lens (TL). Before the sample an objective first magnifies and focuses the excitation beam, second collecting the scattered signal and directing it to a dichroic mirror (DM) that filters the excitation source intensity. The DM separates the MPM excitation and scattered signals, i.e. the NIR and visible PL are separated efficiently in a low-pass DM. Other filters, such as neutral density (ND) filters, and color filters (F), such as NIR filters, adjust the power and filter the spectral range according to the desired spectrum, respectively. After the color filter the beam reaches the detector, which typically consist of a photomultiplier tube (PMT).

As a result of imaging using raster scanning, i.e. point detection, it can be advantageous using wide-field non-descanned detectors, such as PMTs, for collecting fluorescence or light rays. Large-area detectors favour MPI by collecting direct and skew rays focused through a high-NA objective [110]. Traditionally, one-photon imaging setups collect diverging epifluorescence using an image detector. Now, MPI signal detection is most commonly conducted using a photomultiplier tube (PMT). PMTs collect reflected light for wavelengths ranging from 300-900 nm
by varying the photocathode material, which also affects the collection efficiency. The cathode material affects the PMT quantum efficiency, which range from 10-40%, so a correct wavelength-to-collection efficiency ratio should be chosen for the sample emission imaging [110]. Other PMT parameters include high sensitivity, small integration times, no readout noise, and wide-field detection. Thus far, charge-coupled devices (CCD) have not competed with PMTs due to a high readout noise. However, on-chip avalanche amplification can reduce readout noise, and avalanche diodes also show greater sensitivity for low illumination conditions [110]. As a result of wide-area detection, PMTs require darkened ambient conditions that reduces background noise, and sensitive cathodes can break easily if exposed to intense light [20].

2.5 Challenges in optical imaging

This section discusses the challenges of multiphoton imaging, such as laser induced photodamage and depth resolved imaging. Photodamage prevents microscope operators from imaging samples in biological imaging using an optimal setup power. The main hurdles of biological imaging is set by dynamic high-resolution imaging depth, i.e. scattering in thick tissues. Most challenges are tissue specific and different solutions are required for optimizing the setup parameters, thus streamlining the power usage and pulse shape will increase the probability of nonlinear processes in the sample. Several measurement specific factors limit multiphoton imaging, including optics, noise, and power usage [25].

2.5.1 Laser induced photodamage

Laser induced photodamage limits the available illumination power, which in order limits the efficiency of nonlinear processes as they are nonlinearly proportional to the intensity [8]. Some applications of MPI can require maximum power usage in order to image thick samples, such as the mouse brain at a depth of 1000 μm. Therefore, it is important to optimize every element of the setup, so the least amount of power can be used.

Photodamage consists of photobleaching and phototoxicity, which can further be divided based on the laser repetition rate [8]. At low repetition rates, linear absorption limits the excitation power. For increasing repetition rates pulsed lasers generate avalanche breakdown and multiphoton ionization. Avalanche breakdown occurs in a molecule while excited by a strong electric field (at the order of 10¹¹ W/cm²), which accelerates naturally occurring electrons to velocities that generate additional electron-hole pairs. The acceleration causes a cascading effect of electrons further impact-ionizing other electrons, which generates heat in each
event. Heat is also generated in defects or by free electrons in defects, acting as hotspots. Hotspots enhance the field locally by lowering the threshold for photodamage in defects or imperfections.

Section 2.1 reviewed 1PI techniques for biological applications. 1PCLSM excite the entire beam path, thus inducing photodamage along the transmission length. Imaging techniques based on 2P effects only excite the focal volume, thus confining damage to the volume and overall reducing photobleaching due to a lower average power. 2PM effects excite fluorophores using NIR photons. Fluorophores which absorption maximum locates in the UV wavelengths are damaged by 1P techniques. Thus, NIR photons excite the same fluorophores at a lower power [8]. However, a lower pulse duration decrease the amount of power per unit area to induce photodamage, i.e. photodamage moves from the illumination cone to the focal volume. Nevertheless, harmonic generation does not concern electronic states, thus harmonic generation imaging studies bio-samples during longer sessions.

Damage leads to altering of the fluorophore structure, such as DNA damage or oxidative stress, and it can even kill the sample cells. Phototoxicity can be reduced by lowering the intensity or laser exposure to the sample. Dailey et al. demonstrated a linear relationship between fluorophore concentration and phototoxicity [15]. Also, König et al. [51] determined linearity between cell damage and 2PEF in 2PLSM, which is related by the power squared and pulse duration. As a result, a lower pulse duration will increase the photodamage. However, Field et al. [25] studied the relationship between transform-limited pulses and bleaching and they concluded temporal pulse shape modification (spherical aberrations for > 50 fs pulses) refines the two-photon excitation process, thus increasing SNR. The 2PEF efficiency increase faster than the bleaching. Finally, not only does wave front control mitigate dispersion but also selectively excites fluorophores by controlling the third-order spectral phase [73].

2.5.2 Depth resolved imaging

Depth resolved optical imaging is limited by scattering and absorption in thick scattering tissue, such as liquid media (blood and water absorption maxima at 1300 nm [59]), and molecule absorption. Section 2.2.3 demonstrated the advantage of using a longer wavelength for depth resolved imaging. Imaging depths up to 1000 µm are possible at wavelengths between 1300-1700 nm, using high-frequency lasers (e.g. femtosecond lasers). However, scattering produces sufficient fluorescence at the specimen surface to cause noise when imaging at depths larger than 1 mm [92]. Greater penetration into the sample result in fewer emitted photons due to scattering, thus ballistic photons form the main signal, and consequently sophisticated improvements must be added to the imaging setup [39]. The ballistic
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photon signal attenuates exponentially with the imaging distance [36], thus larger excitation powers are used which increase the risk for photodamage. Therefore, it is crucial to optimize the measurement setup in order to mitigate the dispersion in the tissue to be imaged. Several techniques can increase collection efficiency of a setup, including advanced optics, high-σp fluorescent probes (e.g. quantum dots), and complementary electronics. Adaptive optics increase the signal strength by maintaining a high signal ratio by compensating for aberrations in the wavefront caused by the optics system and specimen. Section 3.5 reviews fluorescent probes used in bio-sciences, including GFP and QDs. Complementary electronics, such as photon counting equipment matching the source frequency, made possible with high-λ electronics was discussed in Section 2.3.

2PLSM has several advantages compared to 1PCLSM, including the usage of a higher wavelength, which causes less phototoxicity in the fluorophores. 2PM only excites fluorophores in the excitation plane due to a square power dependence. A 1PCLSM illuminates the entire beam path volume, proportional to the fluorescence excitation, which reduces the 3D sectioning capability [89]. Although confocal photobleaching is larger than that for 2PM, the 2PM photodamage can be larger in the focal volume [78]. Drummond et al. [22] concluded longer wavelengths centered at NIR wavelengths, such as 780 nm, can also generate 3PEF which corresponds to DNA absorption maximum of 240 nm and can result in permanent DNA damage [91]. High-frequency lasers are expensive and require further development for increasing mobility and reducing cost before they can be introduced in the industry. Yet, they are simply introduced into an linear CLSM setup, only requiring few additional optics for basic imaging. Fluorescence methods [2] are most suitable for in vitro applications compared with X-ray and MRI, which excel in depth imaging and spatial resolution. Different processes require different parameters, so the investigated tissue should be tailored according to the method required. All in parallel with the development of more efficient labeling markers.

2.6 Summary

This chapter reviewed several linear and nonlinear optical imaging techniques applied to bio-samples, including one, two, and three-photon imaging, as well as CARS. The chapter also reviewed non-intensity based imaging techniques, such as FLIM and FRET. Theses imaging techniques are challenged by several factors that limit depth and power usage in biological imaging, such as scattering tissue and photodamage limits.

Two-photon imaging has become the technique of choice for imaging highly-scattering thick samples. Several differences of linear and nonlinear imaging techniques can be based on the excitation source. Multiphoton excitation sources
operate using longer red to IR wavelengths, as well as pulsed operation at a femtosecond scale. The longer wavelengths penetrate deeper into samples due to reduced scattering according to Rayleigh theory. Three-photon imaging techniques image up to 1000 μm in scattering brain samples compared with the 100 μm of a one-photon confocal microscope. A pulsed mode generates multiphoton excitation in the objective focal volume, thus photons only excite the sample in the focal plane enabling high-resolution 3D imaging. One-photon techniques excite molecules through the beam path. As a result, imaging with the help of stimuli is impossible, and the confocal microscope must filter out-of-focus photons using a pinhole.

Pulsed operation result in a lower average power in the sample. However, photodamage is greater in the multiphoton focus compared with one-photon confocal imaging. Therefore, limited power usage is necessary. Nevertheless, lower average power and deeper penetration enable depth resolved in vivo imaging of tissue. Moreover, multimodal label-free nonlinear imaging generate several sources which can be combined for correlative analysis of tissue.
Chapter 3

Optical properties of bio-materials

This chapter presents fundamental scattering and absorption theory applied to bio-samples, such as moss and the mouse retina. Section 3.1 provides an overview of linear and nonlinear optical properties of moss and the mouse retina. Section 3.2 and 3.3 discusses photon propagation in scattering tissue. Finally, Section 3.4 and Section 3.5 discusses endogenous and exogenous labeling of tissue in order to provide contrast against the background. The section also lists common probes and their application for optical imaging, such as brightness in terms of photon absorption cross-sections.

Photon propagation in biological materials differ based on their scattering and absorption coefficients. The coefficients consider various material parameters, including molecule emission spectrum maxima, fluorophore action cross-section and structure. Multiphoton imaging can excite intrinsic PL in bio-tissues. On the other hand, highly scattering specimen can possess weak intrinsic fluorescent molecules which must be label by extrinsic fluorophores.

3.1 Bio-samples characteristics

This section describes structure, optical properties and application of moss and the mouse retina for optical imaging. First, the section discusses the useful nonlinear properties of moss leaf structure, which consist of cells, such as chloroplast that emit SHG. Chloroplast are photosynthesis processing organelles, which also emit strong SHG. Second, the section overviews the mouse retina, which is a complex neural tissue containing several circuits for processing visual input. The retinal tissue is structured in a layered signal pathway that consists of several neurons. Neuron mapping conducted by a multiphoton microscope shows cell bodies, action potentials and neuron connections.
3.1.1 Nonlinear characteristics of moss

The moss plant *Plagiomnium affine* is a thyme-moss species that suits MPI due to its nonlinear properties, such as the leaves containing fluorophores that emit strong SHG, but also as a result of a suitable thickness. The moss leaf has a thickness of one cell layer, which suits the 2PM scattering length (ca 200 μm). The moss leaves also contain the photosynthesis conducting organelle chloroplast, which emit strong SHG. The chloroplast SHG originates in the non-centrosymmetric stacked thylakoid membranes. Another source for SHG includes starch granules contained within the chloroplast membranes, which are generated during the day cycle for storing excess energy. Generation of starch is halted by dark-adapting the plants. Starch can introduce uncertainty in chloroplast SHG imaging, since both SHG signals overlap.

Therefore, Reshak et al. [76] dark-adapted moss in order to measure time dependent chloroplast SHG, undisturbed by starch signals. The paper also measured the power-induced torque caused by chloroplasts birefringence rotations, which is starch independent. Torque is proportional to the power and radius of the particle, thus the phenomenon is SHG independent. The reason for SHG imaging uncertainty arise due to starch granule SHG demonstrated by the work of Chu et al. [13], who showed multiphoton images of starch containing rice leaves. Rice leaves contain chloroplast and starch granules, which emit mixed signals consisting of PL and SHG. The structural orientation studies showed SHG emitting tissue by varying the excitation source polarization. SHG can be combined with THG for additional details about the structure, such as interface boundaries due to cell walls. Orderly, but non-centrosymmetric granal thylakoid membranes, such as chloroplasts, emit SHG signals. Additionally, Garab et al. [26] showed subcellular particles, such as organelles like chloroplast, possessing intrinsic birefringence arising from the stacked membranes and grana. A polarization controlled laser can induce torque in the particle using circularly polarized light, and orientation changes by linearly polarized light. This concept could be applied for manufacturing micromotors.

Figure 3.1 shows a SHG image of chloroplast of the moss *Plagiomnium affine* [76]. As shown in the figure, chloroplast emit strong SIHG and no starch granules distort the signal caused by the plant being kept in the dark for three weeks. This result will be compared later to images generated in this thesis in Chapter 5.

3.1.2 Properties of the mouse retina

The retina conducts various preprocessing functions as an extension of the brain in the back of the eye. Figure 3.2 shows how five neurons classes are distributed among ten layers which form the retina [23]. Retinal function is further decided
by neuron shape and size, and up to 60 morphological subclasses generate unique pathways for processing visual input. The eye focuses lights on the outer-most part of the retina, the rods and cones, which transform light, visual input, to data. Several neuron combinations create parallel synaptic pathways for the visual stimulus [7]. Parallel circuits preprocess simultaneous visual input, including brightness and motion. Not only visual stimuli, but physical functions start in the retina, such as the circadian cycle and pupil size control [95]. Retinal neurons process visual input by several methods, such as excitation and inhibition of neighboring neurons. The modified input is relayed to the ganglion cell layer where information is encoded by Retinal ganglion cell (RGCs) to various parts of the brain [23].

Therefore, mapping the retina using optical imaging reveals various details, including single-neuron processing and retinal-brain connectivity [7]. Furthermore, mapping the retinal circuitry can aid detecting and predicting various medical conditions, such as Macular degeneration. As shown in Fig. 3.2, a layered structure processes visual input starting from the photoreceptors, and depending on the type of neuron, the signal proceeds to the inner nuclear layer via retinal ganglion cells to the optic nerve leading to the brain [23]. The retinal neurons can be imaged and electrophysiologically measured using optical imaging techniques and electrodes, respectively. The retinal tissue absorption maxima typically ranges between 400-700 nm and several endogenous fluorophores are found in the retina,
including A2E, NADH and serotonin. Further, neurons which appear near invisible to optical microscopes can be labeled by exogenous fluorescent probes, reviewed in Sec. 3.5. Not only, collagen and microtubules of the sclera emit SHG, which provides an additional signal source for multimodal imaging.

### 3.2 Photon propagation in scattering tissues

This section discusses the effect of scattering theory on NIR photons propagating in highly scattering tissue. The section also compiles the depth resolving abilities of the optical imaging techniques.

Imaging depth for confocal imaging in bio-tissue is modeled using several parameters, including the scattering and absorption coefficients of fluorophores. Multiphoton imaging improved the imaging depth in scattering tissue by shifting to a longer wavelength excitation in the NIR region, which scatter less in thick tissue. The propagation of photons in tissue is hindered by particles about equal or greater in size than the wavelength, i.e. scattering, which approximated by Mie Theory. Photon scattering from particles smaller than the wavelength can be described by Rayleigh scattering, and tissue scattering strength by the mean free path. Scattering losses are inversely proportional to the fourth power of the wavelength. As a result, scattering losses of a 2PM setup are reduced compared to a 1PCLSM, since NIR excitation of 2PM attenuates weaker than 1PEF by a magnitude of one [85]. For example, Schwille et al. [80] compared 1PE and 2PEF induced diffusion in the tobacco leaf epidermis, and the results showed that the highly scattering plant walls reduce the diffusion in the 1PEF case. The results also show that 2PEF induces less scattering and greater absorption. Moreover, 2PEF makes efficient use of all scattered photons, unlike confocal microscopes that filter both useful and stray photons using a pinhole. 2PM excitation photons are nearly only absorbed by pigments in the focal volume; thus, all emitted photons are useful for imaging. Scattered photons are not sufficiently concentrated for two-photon excited processes.

Imaging depth is, in addition to Rayleigh scattering and Mie theory, affected by the tissue state, such as health and age. This imaging depth can be estimated using a maximum depth formula \( z_{\text{max}} \) [92]

\[ z_{\text{max}} = l_s \ln(P_{\text{ave}} \gamma \sqrt{\frac{1}{\tau f}}), \]  

(3.1)

where \( l_s \) is the scattering length that is dependent on wavelength and tissue coefficients, \( P_0 \) (or \( P_{\text{ave}} \)) is the average surface power, \( f \) and \( \tau \) are the laser repetition rate and pulse width, respectively. Moreover, the imaging depth is logarithmically limited by the laser power, and collection efficiency and two-photon
Figure 3.2: Structure of the vertebrate retina, such as the mouse retina. The schematic shows the layered structure as follows: IS+OS - Inner+Outer segment, ONL - Outer nuclear layer, OPL - Outer plexiform layer, INL - Inner nuclear layer, IPL - Inner plexiform layer, GCL - Ganglion cell layer, NFL - Nerve fiber layer, r - Rods, c - Cones, h - Horizontal cells, b - Bipolar cells, a - Amacrine cells, g - RGCs (Courtesy of ref. [23])
advantage, that is $\gamma$, must also be considered. Similarly, the two-photon excited fluorescent power as a function of depth $z$ falls off as

$$F_{2PEF} \propto e^{-2z/L_s},$$  \hspace{1cm} (3.2)

When calculating $z_{max}$, mainly ballistic photons are considered because scattered photons by two-photon excitation scarcely contribute to the two-photon absorption. The maximum imaging depth $z_{max}$ scales linearly with scattering length. As the depth increases, the PSF, thus the resolution is degraded in addition to degrading using a longer wavelength. As a result, imaging depth is specimen specific, such as the maximum imaging depth of human skin is ca 200 $\mu$m-300 $\mu$m for 2PM while imaging in the brain can reach depths up to 500 $\mu$m. Furthermore, scattering generated surface fluorescence, such as fluorescence by GFP expressing specimen, limits the imaging depth at greater depths, which is important for in vivo imaging through the skull [36]. Theer et al. [93] imaged brain slices using a 3PM and reached depths of 1000 $\mu$m, similar values for CLSM is 120 $\mu$m [16]. Thus, the improvement in imaging depth from 1PCLSM to 3PM has increased tenfold from 100 $\mu$m to 1000 $\mu$m [36]. There are other options for increasing $z_{max}$, such as adaptive optics (under filling the back focal plane of the objective) and regenerative amplifiers [93].

### 3.3 Molecular absorption

The previous section presented scattering theory in bio-tissues, which affect the photon propagation of optical microscopes. Photons that avoid scattering must be absorbed; thus, the excitation wavelength must match the absorption spectrum of the targeted fluorophores. This section discusses photon absorption in molecules for biological imaging. Bio-imaging using multiphoton techniques utilize the "optical window" optimal for NIR photons.

2PM generates contrast by the simultaneous absorption of two photons in fluorescent molecules (within a span of femtoseconds [66]) by 2PEF. The 2P absorption energy corresponds to the absorption bandgap of the fluorophore, which generates contrast by the emission of a photon with the same energy. Contrast can be generated either by intrinsic fluorophores or artificial labeling, which is discussed in Section 3.5. Fluorescent molecules generate contrast against the background, such as intracellular medium or water due to differing values of refractive index. Matching the laser source wavelength to the excitation band of fluorophores in biosample imaging yields optimal results, since the emission is maximized. Several matches of wavelength to absorption cross-section of molecules have already been characterized elsewhere [103]. A method for characterizing two-photon excitation absorption probability is the (two) photon absorption cross-section $\sigma$, measured
in Göppert-Mayer (GM), which can be calculated using 2.1. The topic is further elaborated in Section 3.4. The equation depends on several parameters of the measurement setup. The fluorophore brightness is measured as a product of the two-photon absorption cross-section \( \sigma_{2p} \) and fluorescence quantum efficiency \( \varphi_F \) [104].

Photopigment of vertebrae, which excite rods and cones, absorb 1P emission efficiently in the 350-700 nm wavelength range [23, 45]. The optical window of the photopigment corresponds to half the typical excitation wavelength of 2PM setup. 2PM has the advantage of a higher wavelength excitation, in the 700-1000 nm NIR range, which makes the retina nearly transparent and avoid excitation of intrinsic fluorescent molecules, thus enabling electrophysiological measurements of the retina. As a result, 2PM image intrinsic chromophores, such as NADH, for assessing cell metabolic states [74] without absorbing in the extracellular medium of the eye before reaching the tissue. Yet, longer wavelengths using 3PM can also excite intrinsic fluorophores, such as serotonin, in the UV range centered at 280 nm, which may be problematic for 1P laser sources since UV can induce phototoxicity in the cells [57]. Because the absorption probability depends on the square of the incident power [66], two-photon processes are unlikely to occur outside the focal volume. Photons that have no contribute to the 2PEF process are re-scattered and too diluted for absorbing 2PM photons, and as a result they are less likely to be absorbed by the bulk for generating background [20]. Nevertheless, 1P absorption (even by NIR photons) by the specimen is unavoidable and can be estimated through the absorption distribution. Unlike 2PM, CLSM excitation is linearly dependent on the depth \( z \), thus the whole illumination path absorbs UV photons and will undergo photobleaching [75].

Several articles [6, 23] use the 910 and 930 nm wavelength, both for deeper penetration, less scattering and fluorophore absorption maxima in the 480 nm range, such as melanopsin, which is expressed in the photosensitive retinal ganglion cells. In this master’s thesis an 810 nm wavelength laser is used, and other articles [29, 105] have utilized the same source for rat brain and retinal sample imaging. Grimes et al. [29] generated contrast by a replacement of the local calcium binding ions using a calcium sensitive dye, which increases brightness and reveals neuron varicosities at a scale of 3 \( \mu \)m, spaced by 30 \( \mu \)ms. The improved neuron emission enabled monitoring spiking activity by observing calcium transport dynamics and concluding that postsynaptic elements function as microprocessors for parallel microcircuits.
3.4 Measurement and application of the absorption cross-section

Section 3.3 described the absorption of photons in bio-tissue. Now, when researchers want to pair a fluorescent dye with a laser excitation wavelength, then the absorption cross-section $\sigma$ is a useful measure for conversion efficiency between photon absorption and PL emission. In other words, it is the measurement of two-photon absorption probability occurring in the molecule \cite{104}. Therefore, this section presents the role of the action cross-section when choosing fluorescent probes for linear and MPI.

Several research groups, such as Herman and Ducuing \cite{37}, and Xu et al. \cite{103} have studied common extrinsic dye and intrinsic fluorophore absorption cross-sections ($\sigma_{2p}$) using 2P spectroscopy. One could think $\sigma_{2p}$ is determined based on doubling the equivalent 1P absorption wavelength, but due to quantum-mechanical selection rules and vibronic coupling of electron states the conversion process differs \cite{85}. The cross-section even blue-shifts in some cases \cite{89}. The laser cross-section is measured in units of Göppert-Mayer (GM), where 1 GM = $10^{-56}cm^4s/photon$, named after Dr. Göppert-Mayer who demonstrated the possibility of nonlinear absorption (2PEF) in her doctoral thesis in 1931 \cite{27}. 2PEF was determined using $\sigma_{2p}$ and the pump intensity squared (Eq. 2.1 \cite{103}). Now, the two-photon absorption cross-section can be derived from Equation 3.3, which models the time-averaged fluorescence photon flux $\langle F(t) \rangle$, which is based on the number of photons absorbed per unit time. The flux is a product of the fluorecence quantum efficiency of the dye $\eta_2$ and the fluorescence collection efficiency of the MPM setup $\phi$ \cite{102}. Here, $\eta_2$ is assumed to be the same as the 1P quantum efficiency and $\phi$ is a function of collection optics, transmission and quantum efficiencies. Another useful measure is the product of two-photon absorption cross section and the fluorescence quantum efficiency, the product is a measure of brightness \cite{52}.

$$\langle F(t) \rangle = \frac{1}{2} \phi \eta_2 C \sigma \frac{g_p}{\tau} \frac{8n \langle P(t) \rangle^2}{\pi \lambda}$$ \hspace{1cm} (3.3)

Xu et al. determined $\sigma_{2p}$ using Eq. 3.3, requiring three parameters, including $\eta_2$, $\phi$, and the incident light spatial distribution (The distribution $J(\nu)$ is further elaborated in Chapter 4). As a result, the spatial and temporal coherence of the excitation light must be determined. As a conclusion, the 2PEF cross-sections provide estimates for fluorescence spectra, i.e. a catalogue for choosing appropriate probes for the excitation source and for calibration purposes.
Table 3.1: Intrinsic and extrinsic fluorophore imaging parameters, including excitation $\lambda$ and $\sigma_{2p}$

<table>
<thead>
<tr>
<th>Fluorophore</th>
<th>Excitation wavelength (nm)</th>
<th>$\sigma_{2p}$ (GM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NADH [104]</td>
<td>700</td>
<td>0.02</td>
</tr>
<tr>
<td>Serotonin [81]</td>
<td>710-830</td>
<td>0.1</td>
</tr>
<tr>
<td>(E)GFP [34, 104]</td>
<td>800-1000</td>
<td>5-60</td>
</tr>
<tr>
<td>Fluorescein [103, 109]</td>
<td>780</td>
<td>38</td>
</tr>
<tr>
<td>Alexa 594 [109]</td>
<td>780</td>
<td>100</td>
</tr>
<tr>
<td>CdSe QDs [52]</td>
<td>Various</td>
<td>2000-47000</td>
</tr>
</tbody>
</table>

3.5 Fluorescent molecules for tissue labeling

Multiphoton techniques can imagine bio-samples in a label-free manner. However, imaging tissue that has a low absorption cross-section can prove troublesome. As a result, artificial dyes label fluorophores extrinsically, which enable multiphoton imaging. This section reviews common fluorescent probes and their application in bio-imaging. Fluorescent probes can be either endogenous or exogenous, i.e. naturally occurring/fused with the molecules or artificial dyes that are externally injected. Tissue labeling using fluorescent probes include exogenous organic, endogenous fluorophores and quantum dots.

Optical microscopes can gain information about scattering tissue by the means of contrast difference by absorption and harmonic generation. Scattering tissue consists of fluorescent molecules, fluorophores, which are either intrinsic, such as nerve tissue, or extrinsic, such as fluorescent probes for labeling tissue. For some tissue, such as nerve fibers in the retina, nonlinear imaging is sufficient for generating signals without the need for exogenous probes, thus gaining label-free information about the specimen [33, 55]. In the cases of nonviable contrast generating tissue, such as retinal neurons, they can be labeled by fluorescent probes. Labeling neurons can be realized by tagging them with intracellular dye fillings using sharp patch electrodes containing fluorescent dye or a calcium indicator for 2PM. The dye introduces artificial contrast, thus enabling morphological identification of neurons, such as the cell body soma, and synaptic arbors in the retina [23]. This section discusses the use of organic dyes, biological fluorophores and quantum dots for labeling bio-samples. Table 3.1 shows and compares several options for labeling tissue, depending on absorption maximum by wavelength and brightness in terms of absorption cross-section.
3.5.1 Generating contrast using organic dyes

Early work in mapping the retina was conducted by Golgi staining [10] and injection of the fluorescent dye fluorescein [84] into tissue. The Golgi method is a reliable technique for staining the entire specimen (i.e. washing of the specimen by adding a counterstain solution to the extracellular medium). However, the method has drawbacks, such as targeted staining being unavailable due to the random nature of the staining. The Golgi method is limited to ex vivo applications (washing of sectioned tissue). Modern labeling techniques, such as the Nissl method, immunohistochemistry and modern dye filled electrodes [65] overcome the drawbacks.

Advancement in the field created Nissl staining, which is a more refined way to identify neurons based on soma size and morphology. Nevertheless, synaptic arbors are still unidentifiable by this method, which is later revealed by immunolabeling. Immunolabeling is the process of generating DNA strand targeting anti-bodies for specific cell compounds that allows for mapping axons and dendrites.

Modern fluorescent dyes, such as Alexa 596 and Fluo-5F, are combined with electrophysiological recordings [105] that simultaneously monitors nervous system function, such as Ca$^{2+}$ influx induced action potentials in rat brain slices, and tissue morphology, such as labeling neurons, i.e. simultaneous data collection with visual observation. Dyes can be engineered [65] to be excited and emit at specific wavelengths, and using multiple dyes and spectral differentiation result in contrast between cells groups. Other methods for contrast enabling probe delivery include iontophoresis, ballistic methods, electroporation, and un-caging compounds by illumination.

3.5.2 Intrinsic fluorescence by biological fluorophores

Green fluorescent protein (GFP) was first derived by Chalfie et al. [11] from the green jellyfish, Aequorea victoria. By fusing GFP with intrinsic protein in the body, complementary DNA expressed in tissue cells will produce a stable fluorescence, which can become heritable as well. Due to modifications between lines, unique GFP expression are born with new lines, which allows for monitoring of cell evolution (neuronal migration) between lines. As a result, the neuron population expression vary between the lines and there can appear a labeling difference between transgenic lines from a couple of neurons to almost the whole population. Several colors have been developed, including cyan, red, and yellow fluorescent proteins, united by the term XFP. Crossbreeding of different XFP transgenic lines create multi-transgenic lines expressing up to three different XFPs at a time, which create contrasting color channels. XFPs label selectively and target the whole neuron, including synaptic pathways and their terminals, other tissue such
as muscle fiber are not targeted. The same specimen can be imaged over a longer period, up to 9 months while remaining harmless, i.e. XFPs induce negligible phototoxicity and interference with cell growth. However, photobleaching can occur, depending on the wavelength used for imaging [11, 24].

XFPs have large action cross-sections of about 200 GM for specific wavelengths, which make them desirable for MPM. XFPs can be engineered to absorb both one- and two-photon wavelengths [104, 110].

Feng et al. [24] labeled the inner layers of the retina and brain neurons using multiple XFP variants, and as a result showing the labeling consistency of the protein. The work demonstrates the usefulness of stable multi-labeling, but also uncertainty in labeling precision of several neuron subsets. Combined recording of spiking activity using stimuli and imaging was conducted by Borghuis et al. [7]. Their results show simultaneous recordings of fluorescence, using a two-photon microscope, and action potential firings in the form of spiking data, using a whole-patch recorder. A genetically encoded calcium indicator (GECI) successfully labels the five major neurons of the retina by suitable promoter and serotype combinations. As a conclusion, they observed a linear relationship between fluorescence and spiking as a response to stimuli while monitoring activity in multiple neuron populations.

Various intrinsic fluorophores are native compounds found in living tissue, including serotonin and NADH. Serotonin is a neurotransmitter natively found in neural tissue, such as serotonin-accumulating neurons in the retina [64, 94]. Previous work suggests serotonin reacts to nonlinear excitation, under steady-state diffusion, by creating photochemical byproducts that emit fluorescence in the visible wavelength range. Multiple processes, including 3PEF, 4PEF and 2PEF (which in combination create a sum of 6P absorption for visible light), contribute to the absorption cross-section and show minimum results of 0.1 GM [81]. Reduced nicotinamide adenine dinucleotide (NADH) is an intrinsic molecule ubiquitous in bodily tissue, which fluorescent emission varies with oxygen concentration. Studies of cellular metabolism is possible by imaging NADH, which is a reporter compound [60]. Studies show NADH has a low $\sigma_{2p}$ of 0.02 that will be overshadowed by extrinsic fluorophores. Kierdaszuk et al. [48] measured NADH and NAMH absorption cross-sections relative to MSB, and Xu et al. [104] showed absorption cross-sections for NADH and several other fluorescent molecules.

### 3.5.3 Imaging quantum dot labeled tissue

Semiconductor nanoparticles, or quantum dots/qdots (QD), emit PL in a variety of colors that can be engineered by varying their size and composition [62]. Moreover, combining several QDs enable multicolor imaging for molecule tracking or dynamics detection. QDs absorb in broad excitation wavelength and emit narrow emission
radiation relative to the bandgap of the semiconductor crystal, which makes an efficient source for fluorescence in multiphoton imaging [1]. The QD fluorescence towers intrinsic fluorescent molecules by several order of magnitude, as shown in Table 3.1, thus discriminating QDs from background noise. QDs emit fluorescence at an impressive rate of 10000 - 50000 GM, thus labeling scattering and thick tissues efficiently. The typical QD structure consists of a semiconductor hetero structure for tunability of absorption and emission, a protective semiconductor layer for prevention of intermittent blinking [67], which originates from the rough crystal surface and finally biological interfacing, such as by conjugating the qdot with antibodies similarly to immunocytochemistry [62]. Larson et al. utilized water soluble QDs encapsulated in an amphiphilic polymer [101] for in vivo imaging while comparing it to fluorescein. The result show that QDs image tissue at greater depths while using less average power than conventional organic dyes [52]. In addition, qdots brings excitation to the near-infrared which filled a required gap among fluorescent probes [62].

Pathak et al. [71] labeled r-MC1 retinal Muller glial cells using antibody fused quantum dots, and imaged the tissue using a 1P confocal microscope. The results prove the viability of QDs for labeling neurons for probing and improving conventional immunocytochemistry contrast and brightness. Imaging single protein structures in neurons by nanometer size QDs is possible, which opens doors for further neurobiological work.

Combining 3PEF and semiconductor nanocrystals, Yu et al. [106] imaged tumor tissue targeted with qdots under highly scattering autofluorescent skin using a 3PM centered at 920 nm, shown in Fig. 3.3c. The ZnS nanocrystals reduce power usage due to a high three-photon action cross-section, achieved by Mn$^{2+}$ doping and resulting in a Stokes shift which red-shifts the emission, distinguishing it from intrinsic PL and lowering photodamage (Fig. 3.3a). Moreover, ZnS are naturally found compounds in the body which make them biocompatible compared to toxic CdTe qdots [12], thus making ZnS qdots desirable for diagnostic applications. The ZnS qdot absorb UV-light and emit orange light at 580 nm, a high $\sigma_{3p}$ increase the brightness by a magnitude of four compared with conventional UV-dyes, as a result generating contrast against intrinsic PL in the blue-green range (Fig. 3.3b).

### 3.6 Summary

This chapter examined scattering and absorption theory of photons in scattering tissue, such as moss and the mouse retina. In addition, nonlinear properties of the samples were reviewed, and their effect on the imaging setup requirements. The chapter also reviewed several fluorescent probes for bio imaging, such as organic dyes, fluorescent proteins and quantum dots.
Figure 3.3: ZnS probe characteristics for in vivo applications. a, Normalized spectra showing how doped ZnS emission separates from collagen SHG and intrinsic fluorescence. b, Photostability as a function of time, ZnS brightness increase due to photochemical annealing. c, Multiphoton image of ZnS QD (3PEF, orange) targeted tumor and collagen (SHG, blue) (Courtesy of ref. [106])

Photons undergo scattering loss proportional to $\lambda^{-4}$ according to Rayleigh scattering. Therefore, multiphoton imaging improve depth resolved imaging using NIR photons compared to 1P techniques by a magnitude of one. In addition, the NIR photons of MPI optimally utilize the "optical window" of bio-tissue using a 1500 nm wavelength to avoid water, UV and infrared absorption in material, analogous to fiber optics. Furthermore, a large range of possible wavelengths can target the absorption maximum of several fluorophores, even in the UV range due to a lower average power at the sample, compared to one-photon techniques.

Multiphoton techniques enable label-free imaging of bio-sample due to an intrinsic 2PEF and SHG in bio-tissue, such as moss and the mouse retina. The nonlinear signals originate in the moss chloroplast and in several retinal structures, such as NADH and serotonin. The re-emitted signal will be far removed from the pump wavelength in the infrared, thus improving SBR. These samples are suitable for MPI due to an average axial thickness of 200 $\mu$m.

When label-free imaging in not an option fluorescent probes generate artificial contrast in tissue. Imaging biological samples require choosing the right probe, since several factors affect the imaging process, including availability (labeling speed), brightness and stability. Engineering of organic probes is required for matching probe properties with the fluorophore and pump wavelength. Each labeling methods has its advantages and disadvantages; dye injected organic dyes label selectively and fast, but are limited by imaging periods, in vitro microinjections and labeling several populations simultaneously. XFPs generate bright, stable fluorescence. However, genetic lines require ambitious upkeep. Several generations of XFPs enable genetic encoding of the protein properties, such as multiple expressions of XFPs and activation at specific signals and events, such as protein-protein interactions (FRET). Quantum dots emit the brightest fluoresces
among current probes (absorption cross-section), which can be engineered to emit a large range of wavelengths, even in the infrared. Fluorescent probes in the infrared has previously been lacking, but sought after since infrared laser can penetrate deeper in biological tissue and is more distinguished among autofluorescence. By changing the QD composition and size, several parameters can be varied, such as water solubility, photo stability and non-toxicity.
Chapter 4

Material and methods

This chapter presents the experimental methods used in the thesis. Section 4.1 describes the sectioning of the moss leaf samples for SHG imaging of chloroplasts. The section also overviews the nonlinear properties of the moss, which is suitable for MPI. Section 4.2 presents and describes the linear commercial and nonlinear in-house measurement setups, including component choice, image reconstruction and objective use.

4.1 Moss sample preparation

This section discusses acquiring, preparation and sectioning of the moss Plagiomnium affine for linear and nonlinear optical imaging. The moss suits nonlinear MPI because its leaves have an average thickness of two cell layers, with a thickness of 100 μm each, which matches the scattering length of 2PM [97]. In addition, moss leaf cells contain chloroplast that emit strong SHG due to stacked membranes, which break the centrosymmetric ordering. The thesis will evaluate the effect of starch granules on chloroplast SHG imaging. Therefore, two types of the moss leaves will be compared, dark-adapted and non-dark-adapted, in order to attempt to observe the conflicting signals.

Two moss samples were collected from the base of a campus building, one sample was dark-adapted during three weeks, the other was maintained under normal light conditions. Dark-adapted moss halts the generation of starch granules contained in chloroplast. Leaf chloroplasts emit strong SHG, which is easily detected and imaged using 2PM. Uncertainties in SHG imaging of chloroplast arise due to starch, which is another source for SHG [13]. As a result, by comparing both moss types, the images can reveal starch SHG signals that introduces uncertainty to chloroplast imaging.

For imaging of moss, a leaf was plucked from the darkened part of the plant
using a pair of tweezers. The leaf was imaged between a microscope slide and cover slip, emerged in water. Water immersion increases the collection of light by matching the refractive index of glass with the water, thus reducing the signal loss due to TIR. SiIG imaging uncertainty was investigated by starch content comparison between fresh moss leaves to dark-adapted moss.

4.2 In-house measurement setup

This section presents the in-house experimental setup used for multiphoton imaging of moss, as well as the linear optical setup for generating reference images. In addition, the imaging and processing software are presented for generating the measurement results.

Figure 4.1 presents the experimental setup used for 2PI of moss. The custom built two-photon microscope comprises a femtosecond mode-locked infrared Ti:sapphire laser (Mai Tai SP, Spectra-physics). The laser is tuned at 810 nm, with a bandwidth of 30 nm, a pulse width of 120 fs at repetition rate of 85 MHz. The excitation source emit an average pump power of 0.4 W, and 0.340 mW was recorded at the sample. Full power was used for moss imaging. An Olympus LUMPlanFl 60x, 0.90 NA water immersion (WA) objective and a 40x 0.5 NA dry objective focuses the beam at the sample. Fluorescence was recorded using a PMT (Hamamatsu, H7884) and power was recorded using a NIR power meter diode. The sample intensity was mapped using a home-made MATLAB software and displayed on a computer.

Figure 4.2 shows a WITec alpha300 optical system used for optical and linear confocal imaging of the sample. The sample was exited using a 532 nm green laser line, and focused through a Zeiss, 20x and 0.4 NA, dry objective. Moss leaf cells were imaged at a depth of 0.1 μm beneath the surface using a pixel dwell time varying between 50 μs and 5 ms, and generating images with a size of 256x256 pixels.

The two-photon microscope scanning optics not shown in Figure 4.1 include a neutral density filter, which attenuates the beam for power optimization at the sample. Next, the attenuated beam is deflected by a pair of raster scanning X-Y galvanometer scanning (GS) mirrors to the focal plane in the tissue. A motorized stage repeatedly adjusts the imaging depth in order to gain 3D data of the specimen. A DC motorized stage, in combination with a CCD camera, moved the LED illuminated sample for depth imaging and planar adjustments. The scanned beam propagates through a beam expander, which generate a collimated beam that slightly overfills the back aperture of the objective for utilizing the whole NA range of the objective [110]. After the objective, a dichroic mirror (DM) spectrally separates the NIR excitation- from the emission beam by selective reflection and
Figure 4.1: Schematics of the nonlinear optical excitation, beam scanning and detection. GS: Scanning galvo mirrors, SL: Scan-lens, TL: Tube-lens, (D)M: (Dichroic) Mirror, Obj: Objective, S: Sample, MM: Motorized stage, F: Filters, L: Lenses.
Figure 4.2: Schematics of the confocal one-photon setup for combined optical imaging. U1: XY positioner, U3: Objective turret and objectives, U6: Ocular camera, U14: Motorized stage, M10: Reflector slider, E3: Laser, L13: PMT. Courtesy of ref. [100].
transmission. In addition, a 750 nm low-pass filter was applied after the DM for filtering the high power laser, removing any traces of the pump wavelength. Appropriate barrier filters specify the desired spectrum before reaching the PMT. The PMT forms the detection element of the setup, which generate a current for data acquisition equipment. A computer processes the signal, while simultaneously controlling the scanning software.

4.3 Image reconstruction

Two-photon images were reconstructed by raster scanning the sample using a pair of X-Y GS mirrors. Raster scanning measures the reflected intensity in a pixel, and by combining several pixels in a bitmap, the image is reconstructed using a computer. Images were constructed by an in-house MATLAB application, and processing of images, such as stack averaging, was conducted in ImageJ (https://imagej.nih.gov/ij/). Images consist of 256x256 pixels unless stated otherwise, and pixel dwell times range between 1-100 ms. Several slices of a sample were imaged by 2 μm steps along the z-axis. High-contrast images were reconstructed in ImageJ by averaging the stack intensity.
Chapter 5

Results and discussion

This chapter presents and discusses the experimental results of the thesis, which is structured as following. Section 5.1 shows linear one-photon (1P) images of dark-adapted moss for identifying the species. The section also co-localizes structures of dark-adapted moss with a fluorescence intensity map, which functions as reference for 2P images. Section 5.2 shows multiphoton images of both dark-adapted and non-dark-adapted moss, including chloroplast SHG and cell wall two-photon excited fluorescence (2PEF/PL). The section also compares the effectiveness of the microscope objectives for 2P imaging. Finally, the section discusses the effect of starch granule SHG, in non-dark-adapted moss, on chloroplast SHG imaging.

5.1 Linear imaging of moss

To obtain a relevant reference image for the identification of moss structures and signals in the 2P images of Section 5.2, this section presents optical images of a dark-adapted moss sample, as well as 1P confocal moss photoluminescence (PL) intensity measurements. The section also co-localizes the structures of dark-adapted moss with a fluorescence intensity map.

The 1P confocal microscope imaged the sample at a depth of 0.1 µm beneath the surface. The moss was kept in darkness for three weeks to reduce starch granules in the chloroplast. Optical images are produced using a confocal one-photon microscope, as described earlier in Chapter 4. Figure 5.1 shows a 1P optical image of the dark-adapted moss sample. As can be seen from the figure, the structure of the dark-adapted moss leaf consists of several cells, separated by cell walls. The cells contain several chloroplasts, which are mainly located along the cell walls. Chloroplasts appear in a varying amount between cells, which can be a result of the 3D structure or the dark-adaption process. Because the moss leaves on average consist of a single layer of cells, the chloroplasts appear at varying
depths along the surface normal.

![Optical image of the dark-adapted moss cell structure](image)

Figure 5.1: Optical image of the dark-adapted moss cell structure

Figure 5.2 shows a side-by-side magnified optical image of the dark-adapted moss cell structure (Fig. 5.2a), and a PL intensity map from the same area (Fig. 5.2b). From the figure it can be observed that cell walls, chloroplasts and PL colocalize well, thus justifying its use as a reference for comparison with 2P images.

Figure 5.3 presents the PL intensity spectrum of the dark-adapted moss, when excited using a 1P 532 nm laser line. Fluorescence is emitted at a longer wavelength than the excitation source due to electron relaxation in the excited state. The next section will excite SHG in the moss leaf samples using the multiphoton setup. SHG is excited in the sample, parallel to PL, that should increase brightness, as well as provide an addition color channel for identifying structures at a micrometer level.

5.2 Nonlinear imaging of moss

The previous section presented one-photon images of dark-adapted moss. In this section, these images are compared to the proposed 2PM setup images of both dark-adapted moss and moss maintained under normal conditions. Both types of moss were imaged using two types of objectives, including both dry and WA objectives in order to determine the effectiveness of these objectives for generating and collecting SHG from moss chloroplasts. The section also attempts
Figure 5.2: Optical image of dark-adapted moss cells (a) and emitted intensity captured with a 1P confocal microscope (b). The chloroplast and cell walls colocalize with the PL intensity, as shown by the yellow markers.

Figure 5.3: Dark-adapted moss cell PL spectra excited using a laser centered at 532 nm. Fluorescence is emitted in the red to infrared region, captured 0.1 um beneath the surface.
to demonstrate starch signal uncertainty by comparing 2P images of dark-adapted moss to moss maintained under normal conditions.

Figure 5.4 shows a 2P image of dark-adapted moss imaged using a dry objective. As shown in the figure, a 2P signal is emitted mostly from the cell walls, which most likely consist of 2PEF. The image agrees well with the 1P image of moss (Fig. 5.1). In this case, the chloroplasts are also located close to the cell walls in the 2P image. The chloroplasts are dimly visible in the high-quality image. This result is inconsistent with that presented by Reshak et al. [76]. Chloroplast normally emit strong SHG, though this is not observed in the image. The dim chloroplasts most likely resulted from a low objective NA and lack of immersion medium.

Next, the setup uses a WA objective in an attempt to increase the collection efficiency of SHG originating from the chloroplast. Figure 5.5 shows a high-contrast 2P image of moss stored in dark conditions, which is imaged using a WA objective. As can be seen in the figure, several chloroplasts can be observed while using a WA objective. Typically, chloroplasts emit a stronger fluorescence as a function of objective NA. In addition, as described in Chapter 2, using water as an immersion medium causes otherwise total-internal reflected rays to reach the objective, thus increasing the contrast. However, the image still differs from that presented in earlier work. The result can be attributed to variations in the experimental setup or imaging parameters, such as magnification and power usage.

Moss stores excess energy due to photosynthesis in starch granules inside the cells. Starch is another source of SHG, which might disrupt intensity measurements of chloroplasts. In order to confirm the uncertainty emitted by starch SHG, 2P images of freshly plucked moss that can contain starch granules are compared to dark-adapted moss. Because starch granules form another source of strong SHG, the thesis will next investigate whether starch SHG introduces uncertainties in chloroplast SHG imaging.

Figure 5.6 presents a multiphoton image of non-dark-adapted moss imaged using a WA objective. As demonstrated by the figure, depth resolved non-dark-adapted moss chloroplasts emit a strong two-photon signal. The image agrees well with the results observed by Reshak et al. (Fig. 3.1). However, based on the result of Chu et al. [13], multiphoton signals consist of both 2PEF and SHG, thus individual starch granule signals can overpower chloroplast radiation. Nevertheless, Fig. 5.6 suggests chloroplast SIHG imaging uncertainty is not present, based on the lack of granules. However, the proposed setup did not use filtering of 2PEF, thus SHG cannot be discriminated from the other signal. Further actions require filtering of the 2P signal in order to discriminate chloroplast 2PEF from starch granule SHG, thus confirming the SHG imaging certainty. As a result, individual starch granules should appear and co-localized with the chloroplast.

Furthermore, dark-adapting moss is not a requirement for two-photon imaging
Figure 5.4: Multiphoton image of dark-adapted moss, captured using a dry objective, 512x512 pixels.
of chloroplast dynamical movements. Starch granule SHG does not saturate the signal, thus the morphology of the chloroplast can clearly be outlined. As a result, birefringence measurements of chloroplast in order to measure torque is possible due to distinct shapes of the particles [26].

As a conclusion, the thesis successfully demonstrated the feasibility of using the multiphoton measurement setup equipped with a WA objective for 2P imaging of biological samples. The 2PM setup generates depth resolved images of chloroplasts in the epi-direction. Thus, the 2PM can be applied to the retina in future work. The thesis also suggests that leaves of the moss Plagiomnium affine must not be dark-adapted for time-dependent chloroplast SIHG imaging since SIHG uncertainty is not present, which should originate from starch granules. Time-dependent chloroplast SHG imaging can be attributed to the rotation of chloroplasts, which reduced the SHG intensity during the measurements. Uncertainty arise due to starch SHG, which was not observed in the results, thus no background SHG signals should interfere with intensity measurements. The thesis results should quicken the process of future imaging, since preparation of the moss sample can be avoided. Furthermore, imaging in the forward direction could reveal additional information about the organelles, as SHG is anisotropic [76]. However, previous work measured the stronger epi-detection compared to forward-emitted radiation [76]. Finally, plant tissue can vary among samples and among species, such as
SHG due to starch granules in the rice leaf can appear brighter compared with moss [13].

5.2.1 Second harmonic generation imaging of moss chloroplasts

The thesis imaged moss chloroplasts using a 2P setup equipped with a dry and WA objective. Initial trials using the dry objective revealed a lack of signal. Based on the results, two conclusions are drawn about the signal strength. First, a high-NA WA microscope objective increase both the 2P collection efficiency and brightness of the moss sample 2P images. Water immersion reduces total-internal reflection of light by matching the immersion medium refractive index with the refractive index of the microscope slide, resulting in additional brightness. In addition, SHG collection efficiency depends on square of the numerical aperture of the objective [23], i.e. how tightly the beam is confined in space. Thus, A large NA improves the collection efficiency of SHG as well as the SBR by using a 0.9 NA WA objective, compared to a 0.6 NA dry objective. Further, Reshak et. al [76] imaged Plagiomnium affine using several microscope objectives and concluded that high-NA WA objectives are the most efficient at collecting the SHG signals.

Second, an alternative to epi-detection measurements was to measure the forward-
transmitted signal, since SHG is coherent and highly directional (anisotropic) a larger portion of the signal can propagate in the forward-direction. Williams et al. [98] showed SHG anisotropy in collagen fibrils, and the results demonstrated that SIIG mainly emits in the forward direction for thick samples, at a scale of the wavelength or larger (for a 800 nm wavelength laser, thickness $t \geq 400 \text{ nm}$). Thin samples emitted SHG equally in the forward and backward direction at a scale of one tenth of the wavelength (ca 40 nm).

To further evaluate the feasibility of the multiphoton setup, previous work [111] imaged several bio-samples, such as celery and moss, using the experimental setup in this work, which further confirmed the microscope effectiveness by imaging celery. Figure A.1, in Appendix A, shows a 2P image of celery generated using a dry objective. As the figure shows, celery lignin in the xylem emit strong 2PEF. The results agree well with previous work conducted by Cox et al. [14].
Chapter 6

Conclusion and future work

The thesis successfully evaluated the feasibility of the proposed two-photon setup used for nonlinear two-photon imaging of bio-samples. Although the two-photon setup is ultimately intended for imaging mouse retina, the feasibility of the proposed setup was evaluated by imaging chloroplasts in the moss leaves of *Plagiomnium affine*, since the moss leave thickness make them suitable for 2PI.

The chloroplasts were imaged using both a dry and water immersion (WA) objective. Use of the dry objective yielded dim nonlinear images of the chloroplasts, presumably due to a low objective NA. In contrast, the use of a WA objective clearly visualized depth resolved chloroplasts. This results agrees well with previous studies [76], demonstrating the applicability of the measurement setup for nonlinear imaging of mouse retina.

Since starch granule SHG can disrupt chloroplast imaging, the thesis also determined the effect of starch signals on chloroplasts. The results suggest that uncertainty due to starch SHG has no effect on two-photon imaging of chloroplasts in the moss leaves of *Plagiomnium affine*. 2P images of dark-adapted moss were compared to 2P images of non-dark-adapted moss, as well as to previous work [13, 76]. Comparison showed no clear signs of starch granule signals within chloroplasts. Since starch granules colocalize with chloroplasts and no starch signals were observed within the organelle, it can be concluded that the imaging process can be simplified by omitting the dark-adaption phase.

The chloroplasts were imaged without optical filtering, which allowed collection of both 2PEF and SHG signals. As a result, chloroplast 2PEF signals can overpower or mix with the starch signal, thus making the starch invisible. Therefore, future work could introduce filters for two-photon imaging of bio-samples, such as mouse retina, in order to discriminate SHG from 2PEF. This would enable only SHG to be visible and starch to appear as bright areas inside the chloroplasts [13]. However, starch content can vary among species and between samples, which complicates evaluation of the starch signals.
The excitation source of the proposed setup was centered at 810 nm, which only generates 2P effects. Ultimately, in the case of 2PM, imaging depth is fundamentally limited by the signal-to-background ratio (SBR) [41]. For this reason, using a source of longer wavelengths could generate three-photon (3P) effects, which would be attractive for several reasons, including deeper penetration, higher resolution and a wider range of native fluorophores.

Three-photon microscopy (3PM) enables depth resolved imaging of neural circuitry in the mouse brain down to a depth of 1000 μm due its wavelength in the range of 1300-1700 nm. 3PM reduces SBR by more narrowly confining the focal volume due to the 3P effect being proportional to $I^6$, as this simultaneously increases the contrast and effective resolution. Longer wavelength 3PM excitation also scatters and absorbs less in the retina, making it preferable for native fluorophores. In contrast, the retina contains few fluorophores that have an absorption maximum in the range of 650-850 nm, which is the 1P equivalent to the 2PEF wavelength.
Bibliography


Appendix A

Appendix

A.1 Further assessment of the measurement setup effectiveness

Initial preparation of the moss sample included glycerol and oil as an immersion medium. Imaging was most successful with either immersion-oil of high refractive index or water as an immersion medium. Glycerol also dehydrates the leaf, making it a nonviable immersion medium.

Imaging a leaf without a cover slip will dehydrate the leaf immediately, thus ruining the sample. In addition, excess power usage, approaching 1 mW, burns the sample. Focusing of the objective can also induce photodamage.

Previous work conducted in 2018 [111] imaged celery using the current multiphoton measurement setup. Figure A.1 shows a multiphoton image of celery, imaged using a dry objective. The results agree well with previous research. As shown in the figure, celery emit two-photon signals, mainly consisting of 2PEF.

![Figure A.1: 2P image of celery](image)

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