Multimodal visible light optical coherence microscopy for ex vivo brain imaging

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Doctor of Philosophy

Submitted by

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Declaration

This doctoral thesis was conducted by Antonia Lichtenegger under supervision of Assoc. Prof. Bernhard Baumann, PhD (BB) at the Center for Medical Physics and Biomedical Engineering, Medical University of Vienna, Austria. BB gathered funding for the PhD position by a grant from the European Research Council (ERC StG 640396 OPTIMALZ). Prof. Christoph K. Hitzenberger, PhD served as a co-supervisor and mentor. The visible light OCM and FI setup presented in this work was built by Antonia Lichtenegger. The Labview code for imaging acquisition was developed by Antonia Lichtenegger. The data post-processing pipeline in Matlab was developed by Antonia Lichtenegger with the help of Marco Augustin.

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Rules of good scientific practice and good animal practice were followed. All experiments in animal subjects were performed in accordance with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research and under a protocol approved by the ethics committee of Medical University Vienna and the Austrian Federal Ministry of Education, Science and Research (Ethics approval number GZ BMBWF/66.009/0272-V/3b/2019).

Further contributions to parts of the thesis and the publications arising from it are acknowledged in the individual articles.
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Abstract

Optical coherence tomography (OCT) and OCT based microscopy (OCM) are non-destructive optical imaging methods. The image contrast is based on the intrinsic scattering of light within the tissue. In this thesis, a novel and multimodal OCM and fluorescence imaging (FI) setup was developed. The system utilized a supercontinuum laser operating in the visible wavelength region (400-700 nm) to achieve a sub-micrometer resolution of 0.8 µm in brain tissue. The setup was based on a Michelson interferometer and a custom-built spectrometer. The A-scan rate of the camera was 30 kHz. To achieve a high transverse resolution of 2-4 µm, high numerical aperture objective lenses (4× - 20×) were implemented in the sample arm. The system specifications were measured, showing a sensitivity of 89 dB with a roll-off of 24 dB/mm. The system was used to investigate microscopic features, such as cell structures and fiber tracts in healthy and pathological ex-vivo mouse and human, brain tissue. As the field-of-view was limited to 250×250 µm² an automatic moving x-y-z-stage was incorporated underneath the sample to acquire large field-of-view images (2.5×2.5 mm²). Image acquisition was performed using a custom-made Labview program. An extensive post-processing pipeline for OCM data processing was developed in Matlab. Standard OCT image processing steps including background removal, resampling to k-space, numerical dispersion compensation and Fourier transformation were implemented. Additionally, attenuation values were calculated and spectroscopic imaging was performed to integrate additional contrast mechanisms. To change from OCM to FI mode, two mirrors had to be flipped in the setup. Multimodal imaging with OCM and FI has the big advantage that tissue specific contrast and morphological tissue information are acquired in the same field-of-view. The setup was further characterized using various phantoms. The multimodal system was utilized to investigate tissue affected by two common brain diseases, Alzheimer’s disease (AD) and intracranial tumors. AD is the most common form of dementia worldwide and one major hallmark of the disease is the formation of extracellular amyloid-beta plaques. Using the multimodal setup, amyloid-beta plaques were visualized due to their hyper-scattering properties in ex-vivo mouse and human brain tissue. Human central nervous system tumor biopsies were retrieved intraoperatively from the surgeon and imaged prior to conventional neuropathologic work-up. OCM revealed the three-dimensional structure of the brain parenchyma and tumorous areas, and FI added tumor tissue-specific
contrast. OCM findings correlated well with malignant hot spots within the brain tumor biopsies evaluated by histopathology. Visible light OCM in combination with FI is a powerful optical imaging modality to investigate microscopic features in ex-vivo brain tissue samples and may provide complementary contrast for various other applications in the field of neuroimaging.
Kurzfassung


Publications arising from this thesis


Abbreviations

2D .............. Two Dimensional
3D .............. Three Dimensional
5-ALA .......... 5-Aminolevulinic Acid
AD .............. Alzheimer’s Disease
APP ............ Amyloid Precursor Protein
A-β ............. Amyloid-Beta
CARS .......... Coherent Anti-Stokes Raman Scattering
CT .............. Computed Tomography
ECi ............ Ethyl Cinnamate
FD ............. Fourier Domain
FT ............. Fourier Transformation
FI ............. Fluorescence Imaging
H&E ........... Hematoxylin and Eosin
IHC .......... Immunohistochemistry
MR ............ Magnetic Resonance
NA ........... Numerical Aperture
OCT .......... Optical Coherence Tomography
PET .......... Positron Emission Tomography
PS1 .......... Presenilin 1
PS2 .......... Presenilin 2
PS ............ Polarization Sensitive
PPIX .......... Protoporphyrin IX
SD .......... Spectral Domain
SHG .......... Second-Harmonic Generation
SNR .......... Signal to Noise Ratio
SS ............... Swept Source
SWITCH .......... System-Wide control of Interaction Time and kinetics of Chemicals
TD ............... Time Domain
TPEF ............ Two-Photon Excited Fluorescence
WFFM ........... Wide-Field Fluorescence Microscopy
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"Nothing in life is to be feared, it is only to be understood. Now is the time to understand more, so that we may fear less."

(Marie Curie)
Chapter 1

Introduction

1.1 Prologue

Optical coherence tomography (OCT) was introduced in 1991 and has since evolved as essential optical imaging technology. In ophthalmology, OCT is one of the most important imaging and diagnostic tools [1]. The technology also spreads into many other application fields, among others neuroimaging [2]. OCT has been used to investigate the brain anatomy, including cellular structures, fiber tracts, and vasculature [3–5]. Further, different brain pathologies have been investigated, such as Alzheimer’s disease or various types of brain tumors [6–8]. OCT or OCT based microscopy (OCM) are promising tools to study ex-vivo and in preclinical settings in-vivo brain tissue to reveal new insights into the function of the brain and its diseases.

This thesis is organized into five chapters in the following way: Section 1.2 will introduce the topic of neuroimaging and commonly used imaging technologies. Section 1.3 will give a brief introduction into the human brain anatomy. The following section 1.4 gives an introduction to Alzheimer’s disease. Section 1.5 is dedicated to intracranial tumors. The use of animal models for preclinical research related to neuroscience will be elucidated in section 1.6. Section 1.7 will explain the basics of optical coherence tomography and will provide details about further applications of OCT. Section 1.8 will focus on fluorescence imaging (FI) and the combination of OCT and FI. Histology is still the gold-standard of analyzing ex-vivo tissue samples. Hence, the last section 1.9 is devoted to this very important topic. Following the introduction, chapter two to four will lead the reader through the three journal papers published in the course of this thesis. The final chapter is dedicated to a discussion, a conclusion and some further prospects of this work.
1.2 Neuroimaging

Technological advancements have largely impacted the field of neuroscientific research. One major aspect of this still rapidly growing research field is the sector of neuroimaging. Multiple new technologies provided new insights into *ex-vivo* and *in-vivo* processes in the brain of human and animal models [9]. The most common *in-vivo* techniques are positron emission tomography (PET), computed tomography (CT) and magnetic resonance (MR) imaging [10–12]. Optical imaging techniques, which are frequently used, are microscopy, fluorescence/light-sheet microscopy, non-linear imaging techniques, such as Raman imaging, and optical coherence tomography [13,14]. Figure 1 gives an overview over the most common imaging techniques, their resolution, and image depth penetration.

![Figure 1: OCT bridges nicely the gap between high resolution imaging, like conventional microscopy and a deep image penetration like in MRI.](image)

PET imaging utilizes a positron-emitting radioisotope to detect two time-coincident high-energy photons. Due to the physics of the emission and detection of the time-coincident photons, PET imaging is a very sensitive and accurate technique to estimate *in-vivo* concentrations of a specific radio-tracer. That is why the technique is nowadays widely used in oncological, cardiovascular and neurological applications [12]. Further, PET imaging has also evolved as a tool for preclinical studies in animal models. However, the technique has some drawbacks, namely the rather poor image resolution, that a radioactive tracer has to be injected for imaging and that there are trade-offs between resolution and noise and the quantitative accuracy of the measurements [15].
The US medical community stated that CT imaging was the greatest advancement in radiology after the discovery of X-rays [11]. Nowadays, it is a frequently used tool for investigations in neurological applications and also in many other fields such as oncology, emergency medicine, or orthopedics [16]. Disadvantages of the technique are the extremely high costs, the complexity of the acquisition, a high dose of radiation is emitted and a rather poor resolution in in-vivo human settings [17]. In µ-CT, resolutions between 1 and 50 µ were reported, however, these results were only achieved in ex-vivo tissue samples or small animal imaging [18]. CT is an imaging technology which uses X-rays to measure the projection of an object from all directions. Out of these projection data the linear attenuation coefficient through the object is reconstructed and a 3D image is retrieved. Over the last decades, the image quality, speed and robustness has improved in order to use the technique standardized in the clinics. A typical CT scanner today has a resolution of around 0.5 mm in all directions [11, 16].

For MR imaging, magnetic fields, magnetic field gradients and radio waves are used to generate images of the organs in the body. The advantage of MR imaging is that in-vivo 3D images of whole body parts are acquired without using any harming radiation. The disadvantages of this imaging technique are the extremely high costs and complexity of the systems and again the low resolution in an in-vivo human setting. [10, 19]. Using µ-MR scanners, cellular resolution was achieved in ex-vivo tissue samples and small animal imaging [20].

Light microscopy is the optimal tool for modern cell biology as the resolution matches the size of the structures in the tissue, and there is a diverse range of available fluorescent markers for specific labeling of tissue features. Microscopic imaging covers a wide range of techniques, amongst those are bright-field, dark-field, polarization, non-linear and fluorescence microscopy. Fluorescence microscopy uses fluorophores, which are molecules that absorb and emit specific wavelengths, to label and image targeted structures [21]. A more detailed introduction is given in section 1.8.

In brightfield microscopy, thin sections are investigated and the absorption, scattering and deflection of light is measured. As this effect is very small in thin samples, either conventional staining has to be performed or phase differences have to be measured to retrieve image contrast [21]. The numerical aperture (NA) is a dimensionless number, which characterizes the range of angles in which an optical system can accept or emit light. It is given through

\[ NA = n \cdot \sin(\theta) \]  

(1.1)

where \( n \) is the index of refraction of the medium and \( \theta \) is the maximal half-angle of the cone of light that can enter or exit the lens. A further important term in microscopy is the Airy disk. It is defined as the best-focused spot of light, a lens with a curricular aperture can make. This value is limited by the diffraction, meaning that under a certain NA and light with a
central wavelength $\lambda$, traveling in a medium with refractive index $n$ the minimum resolved distance $d_{\text{limit}}$ is given through:

$$d_{\text{limit}} = \frac{\lambda}{2 \cdot n \cdot \sin(\theta)} = \frac{\lambda}{2 \cdot NA}.$$  \hspace{1cm} (1.2)

The numerical aperture and the focal length $f$ of a lens are related through

$$NA = \frac{n \cdot D}{2 \cdot f}$$  \hspace{1cm} (1.3)

with $D$ the entrance pupil. The theoretical resolution of non-confocal microscopy in the $x$-$y$ plane, which is the plane orthogonal to the light beam going in direction $z$, is given through

$$\Delta_{x,y} = \frac{4 \cdot \lambda}{\pi} \cdot \frac{f}{D} = \frac{1.22 \cdot \lambda}{2 \cdot NA}.$$  \hspace{1cm} (1.4)

In $z$-direction, the resolution is given through

$$\Delta_z = \frac{2 \cdot \lambda \cdot n}{NA^2}$$  \hspace{1cm} (1.5)

Using a confocal microscope, see section 1.8, both axial and transverse resolution are improving as the pinhole radius is set somewhat smaller than the Airy disk and

$$\Delta_{x,y,\text{confocal}} = \frac{0.8 \cdot \lambda}{2 \cdot NA}$$  \hspace{1cm} (1.6)

and

$$\Delta_{z,\text{confocal}} = \frac{1.4 \cdot \lambda \cdot n}{NA^2}$$  \hspace{1cm} (1.7)

are derived \cite{22,23}.

For light sheet-microscopy, the problem of out-of-focus-light was eliminated by using a plane orthogonal to the imaging plane for illumination. Finally, superresolution microscopy as a rather new development achieves resolutions below the diffraction limit, however special tissue preparation steps, microscopes and fluorophores are required making in-vivo cell imaging difficult \cite{21}.

Nonlinear microscopy refers to the general term of any microscope which is based on nonlinear optics. There are two types: incoherent and coherent nonlinear optical microscopy. Coherent setups produce signals with a predefined phase and the power is proportional to the concentration of radiating molecules squared. Examples are second-harmonic generation (SHG) or anti-Stokes Raman scattering (CARS) microscopy. This is in contrast to incoherent nonlinear microscopy, where the produced signal phase is random. A typical example is multi-photon fluorescence microscopy or two-photon excited fluorescence (TPEF) microscopy \cite{24}.
Optical coherence tomography or OCT based microscopy has emerged as a powerful optical imaging modality bridging the gap between high resolution and low imaging range, and lower resolution and high imaging range modalities. Especially in ophthalmology, OCT is nowadays the most important imaging and diagnostic tool [1]. OCT/OCM has also gained popularity in the field of neuroimaging and over the last decades the number of publications has been constantly rising, see Fig. 2.

Figure 2: Number of publications in the field of OCT/OCM in neuroimaging. (PubMed search term: ((optical coherence tomography OR optical coherence microscopy) AND (brain OR neuroimaging)), search conducted on the 10.7.2019, URL: https://www.ncbi.nlm.nih.gov/pubmed?term=((optical coherence tomography OR optical coherence microscopy) AND (brain OR neuroimaging)))

1.3 The human brain

In all vertebrate and invertebrate animals, the brain is the center of the central nervous system. In humans, the brain and the spinal cord make up the central nervous system. The brain comprises the cerebrum, the cerebellum and the brainstem. The brain basically controls most of the activities of the human body. The cerebrum, the biggest part of the brain, is divided into two cerebral hemispheres. It consists of a cerebral cortex built up by grey matter, which covers the white matter. Both hemispheres are connected by commissural nerve tracts. The cortex is further divided into two major functional areas: the motor and sensory cortex [25]. These areas are divided into four different regions: the frontal, the parietal, the
temporal and the occipital cortex, see Fig. 3 (a). The frontal cortex is responsible for motor functions, motivation, aggression, smell and the mood, the parietal cortex processes the sensory informations, the temporal cortex is responsible for the smell, hearing, memory and abstract thoughts and the occipital cortex processes visual inputs [26].

Figure 3: The human brain. (a) The human brain consist of the brainstem, the cerebellum and the cerebrum. The cortex is further divided into four different regions the frontal, the parietal, the temporal and the occipital cortex. A size comparison between a human and a mouse brain. (b) A Klüver-Barrera stained histology image showing the six layers of the cerebral cortex. Grey matter is stained pink and the myelin structures in the white matter are stained blue. (c) The direction, cross-section and division nomenclature in the human brain. Images were retrieved partly form https://smart.servier.com on the 15.07.2019.

Neurons, glial cells, neural stem cells and blood vessels are making up the structure of the brain [25]. The grey matter of the cerebral cortex consists of six layers, which are built up by different cell types and connections. The outer most one is called the molecular layer, followed by the external granular layer, the pyramidal layer, inner granular layer, ganglionic or inner pyramidal layer and the multiform layer [27]. Fig. 3 (b) shows a typical Klüver-Barrera stained histology image of a cortex region, including white matter. Grey matter is mostly built up by cell nuclei stained in pink and white matter consists mostly of myelin structures stained in blue. The six layers of the cerebral cortex are indicated. To orientate in the brain, commonly used directions, cross-sections and divisions are shown in Fig. 3 (c) [28].
The mouse brain has a similar structure as compared to the human brain. That is why mouse models are often used for preclinical brain studies, see section 1.6. Of course, the mouse brain is much smaller than the human brain, as shown in Fig. 3 (a). The cerebral cortex of a mouse brain contains 8-14 million neurons, while the human one includes more than 10-15 billion neurons [29].

1.4 Alzheimer’s disease

Alzheimer’s disease (AD) is the most common form of dementia worldwide. One out of nine Americans above 65 years is affected by this disease. Patient numbers are expected to double over the next 20 years leading to not only a financial but also a huge social burden in our ageing society. Even though the disease was discovered already 100 years ago, there is still no effective treatment [30]. Additionally, the definite diagnosis of the disease can only be done post-mortem. Histologic analyses of different regions of the brain have to be performed to confirm the presence of amyloid-beta (A-β) plaques and neurofibrillary tangles the two major hallmarks of the disease and as a consequence the neurodegeneration in the cerebral cortex [30,31]. Figure 4 (a) and (b) show typical histological images of the most important hallmarks of AD, the amyloid-beta plaques (a) and the neurofibrillary tangles (b), respectively. Figure 4 (c) gives an overview over the areas in a human brain affected by extracellular amyloid-beta plaques and intracellular tau-tangles in the different stages of AD. First, the medial temporal, then the lateral and frontal and finally the occipital lobe in the human brain is affected [32].

The causes of the multifactorial disease of AD are 70% genetic and 30% environmental. The genes associated with this genetic factors can be divided into a familial AD and sporadic AD. The APP (amyloid precursor protein), PS1 (Presenilin 1) and PS2 (Presenilin 2) genes are responsible for the presence of familial AD. It is believed that the apolipoprotein E gene is related to the sporadic form of AD [33].

1.4.1 Pathology

The disease is characterized by the degeneration of neurons, the deposition of intracellular neurofibrillary tangles and extracellular accumulation of pathological amyloid-beta protein. The manifestation of these plaques was shown to be the initial event in AD [34]. There are different types of amyloid-beta plaques, namely diffuse, compact and dense-cored or neuritic plaques [35]. For the diagnosis of AD post-mortem, it is essential to identify the existence of neuritic plaques, using the appropriate staining method [36]. The plaques are composed of aggregated A-β-peptides, which originate from the amyloid precursor protein (APP). It is believed that these extracellular plaques have a negative impact on the cell communication causing the synapse loss and decrease of neuronal plasticity [37].
a mouse model of AD it was shown that following the development of plaques the formation of extracellular fibrils occurs [38]. In principle, these fibrils consist of the cytoskeletal, microtubule-associated protein and are associated with the assembly and stabilization of microtubules. In AD-affected brains, the microtubules are moving into the intracellular neuronal space leading to a destabilization and collapse of the cytoskeleton [39].

### 1.4.2 Diagnosis and therapy

The most commonly used neuropathological classification was first introduced in 1991 by Braak and Braak to classify \textit{ex-vivo} tissue affected by AD into six different stages [40]. Further, in 1984, the National Institute of Neurological and Communicative Disorders and Stroke-Alzheimer’s disease and Related Disorders Association first published criteria for diagnosing AD, and in 2007, a new framework was proposed by an international working group, which was again refined in 2010. The new criteria stated that AD could be diagnosed if there is evidence of a specific memory deficit which cannot be improved by curing and the presence of certain biomarkers [41–43].

However, there is still no definite \textit{in-vivo} diagnosis of AD. Imaging possibilities, which have been used for \textit{in-vivo} diagnosis attempts include MRI and CT [44,45]. Liquor diagnostics for biomarkers or genetic markers have been used trying to diagnose AD more precisely and in earlier stages [46]. Until now, there is also no curative treatment possibility. Current treatment focuses on delaying the progression of the disease [47].
1.4.3 CAA

Cerebral amyloid angiopathy (CAA) is a form of angiopathy where amyloid deposits are formed in the walls of blood vessels in the central nervous system. More precisely, the amyloid deposits can be found in the walls of leptomeningeal and cortical arteries, arterioles, and less often capillaries and veins [48]. Amyloid-beta is mostly accumulated in the tunica media and tunica externa [49]. CAA can occur in patients with no history of dementia, the disease is however more common in patients affected by AD. CAA occurs mostly sporadic in the elderly whereby hereditary forms are rarely [48,50]. Figure 5 (a) shows a schematic drawing of a cerebral artery and the typical locations, tunica externa and media, where amyloid-beta deposits occur. Figure 5 (b) and (c) show histology images of brain tissue and a cerebral vessel affected by CAA, stained with Congo red and the counter staining hematoxylin. The A-β accumulations are marked with red arrows. Latest research findings have showed that CAA is increasingly recognized as a major contribution of AD pathology [51,52].

![Figure 5: Cerebral amyloid angiopathy. (a) Schematic drawing of a cerebral artery with the arterial wall structure. (b) - (c) Histology images with (Congo red and hematoxylin staining) amyloid-beta accumulation in the arterial wall structure, indicated by red arrows.](image)

1.5 Central nervous system tumors

Central nervous system tumors are rather common, with more than 40,000 cases a year in the United States [53]. They cause a substantial morbidity, even tough half of the tumors are benign. Usually, biopsies are taken during surgery and the treatment depends on the resulting histological diagnosis. For benign tumors usually surgical or radiation therapy, and for malignant brain tumors additionally chemotherapy are often used. Metastases of
the brain are frequent and morbid complication of solid tumors. Metastases are frequently
treated by surgery and radiation therapy [53]. Glioma is the most common primary intracra-
nial tumor [54].

1.5.1 Glioma

These tumors are accounting for 1.4 percent of all cancers in the United States, resulting in
12,500 deaths each year. The cause and risk factors are still unclear. Studies have showed
a slight male predominance and that mostly people aged between 50 and 70 years are
affected by the disease [54].

Pathology

Gliomas originate from glial cells, more precisely astrocytes, oligodendrocytes, or ependy-
mal cells. Depending on the cell type, a glioma is denoted as astrocytoma, oligodendro-
glioma, or ependymoma, respectively, see Fig. 6. Gliomas are classified according to the
World Health Organization (WHO) criteria into grades I through IV. Figure 6 gives an over-
view over the different types according to their grading.

Figure 6: Overview over gial cells involved in glioma development and the resulting tu-
mor entities. H&E stained histology images for ependymomas, oligodendrogliomas, ast-
rocytomas and glioblastoma multiforme, respectively. Images were retrieved partly form
Grade I gliomas typically have a good prognosis, they are rather rare and more common in children [55]. Type II are characterized by hypercellularity and have a median survival time of 5-8 years. Grade III astrocytomas are characterized by hypercellularity, as well as nuclear atypia and mitotic figures. The median survival time is 3 years. Grade IV gliomas are also known as glioblastoma multiforme (GBM). They are the most aggressive primary brain tumors and have a median survival time of only 15 months. They are characterized by hypercellularity, nuclear atypia, mitotic figures, and evidence of angiogenesis and/or necrosis. Figure 6 shows typical histology images for grade I-IV glioma [54].

Diagnosis and therapy

To diagnose gliomas, PET, MR and CT are the standard imaging technologies [56]. Further a neurological exam can be performed to check for vision, hearing, balance, coordination, strength or reflex problems. A problem in one or in more of those areas may help to identify the region in the brain which is affected by the tumor [57]. However gliomas develop extremely fast such that immediate surgery, followed by radiotherapy and concomitant temozolomide chemotherapy is the most important current treatment approach [58].

![Figure 7: Fluorescence-guided surgery using 5-aminolevulinic acid. (a) Tumorous area, infiltration zone and tumor associated brain parenchyma investigated under a white light microscope. (b) Using a blue light fluorescence surgical microscope, tumorous areas can be visualized by their reddish color. Histology confirms the findings after surgery. Images were taken and modified with permission from Kiesel et al. and Widhalm et al. [59,60].](image-url)
1.5.2 5-ALA imaging

During surgery, the differentiation between tumor tissue and brain parenchyma is of utmost importance to enable maximal safe resection. However, using conventional white-light microscopy during the surgery makes it difficult to differentiating between brain parenchyma and the malignant margins of the tumor, see Fig. 7 (a) [60]. Fluorescence-guided surgery using 5-aminolevulinic acid (5-ALA) was introduced. The patient is administrated the 5-ALA fluorescence tracer orally. In the human hemoglobin metabolic pathway 5-ALA is produced as a natural metabolite. Hence, this fluorescence tracer can also penetrate the blood brain barrier. 5-ALA is taken up by the malignant tumor cells and is metabolized into protoporphyrin IX (PPIX) a fluorescent metabolite. Exciting this fluorescent metabolite with blue light (at around 405 nm) leads to a violet-red fluorescence, see Fig. 7 (b). This technique enables a more specific identification of malignant areas during a surgery [61,62]. There has been a large range of studies showing the benefits of using 5-ALA as an additional tool in glioblastoma surgeries [59]. There is still a lot of research needed to answer the question if this tracer will also help to identify low grade glioma or metastasis [63].

1.6 Animal models for preclinical imaging

Animal models for preclinical research are fundamental for modern neuroscience research. The rapid developments in animal models have led to a better understanding of many neurodegenerative diseases [64]. With the help of animal models, the underlying mechanisms and new views on specific human diseases can be studied in detail and treatment possibilities can be tested before being translated to clinical use [65]. There is huge variety of animal models for all kinds of diseases, such as worms, fish, rodents and primates. It is crucial to choose the right model for a specific scientific question. The field of preclinical imaging is divided into animal models for basic scientific studies and for preclinical drug development [64].

For Alzheimer’s disease exists a large variety of preclinical animal models. In AD, it is of utmost importance to better understand the pathogenesis and processes involved [66]. Animal models, which have been developed are among others Drosophila melanogaster, Caenorhabditis elegans as well as zebrafish. These animal models are easy to breed, however they have a rather simple brain structure and their physiology is far from the human one [67]. Mostly, transgenic mice that express human genes resulting in the formation of amyloid plaques and neurofibrillary tangles are used nowadays [68–70]. The advantages of mouse models are that mice are easy and relatively cheap to breed and that over the last decades a variety of new transgenic models have been developed to study AD. Mouse models in AD research opened the possibilities to answer questions which were impossible to investigate in humans before [67]. Mice have been used to better understand aspects
of the disease progression and the physiological functions of the APP protein and the PS1 gene, which are crucial for drug development [71, 72]. One reason for the big success of mouse models is AD research is their similar number of genes and brain morphology compared to humans. Still, until now no existing mouse model exhibits all features of AD as we know it in human patients. Further, mouse models are genetically modified to express familiar forms of AD, applying these results to sporadic AD may be incorrect [73]. One also has to keep in mind that a mouse has a way shorter life cycle compared to a human, which results in a different development of AD. The mechanisms of AD are still introduced through genetic modification and are therefore believed to be not as complex as in humans [67].

One example of a typical mouse model of AD, which was also used used in this thesis, expresses the amyloid precursor protein and presenilin 1 mutation. This mouse model is therefore genetically modified based on the background of familiar AD in humans. As a consequence, the model shows a rapid development of A-β plaques in various cortical regions [74, 75].

1.7 Optical coherence tomography/microscopy

1.7.1 Principles of OCT/OCM

Optical coherence tomography (OCT) is an optical imaging technique which was first introduced in the 90s [76]. High-resolution, cross-sectional and 3D volumetric OCT images can be acquired in real time. As shown in Fig. 1, OCT nicely bridges the gap between high resolution and low penetration, and low resolution and deep penetration imaging. OCT systems normally provide an axial resolution of 1-100 μm and the imaging depth is limited to a couple of millimeters. The internal microstructure of a tissue is revealed by measuring the back-scattered and back-reflected light [77]. OCT is based on low-coherence interferometry, where correlation measurements are performed by interfering light backscattered from the investigated tissue with a reference light beam. A low coherent light source is characterized by statistical phase discontinuities over a distance known as the coherence length. Figure 8 (a) shows a simplified time domain OCT setup based on a Michelson interferometer [1].

The intensity $I$ and the electrical field $E$ are related through

$$I = \frac{c}{4 \cdot \pi} \cdot \frac{\epsilon}{\mu_m} \langle E^2 \rangle$$  (1.8)

with the temporal average $\langle \ldots \rangle$, $c$ is the speed of light, $\epsilon$ the dielectric constant and $\mu_m$ the magnetic permeability. A broadband light source emits a beam which is split up into a reference beam $I_r$ and a signal beam $I_s$. The resulting output intensity $I(z)$ is given by

$$I(z) = I_r + I_s + 2 \cdot \sqrt{I_r \cdot I_s} \cdot |\gamma(\Delta z)| \cdot \cos(2 \cdot k_0 \cdot \Delta z)$$  (1.9)
where $\Delta z$ is the path length difference between the signal and the reference arm of the interferometer and $k_0 = \frac{2\pi}{\lambda}$ is the central wavenumber of the spectrum and $\lambda$ the central wavelength. The last term in equation (1.9) is the cross-correlation term which scales with the signal strength of sample and reference arm $\sqrt{I_r \cdot I_s}$. The term $\gamma(\Delta z)$ is the complex degree of coherence. An incoherent light source is defined with $|\gamma(\Delta z)| = 0$ and then no interference is possible. If $|\gamma(\Delta z)| = 1$, a coherent light is defined and interference is possible for all path length differences. For low-coherence interferometry, $0 < |\gamma(\Delta z)| < 1$, interference is observed when the reference arm length and the sample arm length are within the coherence length $\Delta z = l_c$, see Figure 8 (b). Essentially, the auto-correlation of the light wave is measured. The coherence length is inversely proportional to the bandwidth of the light source and defines the axial resolution. Shorter coherence lengths results in finer resolutions [1].

![Figure 8: The different types of OCT setups and the resulting measurements. (a) A schematic drawing of a time-domain OCT setup. (b) The measured interference signal from a TD-OCT setup. (c) Schematic drawing of a spectrometer and swept source based OCT setup. (d) The interference signal detected by SD/SS-OCT and the resulting depth scan after Fourier transformation.]

A single depth scan (z-direction) in OCT is called an A-scan, see Fig. 9 (a). If the beam is also scanned in lateral direction (x-direction), a B-scan or cross-sectional image is acquired. If the beam is also scanned in the second lateral direction (y-direction), a whole volume (a so-called C-scan) is acquired. For the acquisition of images various schemes for scanning the tissue can be used. A common scanning pattern used in OCT systems is point wise...
scanning, where the beam is raster scanned point by point over the tissue [1]. In the recent years, line field and full-field OCT were developed to increase the imaging speed owing to the parallel acquisition [78, 79].

1.7.2 Fourier domain OCT

OCT was first introduced in 1991 by Huang et al. to perform biomedical imaging using a time domain OCT setup [76]. In TD-OCT mechanical scanning of the reference mirror is needed to ensure interference at different depth positions, see Fig. 8 (a). In the second generation of OCT setups, Fourier domain (FD) OCT was introduced. It was shown that FD-OCT systems have a significant sensitivity advantage compared to TD-OCT [80]. One can distinguish between spectrometer based and swept source (SS) based FD-OCT systems, see Fig. 8 (c). In spectrometer based OCT setups a broadband light source is utilized and the interference spectrum from the interferometer is collected using a spectrometer. In SS-OCT, a narrow-band light source sweeps though a broad spectral range and the interference signal is measured as a function of time. In both systems, a beam is emitted from the light source to the reference and sample arm. The first beam is reflected from the reference mirror, which is in a fixed position. The second beam in the sample arm is backscattered and backreflected from internal structures of the tissue. The resulting interference pattern from sample and reference arm can be measured for example using a spectrometer [1]. The recorded interference spectrum can be described according to the spectrum $S$ (spectral density)

$$S(k, \Delta z) = S_r(k) + S_s(k) + 2 \cdot \sqrt{S_r(k) \cdot S_s(k)} \cdot \Gamma(\Delta z) \cdot \cos(2 \cdot k \cdot \Delta z).$$  \hspace{1cm} (1.10)

The third term contains the interference information and is scaled by the spectral densities $S_r, S_s$ backscattered from the reference and sample arm, respectively. Different echo delays will result in different modulation frequencies, see Fig. 8 (d). The interference term is sinusoidally modulated across wavenumber ($k$) and each $\Delta z$ produces a different modulation frequency. Hence, interference signals for different $\Delta z$ can be recorded simultaneously. The third term is scaled with $\Gamma(\Delta z)$, the spectral degree of coherence, which means that the widths of the detected peaks are determined by the bandwidth of the light source. For measuring the echo delays a rescaling to linear frequency or wavenumber has to be performed followed by a Fourier transformation (FT)

$$\mathcal{F}[S(k, \Delta z)] \rightarrow \tilde{I}(\Delta z)$$  \hspace{1cm} (1.11)

see Fig. 8 (d) [1].

A spectrometer consists of a diffraction element, such as a grating, which splits up the spectrum in its single components and a line-scan camera is used to read the spectral data.
The speed of the line scan camera determines the A-scan rate. Depending on how many A-scans are acquired in a single volume, the imaging speed can be calculated. In FD-OCT the reference mirror does not have to be scanned, thus a much higher imaging speeds can be achieved [81].

However, imaging artifacts appear, which are not present in TD-OCT. FD-OCT measures axial scans from a reference delay path, the so-called zero delay. FD-OCT only measures the real part of $S(k, \Delta z)$. It is not possible to distinguish echos from positive or negative zero delays $\Delta z$ as the relationship $\cos(2 \cdot k \cdot \Delta z) = \cos(-2 \cdot k \cdot \Delta z)$ applies. It means that the magnitude of the Fourier transformation is symmetric for positive and negative values. Using a spectrometer with $N$ pixels will result in $N/2$ pixels after FFT. Further, a folded mirror image is produced when the sample is located at or too closely to the zero delay [1,82].

### 1.7.3 Performance of an OCT setup

When characterizing an OCT setup, system and image parameters have to be evaluated, amongst others the sensitivity and the roll-off, see Fig. 9 (b) and (c) [1].

![Figure 9: The specifications of an OCT setup. (a) A single depth scan is called an A-scan. When the beam is scanned in lateral direction, a B-scan and if the beam is scanned in the other lateral direction, a C-scan is acquired. (b) The roll-off measurement. (c) The signal-to-noise ratio (SNR) measurement. (d) The trade-off between high transverse resolution ($\Delta_{x,y}$) and a large depth of field ($b$). $\Delta_z$ is axial resolution defined by the used spectrum.](image)

The signal-to-noise ratio (SNR) is defined as the ratio between the mean-square peak signal power ($\langle I^2 \rangle$) and the standard deviation of the noise ($\sigma_{noise}^2$), see Fig. 9 (c).

$$SNR = \frac{\langle I^2 \rangle}{\sigma_{noise}^2}$$  \hspace{1cm} (1.12)
It is usually expressed in decibel units using $10 \cdot \log_{10}(SNR)$. The sensitivity ($\Sigma$) is defined over the smallest detectable back-scattered sample arm signal $R_{s,min}$

$$\Sigma = \frac{1}{R_{s,min}} |_{SNR = 1}. \quad (1.13)$$

It denotes the minimum detectable reflected optical power compared to a perfect reflector, i.e. the weakest signal that can still be measured. A good SNR is obtained under a shot-noise-limited regime, where the overall system noise is dominated by the photon shot noise arising back from the sample arm [80]. The detection sensitivity decreases as a function of the measurement range, which is called the roll-off, see Fig. 9 (b). There are multiple factors responsible for the roll-off. Most importantly, the detector of a spectrometer has a finite pixel width. The roll-off is therefore a convolution of a rectangular function, representing the pixel size, and the interferometer signal in the frequency domain, resulting in a depth-depended signal reduction by a sinc function. Echos, which are far from the zero-delay, have a smaller SNR as they produce progressively high frequency spectral oscillations. In general, the spectral resolution is limited by the camera pixel size, the electronic cross talk, the resolution of the diffraction grating and the optical aberrations in the spectrometer lens [80].

Further parameters, which characterize an OCT setup, are the field of view, the axial and the transverse resolution. The axial resolution is given by the coherence length which in comparison to conventional microscopy is defined by the spectral characteristics of the light source. In the case of an Gaussian spectral shape, the axial resolution is defined as

$$\Delta z = \frac{2 \cdot \ln(2)}{\pi} \frac{\lambda^2}{\Delta \lambda} \quad (1.14)$$

where $\lambda$ is the central wavelength and $\Delta \lambda$ the full-width-at-half-maximum of the power spectrum and $\Delta z$ is the full-width-at-half-maximum of the coherence function. This formula states that the axial resolution is inversely proportional to the bandwidth of the light source. The lower the central wavelength and the broader the spectrum the better the axial resolution [1]. For the transverse resolution $\Delta x,y$ the same principle as in microscopy applies (see equation (1.4))

$$\Delta x,y = \frac{4 \lambda}{\pi} \cdot \frac{f}{D} \quad (1.15)$$

where $f$ is the focal length of the objective lens and $D$ the diameter of the incident beam or the entrance pupil. That means that a higher transverse resolution is achieved when a higher NA is used. However, there is a trade-off between high transverse resolution and the depth of field or the confocal parameter $b = 2 \cdot z_R$ ($z_R$ is the Rayleigh range), see Fig.9
The confocal parameter \( b \) is defined as
\[
b = \frac{\pi \Delta^2 \Delta_{x,y}}{\lambda}.
\]

Figure 9 (d) illustrates that the higher the transverse resolution is the shallower the depth of field. For low NA’s, the confocal parameter is normally much larger than the coherence length \( b > \Delta_z \). In the special case of very high NA focusing \( b \leq \Delta_z \), such that a combination of confocal as well as coherence gating is achieved. [1, 83].

Dispersion is an optical phenomenon in which the phase velocity of a wave depends on its frequency or wavelength [84]. As OCT is working with broad spectral light sources, dispersion compensation plays a crucial role to achieve a good image quality. Dispersion is mostly introduced by using different optical components in the reference and sample arm, for example through the objective lens. A possibility to reduce the dispersion mismatch between the sample and reference arm is to use optics with similar refractive properties in both arms, for example by using glass prisms in the reference arm to compensate for sample arm optics [85]. Various methods have been developed to compensate the dispersion in FD-OCT numerically in post-processing [86–89].

1.7.4 OCT applications in neuroimaging

Nowadays, OCT is one of the most important imaging and diagnostic tools in ophthalmology [90]. OCT has also been used in several other disciplines over the last decades [1]. Application fields include neuro-, skin, endoscopic and oncology imaging [2, 91–93].

Figure 10: Ex-vivo imaging of brain features using OCT/OCM. (a) Whole mouse brain imaging reveals fiber tracts in the en-face projection [4]. (b) Cellular structure are investigated in a B-scan of the cerebral cortex [3]. (c) Full field OCT reveals the cerebellar granular layer structures [94]. (d) White fiber tracts are imaged in high resolution in en-face projections [95]. Images were taken and modified with permission of the corresponding authors and publishers (© The Optical Society of America).
In neuroimaging, OCT/OCM was used to image cellular structures, fiber tracts and vasculature [3–5,96], see Fig. 10. OCT was utilized to investigate neurodegenerative diseases such as AD and brain tumors [6,8,94,95,97,98]. The visualization of Aβ plaques in a mouse model was first demonstrated in 2012 [6]. The OCM setup used a Bessel beam illumination around 800 nm to overcome the limited Rayleigh range due to the high numerical aperture objective lenses [6]. To achieve a higher axial resolution, a visible light extended focus OCM setup was utilized to visualize Aβ plaques in brains of an AD mouse model [95]. Further, plaques were visualized and their birefringent properties were analyzed using a PS-OCT setup operating at 840 nm [97]. Full field OCT was used for label-free imaging of tumorous glial and epileptic margins [94]. An ultrahigh resolution OCT setup operating at around 800 nm was used to investigate various types of brain tumors including fibrous, transitional meningioma and ganglioglioma. Morphological features were identified and results were compared to standard H&E staining [98]. OCT in combination with attenuation calculation enabled the detection of mouse and human brain cancer infiltration ex vivo and in vivo [8]. A rather new application field is the use of OCT during brain tumor resection. Since OCT offers the opportunity to investigate the tissue morphology in 3D, in real time and non-destructively, it might be a promising tool to be integrated into a surgical microscope for imaging during brain tumor resection [99–103].

1.7.5 Visible light OCT/OCM

The axial resolution of an OCT setup depends on the used light source. The axial resolution can be improved by using a broader spectrum located in a lower wavelength region. To date, most OCT setups operate in the near infrared region because of the deeper penetration of light into the tissue and because of the availability of commercial light sources. In the last years, a lot of research groups have focused on visible light OCT/OCM imaging to achieve sub-cellular resolution [104]. Table 1.1 gives an overview of recently developed visible light OCT setups and their specifications. The first visible light OCT setup was introduced in 2002 [105]. Since then, the system design and data processing algorithms have been greatly improved [109,112]. Visible light OCT setups have been used to investigate phantoms [108,113–115] and to perform in-vivo measurements [116,117]. Setups were also used to conduct studies in animal experiments [110,118,119] and in patient imaging [107,120]. Various tissue types have been investigated such as the retina [121], the brain cortex [95,122–125] and the female reproductive tract [126]. Furthermore, visible light OCT setups were also used in combination with fluorescence imaging to gain multimodal contrast [106,127].
<table>
<thead>
<tr>
<th>Authors</th>
<th>Year</th>
<th>(\Delta_z)</th>
<th>(\lambda)</th>
<th>(\Delta\lambda)</th>
<th>Sensitivity</th>
<th>Roll-off</th>
<th>A-scan rate</th>
<th>Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>Povazay et al. [105]</td>
<td>2002</td>
<td>0.75</td>
<td>750</td>
<td>325</td>
<td>87 (1.0 mW)</td>
<td>-</td>
<td>-</td>
<td>In-vitro cells</td>
</tr>
<tr>
<td>Jiang et al. [106]</td>
<td>2014</td>
<td>5.80</td>
<td>480</td>
<td>50</td>
<td>85 (0.5 mW)</td>
<td>-</td>
<td>-</td>
<td>Human Retina</td>
</tr>
<tr>
<td>Yi et al. [107]</td>
<td>2015</td>
<td>0.97</td>
<td>564</td>
<td>115</td>
<td>86 (0.2 mW)</td>
<td>12</td>
<td>25</td>
<td>Human Retina</td>
</tr>
<tr>
<td>Nishi et al. [108]</td>
<td>2016</td>
<td>0.69</td>
<td>625</td>
<td>260</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Semiconductor</td>
</tr>
<tr>
<td>Chong et al. [109]</td>
<td>2017</td>
<td>1.40</td>
<td>560</td>
<td>100</td>
<td>94 (0.1 mW)</td>
<td>5</td>
<td>10</td>
<td>Human Retina</td>
</tr>
<tr>
<td>Pi et al. [110]</td>
<td>2017</td>
<td>1.70</td>
<td>560</td>
<td>134</td>
<td>89 (0.8 mW)</td>
<td>6</td>
<td>-</td>
<td>Rat Retina</td>
</tr>
<tr>
<td>Marchand et al. [95]</td>
<td>2017</td>
<td>0.70</td>
<td>647</td>
<td>246</td>
<td>-</td>
<td>-</td>
<td>20</td>
<td>Mouse Brain</td>
</tr>
<tr>
<td>Harper et al. [111]</td>
<td>2018</td>
<td>1.00</td>
<td>550</td>
<td>300</td>
<td>96 (1.0 mW)</td>
<td>15</td>
<td>25</td>
<td>Mouse Retina</td>
</tr>
</tbody>
</table>

Table 1.1: Summary of representative visible light OCT setups. \(\Delta_z\) denotes the axial resolution, \(\lambda\) the central wavelength and \(\Delta\lambda\) the full-width-at-half-maximum of the spectrum. The sensitivity is given with the power at the sample in parentheses.

### 1.7.6 Spectroscopic Imaging

Spectroscopic imaging is a functional extension of OCT, in which the spectroscopic information of the back-scattered and back-reflected light is measured. This is achieved by digital signal processing, without changing the optical setup [128]. Using spectroscopic OCT the gray scale contrast of OCT can be overcome and a contrast enhancement is performed [129]. One of the most widely used applications of spectroscopic OCT is the measurement of hemoglobin concentration and blood oxygen saturation levels which are important biomarkers for retinal diseases [114, 130, 131]. The technique was also utilized for the detection of atherosclerotic plaques [132, 133]. Further, spectroscopic OCT was used to investigate burn injuries and distinguish burned tissue from healthy tissue to evaluate the severity of the skin injuries [134]. Recently, exogenous contrast agents were used to increase the contrast gained by near-infrared spectroscopic OCT, for example through absorbing dyes [135], gold nanocages [136] or gold nanostructures [137]. Visible light OCT setups have been used to perform high resolution, spectroscopic, in-vivo retina and skin imaging [111, 112, 138].

### 1.7.7 Attenuation coefficient calculation

In general when light interacts with a material, it gets attenuated. For OCT imaging this effect is mainly driven by two mechanisms: scattering and absorption. Using OCT makes it possible to estimate the attenuation coefficient, revealing valuable additional information about a specific tissue.

A coherent light beam propagates through a medium following the Lambert-Beer’s law

\[
I(z) = I_0 e^{-\mu z}
\]  

(1.17)
where \( I(z) \) is the resulting irradiance after the beam has traveled through the tissue over a distance \( z \) with an incident beam of \( I_0 \). The attenuation coefficient \( \mu \) describes how fast light is attenuated, so for large \( \mu \) values the result is a quick exponential decay. The attenuation coefficient is dependent on the wavelength, \( \mu = \mu(\lambda) \). There are multiple ways to extract the attenuation coefficient from OCT data. One of the simplest ways is based on fitting an exponential curve to the axial OCT signal decay. However, this method relies on the availability of a sufficient amount of pixels along a depth profile. A more advanced approach, the so called depth-resolved method, also used in this work was proposed by Vermeer et al. in 2014 [139]. From the Lambert-Beer’s law, an equation for the attenuation coefficient can be derived

\[
\mu(z) = \frac{I(z)}{2 \int_z^{\infty} I(u) \, du}.
\]  

which can be discretised in the following form

\[
\mu[i] = \frac{I[i]}{2\Delta \sum_{i+1}^{\infty} I[i]}
\]  

for practical use, where the attenuation value is evaluated for each single pixel with index \([i]\) and \( \Delta \) is the axial pixel size. To calculate the attenuation correctly, the signal has to be corrected for the roll-off and the noise floor. For the noise floor, a background image can be acquired, by blocking the sample arm and subtracted from the measured data. The roll-off can either be measured directly or modeled, for example using a Gaussian curve. Each A-line intensity profile has to be divided by this signal decay to compensate for the roll-off [139].

There are several reports of applications in which the attenuation coefficient improved the tissue contrast in OCT imaging [8, 140–142]. One very promising approach which was shown by Kut et al. was to distinguish malignant from healthy brain tissue using the attenuation coefficient [8]. Further, lipid-core atherosclerotic plaques were identified using a so-called index of plaque attenuation [141]. Even in 3D tumor spheroids, necrotic areas could be detected using the intrinsic optical attenuation [142].

1.8 Fluorescence imaging

Fluorescence imaging (FI) is a powerful tool in biological research. Especially in neuroscience, FI is used to study the cell and its molecular components. The technique allows to study specific physiological processes such as protein location and associations, motility, and ion transport and metabolism with sub-cellular resolution [143]. A large variety of FI techniques have been developed over the last decades, providing a window to the living cell. Some of the most well known techniques are wide-field fluorescence microscopy
techniques, confocal microscopy, two-photon fluorescence microscopy and light-sheet microscopy [144–147].

Figure 11: The principle of confocal microscopy. A light source with an excitation filter is used to excite target fluorophores. The emitted fluorescence light passes through a dichroic mirror and an emission filter. A pinhole is used to eliminate out of focus glare before the detector.

Confocal microscopy acquires 3D images in high resolution of biological and non-biological samples. The out-of-focus glare is eliminated by a pinhole confocal with the excitation pinhole in front of the detector. A schematic drawing of a confocal microscopy setup is shown in Fig. 11. A light source and an excitation filter ensures the right illumination color. For single photon fluorescence, the fluoroscopes absorb the excitation light, located in a lower wavelength region and emit light in a longer wavelength region. A dichroic filter guides the excitation and emission light which are both located in two different wavelength regions into two different paths. In front of the detector an additional emission filter eliminates unwanted fluorescence from other wavelength regions [148]. Fl was used in many applications in the field of neurosciences [149–153].

1.8.1 Combining OCM and Fl

The combination of the two complementary techniques of OCT and FI enables to retrieve the structural morphology of the tissue by OCT and at the same time investigate the tissue specificity, based on a biochemical or metabolic contrast by Fl [154]. Most combined systems so far have been based on rather complex optical layouts, with two separated light sources and paths for OCM and Fl imaging and were mostly designed to work for one specific fluorescent marker. The OCT systems of these setups were utilized to inves-
tigate retinal tissue and mostly used a spectrum located in the near infrared wavelength region [154–162]. A dynamic full-field OCT setup using two different light sources was developed for the simultaneous acquisition of fluorescence and OCT data for zebrafish imaging [162]. An OCT system operating at 1300 nm combined with a FI setup with an excitation wavelength at 750 nm was utilized to investigate mouse colon tissue ex-vivo and distinguish healthy and cancerous areas [154]. Recently, a supercontinuum source was used for combined OCT and fluorescence imaging of fluorescently labeled ocular structures in transgenic mice [161].

1.9 Histology

Histology is the study of the microscopic structures of a biological tissue and a gold-standard method for ex-vivo tissue examinations in diagnosis. There are two established ways to create histology sections which are shown in Fig. 12. One option is to first fixate the tissue, for example using formalin, and then embed the tissue in a block of paraffin wax in a cassette. This block is afterwards used for sectioning. Typically, a few micrometer thick slices are sectioned using a microtome. The second option is to freeze the tissue sample embedded it in a substance for example O.C.T (Optimal cutting temperature compound) and perform frozen sectioning. Typically, slightly thicker sections are cut using a cryotome. As most cells are transparent and therefore colorless, staining is needed to achieve image contrast [163].

![Diagram of histology process](image)

Figure 12: Overview of the typical steps involved in histology. The brain tissue is either fixed using for example formol or being frozen in optimal cutting temperature compound (O.C.T.). The formol fixated tissue is being embedded in paraffin in a cassette and micrometer thick sections are sliced using a microtome. After mounting, dewaxing and staining is performed. The frozen tissue is directly sectioned using a cryotome before being mounted and stained.
1.9.1 Staining techniques

In histology, staining is used to highlight specific features of the tissue and enhance the image contrast at the same time. Either fluorescent or non fluorescent markers can be used. There is a huge variety of typical stains used in routine histology analysis. The most common one is H&E staining, where hematoxylin (H) stains the nuclei and the endoplasmic reticulum in the typical bluish color, while eosin (E) stains the plasma, the collagen and the endoplasmic reticulum in the typical pinkish color [164]. In modern histology advances have been made in immunohistochemistry (IHC), antibody binding and electron microscopy [165].

Immunohistochemical staining is used to visualize specific compounds. IHC is for example used to label amyloid-beta accumulations in AD affected brain tissue. To confirm the presence of senile plaques additionally, Congo red staining or silver staining methods have to be performed [36, 166]. If Congo red binds to amyloid fibrils, the absorbance peak shifts from 490 nm to 540 nm [167]. For fluorescent labeling of amyloid-beta compounds typically used stains are curcumin, Thioflavin-S or IHC [168]. Additionally, a large variety of IHC dyes exists to label the second hallmark of AD, the aggregation of tau protein in brains [169].

1.9.2 Optical clearing techniques

Any biological tissue will be composed mostly of water embedded in a morphological structure formed by lipids and proteins. When light propagates through the tissue, all these components interact differently depending on their refractive index. This mixture of components of small size with different refractive indices leads to a lack of transparency in the tissue. The thicker and less transparent the tissue is, the less information will be gained by optical imaging such as microscopy. [170]. One solution is to slice the tissue in thin sections and analyze each sequentially and reconstruct the whole structure in the end. However, this procedure is time-consuming, artifacts can easily be introduced and a massive amount of data is produced [171]. In order to make large fixed biological samples transparent optical clearing techniques were developed over last years. Optical tissue clearing is removing, replacing or modifying the components of a tissue, such as the lipids and proteins to homogenize the refractive index [170, 172]. Modern clearing techniques can be divided into two sub-groups: solvent-based clearing and emerging aqueous-based techniques. For the solvent-based clearing technique, dehydration with lipid solvation and clearing by refractive index matching to the remaining dehydrated tissue’s refractive index has to be performed. On the other hand, aqueous-based techniques utilize either passive immersion in a solution that is refractive index matched to the tissue, or lipid removal followed by hydration of the sample to lower the refractive index of the remaining tissue, or passive or active removal of lipid, followed by immersion in a refractive index matched medium [170]. In this thesis a
clearing technique called SWITCH (system-wide control of interaction time and kinetics of chemicals) was utilized. SWITCH clearing belongs to the family of the hydrogel embedding methods. Simplified, the procedure of optical clearing with SWITCH involves stabilization of proteins, lipid extraction, and finally a solution with a refractive index of 1.38-1.45 is used to immerse the sample. [173].
1.10 Aims of the thesis

The aims of this thesis were the following:

1. To investigate microscopic structures in *ex-vivo* brain tissue, a high axial and transverse resolution is needed. Therefore, an optical coherence microscopy system operating in the visible wavelength range shall be designed and built, to investigate *ex-vivo* brain tissue with sub-micrometer resolution. A comprehensive data post-processing pipeline shall be developed. The spectroscopic possibilities of the visible light OCM system shall be investigated for *ex-vivo* brain imaging. The attenuation coefficient as alternative contrast possibility shall be used to differentiate brain pathologies.

2. The penetration depth of visible light is rather limited in brain tissue imaging. Optical tissue clearing as a tool to increase the penetration depth shall be investigated for imaging *ex-vivo* brain tissue with visible light OCM. The optical clearing method shall be optimized to increase the penetration depth while still keeping efficient sensitivity for imaging.

3. The visible light OCM setup shall be used to visualize the pathology of *ex-vivo* brain tissue affected by Alzheimer’s disease. The scattering properties observed by visible light OCM of amyloid-beta plaques as one hallmark of the disease shall be analyzed. The visible light OCM system shall be used to analyze the plaque development over time in a mouse model of AD. The development of plaques in human and mouse brain tissue shall be compared. The pathology of cerebral amyloid angiopathy, a common disease occurring in AD patient shall be investigated.

4. The field-of-view using high numerical aperture objective lenses is rather limited. An automatic moving x-y-z translation stage shall be used to perform large field-of-view mosaic imaging.

5. OCM reveals the 3D structure of brain tissue based on the intrinsic scattering of light within the tissue. Fluorescence imaging can provide additional contrast from exogenous fluorophores. A fluorescence imaging extension shall be integrated into the visible light OCM setup to perform multimodal imaging. A shared light source shall be used for OCM and FI imaging.

6. The advantages of a combined visible light OCM and FI setup shall be evaluated with the help of test targets. The multimodal setup shall be utilized to investigate AD and glioma brain tissue samples.

Aims one to three were investigated in the first publication. A visible light optical coherence microscopy setup was developed. Specification measurements were performed
to characterize the system. A processing pipeline for the OCM data acquisition and post-processing, including attenuation calculation and spectroscopic imaging was introduced. Healthy human brain tissue was imaged, and white and grey matter were distinguished based on their different scattering properties. As a next step, ex-vivo human Alzheimer’s disease brain tissue was investigated and amyloid-beta plaques were visualized. Attenuation and spectroscopic data were utilized to identify and analyze AD brain tissue. Finally, brain tissue of an AD mouse model was investigated. To increase the penetration depth, optical tissue clearing was performed and the effect on the OCM data was evaluated.

Aims three to four were investigated in the second publication. An automatic x-y-z stage was integrated into the sample arm to perform images of a large field-of-view. CAA and AD pathology was visualized in mouse and human brain, respectively. Additionally, several objective lenses with magnifications of $4\times$ to $20\times$ were utilized to image different features of ex-vivo brain tissue. Large field of view images of brains of young and old mice were acquired to perform an analysis of plaque load and size. The plaque size and load were compared to those found in human brain tissue. Cerebral vasculature was investigated in ex-vio human brain tissue of human CAA patients.

Aims five to six were investigated in the third publication. A fluorescence imaging channel was integrated into the existing setup to perform multimodal imaging. A simple optical design with a single shared light source and illumination path enabled the visualization of the morphology with tissue specific contrast. By imaging two different phantoms, a system characterization was performed. The setup was used in two neuroimaging applications. First, brains of an AD mouse model were stained with curcumin and A-$\beta$ plaque imaging was performed. Second, 5-ALA positive and negative central nervous system tumor tissue samples retrieved intraoperatively were imaged prior to conventional neuropathologic work-up. Attenuation values were calculated from OCM data sets. Attenuation and fluorescence data enabled the differentiation between malignant areas and brain parenchyma.
Chapter 2

Spectroscopic imaging with spectral domain visible light optical coherence microscopy in Alzheimer’s disease brain samples

In the first peer-reviewed journal paper incorporated in this work, the newly developed visible light optical coherence microscopy system was introduced. First, the supercontinuum light source and the optical components, which were incorporated into the setup, were described in detail. The broadband spectrum provided by the supercontinuum laser operating in the visible wavelength region (400-700 nm) enabled sub-cellular axial resolution in brain tissue. Specification measurements were performed to characterize the system regarding optical resolution, SNR and roll-off. The data acquisition and post-processing pipeline was introduced. Additionally, to standard OCT post-processing steps, attenuation coefficients were calculated and a spectroscopic image analysis using different wavelength windows was introduced.

The system performance was evaluated for mouse and human brain tissue. Ex-vivo control and AD diseased human brain tissue samples were investigated. First, ex-vivo control human brain tissue was utilized to show the possibility to differentiate white and grey matter based on their different scattering and attenuation properties. In brain tissue of AD patients, amyloid-beta plaques were visualized in the OCM intensity and spectroscopic images as hyper-scattering features compared to the adjacent brain parenchyma. A comparison between intensity and attenuation values of grey matter, white matter, and plaques showed statistically significant differences. Further, using Congo red staining of amyloid-beta plaques, the spectroscopic properties of neuritic plaques, grey matter, and white matter, were investigated.

The last part of the paper focuses on an investigation of brain tissue of an AD mouse model. As the penetration of visible light into brain tissue is rather limited, optical tissue
clearing was established. A clearing method called SWITCH was utilized [173]. The optimal clearing time for effectively increasing the penetration while still keeping sufficient OCM image contrast was investigated. Untreated, cleared and Congo red stained cleared mouse brain tissue was imaged and A-β plaques were successfully visualized.
Spectroscopic imaging with spectral domain visible light optical coherence microscopy in Alzheimer’s disease brain samples

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Abstract: A visible light spectral domain optical coherence microscopy system was developed. A high axial resolution of 0.88 μm in tissue was achieved using a broad visible light spectrum (425 – 685 nm). Healthy human brain tissue was imaged to quantify the difference between white (WM) and grey matter (GM) in intensity and attenuation. The high axial resolution enables the investigation of amyloid-beta plaques of various sizes in human brain tissue and animal models of Alzheimer’s disease (AD). By performing a spectroscopic analysis of the OCM data, differences in the characteristics for WM, GM, and neuritic amyloid-beta plaques were found. To gain additional contrast, Congo red stained AD brain tissue was investigated. A first effort was made to investigate optically cleared mouse brain tissue to increase the penetration depth and visualize hyperscattering structures in deeper cortical regions.

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References and links


1. Introduction

Alzheimer’s disease (AD) is the most common form of dementia and affected over 48 million people worldwide in 2015 [1]. One hallmark of AD are plaques formed out of amyloid-beta protein. Based on the presence of amyloid-beta the concept of preclinical AD has been developed [2]. Aside from research on novel diagnostic markers and therapeutic approaches, current research is focusing on unraveling the pathogenesis of AD which is still poorly understood [3].

Many imaging techniques, such as magnetic resonant imaging (MRI), positron emission tomography (PET) and computed tomography (CT), are important diagnostic tools in clinical routine and are frequently used in neuroscience research [4]. Complementing these techniques, optical coherence microscopy (OCM) could be a powerful imaging method for many in vivo and ex vivo studies, as it provides the possibility of low cost, real time, three dimensional (3D) imaging with micrometer resolution [5]. OCM is based on low-coherence interferometry and an objective lens is focusing the light onto the sample to achieve high transversal resolution [6]. The potential of OCM has already been demonstrated for diverse neuroimaging applications [7–15]. For instance, the microstructure of tumors was investigated with the help of an OCM system. In several studies, OCM provided contrast between healthy and cancerous tissues based on the difference in the backscattered and backreflected light intensity as well as on the assessment of the attenuation coefficients [7, 16, 17]. These OCM systems provided an axial resolution of 5.0 μm, 6.4 μm and 1.5 μm, respectively. An even higher axial resolution of 0.9 μm was achieved using a very broad spectrum located in the near infrared around 800 nm and enabled the investigation of subtle structural features of healthy and cancerous brain tissue [14]. An OCM system working in the near infrared range at 1310 nm was developed for deep brain tissue imaging of the cerebral cortex [8]. For some applications, OCT could even serve as an alternative to classical histology. A histological analysis requires delicate and precise slicing and staining of the tissue. This process is time consuming and prone to artifacts, such as deformations due to the tissue handling. Unlike histology, OCT offers the option of direct 3D tissue assessment. Brain tissue was investigated by polarization sensitive OCT (PS-OCT). The birefringent behavior of myelinated structures in the WM was exploited to perform tractography, i.e., orientation mapping of myelinated fibers [9, 11, 18]. Measurements by a PS-OCT system enabled the visualization of neuritic amyloid-beta plaques in brain tissue of human AD patients and provided information about the polarization characteristics of these pathological structures [12]. Using extended focus OCT, label free imaging of cerebral amyloid-beta plaques was demonstrated in a mouse model.
Traditionally, light sources working in the near infrared region have been used to perform OCT, as the light can penetrate deeper into scattering tissue when working at longer wavelengths such as 1300 or 1700 nm [19]. Recently, imaging was also realized using visible light to perform visible light optical coherence tomography [20–24]. Major advantages of using visible light for OCT compared to near infrared light are a higher axial resolution due to a shorter central wavelength (given the same spectral bandwidth) and a stronger backscattering signal [21]. Using a very broadband light spectrum opens the door to spectroscopic imaging possibilities [21,25–27]. Using spectroscopic visible light OCT for imaging stained tissue could be of particular interest to further increase the contrast of specific structures and compounds, similar to histological practice but without the need for sectioning [28]. Most stains and dyes, including fluorescent dyes, are designed for use under visible light and thus could be assessed by visible light OCT [29]. One drawback when working with visible light is the rather low penetration depth. In brain tissue, the penetration of visible light is limited to a couple of hundred micrometers due to strong scattering and attenuation effects [19]. One way to increase the light penetration in ex vivo tissue is to use optical clearing techniques [30]. In 2015, Murray et al. introduced an advanced clearing technique called SWITCH (system-wide control of interaction time and kinetics of chemicals) that has been successfully applied to mouse and human brain tissues [31].

In this article we present a spectral domain visible light OCM system providing sub-micrometer axial resolution with a broadband spectrometer operating at an A-scan rate of 30 kHz. We successfully apply it to the imaging of human brain tissue with intrinsic contrast to explore its spectroscopic imaging capabilities. In addition to OCM imaging of normal brain tissue, we showcase that visible light OCM can visualize AD-related amyloid-beta plaques in ex vivo AD brain tissue based on their inherent scattering contrast as well as by spectroscopic detection of an amyloid specific stain routinely used in neuropathology. A closer look into the distribution of these plaques in specific brain regions might help to further investigate AD and hence to gain a better understanding of the disease and to hopefully find new leads for treatment [2]. Finally, we demonstrate the first application of SWITCH clearing for visible light OCM imaging in mouse brain tissue with nearly doubled penetration depth.

2. Methods and materials

2.1. Visible light optical coherence microscopy setup

Imaging was performed with a visible light spectral domain OCM system [32]. A sketch of the system is shown in Fig. 1(a). The light source was a supercontinuum laser (NKT Photonics, SuperK EXTREME EXU-6) with an emission spectrum ranging from ultraviolet (380 nm) to near infrared (2350 nm). A variable bandpass filter (NKT Photonics, SuperK V ARIA) was used to crop the spectrum to the visible light range (420 – 700 nm). The detected spectrum, 425 – 685 nm, had a central wavelength of $\lambda_c = 555$ nm, a full-width at half maximum (FWHM) of $\Delta \lambda_c = 156$ nm and a total bandwidth of $\Delta \lambda = 260$ nm, see Fig. 2(a). The input light was attenuated after the variable bandpass filter before entering the interferometer, such that the sample arm power was 0.8 mW. The beam entered the system through a reflective collimator and passed a Glan-Thompson polarizer before being split into reference and sample arm by a 70:30 beamsplitter. In the reference arm, a variable neutral density filter was used to control the reference power and glass prisms (BK7, UV-Fused Silica) were inserted to compensate for dispersion effects. The sample arm, see Fig. 1(b), comprised a microelectromechanical mirror (MEMS) scanner (Mirrorcle Technologies, Inc.) to perform the raster scanning. Sawtooth functions with a fast horizontal x axis and a slow vertical y axis were applied for scanning. A telescope expanded the beam diameter to 3.6 mm ($1/e^2$) to fit the aperture of the objective (Olympus UMPLFLN 10XW) with a 10× magnification to focus the beam onto the tissue. Backscattered and backreflected light from the sample and reference arm was interfered at
the beam splitter and coupled into a photonic crystal fiber (NKT Photonics, LMA-5), which provides single mode transmission of almost the full visible spectrum, leading to a custom-made spectrometer, Fig. 1(c). The spectrometer included a reflective collimator, a diffraction grating with 1800 lines/mm and a custom made lens to focus the beam down to a CMOS line scan camera (Basler, spL8192-70km, 12-bit, pixel size 10 μm × 10 μm) with 8192 pixels, enabling a spectral resolution of ~ 0.03 nm. The camera was running at a rate of 30 kHz and the quantum efficiency data are shown in Fig. 2(a) as a dotted line. Spectral data were collected by a frame grabber (National Instruments, NI PCIe-1473R).

Fig. 1. The spectral domain visible light OCM system. (a) Sketch of the system with BF (Bandpass Filter), BS (Beam Splitter), C (Collimator), DC (Dispersion Compensation), DG (Diffraction Grating), F (Filter), FG (Frame Grabber), L (Lens), LSC (Line Scan Camera), M (Mirror), MEMS (Microelectromechanical Mirror), MEMS C (MEMS Control), NDF (Neutral Density Filter), O (Objective), P (Polarizer), PC (Computer), RM (Reference Mirror), SLS (Supercontinuum Light Source). (b) Image of the sample arm. (c) Image of the spectrometer.

Specification measurements were performed to characterize the system. An axial resolution of 1.2 μm in air was measured with a mirror as the sample, which corresponds to 0.88 μm in brain tissue assuming a group refractive index of 1.36 [33], see Fig. 2(b). The axial resolution over the whole depth range is shown in Fig. 2(c). The side lobes in the point spread function are a result of spectral modulations caused by the used optical components and the variable bandpass filter. A transversal resolution of 2 μm was measured by imaging a US Air Force resolution test target (Edmund Optics), Fig. 2(d). The theoretical transversal resolution was calculated as 1.8 μm. Resulting from the spectral resolution of ~ 0.03 nm, the imaging depth in air was 1.8 mm (1.3 mm in tissue). The theoretical depth of focus was 44 μm (NA = 0.1). Further specification measurements revealed a sensitivity of 89 dB close to the zero delay and a roll-off of 24 dB/mm, Fig. 2(e). For every OCM volume scan 500 × 500 A- and B-scans were acquired. The field of view varied from 0.25 × 0.25 mm² to 0.5 × 0.5 mm² for different acquisitions. One acquisition took 8.3 seconds.

2.2. Brain samples

2.2.1. Human brains

Post mortem, formalin fixed human brain samples of one control subject and two patients diagnosed with end-stage AD (Patient 1: female, 78 years, showed additionally cerebral amyloid angiopathy and subarachnoid hemorrhage; Patient 2: female, 88 years, showed additionally
complex tauopathy, argyrophilic grain disease and TDP-43 proteinopathy) were investigated. The human brain samples were provided by the Neurobiobank of the Medical University of Vienna (ethics approval number 396-2011). Specimens were obtained from patients who underwent autopsy at the Medical University of Vienna. The human control brain tissue was taken from the frontal cortex. AD brain tissue was investigated at both the frontal and temporal cortex. For spectroscopic imaging cortical pieces of $2 \times 2 \times 2 \text{ mm}^3$ were cut out from the AD brain of patient 1. These samples were stained in Congo red for 120 min and cut in half, and the sectioned surface was imaged by OCM. For comparison with the gold standard histological slices were created. For distinguishing grey matter (GM) from white matter (WM) in the human control brain tissue, Klüver-Barrera staining was used, which stained myelin structures blue and GM pink. To visualize amyloid-beta plaques the AD brain tissue was stained using Congo red (Highman method). This stain specifically attaches to amyloid [35].

2.2.2. Mouse brains and optical clearing

Since the penetration depth in visible light OCM was limited to a couple of hundred micrometers, optical clearing of the brain tissue was performed for several samples. For this purpose the brains from healthy wild type C57BL/6 mice, 22 and 26 weeks old and one brain of an AD mouse model (APP-PS1 (amyloid precursor protein - presenilin 1 protein), 22 weeks old, female, Professor M. Jucker, Hertie Institute of Clinical Brain Research (HIH), University of Tuebingen, Germany) were used. All mice were sacrificed by cervical dislocation and the brains were carefully removed and optically cleared following the SWITCH protocol steps [31]. An overview of the steps performed for imaging the mouse brain is shown in Fig. 3(a). Following extraction, the mouse brain was fixed using a 4% paraformaldehyde solution for one week. It was then put into a shaker with the 4°C fixation-OFF solution, as described in the SWITCH protocol [31]. After two more days in the fixation-ON solution, the brain was washed two times for six hours in 4% glycerin and 4% acetamide in phosphate buffered saline and was put for one night in the inactivation solution. For the clearing step, the brain was put into the thermal clearing solution at 37°C. For a first experiment, the brain was kept in the thermal clearing solution for two days and
measurements were taken after each day. In a second experiment the brain was only kept for a few hours in the thermal clearing solution and measurements were taken every 30 minutes. The AD mouse brain was cleared for 60 minutes. Animal experiments were approved by the local ethics committee and by the Austrian Federal Ministry of Science, Research and Economy under protocol BMWFW-66.009/0360-WF/V/3b/2016.

2.3. Data acquisition and processing

Data acquisition was performed in LabView (LabView 2015, Version 15.0, 64-bit, National Instruments) and the data were stored in a 16-bit binary format for performing further post-processing steps in Matlab (MATLAB, R2015b, MathWorks). After resampling the spectral data to k-space, background removal was performed. Numerical dispersion compensation was applied as described by Wojtkowski et al. [36] and Choi et al. [37]. By Fourier transforming these data, three dimensional OCM images were computed. En-face images were generated by calculating mean projection images at various depths within the tissue.

To further analyze the data, multiple post processing steps were performed which are summarized in a graphical overview in Fig. 3(b).

![Fig. 3. Workflow for imaging mouse brains using OCM and the post processing pipeline. (a) The first step was to extract the mouse brain, which was then fixed and clearing was performed. Imaging of the optically cleared brains was conducted and the results were analyzed. (b) Processing pipeline for attenuation en-face maps and spectroscopic OCT images. Both attenuation coefficients were calculated for 3D OCM intensity data and en-face maps were computed. For each A-scan of one B-scan the original spectrum was filtered by the chosen number of Gaussian windows to create the spectroscopic B-scans which could be combined to a spectroscopic B-scan.]

2.3.1. Spectroscopic analysis

Different types of spectroscopic images were generated. Here, the general idea was to create Gaussian windows located in different wavelength regions \( \lambda_i \) of the spectrum and to keep the axial resolution \( \Delta z \) for each band \( \Delta \lambda_i \) constant,

\[
\Delta z = \frac{2 \cdot \ln(2) \lambda_i^2}{\pi \Delta \lambda_i}.
\]

(1)
A general model was established based on the given FWHM bandwidth $\Delta \lambda_i$ of the whole spectrum and the manually chosen central wavelengths of the Gaussian windows $\lambda_i$ as inputs.

$$\Delta \lambda_1 = \frac{\Delta \lambda_i}{1 + \frac{1}{\Delta \lambda_1^2} \left( \sum_{i=2}^{N} \Delta \lambda_i^2 \right)}$$

$$\Delta \lambda_{i+1} = \frac{\lambda_{i+1}^2}{\lambda_i^2} \Delta \lambda_i, \ i = 1...N - 1$$

By using equation (2) and (3), the FWHM bandwidths $\Delta \lambda_i$ of the Gaussian windows can be calculated. The same results could be achieved by first resampling to k-space and then splitting up the spectrum in equal parts. The advantage of the approach described above is that it can be used intuitively as a toolbox only requiring the choice of select wavelength regions in order to perform spectroscopic imaging. Three different spectroscopic approaches were used with two, three and seven Gaussian windows. Table 1 gives an overview of the used total FWHM bandwidths, the central wavelength values, the calculated bandwidths and the constant axial resolutions.

<table>
<thead>
<tr>
<th>Table 1. Parameters for spectroscopic imaging approaches.</th>
</tr>
</thead>
<tbody>
<tr>
<td>First Approach:</td>
</tr>
<tr>
<td>$\Delta \lambda_i = 156$ nm, $\Delta z = 2.7$ $\mu$m</td>
</tr>
<tr>
<td>$\lambda_1 = 520$ nm, $\lambda_2 = 560$ nm, $\lambda_3 = 600$ nm</td>
</tr>
<tr>
<td>$\Delta \lambda_1 = 45$ nm, $\Delta \lambda_2 = 52$ nm, $\Delta \lambda_3 = 59$ nm</td>
</tr>
<tr>
<td>Second Approach:</td>
</tr>
<tr>
<td>$\Delta \lambda_i = 55$ nm, $\Delta z = 4.9$ $\mu$m</td>
</tr>
<tr>
<td>$\lambda_1 = 500$ nm, $\lambda_2 = 600$ nm</td>
</tr>
<tr>
<td>$\Delta \lambda_1 = 23$ nm, $\Delta \lambda_2 = 32$ nm</td>
</tr>
<tr>
<td>Third Approach:</td>
</tr>
<tr>
<td>$\Delta \lambda_i = 156$ nm, $\Delta z = 6.2$ $\mu$m</td>
</tr>
<tr>
<td>$\lambda_1 = 500$ nm, $\lambda_2 = 520$ nm, $\lambda_3 = 540$ nm, $\lambda_4 = 560$ nm, $\lambda_5 = 580$ nm, $\lambda_6 = 600$ nm, $\lambda_7 = 620$ nm</td>
</tr>
<tr>
<td>$\Delta \lambda_1 = 18$ nm, $\Delta \lambda_2 = 19$ nm, $\Delta \lambda_3 = 21$ nm, $\Delta \lambda_4 = 22$ nm, $\Delta \lambda_5 = 24$ nm, $\Delta \lambda_6 = 25$ nm, $\Delta \lambda_7 = 27$ nm</td>
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For the first approach, three Gaussian windows were created such that the central wavelengths were located in the blue, green and red spectral range. By multiplying the original spectrum by these three Gaussian windows, three B-scans encoding blue, green and red light were generated after Fourier transformation. In order to achieve similar power in the three color channels, the three sub-spectra were normalized before Fourier transformation. The spectra were normalized in such a way that in the end all had the same total spectral energy (i.e. the same area under the spectral profile). Additionally in each A-scan the wavelength depended roll-off was compensated [38]. The combination of the three B-scans resulted in an RGB image, in which regions appearing white indicate equal contributions of all three spectral bands. While the chosen Gaussian windows were overlapping in the first approach, see Fig. 7(d), the idea behind the second spectroscopic image approach was to use only two Gaussian windows which were not overlapping and therefore each image was completely independent of the other (see Fig. 7(e)). The first Gaussian window was located in the green and the second in the red spectral region. Finally also a set of seven Gaussian windows covering the whole spectrum was generated, see Fig. 6(a).
2.3.2. Attenuation analysis

In another processing step, the light penetration characteristics were analyzed by extracting the attenuation behavior using two different approaches. The intensity of the light amplitude \( I \) in a homogeneous medium follows the Lambert law of the form

\[
I(z) = I_0 \exp(-\mu_t z) \tag{4}
\]

where \( z \) denotes the depth in mm, \( \mu_t \) the total attenuation coefficient in \( mm^{-1} \) and \( I_0 \) the input intensity [39]. An exponential decay model was used to fit the attenuation coefficient for each A-scan in a certain depth beneath the surface and will henceforth be called the global attenuation coefficient. Furthermore a discrete model proposed by Vermeer et al. [39] was used to create B-scans containing the attenuation characteristics by calculating the attenuation coefficients \( \mu_t[i] \) for each pixel \( i \) from the intensity values \( I \), the total number of pixels along an A-scan \( N \) and the pixel size \( \Delta \) in mm by

\[
\mu_t[i] = \frac{1}{2\Delta} \log \left( 1 + \frac{I[i]}{\sum_{i+1}^{N} I[i]} \right). \tag{5}
\]

This attenuation coefficient is termed as the local attenuation. The advantage of the second method is that the attenuation can be calculated pixel wise.

2.4. Statistical analysis

Box and scatter plots were generated to visualize differences in the results. Two-sample t-tests in combination with Bonferroni correction were performed for statistical analysis. The significance level was defined as \( p < 0.05 \).

3. Results

3.1. Imaging of control human brain tissue

In order to demonstrate the performance of the OCM system for ex vivo imaging of biological tissue, initial experiments were performed in formalin fixed post mortem human brain samples. Figure 4 shows results for the investigation of control brain tissue using visible light OCM. Figure 4(a) shows the scanned part of the tissue including both GM and WM as well as the corresponding histological image. To further quantify the backscattered intensity difference between GM and WM, the intensity values in a slab extending over 200 \( \mu m \) in one B-scan of WM and GM were extracted, respectively. The average intensity of the backscattered signal was higher in the region of WM compared to GM in the first 200 \( \mu m \) of the tissue. For this region the global attenuation coefficient was also extracted for each A-scan by fitting the exponential model Eq. (4) to the depth profile line. A scatter plot, consisting of 400 data points (200 for WM and 200 for GM), displaying intensity vs. global attenuation coefficients is shown in Fig. 4(b). Clusters representing GM and WM can be clearly identified. Figure 4(c) shows an average projection en-face intensity image generated over the first 100 \( \mu m \) below the surface of the brain tissue. A 3D inverted grey scale rendering of the OCM intensity data and a B-scan of a region of WM and GM is shown in Fig. 4(d). Deeper light penetration into the region of grey matter compared to white matter was observed. The local attenuation values were calculated for each pixel in the volume using Eq. (5). A local attenuation en-face map was created over the first 100 \( \mu m \) below the surface shown in Fig. 4(e).

3.2. Imaging of brain tissue of human AD patients

In the next step, we investigated the capability of the system to visualize micrometer scale lesions in pathologic tissue based on their intrinsic optical properties. Cortical tissue of a human brain affected by AD was imaged using OCM, see Fig. 5(a). In the 3D OCM image (Fig. 5(b)),...
hyperscattering structures can be observed, which correspond to neuritic amyloid-beta plaques found in GM in histology. Figure 5(c) shows the histological image of a Congo red stained GM region of the corresponding tissue sample including a neuritic plaque. A zoom into a region of plaques is shown in Fig. 5(d). These structures are also clearly visible in the en-face projection image (mean intensity projection over the first 200 $\mu$m in the brain tissue), see Fig. 5(e).

When looking at the spectroscopic B-scan (Fig. 5(f)) generated using three Gaussian windows in the red, green and blue range, the plaques appear highly scattering in all wavelength ranges hence showing up as white in the image. The investigation showed that the plaques are visible even without additional spectroscopic analysis. Furthermore a transition from white to yellow and to red was observed due to the difference in penetration of the different wavelengths. To quantify the difference between the optical properties of GM, WM and plaques, intensity values and local attenuation coefficients were extracted from GM, WM and plaque regions, respectively. For WM and GM each 200 data points were extracted from a region of 100 $\mu$m $\times$ 200 $\mu$m beneath the tissue surface. Likewise 200 data points were collected from various plaques. The results are visualized in two box plots in Fig. 5(g). It can be observed that the overall intensity of light backscattered from WM was higher than in GM and highest in plaques. Similar to the control brain, the attenuation coefficients were greater for WM than for GM. For the plaques, a very broad spread of attenuation coefficients can be observed. Statistically significant differences ($p < 0.05$) were found when comparing intensity and attenuation coefficients for WM compared to GM, WM compared to plaques and GM compared to plaques, respectively, indicated with asterisks in Fig. 5(g). To correct for multiple comparisons the Bonferroni correction was applied.

The spectroscopic differences between WM, GM and plaques were investigated with a higher number of Gaussian windows. Seven Gaussian windows were generated using equation (2) and (3) shown in Fig. 6(a). B-scan images were computed for each of these windows. Out of these intensity B-scans, local attenuation coefficient images were calculated and finally average
3.3. Imaging of stained AD human brain tissue

To demonstrate the potential of visible light OCM to image intact tissue processed by a standard staining protocol, AD brain tissue was stained with Congo red, see Fig. 7(a). In the OCM image (Fig. 7(b)), the highly scattering plaques can be distinguished from brain tissue based on the higher signal intensity. The stained AD brain tissue was analyzed by creating spectroscopic images with three and two Gaussian windows. Figure 7(c) shows the average intensity and the attenuation coefficients in GM and plaques in the red, green and blue wavelength region respectively. For averaging, a region of 100 µm x 100 µm in GM (4000 data points) and the plaque region indicated by a black arrow in Fig. 7(d), was chosen (350 data points) for each wavelength.

Different characteristics for the three color regions between GM and plaques can be observed. Obvious from the photograph of the stained AD brain tissue, (Fig. 7(a)) a stronger scattering of red light in the higher wavelength regions, can be observed. The same behavior is reflected in the results, leading to higher backscattered intensity values and stronger attenuation of higher wavelengths. Also in the stained tissue, the plaques exhibited a higher intensity and also a stronger attenuation compared to GM. The spectroscopic B-scans are shown in Fig. 7(d) for three windows and Fig. 7(e) for two windows. In the combined spectroscopic B-scans Fig. 7(d), the plaques stand out as highly scattering features. In Fig. 7(d), the plaques appear in the red and
Fig. 6. Spectroscopic evaluation of AD brain tissue. (a) The seven Gaussian windows used for the spectroscopic analysis. (b) Intensity against wavelength for WM, GM and plaques with the wavelength dependent standard deviations of the data represented by shaded bands. (c) Local attenuation coefficients against wavelength for WM, GM and plaques with the wavelength dependent standard deviations of the data represented by shaded bands. (d)-(g) B-scans of the same region as seen by different wavelength regions, (d) $\lambda_1 = 560 \text{ nm}$, (e) $\lambda_2 = 580 \text{ nm}$, (f) $\lambda_3 = 600 \text{ nm}$, (g) $\lambda_4 = 620 \text{ nm}$. Red arrows indicate the plaques and the green arrows mark the plaques where the values were extracted from.

partly in the green channel as the Gaussian windows are overlapping. It can also be observed that longer wavelengths can penetrate deeper into the tissue while the lower wavelength components of the spectrum only penetrate a few tens of micrometers deep. The difference between Fig. 7(d) and Fig. 7(e) is that in the latter only two non overlapping Gaussian windows were used as an input for filtering the broadband visible light spectrum. In Fig. 7(e) the red channel is predominantly picking up the plaque structures as an effect of Congo red staining. In Fig. 7(f) a plot of intensity vs. local attenuation coefficient is shown. Here, plaque structures and GM can easily be distinguished based on their spectral characteristics.

3.4. Imaging of optically cleared mouse brain tissue

Optical tissue clearing was implemented in order to expand the imaging performance of visible light OCM for deep tissue regions. Figure 8 shows results of the investigation of control mouse brain tissue optically cleared using the SWITCH approach. In Fig. 8(a), an image of a formalin fixed mouse brain hemisphere is shown. Figure 8(b) shows the mouse brain after one day in thermal clearing solution. A more brownish hue of the brain and an increased transparency of the superficial tissue structures can be observed. Figure 8(c) shows the same brain after two days in the optical clearing solution with an even more brownish color and again increased transparency. OCM data sets were acquired in the prefrontal cortex of the mouse brain for each of these three steps.

One B-scan was analyzed at each of the three clearing steps and a decrease in average intensity was observed, see Fig. 8(d). For the analysis for each step 2000 data points were extracted from a region of $100 \mu m \times 100 \mu m$ beneath the tissue surface. OCM B-scan images before clearing (Fig. 8(e)) and after one day in the thermal clearing solution (Fig. 8(f)) were acquired. Before clearing, the penetration was limited to approximately $100 \mu m$, whereas after one day of optical
clearing, the penetration was already increased to almost 200 μm. Some structural details can even be observed at a depth of 200 μm, see Fig 8 f). When continuing the clearing process after day one, the backscattered intensity after the surface reflection reached the level of the background such that the contrast was no longer sufficient for OCT imaging. For each step 200 data points were extracted in a region of 100 μm × 100 μm beneath the tissue surface and the global attenuation coefficients were calculated. Two example fits for A-scans before and after clearing can be seen in Fig. 8(e) and Fig. 8(f). The global attenuation coefficient was decreasing as expected, leading to a deeper penetration of the light into the tissue due to less scattering, see Fig. 8(g).

To explore the optimal clearing time, another experiment was performed. A whole mouse brain was imaged before starting the clearing process. Then the brain was put into Thermal Clearing Solution at 37°C. OCT measurements were taken approximately every 30 minutes at the same location over a time frame of approximately 5 hours. An area covering 200 × 100 pixels (100 μm (x) × 50 μm (z)) beneath the tissue surface was chosen to extract the mean amplitude in x direction. The time course of these values during the optical tissue clearing process is plotted in Fig. 9. A trend of decreasing amplitude over time can be observed, as shown by the green dashed line in Fig. 9.

Finally an AD mouse brain was extracted, fixated and sliced to 1 mm thick coronal sections (Fig. 10(a)) and optically cleared for 60 minutes (Fig. 10(b)). After clearing, the brain was stained for 10 minutes using Congo red (Fig. 10(c)). Imaging was performed at each step in the optical clearing and staining process. When looking at the spectroscopic image (Fig. 10(d)), the plaque structures were identified as highly scattering. OCT images reconfirmed the doubling of penetration depth (Fig. 10(e) and Fig. 10(f)) and therefore the highly scattering structures in deeper cortical areas were visible. In Fig. 10(g) a B-scan of the Congo red stained brain tissue is shown. When performing the spectroscopic analysis with two separated spectral bands, the
Fig. 8. Investigating the cleared mouse brain. (a) Mouse brain before optical clearing. (b) Mouse brain after 1 day in thermal clearing solution. (c) Mouse brain after 2 days in thermal clearing solution. The black square indicates the area scanned by OCM. (d) Mean OCM amplitude extracted from a region of 100 μm × 100 μm beneath the surface versus clearing steps with the standard deviations of the data represented by shaded bands. (e) OCM B-scan structural image before clearing with an A-scan and the fitted global attenuation line. (f) OCM image after one day in Thermal Clearing Solution and zoom into a structural detail (potentially a vessel) in a deeper tissue area. (g) Mean global attenuation in a region of 100 μm × 100 μm beneath the surface versus clearing steps with the standard deviations of the data represented by shaded bands. After imaging the values were normalized with respect to the measurement before performing the optical clearing.

additional structures only appeared in the red channel (Fig. 10(h)) and were not visible in the green one (Fig. 10(i)).

4. Discussion

In this article, a visible light spectral domain optical coherence microscopy system was used to investigate brain tissue samples. The visible light OCM system presented in this paper was based on a supercontinuum laser feasible for OCT imaging. With a bandwidth of 260 nm centered at 555 nm an ultrahigh resolution of 0.88 μm was achieved thereby providing access to imaging subtle structural details in healthy and pathological brain tissue such as amyloid-beta plaques. OCT based on visible light was shown to be beneficial for imaging with extremely high axial resolution as well as for performing spectroscopic measurements [20–24, 26, 40, 41]. Ultrahigh axial resolution can be achieved by selecting a light source providing a short central wavelength and/or a very broad spectral bandwidth. In the past, visible light OCT was demonstrated for imaging subcellular components in human cells, small animals, as well as the retina and skin in humans. Thereby, axial resolutions from 0.75 μm to 2.0 μm were reported [20–24, 26, 40]. Owing to the rather high relative intensity noise of the supercontinuum laser, the system sensitivity was relatively low at 89 dB compared to the use of a standard superluminescent diode operating in the near infrared [23, 42, 43]. Similar sensitivity values were previously reported for visible light OCT systems based on supercontinuum generation [20–22, 44]. Also there is still some potential in increasing the signal in the short wavelength region, which was limited by the cutoff wavelength of 425 nm for the photonic crystal fiber (NKT Photonics, FD1-PM) currently used for light delivery. By using a different fiber, the spectral range could be expanded to include more of the blue spectrum, potentially even into the ultraviolet [45]. Finally the spectrum was
subject to a fixed modulation pattern in the blue wavelength region, which was caused by the
wavelength filter as can be observed in Fig. 2(a). A custom filter set could be used to avoid these
modulations and thus to achieve a smoother spectral envelope. Despite these limitations, the
OCM setup reported in this article proved considerable advances in terms of covered bandwidth,
spectral resolution and imaging speed based on a custom spectrometer design. The A-scan rate of
30 kHz would facilely enable in vivo OCM imaging of small animals in developmental studies,
thereby facilitating spectroscopic imaging of dynamic processes.

The imaging performance of the OCM system was evaluated by distinguishing grey and white
matter. As expected from previous works in the near infrared [9, 10, 16], our investigations
revealed that GM and WM also provided intrinsic contrast in the visible range. Significantly
different penetration depths were observed in OCM images of WM and GM. WM structures are
densely myelinated and therefore highly reflective, leading to strong backscattering observed at the tissue surface [8]. In contrast GM is mostly built up by cell bodies which are less scattering and hence lead to lower backscatter intensity near the brain surface [8]. Owing to the lower attenuation, light can penetrate deeper into GM compared to WM. As a result, WM and GM were easily distinguishable in OCM en-face attenuation images (Fig. 4(e)). A quantitative comparison of intensity values and attenuation coefficients revealed statistically significant differences between WM and GM (p < 0.001). The measured mean global attenuation coefficients of 0.39 (±0.06) mm⁻¹ for WM and 0.35 (±0.03) mm⁻¹ for GM, both calculated over the whole spectrum, were in good agreement with values reported in the literature, ranging from 0.1 to 0.9 [46–49]. However, there is a large variety in the calculated attenuation coefficients. One reason is the lack of an additional calibration step before measuring. For the future a phantom of known attenuation will be used for calibration. An example for a phantom used for calibration was shown by [50]. Furthermore the attenuation of non-fixed and formalin fixed tissue differs considerably [51]. Lastly, errors introduced by tissue distortions or incorrect surface segmentation can also affect the calculations. Recently, clusters of scatter intensity and attenuation characteristics were introduced as a sensitive method for differentiating WM and GM [52]. Consequently, a more specific differentiation between WM and GM was also observed by clustering intensity and attenuation coefficients in the visible spectrum (Fig. 4(b)). The combination of different optical properties in such clusters could enable automated differentiation and segmentation of cerebral tissue structures.

Investigating amyloid-beta plaques and in particular their accumulation behavior and structure is an important topic in neuroscience research since it is believed that amyloid-beta plaques are a biomarker for Alzheimer’s disease [2, 53, 54]. Various imaging techniques have been demonstrated for imaging cerebral amyloid-beta plaques in situ [55–58]. Since the size of amyloid-beta plaques is only in the order of a few tens of micrometers [59], high spatial resolution is required for imaging. Using molecular-targeting vectors labeled with MRI contrast agents and extremely high field strengths, it is possible to visualize single plaques using MRI [60]. Optical imaging techniques are usually less complex and more affordable. Hence a great variety of optical microscopy approaches has been demonstrated for imaging amyloid-beta plaques in the brain [58, 61, 62]. One drawback of many microscopy techniques is that histological tissue preparation including sectioning and staining is required prior to imaging. OCM provides rapid 3D microscopy imaging of biological tissue based on intrinsic contrast and has recently also been demonstrated for the visualization of cerebral amyloid-beta plaques using two different approaches. In a mouse model of AD, plaques were investigated by an extended-focus OCM system operating at 850 nm [13]. The setup featured an axicon lens in the sample arm to create a Bessel-like illumination beam for an extended focus. Using this illumination in combination with a Gaussian beam detection similar to dark-field microscopy, amyloid-beta plaques could be assessed both in vivo and in vitro [13]. Sub-micrometer resolution imaging of amyloid-beta plaques in a mouse model of AD was recently demonstrated by a visible light OCM setup which was also based on a Bessel-like illumination beam [63]. This work emphasized the need of ultrahigh resolution when visualizing amyloid-beta plaques. Especially in early stages of AD, for example to follow up plaque growth, micrometer-scale resolution may be necessary. Visible light provides this very high resolution and at the same time the possibility to perform spectroscopic imaging. In a different approach, the polarization behavior of amyloid-beta plaques in human brain samples was investigated by a PS-OCM system operating at 840 nm [12]. Based on their intrinsic birefringence, the three dimensional distribution of neuritic plaques was successfully visualized. In line with these earlier OCM approaches, the system presented here showcased the visualization of neuritic amyloid-beta plaques in brain samples of AD patients. Other than the three methods described before, the visible light OCM layout is relatively simple, and the setup and alignment of its optical components was straightforward. Based on the inherent
hyperscattering properties of the plaques under visible light, the new approach enabled ultrahigh resolution imaging of amyloid-beta plaques in combination with spectroscopic imaging and attenuation coefficient analysis. The size and location of the plaques were also confirmed by histology (Fig. 5(c)). The imaging data in AD brain samples suggest that OCM could be utilized to collect more information about the location, distribution and plaque size variation in different areas of the brain and at different stages of the disease.

The broad spectral bandwidth opened the door to a spectroscopic image analysis of the data. Multiple spectral windows made it possible to investigate the relationship between wavelength, intensity and attenuation. For longer wavelengths, a trend towards more overall backscattering intensity was observed. Moreover the plaques, WM and GM can be distinguished based on the intensity of the backscattering signal. The spectroscopic analysis revealed a similar behavior of increasing attenuation with wavelength until 550 nm and decreasing attenuation at longer wavelengths as reported in [46, 47, 64]. This attenuation phenomenon may be explained by a combination of a continuous decrease of backscattering over wavelength and the possible absorption peak of hemoglobin at around 550 nm [47]. More investigations will be conducted to evaluate the influence of chromatic aberrations on the results. In future studies, visible light OCM may be used to automatically detect and distinguish GM, WM and plaque structures in brain tissue of AD patients or even in vivo in preclinical studies using AD models. The presented visible light OCM system may also be applied to an automated assessment of brain tumors - similar to a recent study using OCT in the near infrared [16] - based on measurements of the attenuation coefficient, or to detect lesions in other neurological diseases, such as multiple sclerosis or Parkinson’s disease.

The spectroscopic imaging capability of visible light OCM provided access to specific contrast generated by tissue staining. Staining with antibodies is a standard method in histopathologic practice and a myriad of commercially available stains and dyes have been established. Since stained tissue sections are usually examined under white light, most of the stains absorb light in the visible range and could therefore be accessible by visible light OCM. In order to demonstrate the concept of imaging stained tissue samples by visible light OCM, we performed Congo red staining of cortical samples of an AD patient, as this stain specifically attaches to amyloid [35]. By performing a spectroscopic analysis of Congo red stained tissue, the stained amyloid-beta plaques were identified based on their specific spectral contrast (Fig. 7). Congo red staining yielded a much stronger signal of the plaques in the red channel. The spectroscopic analysis revealed that the attenuation is first increased and decreased after 500 nm for grey matter. The observed chromatic attenuation may be a result of a combination of two effects: On one hand a decrease in scattering with increasing wavelength, on the other hand the absorption behavior of Congo red itself [65]. Note that the mean values for intensity and attenuation were calculated over a region of 100 $\mu$m $\times$ 100 $\mu$m of the tissue. Red light can penetrate deeper into tissue, resulting in the average intensity remaining higher for longer wavelengths. Therefore, the observed measurements seem plausible. Depending on the spectral signature of a stain, dedicated spectral windows can be tailored for the targeted detection of select structures or compounds. Also fluorescent dyes are common in histopathology and could be detected via their specific absorption band in the visible range. Furthermore, the implementation of an additional channel for detecting fluorescence would be straight forward in visible light OCM because the same light source and illumination path could be used for both modalities. By using standard dyes, the concept of visible light OCM could therefore be exploited to provide three dimensional images with molecular contrast without the need for slicing.

Imaging optically cleared brain tissue provides the possibility to increase light penetration into tissue. The disadvantage of performing optical coherence microscopy with visible light rather than with conventionally used near infrared light is the reduced penetration depth [19]. To overcome this limitation in ex vivo samples, an effort was made to increase the penetration
depth by optical tissue clearing. Optical clearing was proven to increase the penetration of light in near-infrared OCT for example in skin tissue, cervical epithelium and brain tissue [66–68]. Tissue clearing is a compromise between penetration and backscattering, as the clearing process takes out the light-scattering lipid bilayers. Therefore, an additional investigation was performed in order to determine the optimal clearing time for visible light OCM imaging in murine cerebral tissue. A whole mouse brain was repeatedly imaged for several clearing steps such that signal intensity could be tracked as a function of time in the OCM images. During this longitudinal imaging process, we observed that it was very important to image a flat surface to achieve a homogeneous backscattering signal. In future investigations, a vibratome could be used to ensure a flat imaging surface [10, 18]. Investigating a cleared AD mouse brain confirmed the increase of penetration by imaging additional hyperscattering structures in deeper cortical areas. Spectroscopic analysis of Congo red stained AD mouse brain tissue demonstrated structures which were specifically stained by Congo red. In future research we are also planning to explore other clearing protocols in order to achieve the best possible image contrast while improving visible light penetration [69]. Combining optical clearing for increased light penetration, staining for specific contrast on top of the intrinsic tissue contrast, and spectroscopic visible light OCM may open a new toolbox for three dimensional deep-tissue imaging with microscopic resolution.

5. Conclusion

A spectral domain visible light OCM system was developed to investigate healthy and AD ex vivo brain tissue of humans and mice. GM and WM were distinguished based on the difference in backscattered and reflected intensity and attenuation coefficients. A spectroscopic analysis of the data was performed. Amyloid-beta plaques in AD brain tissue were visualized. The analysis of the results showed a statistically significant difference between plaque structures, GM and WM. A spectroscopic analysis of GM, WM and plaques was performed using two to seven spectral windows to characterize the wavelength dependence of these three structures. In order to demonstrate the potential of visible light OCM for imaging with contrast provided by stains commonly used in histopathological practice (in addition to the intrinsic contrast) Congo red stained brain tissue was imaged and the spectroscopic behavior of stained tissue was analyzed. Optical clearing increased the penetration depth and structures in deeper brain areas became visible. OCM results were in good agreement with histology. In the future more brain samples shall be investigated to get more data on the distribution and size of the plaques in various brain regions and at different stages of the disease.

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Disclosure

None.
Chapter 3

Assessment of pathological features in Alzheimer’s disease brain tissue with a large field-of-view visible-light optical coherence microscope

In the second peer-reviewed journal paper incorporated in this work, additional hardware and software extensions were integrated into the visible light OCM setup. As the field of view was rather limited when working with high NA objective lenses, an automatic x-y-z translation stage was placed underneath the sample to perform large field-of-view imaging. Additionally, several objective lenses with magnifications from 4× to 20× were utilized to visualize different features of ex-vivo brain tissue. A water-immersion objective reduced reflection artifacts from the tissue surface such that the image contrast was further increased. The processing pipeline for ex-vivo mouse brain imaging was introduced. The pipeline ensures high quality and repeatable brain tissue processing for visible light OCM imaging.

Different objective lenses were used to image plaques, cellular structures, white matter tracts as well as vascular features in mouse brain tissue. Large field-of-view images of brain sections from two young (8 weeks) and two old (64 weeks) AD mice were acquired. As a proof of concept, a first plaque load analysis was performed, which revealed an increase over age also observed in histology studies. The plaque diameter in micrometers was evaluated in histological and OCM data and showed a good agreement.

As the mouse model is used as a preclinical model to study AD in humans, a comparison between Aβ plaques found in mouse and human brain tissue was conducted. The contrast to noise ratio between plaques and surrounding brain parenchyma was evaluated and the plaque load and volume were compared. The results showed that the plaque load in the mouse brains was significantly higher and the plaques tended to be larger in human brain tissue.
CAA affected regions of ex-vivo human AD brain tissue were imaged. The A-β accumulations in arterial walls of affected ex-vivo human brain samples were visualized as hyper-scattering and strongly attenuating regions.
Assessment of pathological features in Alzheimer’s disease brain tissue with a large field-of-view visible-light optical coherence microscope

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Assessment of pathological features in Alzheimer’s disease brain tissue with a large field-of-view visible-light optical coherence microscope

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Abstract. We implemented a wide field-of-view visible-light optical coherence microscope (OCM) for investigating ex-vivo brain tissue of patients diagnosed with Alzheimer’s disease (AD) and of a mouse model of AD. A submicrometer axial resolution in tissue was achieved using a broad visible light spectrum. The use of various objective lenses enabled reaching micrometer transversal resolution and the acquisition of images of microscopic brain features, such as cell structures, vessels, and white matter tracts. Amyloid-beta plaques in the range of 10 to 70 μm were visualized. Large field-of-view images of young and old mouse brain sections were imaged using an automated x−y−z stage. The plaque load was characterized, revealing an age-related increase. Human brain tissue affected by cerebral amyloid angiopathy was investigated and hyperscattering structures resembling amyloid beta accumulations in the vessel walls were identified. All results were in good agreement with histology. A comparison of plaque features in both human and mouse brain tissue was performed, revealing an increase in plaque load and a decrease in reflectivity for mouse as compared with human brain tissue. Based on the promising outcome of our experiments, visible light OCM might be a powerful tool for investigating microscopic features in ex-vivo brain tissue.

Keywords: optical coherence microscopy; Alzheimer’s disease; imaging system; visible light; supercontinuum laser.

1 Introduction

One out of nine Americans aged above 65 years is suffering from Alzheimer’s disease (AD), making it the most common form of dementia worldwide. As our society is facing an aging population, the cases of AD will double in the next 20 years, leading to a considerable financial and social burden. With disease progression, patients are losing their ability to remember and in the end are dependent on care-giving. To diagnose AD while the patient is still alive, clinicians have to exclude other diseases with similar symptoms and check for cognitive and behavior changes with neurophysiological tests. Ultimately, AD can only be diagnosed by a histological analysis of the brain tissue postmortem. However, as a step toward treatment of the disease, an early diagnosis is crucial.

At the cellular level, the disease is characterized by the degeneration of neurons and the formation of neurofibrillary tangles composed of tau protein. Extracellular plaques composed of amyloid-beta (Aβ) protein are also formed. In humans, the Aβ plaques that are 10 to 200 μm in diameter are considered a hallmark of the disease and have been subject to many investigative studies. However, further research is needed to thoroughly understand the formation and accumulation of the Aβ plaques in various brain regions and stages of the disease. For example, there are diverse opinions regarding the contribution of plaque formation to the etiology of the disease.

Genetically modified animal models can help to gain a better understanding of the underlying mechanisms of the disease. Mouse models, in particular, have been extensively used as their breeding and handling is rather easy and cheap, and the genetic modification techniques are well established. In the past decades, a variety of mouse models have been developed, which reproduce different aspects of AD-related pathologies. The mouse model used in this paper exhibits an overexpression of the amyloid precursor protein (APP) and a presenilin 1 (PS1) mutation accompanied by a rapid development of Aβ plaques in various cortical regions.

Many imaging techniques have been used to investigate Aβ plaques in vivo and ex vivo in both humans and animal models. In clinical research, computed tomography (CT) and magnetic resonance imaging (MRI) have been routinely used to explore potential biomarkers for the disease. In preclinical research, positron emission tomography (PET) and CT were used to study amyloid protein accumulation in whole brains of rodents; however, due to its high resolution in soft tissue, MRI has been the preferred tool for many studies. Resolutions as low as 35 μm were achieved using a mouse model with MRI On the flipside, these neuroimaging techniques are rather expensive, time consuming, and complex. For much higher resolution, two-dimensional (2-D) and three-dimensional (3-D) images can
be acquired using optical microscopy techniques, such as confocal microscopy or light sheet microscopy. In light sheet microscopy, optical clearing is frequently used to increase the penetration depth of light into the tissue to acquire 3-D images with micrometer-scale resolution. Using this method, large and densely sampled datasets are created; however, the acquisition time is often fairly long. A variety of techniques to perform optical clearing have been developed. In 2015, the clearing technique SWITCH was introduced, whose major advantages are the preservation of the tissue size, the possibility to label tissue-accumulations were identified as highly scattering features in the brain tissue. Ex-vivo brain tissue affected by cerebral amyloid angiopathy (CAA) was investigated and A-β accumulations were imaged in vessel walls. Additionally, by implementing an automatic x − y − z stage, large area scans were acquired. A first effort was made to quantify the plaque load in sections of young and old AD mouse brains. Finally, a comparison of A-β plaques observed by OCM in human brains and their counterparts in mouse brain tissue was performed.

2 Methods

2.1 Visible-Light Optical Coherence Microscope

The visible light OCM system used in this work is described in detail elsewhere and thus is only explained briefly in the following. The sketch of the OCM setup is shown in Fig. 1(a). The OCM system comprises a free-space Michelson interferometer and a homemade spectrometer acquiring spectral data at an A-scan rate of 30 kHz. A supercontinuum laser (NKT Photonics SuperK EXTREME EXU-6) in combination with a tunable filter box (NKT Photonics SuperK VARIA) delivers the visible light spectrum, centered at 555 nm with a full width at half maximum of 156 nm. The resulting axial resolution was measured using a mirror as a sample to be 1.2 μm in air, which corresponds to 0.88 μm in brain tissue assuming a group refractive index of 1.36. The original system was modified in several ways. Objectives with 4× (UPLFLN 4X, Olympus), 10× (UPLFLN 10X, Olympus), and 20× (UPLFLN 20X, Olympus) magnification were used to acquire images with different fields of view and thus with different transversal resolutions. Transversal resolutions from 8 to 2 μm were measured using the US Air Force 1951 resolution test target and the field-of-view varied from 1000 × 1000 μm² down to 300 × 300 μm², respectively. The theoretical depth of focus values were calculated to be 711, 177, and 44 μm, respectively. While imaging the focus was
set at the tissue surface. For each objective, the length of the reference arm and the focus had to be adjusted, and dispersion compensation was optimized both in hardware and in postprocessing.

Imaging was also performed with a 10x water immersion objective (UMPLFLN 10XW, Olympus) to reduce reflection artifacts from the superior surface of the tissue. Additionally, two motorized stages (MLS203-1 and MZS300-E, Thorlabs) moving in x-, y-, and z-directions, respectively, were integrated into the sample arm and automated for scanning large fields-of-view up to several square millimeters. The maximum scanned area was 2.5 x 2.5 mm². A custom-made LabView (LabView 2015, Version 15.0, 64-bit, National Instruments) program controlled the automatic x – y stage motion and the focus was set with the z-stage. Furthermore, a water-filled glass cuvette was integrated into the reference arm for improved balancing of dispersion mismatch introduced by the tissue and the water between the tissue and the immersion objective. A sensitivity of 91 dB with a sensitivity roll-off of 24 dB/mm was measured where the shot noise limit was calculated to be 94 dB.

2.2 Tissue Processing Pipeline

A mouse tissue processing pipeline was developed, which is shown in Fig. 1(b). After carefully extracting the brain, the tissue was fixed in 4% paraformaldehyde for seven days. The fixed brain tissue was embedded in a 5% agarose gel. This block was sectioned into 200-μm thick slices using a vibratome (Vibratome Series 1000 Sectioning System, The Vibratome Company). The sections were then glued onto glass slides. Then optical clearing was performed for 15 min at 37°C following the SWITCH protocol. In the previous works, the advantages of combining OCM with optical clearing were explored. All the duration of 15 min used here was found to be optimal for optical clearing to reach deeper light penetration into the tissue while keeping enough contrast for OCM imaging. All results were compared with histological images. One hemisphere of the brain was processed following the steps explained above while the other was used for histology. Results from OCM imaging were then compared with histological images gained at a similar position in the contralateral hemisphere. For these images, the hemisphere was embedded in paraffin and sectioned into 3-μm coronal slices using a microtome. Immunohistochemistry against Aβ was performed on every third section (Dako Beta-Amyloid 1:50, Clone 6F/3D, Detection system Dako EnVision). Some of the remaining sections were stained with Congo red to confirm Aβ plaque findings. In all images, hematoxylin was used as a nuclear staining. Micrographs were acquired with a slide scanner (C9600-12, Hamamatsu) and after acquisition the plaques were segmented automatically in the micrographs using the “ColSeg” plugin of Fiji. To analyze the plaques in AD-affected human brain tissue, a small piece was excised from the cortex, embedded in agarose gel, and cleared following the same procedure as described above. The surface was imaged using OCM and afterward histology was performed to confirm the results. For imaging the CAA-affected arteries, no clearing step was conducted.

2.2.1 APP-PS1 mouse brain samples

Heterozygous breeding of APPswe, PSEN1dE9 (APP-PS1, MMRRRC stock number 34829, The Jackson Laboratory) mice was established. Brains of both the APP-PS1 mouse model and their healthy litter mates were investigated. Animal experiments were approved by the local ethics committee and by the Austrian Federal Ministry of Science, Research and Economy under protocol BMWFW-66.009/0360-WF/V3b/2016. Mice with an age of 8 (N = 2), 51 (N = 2), and 64 (N = 2) weeks were sacrificed and investigated.

2.2.2 Human brain samples

The human brain samples were provided by the Neurobiobank of the Medical University of Vienna (ethics approval number 396-2011). Specimens were obtained from patients, who underwent autopsy at the Medical University of Vienna. Formalin-fixed en-vivo brains of one control patient and two late stage AD patients were investigated primarily in the frontal cortex. In both pathological cases, CAA was previously confirmed by histology.

2.3 Data Acquisition and Postprocessing

An imaging protocol consisting of 8192 x 500 (A-scans) x 500 (B-scans) pixels per volume was chosen. The raw data were acquired using a custom-made LabView program. The postprocessing pipeline was implemented in MATLAB (MATLAB, R2015b, MathWorks). An additional step of spectral shaping was integrated to reduce side lobe effects in the images after resampling linearly the data to k-space. Attenuation images were calculated as described in previous works. For the OCM en-face projections, the intensity values over the first 50 μm underneath the tissue surface were averaged unless otherwise stated.

2.3.1 Plaque load evaluation in large field-of-view images

For the large field-of-view images, the plaque load (plaques per square millimeter) was evaluated. In the OCM intensity en-face projections, the plaques were manually segmented. To have a fair comparison to histology, five consecutive histological slices with two slices in between (accounting for the 50 μm averaged in OCM) were chosen in a similar brain region in the corresponding contralateral hemisphere. The histological images were registered using the Fiji “StackReg” tool, and the plaques were segmented using color deconvolution and thresholding in Fiji. The union of these resulting five binary images was automatically analyzed using Fiji. The plaque diameter and the total plaque number were extracted.

2.3.2 Comparing plaques in human and mouse brain tissue

The plaques in the OCM intensity volumes were segmented manually using the segmentation paint brush tool in ITK-Snap. The plaque load (plaques per cubic millimeter), the plaque volume (μm³), and the intensity values in the OCM images were evaluated using Fiji and MATLAB. For the intensity analysis, 25 randomly selected plaques in each volume for human and mouse brain tissue were analyzed by its contrast-to-noise ratio (CNR) that was calculated using the mean intensity in the surrounding brain parenchyma (μB),
and the variance \((\sigma_P^2, \sigma_B^2)\) of these intensity values. The surrounding background in the brain tissue was chosen at the same depth as the plaques.

### 2.4 Statistics

To compare the extracted brain features, box plots and histograms were generated for a descriptive analysis. Two-sample Kolmogorov–Smirnov tests with Bonferroni correction were performed in MATLAB for statistical analysis, with a significance level of \(p < 0.05\).

### 3 Results

#### 3.1 Investigating Microscopic Features of Mouse Brain Tissue

Using different objectives with 4x, 10x, and 20x magnifications, imaging of mouse brain tissue was conducted and plaques, cellular structures, white matter tracts as well as vascular features were visualized. APP-PS1 mouse (8 to 64 weeks old) brain sections were imaged using the three magnification levels and A-\(\beta\) plaques ranging from 10 to 70 \(\mu\)m in diameter were observed in the cortex. Figures 2(a)–2(c) show OCM en-face projections of different regions at different scales. In all images, examples of A-\(\beta\) plaques are marked with red arrows. An immunohistochemically stained image of a neighboring region from the same mouse brain is shown in Fig. 2(d). Representative B-scans at the three magnifications including plaques are shown in Figs. 2(f)–2(h). An en-face projection using a water immersion 10x objective is shown in Fig. 2(l). Imaging with the water immersion objective improves visibility of smaller plaques that exhibit less contrast [Fig. 2(b)] as compared with the image taken without water immersion [Fig. 2(b)].

By optical clearing brain tissue, the penetration depth of light was doubled which makes it possible to image thicker volumes with a single acquisition.\(^{26}\) One advantage when imaging AD-affected brain tissue with OCM is the ability to visualize plaques quickly in 3-D. Figures 1(i)–1(k) show OCM en-face projections over 20 \(\mu\)m in the tissue at different depth positions acquired with the 10x water immersion objective. In these projections, plaques can be observed throughout the whole OCM volume. In Fig. 2(e), a color-encoded depth projection shows the appearance of plaques in another dataset in a region over 200 \(\mu\)m. An attenuation image [Fig. 2(m)] calculated from Fig. 2(l) shows that the attenuation in plaques is higher compared with the surrounding tissue.

To highlight the advantages of imaging with a water immersion objective, the same position in the mouse brain was first imaged using the 10x magnification objective without water and then with water immersion. The results are shown in Figs. 3(a)–3(d). Especially in the B-scan image without water immersion, Fig. 3(b) reflections from the first surface are clearly visible, which are suppressed in Fig. 3(d). Details are obscured by the reflections [this is marked by yellow arrows in
Figs. 3(b) and 3(d)]. Also in the en-face projection Fig. 3(a) artifacts appear. Control and APP-PS1 mouse brain sections were imaged to investigate the advantages of using various objectives. The same position in a mouse brain cortex was imaged with 4×, 10×, and 20× magnification. The results are shown in Fig. 4(a). Using the 4× magnification, an overview of the imaged region is achieved. Going to 10× magnification, amyloid-beta plaques can clearly be identified for further analysis, for example, for plaque load evaluation as it was done in this article. Using 20× magnification, the structure of the plaques can be investigated in greater detail; however, the field of view is very limited.

Further microscopic features were investigated using the three magnification levels. Figure 4(b) shows white matter tracts imaged with the 4× magnification objective. In Fig. 4(c), the vasculature of a brain region imaged by a 10× magnification objective can be seen. Figures 4(d)–4(e) show OCM images taken with the 10× water immersion objective. Figures 4(d) and 4(e) show en-face projections over the first 50 μm underneath the tissue surface where white matter tracts are clearly visible. In Fig. 4(d), even smaller fiber tracts can be identified, which are also observed in the B-scan image as regions with higher intensity. When investigating brain tissue with 20× magnification, see Fig. 4(f), Aβ plaques can be resolved at higher transversal resolution and cellular structures can be observed as hyposcattering spots. For comparison, Fig. 4(g) shows a histological image of an adjacent region in the same mouse brain.

3.2 Large Field-of-View Images of Mouse Brain Tissue

Large field-of-view images of brain sections from two young (8 weeks) and two old (64 weeks) AD model mice were acquired. A coronal section in the cortex and the hippocampal region was imaged. A first analysis of the plaque load was performed as a proof of concept. In Fig. 5(a), a large field-of-view en-face image of a brain section from a 64-week-old AD mouse
acquired with the 10× water immersion objective is shown. In total, 49 tiles were automatically acquired and manually stitched together to cover an approximate area of 2.5 × 2.5 mm². An immunohistochemically stained section of the same brain region is shown in Fig. 5(c), with a zoom-in into the cortex and the hippocampal formation. In both the OCM intensity images and the histologic section, anatomical features such as the cortex, the hippocampal formation, and the thalamus can be identified. At the bottom of the large field-of-view images, large white matter fiber tracts can also be observed. A large number of amyloid-beta plaques can be identified in the OCM intensity images as hyperscattering structures [Fig. 5(a), marked with red arrows] as well as in the histologic sections as dark brown spots [Fig. 5(c) marked with red arrows]. In Fig. 5(b), a similar brain region was imaged in an adolescent mouse (8 weeks) using a total of 36 tiles. Less overlap was chosen to cover the same area as compared with the scan of the old mouse brain to reduce the acquisition and processing time. Two additional brain sections (one 8-week old and one 64-week old) were imaged using 36 tiles. To have a quantitative comparison of the plaque load, the plaques were manually counted in the OCM intensity images as well as automatically counted in the histology images using Fiji. The mean values were compared for all four brains. As the en-face OCM data were averaged over 50 μm in the tissue; for the histological evaluation, a stack of slices was averaged to cover the same depth. In contrast to the surrounding tissue is rather low. In the older mice, an average of 23 plaques/mm² were counted in the OCM image mosaic and 24 plaques/mm² in the histology image. The imaged positions were acquired in similar regions and the results from OCM imaging and histology agree well. For the young mice, an average of 17 plaques/mm² were counted in the OCM image, compared with 16 plaques/mm² in the histology image. In total, this results in an increase of 35% for the plaque load in OCM and 50% in histology in aged compared with young mouse brains. These values are in good agreement with numbers found in the literature for APP-PS1 mice of similar age. In both histology and OCM images, the plaques appear smaller in younger mice. For quantification histograms are shown analyzing the plaque diameter in the 8- and the 64-week-old mouse brains. The trend of bigger plaques in the older mice can be shown in Fig. 5(d) and significant differences were found in OCM and histology analysis (p < 0.01). From the histogram, it seems that plaques imaged with OCM in the 64 weeks old mouse appear to be bigger compared with histology. The statistical analysis resulted in a significant difference between plaque size in the 64-week-old mice measured with OCM compared with histology (p < 0.01); however, for the 8-week-old mice no difference was found (p = 0.07).

### 3.3 Comparing Plaques in Human and Mouse Brain Tissue

A-β plaques in human brains and in brains of the AD mouse model were manually segmented. Based on the size, number, and CNR values gained from 10 datasets (five data sets from
In this article, a visible light OCM system was utilized to investigate brain tissue of human AD and CAA patients and of a mouse model of AD. The high axial resolution of this OCM prototype resulted from the broad spectral bandwidth (255 nm) of the light source, centered in the visible wavelength range (425 to 680 nm). The OCM system enabled the imaging of anatomical features with submicrometer resolution up to 200-µm deep in tissue. Through the ability of the system to use a variety of objectives and an automated x-y-z sample stage, wide field-of-view images with different magnifications were acquired. Using water immersion objective reflections in histologic sections of the investigated CAA brains, A-β accumulations were found, see Figs. 7(b) and 7(c).

In Fig. 7(d), an en-face projection shows the A-β accumulations in the vessel wall. Such accumulations are marked with yellow arrows, whereas A-β plaques in the surrounding brain tissue are marked with red arrows. Figure 7(e) shows the histological section of a control brain where no A-β accumulations can be found. All OCM images were taken with the 10x magnification water immersion objective. In Fig. 7(f), the A-β accumulations in the arterial wall can be clearly identified through higher attenuation values. Figure 7(g) shows the corresponding intensity B-scan. In the OCM images, the vessels can be identified by their specific wall structure known from histology. In the representative B-scans in Figs. 7(h)–7(i), A-β accumulations in the arterial walls and A-β plaques can be seen as highly scattering features. Figure 7(j) shows an OCM B-scan image of a control brain in which no A-β accumulations were found in the arterial wall.

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from the supreme tissue surface can be oppressed. This leads to a better image contrast and additional details are revealed, as it is shown in Figs. 3(a)–3(d). A-β plaques, A-β accumulations in vessel walls, vascular features, cellular structures, and white matter fiber tracts were investigated. Unlike the surrounding brain tissue, white matter fiber tracts are highly scattering structures and therefore appear bright in the OCM intensity images. The same behavior has been previously described by other groups.27,38,52,53 The different magnifications enabled the visualization of white matter tracts in different scales, see Fig. 4. Cellular structures were successfully imaged with the visible light OCM system, appearing as hyposcattering structures in the image [Fig. 4(g)]. This is also in agreement with previous reports, although there are some controversial discussions if the investigated structures are cell nuclei or cell bodies.27,35,52,54,55

The big advantage of using various magnifications is that the image can be acquired. The stage can be used to acquire image ranges between 8-weeks-old and 64-weeks-old mice, which is in good agreement with values reported in the literature.20,48,49

As an extension to the system, an automatic motorized z stage was integrated in the sample arm to enable acquisitions of large field-of-view images over several square millimeters. In principle, with the custom-made software and the stage, fields-of-view into the square centimeter range could be acquired. The stage can be used to acquire image ranges up to 11.0 cm × 7.5 cm.

The OCM system was utilized to acquire images in brain sections of young and old AD mice. Age-related plaque load increase was evaluated by assessing the number of plaques per cubic millimeter. The neuritic plaques were counted both in OCM images and corresponding histologic sections. As the results of both approaches agreed well, visible light OCM may be considered as a suitable tool for analyzing the plaque load. A 35% to 50% increase of plaque load was observed between 8-weeks-old and 64-weeks-old mice, which is in good agreement with values reported in the literature.20,48,49

From histology and OCM images, it was observed that the plaques seemed to be smaller and fewer in younger mouse brains compared with the older ones. A histogram analysis showed that in the 64-week-old mice significantly bigger plaques appear (p < 0.01). The histogram analysis also showed that plaques appeared bigger when imaged with OCM and had a broader distribution of the diameter when compared with micrographs of histological sections. For the 64-week-old AD mouse, this diameter difference was statistically significant (p = 0.01), however this was not the case for the 8-week-old mice (p = 0.29).

The difference may be explained by the different contrast mechanisms underlying the two modalities. Immunohistochemical staining specifically marks amyloid-beta such that it is possible to precisely segment the plaques in the histology images. In OCM imaging, the contrast is based on the scattering differences between structures. As the density of amyloid fibrils is not constant within the plaques,25 this may lead to a fuzzier border between plaques and the surrounding brain parenchyma, resulting in a broader distribution in diameter and a bigger size of the

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Fig. 7 OCM of CAA. (a) Schematic drawing of a cerebral artery with the arterial wall structure. White arrows indicate the most frequent positions where A-β accumulates. (b)–(c) Histological images (Congo red and hematoxylin staining). A-β accumulation in the arterial wall structure and A-β plaques can be seen. (d) En-face projection over the first 50 μm underneath the tissue surface. (e) Histological image (Congo red and hematoxylin staining) of a control cerebral artery, where no A-β accumulations can be found. (f) Attenuation B-scan image. (g) Corresponding intensity B-scan of (f). (h)–(i) Intensity B-scans acquired in CAA-affected brains. (j) OCM B-scan image of the control cerebral artery. Yellow arrows indicate the A-β accumulation in the arteries and red arrows A-β plaques in the surrounding tissue. Examples of manually segmented accumulations are included as well.
segmented plaques. In the literature, a similar topographic distribution of amyloid-beta plaques in human and mouse brains was observed; however, at a different time scale as the life cycles are very different. For the future, it would be interesting to further characterize an optimal age of the mouse model that realistically represents the plaque accumulation of AD patients. Based on our promising observations, we plan to thoroughly analyze brain sections of AD mice at different ages in a cross-sectional study to further characterize the mouse model. Also, until now, the stitching of the OCM images was performed manually and only for en-face projections, but mosaicing of OCM volumes may be fully automated by advanced processing routines in the future. One problem that has to be overcome is to automate the z-stage adjustment to set the focus. A software will be implemented, which will detect the first surface of the tissue and then set the focus accordingly.

A-β plaques in the range of 10 to 70 µm in diameter were visualized as they appear as strongly scattering structures compared with the surrounding cortical tissue. The size range and the localization of the plaques mainly in the cortex and the hippocampal formation are in agreement with values reported in the literature. In the attenuation images [Figs. 2(m) and 7(f)], smaller plaques may often be visualized more clearly than in the corresponding OCM intensity images [Figs. 2(l) and 7(g)]. Hence, attenuation analysis may be a useful tool toward an automated segmentation of plaques in brain tissue. Furthermore, the results of our OCM measurements were in good agreement with observations in histologic sections of the same sample. A-β plaques can be categorized into neurotic and diffuse plaques. Staging of the plaques based on the OCM image contrast could be investigated in the future. The chemical composition of the plaques and probably the resulting different refractive index could be an explanation for the contrast in the intensity images. Another source of the contrast of the plaques in the OCM intensity images could be the specific backscattering profile of the plaques, which was described in a mathematical model by . As mentioned in the methods, when changing between objectives, the dispersion mismatch had to be corrected in postprocessing. This could be overcome using the same objectives in both the sample and reference arm or by having two reference arms and switching between them, as it was shown by .

A comparison of the CNR, size, and load between human and mouse brain tissue was performed. Our results suggest that mouse brain tissue exhibits a higher plaque load when compared with human brain tissue in the cortex. For the plaque size, the human brains tend to have bigger plaques (human 6600 ± 1700 µm³, mouse 4500 ± 1200 µm³) but no significant difference was found by OCM. However, until now only two patients and two mice were imaged and in the future more datasets will be acquired and evaluated. Still, similar results were found in the literature. The CNR in human plaques was significantly higher compared with murine plaques. Literature suggests that the chemical composition of human and mouse plaques is different. In human brains, more dense plaques are developed compared with those in the brains of AD mouse models, which might affect the light scattering properties and thus could be an explanation for the higher CNR values observed in the OCM intensity images. Another reason for the observed higher intensity values in human tissue could also be the longer fixation time of the tissue. One approach could be to compare fresh samples of human and mouse brain tissue. For our analysis, plaques in the cortex region were imaged, in the future the A-β plaques in other brain regions will be analyzed to track temporal changes in the plaque distribution.

CAA is a common pathology diagnosed in AD patients and is characterized by A-β accumulation in the arterial walls.CAA-affected ex vivo brain tissue of human patients was investigated and hyperscattering regions were observed in the arterial wall regions. The results were compared with histology showing good agreement.

The next upgrade to the OCM system will be the addition of a fluorescence detection channel to simultaneously acquire coregistered OCM and fluorescence images. The current gold standard to confirm the observation of plaques is to perform histology. In the future, the fluorescence channel could confirm plaque findings. Particularly interesting would be multimodal OCM/fluorescence imaging of curcumin or thioflavin-S-stained brain samples to expand specificity and contrast for the A-β plaques. Further it would be interesting to investigate tau labeling to explore the possibilities to detect the neurofibrillary tangles. The big advantage of using visible light OCM in combination with fluorescence imaging is that the same light source and illumination path can be used such that the exact same position can be imaged simultaneously.

The presented visible light OCM system has a large variety of potential application fields in neuroimaging. One possibility would be to use the system to investigate brain tumors, to be able to differentiate between healthy and tumorous brain areas. This could be done by using the intensity images and their structural information as shown by , by analyzing the attenuation images, as it was shown by , or by the different scattering properties of the tissue as it was shown by . Through the high axial and transversal resolution of the system, it would be especially interesting to image the microscopic structure of different types of tumors. OCM could also be used to investigate pathological features of other neurological diseases such as multiple sclerosis or Creutzfeldt-Jakob disease. In summary, visible light OCM may be a versatile and powerful tool for neuroimaging.

5 Conclusion

Ex vivo brain tissue of patients diagnosed with AD and of a mouse model of AD were investigated using a visible light OCM system. Microscopic anatomical features such as cellular structures, vascular features, and white matter fiber tracts were imaged. Furthermore, amyloid-beta plaques with a diameter in the range of 10 to 70 µm were identified in the cortex. In CAA-affected brain tissue, A-β accumulations were observed in the arterial walls. OCM results were hereby in good agreement with histology. Large fields of view of young and old mice brain sections were imaged using an automated motorized x − y − z stage. A first effort was made to quantify the plaque load using OCM and histology, showing an age-dependent plaque load increase over time. A comparison of the plaque intensity, size, and load in human and mouse cortex tissue was also performed. The analysis showed a significant difference in plaque load and intensity between human and mouse brain tissue. Visible light OCM is a powerful tool to investigate microscopic features in ex vivo human and mouse brain tissue samples and could be extended to many other application fields.
Disclosures
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Chapter 4

Revealing brain pathologies with multimodal visible light optical coherence microscopy and fluorescence imaging

In the third peer-reviewed journal paper incorporated in this work, the multimodal visible light OCM and fluorescence imaging (FI) setup was presented. OCM provides the morphology or 3D structure of the investigated tissue and FI provides additional tissue specific contrast. The multimodal approach allowed to acquire OCM and FI data sequentially with the same field of view. The aim was to create a setup based on one shared light source for OCM and FI imaging, which was adaptable and easy to use. For this purpose, simply two mirrors in the setup had to be flipped to change between the two modalities. In the FI channel, a filter cube enabled the flexibility to image a wide range of typical fluorescent markers used in modern histology analysis. Specification and phantom measurements were performed to characterize the system. Three different stains and phantoms were investigated to demonstrate the flexibility of the setup.

The multimodal setup was used to investigate two common brain diseases, AD and central nervous system tumors. For AD imaging, curcumin stained brain slices of a mouse model of AD were examined. Amyloid-beta plaques were specifically identified using FI. In the co-registered OCM images the plaques appeared as hyper-scattering structures in 3D.

For human brain tumor imaging, biopsies were imaged prior to conventional neuropathologic work-up. FI detected 5-ALA fluorescence in the tumors while OCM visualized the three-dimensional structure of the brain parenchyma and tumor affected areas. Attenuation coefficients were computed from the OCM data. Attenuation and the fluorescence intensity values were analyzed and showed a statistically significant difference between 5-ALA positive and negative brain tissues. These results were in agreement with the histological analysis of the same regions.
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Abstract. We present a multimodal visible light optical coherence microscopy (OCM) and fluorescence imaging (FI) setup. Specification and phantom measurements were performed to characterize the system. Two applications in neuroimaging were investigated. First, curcumin-stained brain slices of a mouse model of Alzheimer’s disease were examined. Amyloid-beta plaques were identified based on the fluorescence of curcumin, and coregistered morphological images of the brain tissue were provided by the OCM channel. Second, human brain tumor biopsies retrieved intraoperatively were imaged prior to conventional neuropathologic work-up. OCM revealed the three-dimensional structure of the brain parenchyma, and FI added the tumor tissue-specific contrast. Attenuation coefficients computed from the OCM data and the fluorescence intensity values were analyzed and showed a statistically significant difference for 5-aminolevulinic acid (5-ALA)-positive and -negative brain tissues. OCM findings correlated well with malignant hot spots within brain tumor biopsies upon histopathology. The combination of OCM and FI seems to be a promising optical imaging modality providing complementary contrast for applications in the field of neuroimaging. © The Authors. Published by SPIE under a Creative Commons Attribution 4.0 Unported License. Distribution or reproduction of this work in whole or in part requires full attribution of the original publication, including its DOI. [DOI: 10.1117/1.JBO.24.6.066010]

Keywords: optical coherence tomography; supercontinuum laser; Alzheimer’s disease; glioma; 5-aminolevulinic acid; protoporphyrin IX

1 Introduction

Optical coherence tomography (OCT) is an optical imaging technique, which was introduced in the early 1990’s. OCT has since become one of the most important imaging and diagnosis tools in ophthalmology. The use of OCT or optical coherence microscopy (OCM) also expanded into a wide range of application fields such as neuro-, skin, and endoscopic imaging. In neuroimaging, OCT has been utilized to investigate diseases such as Alzheimer’s disease (AD), Parkinson’s disease, and a variety of brain tumors. However, in comparison to histology, OCT often lacks tissue-specific contrast of anatomical structures since conventional OCT image contrast is mainly based on light scattering. Current research in the OCT community has therefore also focused on validating and/or combining OCT with other established imaging modalities. One promising possibility is to combine OCT and fluorescence imaging (FI).

FI provides tissue specificity based on biochemical or metastatic contrast through either autofluorescence or exogenous fluorescent dyes. A combination of OCT and FI may enable the assessment of tissue morphology by OCM imaging with complementary biochemical tissue information retrieved by FI. Multimodal OCT and FI approaches have been implemented in different ways by research groups over the past years. However, most presented OCM/fluorescence systems thus far have been based on rather complex optical layouts, which were designed to only work for one specific fluorescent dye and often relied on two separate light sources and paths, one for OCM and one for FI. Also, most of the OCT subsystems were operated in the near-infrared wavelength region and were designed for imaging the retina. Recently, using supercontinuum lasers working in the visible light spectrum, submicrometer axial resolutions for imaging the eye, cells, and brain tissue were achieved.

AD is characterized by the degeneration of neurons, the formation of extracellular plaques composed of amyloid-beta protein, and the accumulation of intracellular neurofibrillary tangles composed of tau protein. In our recent work, we investigated neuritic amyloid-beta plaques in AD brain tissue using a high-resolution visible light OCT setup. In histology, which is the gold standard for confirming these structures in neuropathology, amyloid-beta plaques can be identified using various stains, such as Congo red or immunohistochemical staining against amyloid-beta. Another possibility to visualize amyloid-beta plaques is the use of fluorescent dyes, such as thioflavin-S or curcumin. Curcumin is a yellow pigment, which is extracted from the rhizome of the plant Curcuma longa and has been used to label amyloid-beta deposits ex-vivo.

Optical imaging plays a crucial role during dissections of brain tumors. In state-of-the-art neurosurgery, the surgeon utilizes an intraoperative microscope to navigate through the procedure and to localize malignant tissue areas to be resected. The most common and most aggressive primary brain tumor is the glioblastoma multiforme, which belongs to a group of tumors called gliomas. Even with the best possible treatment,
in most cases median survival does not exceed 15 months. Surgery followed by radiotherapy and concomitant temozolomide chemotherapy is the most important current treatment approach.\textsuperscript{49} To enable maximal safe resections, intraoperative differentiation of tumor tissue and brain parenchyma is of utmost importance.\textsuperscript{50} Recently, 5-aminolevulinic acid (5-ALA) FI has emerged as a powerful intraoperative modality capable of detecting high-grade glioma. The patient orally intakes the 5-ALA tracer prior to surgery. 5-ALA is then metabolized in the tumor cells to the fluorescent molecule protoporphyrin IX (PPIX).\textsuperscript{51} During the operation, a blue light source, integrated in the neurosurgical microscope, is used to excite the fluorophore, making it easier for the surgeon to identify the malignant areas.\textsuperscript{52–54} However, FI lacks morphological information about the tissue. It was already shown that OCT is a promising technique to investigate tumorous brain tissue.\textsuperscript{12,55–59} Kut et al.\textsuperscript{12} evaluated the attenuation coefficient extracted from the OCT data to distinguish between healthy tissue and tumors at different stages. Researchers have focused on integrating OCT into surgical microscopes.\textsuperscript{60–62}

In this work, we present an adaptable, multimodal visible light OCM and FI setup. Our system is based on a supercontinuum laser operating in the visible wavelength range as a single, shared light source for both OCM and FI. By using a visible spectrum and appropriate spectral filter sets, the presented setup is able to excite various fluorescence dyes. In the OCM channel, the broad spectrum provides a high axial resolution to investigate microscopic features in the tissue. We present imaging of amyloid-beta plaques, in \textit{ex vivo} brain tissue of a mouse model of AD, using curcumin-based fluorescence contrast and visualize the three-dimensional structure of the brain tissue using the OCM channel of our multimodal setup. Finally, we investigate \textit{ex vivo} tumor biopsies with our multimodal setup. We identify the malignant regions with the FI setup and assess the morphological information of the brain parenchyma with the OCM. Finally, we evaluate the attenuation coefficients retrieved from the OCM measurements and perform a comparison to histology.

\section{Methods}

\subsection{Combined Visible Light Optical Coherence Microscopy and Fluorescence Imaging Setup}

A multimodal visible light optical coherence microscope (OCM) and FI setup was developed. The OCM subsystem was first introduced in 2017.\textsuperscript{6} A sketch of the setup is shown in Fig. 1(a).

![Fig. 1](https://www.spiedigitallibrary.org/journals/Journal-of-Biomedical-Optics on 04 Jul 2019
Terms of Use: https://www.spiedigitallibrary.org/terms-of-use)
The system is based on a free-space Michelson interferometer and a custom-built spectrometer. A supercontinuum laser in combination with a variable filter box (NKT Photonics SuperK EXTREME EXU-6 and VARIA) provided a broad visible spectrum (400 to 700 nm). An axial resolution of 0.88 μm in brain tissue was measured assuming a refractive index of 1.36. A 20x magnification objective lens was utilized (Olympus, UPLFLN 20X) leading to a measured transversal resolution of 2 μm and a theoretical depth of focus of 44 μm. The A-scan rate of the system was 30 kHz. To switch from OCM to FI, two flipping mirrors (F.M.), see Fig. 1(b), were used to deflect the beam. A zoom-in photograph of the combined setup in Fig. 1(c) shows the part where the two mirrors can be flipped. For FI, a variable combination of excitation filter (Ex.F.), dichroic mirror (D.M.), and emission filter (Em.F.) can be chosen and inserted in a filter cube (Olympus U-MF2) and may therefore enable flexible imaging of a variety of fluorophores. The light beam first passed through an excitation filter (Thorlabs MF 434-17, central wavelength = 434 nm, bandwidth = 17 nm) and was reflected by a dichroic filter (Thorlabs MD 434) and scanned across the sample. The beam in the sample arm was raster scanned using a microelectromechanical mirror scanner (MEMS, Mirricle Technologies, Inc.). The line scan camera (Basler spLS192-70km) in the spectrometer, the MEMS and the PMT were synchronized using a custom-made LabView [Version 15.0 (64-bit)] program. The photons emitted by sample fluorescence passed through an emission filter (Curcumin: Thorlabs MF 530-43, central wavelength = 530 nm, bandwidth = 43 nm; 5-ALA: MF 630-69, central wavelength = 630 nm, bandwidth = 69 nm). The fluorescent signal was focused by a lens onto a photomultiplier tube (Thorlabs, PMMT02, PMT), whose voltage output was digitized using a data acquisition card (National Instruments PCIe-6321). A pinhole with a diameter of 700 μm was placed in the focal plane before the PMT to reduce out-of-focus light. For OCM imaging, the power at the sample was measured to be 0.8 mW and for FI 0.2 mW.

2.2 Tissue Preparation

2.2.1 Phantom imaging

To validate the setup, three fluorescence phantoms were imaged. For the first phantom, mounting medium (Aqua-Poly/Mount, Polysciences) was mixed (concentration 10%) with a curcumin powder (Sigma-Aldrich). This mixture was stuck onto paper and a region including both paper and curcumin was imaged. As a control case, only mounting medium on paper was imaged. Second, to verify the system’s 5-ALA imaging capabilities, a fluorescence reference target (Starna Scientific, Protoporphyrin IX in PMMA, concentration 20%) was utilized (data not shown). For the third phantom dragon-green micro-beads (polystyrene-based microspheres dyed with dragon-green fluorophore) were imaged, and the results are shown in Figs. 1(d)-1(e). Figure 1(d) shows the OCM en-face image averaged over 10 μm in depth as well as a representative B-scan. Figure 1(e) shows the corresponding FI image including a zoom-in. Figure 1(f) shows an overlay of the OCM (red) en-face projection and the FI image (green).

2.2.2 Curcumin-stained mouse brain tissue

Heterozygous breeding of an AD mouse model, APPswe, PSEN1de9 (APP-PS1, MMRRc stock number 34829, The Jackson Laboratory) was established. Animal experiments were approved by the local ethics committee and by the Austrian Federal Ministry of Education, Science and Research under protocol BMWFU-66.009/0279-WFV/3b/2018. One mouse was sacrificed at the age of 63 weeks. The brain was carefully removed and for one hemisphere a vibratome (Vibratome Series 1000 Sectioning System, The Vibratome Company) was used to cut 100-μm-thick brain sections. The slices were cleared for 15 min following the SWITCH clearing protocol stained with curcumin, and imaged using a laser scanning microscope (Confocal Microscope LSM 700 Zeiss). The other hemisphere was embedded in paraffin and 3 μm thick slices were stained with curcumin and imaged by a conventional Olympus fluorescence microscope (BX51). Amyloid-beta plaques in consecutive sections were then visualized by immunohistochemical [anti-Aβ antibody (clone 6F/3D, diluted 1:100, Dako)] staining for neuropathological confirmation of findings.

2.2.3 Tumor and control human brain samples

Brain tumor samples were retrieved intraproactively. The surgeon used a surgical fluorescence microscope to identify 5-ALA-positive areas and resected the tumor-associated tissue. During tumor resection, 5-ALA-positive and/or -negative tissue samples were routinely collected. Half of the biopsy samples was directly processed for routine neuropathological work-up and the other half was imaged with the multimodal setup (Ethical approval EK 419/2008 - Amendment 04/2018). The fluorescence images were acquired before the OCM volumes to prevent photo bleaching. After OCM and FI, the samples were prepared for histology. Hematoxylin and eosin staining was performed and micrographs were acquired with a slide scanner (Hamamatsu NanoZoomer 2.0 HT). In total, 12 biopsies of 6 patients (age range 47 to 65 years) diagnosed with brain tumors were investigated. According to the surgeon six samples showed strong 5-ALA-positive fluorescence, three samples showed vague 5-ALA-positive fluorescence and three samples were 5-ALA negative. A detailed description of all biopsies can be found in Table 1.

2.3 Data Acquisition and Postprocessing

Processed OCM volumes consisted of 4096(z)×500(x)×500(y) pixels and the fluorescence images comprised 500(z)×500(x)×500(y) pixels. The field of view was 200×200 μm². Data were acquired using a custom made Labview program (LabView 2015, Version 15.0, 64-bit, National Instruments). The OCM data were processed following the steps described by Lichtenegger et al., and en-face projections were generated by averaging intensity over various depths. Fiji was used to generate composition images of FI and OCM results.

2.3.1 Data processing of the brain biopsy measurements

An overview over the data processing pipeline for the tumor samples is shown in Fig. 2. After FI [Fig. 2(1)] and OCM [Fig. 2(3)] acquisition, surface flattening was performed, and attenuation maps [Fig. 2(4)] were generated following previous work. A region of interest consisting of 100 B-scans was chosen manually and for each B-scan, the average attenuation coefficient was calculated [Fig. 2(5)]. The respective average fluorescence signal [Fig. 2(2)] was calculated to create a scatter plot [Fig. 2(6)]. The fluorescence data [dataFI(n), n = 1…N,
The detailed information of the 12 biopsies of 6 patients. The 5-ALA status and the definite diagnosis of patient records are shown. 5-ALA − corresponds to no fluorescence, 5-ALA + to vague, and ++ to strong 5-ALA fluorescence.

<table>
<thead>
<tr>
<th>Patient ID</th>
<th>Biopsy (n)</th>
<th>Fluorescence status</th>
<th>Definite diagnosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>1</td>
<td>5-ALA −</td>
<td>Adjacent brain parenchyma</td>
</tr>
<tr>
<td>I</td>
<td>2</td>
<td>5-ALA ++</td>
<td>Compact tumor and infiltration zone</td>
</tr>
<tr>
<td>II</td>
<td>3</td>
<td>5-ALA ++</td>
<td>Compact tumor and infiltration zone</td>
</tr>
<tr>
<td>II</td>
<td>4</td>
<td>5-ALA ++</td>
<td>Diffuse tumor infiltration and necrotic tissue</td>
</tr>
<tr>
<td>III</td>
<td>5</td>
<td>5-ALA ++</td>
<td>Infiltration zone</td>
</tr>
<tr>
<td>IV</td>
<td>6</td>
<td>5-ALA −</td>
<td>Adjacent brain parenchyma</td>
</tr>
<tr>
<td>V</td>
<td>7</td>
<td>5-ALA +</td>
<td>Physiological tissue and infiltration zone</td>
</tr>
<tr>
<td>V</td>
<td>8</td>
<td>5-ALA +</td>
<td>Physiological tissue and infiltration zone</td>
</tr>
<tr>
<td>V</td>
<td>9</td>
<td>5-ALA ++</td>
<td>Compact metastatic tissue</td>
</tr>
<tr>
<td>VI</td>
<td>10</td>
<td>5-ALA −</td>
<td>Adjacent brain parenchyma</td>
</tr>
<tr>
<td>VI</td>
<td>11</td>
<td>5-ALA −</td>
<td>Adjacent brain parenchyma</td>
</tr>
<tr>
<td>VI</td>
<td>12</td>
<td>5-ALA ++</td>
<td>Compact tumor and infiltration zone</td>
</tr>
</tbody>
</table>

Table 1

Fig. 2 The data processing pipeline. First, OCM and FI images are acquired. Attenuation maps are generated from the OCM data. Attenuation coefficients are averaged in a chosen region of interest. The results from the attenuation analysis are then compared in a scatter plot with their respective averaged FI values.

\[ N = 12 \text{ biopsies}) \] was normalized using \( \text{normFI}(n) = \frac{\text{dataFI}(n) - \text{min(dataFI)}}{\text{max(dataFI)} - \text{min(dataFI)}} \). To calculate the maximum \( \text{max(dataFI)} \) and minimum \( \text{min(dataFI)} \), all data sets were used. The resulting clusters (each point represents one B-scan) were visualized in a scatter plot (5-ALA-positive/negative and infiltration zone). For the statistical analysis, mean values of attenuation and FI data were calculated for each of the 12 samples. Mann–Whitney U tests with Bonferroni correction were performed to test for the equality of the distributions in attenuation and fluorescence data using a significance level of \( p < 0.01 \).

Further cell counting in OCM volumes was performed. Subvolumes consisting of regions of interest of 120 × 200 × 20 \( \mu m^3 \) were evaluated. For each volume three times the same area was evaluated. The cells, appearing as hyperscattering regions in the intensity volumes, were manually annotated using ITK-Snap. The binary output files were then used to perform automatic cell counting using the three-dimensional (3-D) objects counter tool in Fiji.

3 Results

3.1 Curcumin Phantom Measurements

A curcumin phantom was imaged to validate the performance of the system. The excitation and emission spectra of curcumin are shown in Fig. 3(a) along with the filters used. In the fluorescence image [Fig. 3(b)], a clear contrast between pure paper and the mounting medium mixed with curcumin is visible. A representative intensity B-scan image and the intensity averaged OCM en-face projection over 50 \( \mu m \) are shown in Figs. 3(c) and 3(d), respectively. Note that it is challenging to distinguish the curcumin from the paper in the OCM en-face image. Figure 3(e) shows an overlay of the OCM en-face image (red) and the corresponding fluorescence image (green).

3.2 Amyloid-Beta Plaque Imaging in Brain Tissue of an Alzheimer’s Disease Mouse Model

Curcumin-stained brain sections of a mouse model of AD were imaged with the combined setup (Fig. 4). An immunohistochemical and hematoxylin-stained histology image of an adjacent brain region is shown in Fig. 4(a). Amyloid-beta plaques appear as brown structures. Figure 4(b) shows an image of a 3-\( \mu m \)-thick brain section stained with curcumin taken with a commercial fluorescence Olympus microscope. The plaques can be identified as regions with increased fluorescence due to curcumin. A similar region in a 3-\( \mu m \)-thick brain section was imaged with the OCM/FI setup, and the FI results are shown in Fig. 4(c). Again, amyloid-beta plaques can be identified as highly fluorescent structures. A 100-\( \mu m \)-thick brain slice was first imaged with a commercial laser scanning microscope [Fig. 4(d)]. A representative OCM B-scan of the vibratome section is shown in Fig. 4(e). A plaque can be identified as a hyperscattering structure. Figure 4(f) shows an OCM intensity en-face projection over 10 \( \mu m \) underneath the tissue surface. A plaque shows up as a highly scattering feature. The corresponding fluorescence image is shown in Fig. 4(g). The focus was set at the same depth as for the OCM acquisition. Finally, a composite image of OCM (red) and fluorescence (green) image is shown in Fig. 4(h). The amyloid-beta plaque, which is present in both the OCM and the fluorescence image, can be observed in a yellowish color. Another plaque, which is present in the fluorescence image, is barely visible in the OCM image. The dark spots in the fluorescence image are cells and appear as hyperscattering in the OCM images.

3.3 Imaging of 5-ALA Brain Tumor Biopsies

5-ALA-positive tumor samples retrieved intraoperatively were imaged prior to routine neuropathologic work-up. In Fig. 5(a),
the excitation and emission spectra of 5-ALA are plotted. The emission (red) and excitation (blue) filter spectra are indicated by color bands.71 In Figs. 5(b)–5(d), histological images are shown for a 5-ALA-negative area, an infiltration zone, and a 5-ALA-positive area. Tumor area and infiltration zone can be distinguished by the densities of malignant cells. In the infiltration zone, areas of physiological brain tissue with infiltrating malignant cells (indicated by green arrows) can be observed. An average en-face projection over 20 μm and a representative B-scan image of a 5-ALA-positive area are shown in Figs. 5(e) and 5(f), respectively. The averaged attenuation map shows lower values in the left lower corner [Fig. 5(g)]. The combined OCM (blue) and fluorescence image (red) in Fig. 5(h), in the same area, shows a stronger fluorescence signal indicating a higher density of malignant cells.72 An average en-face projection over 20 μm and a representative B-scan image of a 5-ALA-negative area are shown in Figs. 5(i) and 5(j), respectively. Compared to 5-ALA-positive tissue, the tissue morphology is more homogeneous and the intensity in the OCM image is increased.

Cell counting was conducted in three OCM data sets, three times each for one tumor area, a nontumorous area, and an infiltration zone. As shown in Fig. 6(a), the cell count in the 5-ALA-positive area and the infiltration zone was 139% and 15% higher than in 5-ALA-negative tissue, respectively. Attenuation maps for all measured OCM volumes were calculated. Figure 6(b) shows the averaged attenuation coefficients over B-scans plotted over the averaged intensity values retrieved from the fluorescence images. For the statistical evaluation mean values for all 12 data sets were evaluated. The 5-ALA-positive tissue exhibits lower attenuation coefficients and higher fluorescence values when compared to 5-ALA-negative area. The mean attenuation values in the infiltration zone compared to the 5-ALA-negative area showed a trend to be lower but no significance was found ($p = 0.014$). The mean attenuation values in the infiltration zone compared to the 5-ALA-positive area showed a trend to be higher but no significance was found ($p = 0.15$). All other results were statistically significant (Table 2) with a $p$-value of <0.01. The mean values of the clusters which were tested for different distributions are indicated by the color bars in Fig. 6(b).

4 Discussion

We developed a combined visible light OCM and FI setup. Our multimodal system presents a compact and simple design to consecutively acquire OCM and FI data by using a single light source for both modalities in comparison to previously reported work.15,18,19,22–24,26–33 Switching from OCM imaging to FI is achieved by simply flipping two mirrors in the setup. Another advantage of our setup is that it has the possibility to detect a
variety of standard fluorescence dyes as the light source spectrum covers the whole visible range. To image specific fluorescent dyes, the system can be equipped with different filter cubes that are readily available for commercial fluorescence microscopes. In order to further improve the system and to enable fast switching between different dyes, it might be an option to assemble multiple filter cubes that could then be integrated in an automated wheel or stage for quick exchange. At the same time, using the broad visible spectrum for OCM, an axial resolution of 0.88 μm in brain tissue was achieved, which enabled imaging on a cellular level. When investigating amyloid-beta plaques, which are in the range of 10 to 200 μm, a high resolution is crucial.

Further technological improvements of the OCM/FI setup could enable dynamic focusing and 3-D FI. While focusing was done manually in the current implementation, setting the focus for FI and OCM imaging in the future could be done using an automatic z-stage. The implementation of such a stage would also enable the acquisition of confocal image stacks of 3-D FI data. For now, the focus was set at the same position as the OCM image, at the top of the surface of the tissue. The penetration depth into brain tissue using visible light OCM was limited to ~100 μm. Investigating optically cleared tissue would increase the penetration depth, as has been shown in our previous work.

Further, to achieve good FI results, a flat surface would decrease the penetration depth, as has been shown in our previous work.

For the data acquisition, first the FI image and then OCM volumes were acquired to prevent bleaching. For all measurements, the same power, pinhole settings, and objective lens were used. The focus was always set at the tissue surface. Furthermore, imaging was performed in a darkened room. The measured FI intensity is also dependent on the biopsy shape and the autofluorescence. As a next step calculating a relative or quantitative fluorescence intensity will be investigated, following, for example, Valdés et al. For that purpose, a fluorescence phantom is needed as a reference to achieve correct relative intensity values.

Two application possibilities of the setup in the field of neuroscience were presented. First, brain tissue slices of an AD mouse model stained with curcumin were imaged. The investigations revealed that with the same light source amyloid-beta plaques can be specifically identified using FI, and the morphology of the brain tissue, including anatomical features, can be investigated by OCM. The plaques were identified in the OCM images as highly scattering structures and in the FI images as highly fluorescent spots. The additional fluorescence channel confirmed that the highly scattering structures observed with OCM are amyloid-beta plaques. The results were in good agreement with literature and histology.

A commercial 20x objective lens was used for imaging. Using higher numerical aperture (NA) would result in a better fluorescence signal. In future, objective lenses with even higher NA will be used to investigate smaller anatomical features with a better fluorescent response.

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amyloid-beta plaques compared to curcumin. As a next step, the FI channel could be utilized to investigate tau accumulations, which could add additional insights in the mechanisms of the disease.

Second, 5-ALA-positive and -negative human brain biopsies from tumor surgeries were investigated prior to neuropathologic work-up. The 5-ALA-positive regions in the samples were visualized by FI and morphological OCM information of the tissue was acquired with minimal delay. For all data sets, attenuation maps were calculated and analyzed. The attenuation in the 5-ALA-positive areas was significantly lower than 5-ALA-negative brain areas, and the fluorescence signal was significantly higher. Due to autofluorescence of the tissue, in some cases [see Fig. 6(b)], it was hard to distinguish the negative and infiltration zone. In these cases, the multimodal approach using fluorescence and attenuation values may provide better contrast. Using in total 12 biopsies, a preliminary analysis is presented in this article; however, a greater sample size will be needed to achieve a more reliable statistical conclusion. Still, the attenuation values and their decrease in tumorous tissue were in good agreement with the literature. Kut et al. described that tumors infiltrate into white matter, breaking down myelin and therefore decreasing its expression. This ultimately leads to a lower attenuation. OCM-based cell counting results showed an increase of 139% from 5-ALA-negative to 5-ALA-positive tissues and 15% increase in the infiltration zone. Our results

Fig. 5 Imaging of 5-ALA-positive and -negative human brain biopsies. (a) Excitation and emission spectra of 5-ALA. (b–d) Histology of a control region, an infiltration zone, and a core tumor zone. (e) Intensity en-face OCM projection over 20 μm in a 5-ALA-positive area. (f) Representative B-scan image of (e). (g) Average attenuation map over 20 μm in a 5-ALA-positive area. (h) OCM en-face projection (blue) overlaid with the FI image (red). The dotted line in panels (e) and (g) outlines the hyperfluorescent region in panel (h). (i)Intensity en-face OCM projection over 20 μm in a 5-ALA-negative area. (j) Representative B-scan image of (i).
showed that malignant tissue exhibits a higher cellularity compared to nontumorous brain tissue. These data were in agreement with histology. The cell counting results from the OCM volumes were plotted over the averaged intensity values retrieved from the FI images. (*Significance level \( p < 0.01 \).) The mean value of each data set is indicated by a yellow dot.

First, curcumin-stained brain slices of a mouse model of AD were imaged. Amyloid-beta plaques, one hallmark of AD, were specifically identified using FI. Consecutively, the brain morphology was investigated in 3-D using OCM, and by FI, the OCM results could be validated. Second, 5-ALA-positive and -negative brain biopsies were imaged. Average attenuation coefficients and fluorescence intensity values showed significances differences between 5-ALA-positive and -negative brain tissue. Cell counting was performed in the OCM data showing a typical increase in cellularity in malignant tissue. This multimodal approach offers the possibility to investigate microscopic, 3-D features using OCM and at the same time gain tissue-specific contrast by FI. Using OCM in combination with FI may therefore be a versatile and powerful tool for many applications in the field of neuroscience.

Disclosures
None.

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Chapter 5

Discussion and Conclusion

5.1 Discussion and future prospects

A multimodal visible light OCM and Fl system was developed for ex-vivo brain tissue imaging. The system provided sub-micrometer axial resolution in brain tissue using a broad visible spectrum (400-700 nm). Objective lenses with high numerical apertures enabled high transverse resolution imaging. Microscopic features such as cellular structures, fiber tracts and vasculature were imaged non-destructively. An integrated automatic x-y-z translation stage enabled the acquisition of large field-of-view image mosaics. The additional fluorescence channel added tissue specific contrast to the morphology gained by the OCM data. Two applications in the field of neuroimaging were investigated. First, human and mouse brain tissue affected by AD was imaged. One hallmark of AD, namely the Aβ plaques, were identified as highly-scattering features in OCM and after staining as fluorescent structures in Fl. Second, central nervous system tumor samples retrieved from surgery were investigated to differentiate between healthy and malignant areas.

5.1.1 Technical aspects

To achieve sub-cellular resolution for OCM, a supercontinuum laser, providing an extremely broad spectrum, from NKT Photonics was utilized. However, supercontinuum lasers typically have a rather high relative intensity noise [174]. For our setup, a sensitivity of only 89 dB was measured. Compared to standard SD-OCT setups working with a superluminescent diode and similar power in the sample arm, which achieve up to 40 dB more sensitivity, this is a rather low value [174, 175]. However, similarly low sensitivity values have been reported from other visible light setups, see table 1.1 [107, 126, 176, 177]. A new generation of low-noise supercontinuum lasers with higher pulse repetition rate was recently developed, and continuing this trend will further push the usage of supercontinuum sources for OCT applications [178]. Spanning from 425 to 2350 nm, these lasers provide an even broader spectral range than used for our application [175]. In theory, the axial resolution could there-
fore still be increased. However, the main limitations are the commercially available optical components which are not suitable for such extreme spectral bandwidths. Instead of expanding the used bandwidth, a central wavelength located in the ultraviolet region was recently used to achieve nanometer scale resolution. However, using ultraviolet light for OCT only a very shallow penetration depth of around 100 nanometers is achieved [179].

For ex-vivo acquisitions, the imaging speed is rather irrelevant as the tissue does not move and no motion artifacts have to be considered. The used A-scan rate of 30 kHz was rather low, compared to recently reported OCT systems with MHz line rates [180]. However, the imaging speed of our setup might already enable preclinical in-vivo applications and could be used to perform in-vivo imaging in mouse brains, for example through a cranial window or a thinned scull to further investigate AD or intracranial tumor related pathologies [181]. Still, a higher A-scan rate would make the acquisition easier as motion artifacts can be suppressed using higher imaging speeds [182].

Large field-of-view en-face images were acquired using a moving x-y translation stage (MLS203-1, Thorlabs, maximal field of view 11.0 cm × 7.5 cm). The acquisition of the mosaic images was controlled by a homemade Labview program and in total between 25-36 tiles were acquired per image. For generating the large field-of-view en-face projections, the stitching was performed manually. The implementation of a fully automatic reconstruction as it was shown by Lefebvre et al. [4] would greatly reduce the workload. Fluorescence data were acquired at the same focus position as the OCM volumes and the focus was set manually using a z-stage (MZS500-E, Thorlabs) integrated underneath the sample holder. Further, the z-stage could also be used to automatically set the focus in small and large field-of-view images to further automate the acquisition. This z-stage could also be used to acquire fluorescence z-stacks comparable to the 3D OCM data.

Commercial microscope objective lenses with different numerical apertures (4-20 × magnification) were utilized to investigate various features in ex-vivo brain tissue. In the current implementation of the OCM/FI setup, the objective lenses were exchanged manually. Instead, an objective wheel as they are often used in commercial microscopes could be integrated into the visible light OCM setup to enable faster switching between different magnifications. The dispersion mismatch has to be corrected by different amounts of glass for each objective lens. One possibility would be to build multiple reference arms and switch between them [4]. Another possibility would be to create a flexible reference arm where the respective dispersion compensation optics are adapted every time the objective lens is switched [95].
Polarization sensitive OCT (PS-OCT) is a hardware-based functional extension of OCT. The polarization state of light can be altered by various light-tissue interactions and thus can be used to exploit additional image contrast [183]. In PS-OCT, the sample is typically illuminated with one or more polarization states and the polarization state of the OCT signal is detected. This information is used to investigate among others the birefringence and depolarization of the tissue. Birefringence is a property found in fibrous tissues and depolarization can be caused by multiple light scattering of particles of various shape, size, property and density [184]. Baumann et al. described in 2017 the visualization of neuritic plaques using a PS-OCT setup working at 840 nm. The birefringent behavior of neuritic plaques in human AD brain tissue and amyloid accumulations in CAA arteries was investigated [97]. Harper et al. introduced visible light PS-OCT imaging in the mouse eye and showed the benefits of performing high resolution PS imaging [111]. Yashin et al. were able to distinguish non-tumorous and tumorous brain tissue using microstructural co- and cross-polarized OCT [185]. Additional polarization optics and a second spectrometer would enable PS data acquisition using the OCM setup presented in this thesis. High resolution polarization sensitive OCM measurements would provide a novel contrast possibility to image brain features and pathology related changes.

5.1.2 Data processing

For this work, an extensive data processing pipeline was implemented, including background removal, rescaling to k-space, spectral shaping, numerical dispersion compensation, flattening, attenuation calculation, spectroscopic image processing and FI. As a next step, fusing of multiple volumes could be implemented to suppress speckle noise to further improve the OCM image quality [3]. Using averaging the image quality could further be increased to detect smaller fiber tracts and cellular features [186]. The fluorescence measurements were performed in a fully darkened room, to ensure repeatable results. Further, the focus and the power at the sample were set to be the same for each acquisition and the tissue was prevented from bleaching. For data evaluation, the fluorescence values were normalized according to the overall minimum and maximum. Until now, 5-ALA fluorescence was only assessed qualitatively which in a next step could be extended to relative, or quantitative evaluations. One possibility to ensure quantitative fluorescence measurements would be the use of a fluorescent reference target [187, 188].

5.1.3 Tissue processing

A crucial topic is the validation of ex-vivo OCM images by the gold-standard, i.e. histology. Here, one difficulty is the one-to-one correlation of OCM and histology images. After OCM imaging, the ex-vivo tissue has to be fixed and embedded. In these steps, motion artifacts can be introduced easily and fixation introduces tissue shrinkage [189].
preparation step is the sectioning. In this process it is extremely difficult to ensure that the same depth position can be cut under a similar angle as in OCT, as the tissue surface is not completely flat. An algorithm could be developed to perform advanced tissue flattening. Hence, it is important to ensure a flat surface for imaging. One option is to combine the OCM imaging directly with a vibratome and perform histology immediately after the acquisition [4, 190]. In line with other literature reports, we also observed in our experiments that the image quality strongly depends on whether fresh, frozen or fixated tissue was imaged [191]. Intensity and attenuation related changes were obtained over time in fresh brain tissue [192]. Imaging frozen tissue sections introduced highly scattering artifacts in the OCT measurements and less information was gained. As the duration of fixation has showed to have an impact on the tissue properties [193], it might be interesting to use the visible light OCM setup to investigate also the influence of fixation time on the image quality.

A broad visible spectrum was utilized to achieve sub-micrometer resolution. Using visible instead of infrared light to perform OCM measurements has the disadvantage of a reduced penetration depth into the tissue [194]. In this thesis, optical tissue clearing was investigated as a tool to increase the depth range for visible light OCM imaging. A recently developed optical clearing method called SWITCH was applied [173]. The experiments showed that there is trade-off between penetration-depth and SNR. Long clearing times result in washing out too many light-scattering lipid bilayers, which are contributing to the contrast in OCM images [195]. One possibility to overcome this limitation would be to introduce an additional labeling or staining step, for example to specifically highlight the vasculature of the tissue [196]. SWITCH performed well in combination with OCM imaging, as the transparency of the tissue could easily be controlled in comparison to other clearing techniques, such as ethyl cinnamate (ECi) where the tissue turned completely transparent already after a few seconds [197, 198].

5.1.4 Applications

Neuritic amyloid-beta plaques are one biomarker of AD and believed to be crucial for the development of the disease [199]. Investigating these structures is an important topic in AD related research [34, 199]. The diameter of the plaques is in the range of 10-200 µm in human patients such that high spatial resolution is required to visualize them [200]. A great variety of optical microscopy approaches have been used to image amyloid-beta plaques most of which however require tissue processing steps such as staining and sectioning to achieve a suitable contrast [201, 202]. Visible light OCM as presented in this thesis offers the opportunity of rapid volumetric microscopy with high-resolution images based on the intrinsic contrast of the brain tissue. Amyloid-beta plaques were identified as highly scattering structures compared to the surrounding brain parenchyma in human, and mouse brain
tissue. The presence of the plaques and their size observed by OCM was confirmed by histology. The attenuation and spectroscopic behavior of the plaques was analyzed. In the attenuation images, often smaller plaques are better visualized. The spectroscopic OCT imaging approach allowed to investigate stained tissue more specifically in 3D images with molecular contrast without the need for slicing. The setup also allowed to perform plaque load studies in large field-of-view images in brains of an AD mouse model. Thus far, the plaques were segmented manually using ITK-Snap, and in order to facilitate the data analysis automatic plaque segmentation could be developed [203]. Then, using the setup, a fast characterization of AD mouse models could be promoted. Finally, using the FI channel also other plaques were specifically identified. Recently, Gesperger et al. showed the possibility to use PS-OCT for a categorization of plaques into diffuse and neuritic plaques [204,205]. As an extension of Gesperger’s study, the visible light OCM setup could be used for high resolution plaque staging based on the OCM contrast in combination with the FI information.

Utilizing the FI channel of the multimodal setup, curcumin, Alexa Fluor 488 and 5-ALA fluorescence labeled structures were imaged. Using a visible light source enables the excitation and investigation of various other fluorescent stains. Alternative staining protocols for both Aβ and tau imaging such as Thioflavin-S staining could be used to image AD related pathology more comprehensively with our setup [168,169].

Tumor biopsies retrieved during 5-ALA-guided surgery were imaged with the multimodal setup prior to neuropathologic work-up. The combination of attenuation values retrieved from the OCM data and fluorescence intensity data enabled the differentiation of malignant areas from healthy tumor-associated brain parenchyma. The attenuation in tumorous tissue was significantly reduced as compared to the brain parenchyma and the fluorescence intensity was significantly increased. Lower attenuation values can be explained by the fact that tumors infiltrating into white matter and breaking down myelin and therefore decreasing its expression [8]. OCM attenuation values were in good agreement with results found in literature [8,185]. We would like to note that in order to achieve repeatable measurements, the focus has to be set at the same position using the same confocal parameter or that this effect has to be corrected for in post-processing [206,207]. When it comes to in-vivo studies, new approaches, such as using artificial intelligence to distinguish malignant and healthy brain areas could be considered [102]. Also, texture analysis of the glioma tissue could be further used for a more sensitive tumor detection [208,209]. To characterize sensitivity and specificity and to achieve reliable statistics, a larger number of samples has to be imaged. Intracranial tumor samples may then also be analyzed according to their specific type and/or grade. Finally, studies have suggested that intra-tumor heterogeneity might be the key to understand failures in glioblastoma treatments, however further research is needed to understand this complex phenomenon [210,211]. Utilizing the x-y-z-stage to
perform large field-of-view images, the intra-tumor heterogeneity could be investigated in glioblastoma samples by visible light OCM.

5.2 Conclusion

In conclusion, in this thesis a multimodal visible light optical coherence tomography and fluorescence imaging setup was presented to perform imaging of *ex-vivo* brain tissue in sub-cellular resolution. Major advantages of OCM compared to conventional histology are that OCM is only based on the intrinsic scattering contrast of light of the tissue and that OCM imaging allows to non-destructively investigate thick tissue sections. Another advantage of our combined setup is that staining can be applied and further tissue specific contrast can be investigated in the same field-of-view. Hence, OCM presents a promising platform for *ex-vivo* tissue analysis in AD and brain tumors. Moreover, OCM could also be used to investigate pathological features of various other neurological diseases such as multiple sclerosis or Creutzfeldt–Jakob disease. In conclusion, in this thesis a multimodal visible light OCM and FI setup was presented which may be a versatile and powerful tool for many applications in the field of neuroimaging.
Bibliography


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