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Shortwave Infrared Imaging
and Its Translation to Clinically-Relevant Designs

by

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Abstract

Visualizing structures deep within biological tissue is a central challenge in biomedical imaging, with both preclinical implications and clinical relevance. Using shortwave infrared (SWIR) light enables imaging with high resolution, high sensitivity, and sufficient penetration depth to noninvasively interrogate sub-surface tissue features. However, the clinical potential of this approach has been largely unexplored. Until recently, suitable detectors have been either unavailable or cost-prohibitive. Additionally, clinical adoption of SWIR imaging has been inhibited by a poor understanding of its advantages over conventional techniques. For fluorescence imaging in particular, there has further been a perceived need for clinically-approved contrast agents. Here, taking advantage of newly available detector technology, we investigate a variety of biomedical applications with SWIR-based imaging devices. We describe the development of a medical otoscope and our clinical observations using this device to evaluate middle ear pathologies in both adult and pediatric populations, showing that SWIR otoscopy could provide diagnostic information complementary to that provided by conventional visible otoscopy. We further describe fluorescence detection of an endogenous disease biomarker in animal models including nonalcoholic fatty liver disease and cirrhotic liver models and models of a neurodegenerative disease pathway. While this biomarker has been known for decades, we describe a method for its noninvasive detection in living animals using near infrared and SWIR light, as opposed to its conventional ex vivo detection. Furthermore, we show that SWIR image contrast and penetration depth are primarily mediated by the absorptivity of tissue, and can be tuned through deliberate selection of imaging wavelength. This understanding is crucial for rationally determining the optimal imaging window for a given application, and is a prerequisite for understanding which clinical applications could benefit from SWIR imaging. Finally, we show that commercially-available near infrared dyes, including the FDA-approved contrast agent indocyanine green, exhibit optical properties suitable for in vivo SWIR fluorescence imaging, including intravital microscopy, noninvasive, real-time imaging in blood and lymph vessels, and tumor-targeted imaging with IRDye 800CW, a dye being tested in clinical trials. Thus, we suggest that there is significant potential for SWIR imaging to be implemented alongside existing imaging modalities in the clinic.

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Chapter 1

Introduction to shortwave infrared biological imaging as a potential clinical technology

1.1 Translating preclinical techniques into clinical technologies

Successfully implementing a new clinical technology is one of the most rewarding accomplishments that a researcher in the biomedical field can attain. With potential rewards like increasing the accuracy of diagnostics, enabling better disease management, and improving patients’ health outcomes, there are many reasons to feel motivated along the journey. The process, however, is long, requires the input of many, and can feel fraught with many setbacks among the forward progress. A technology traverses from basic research to drug discovery to preclinical tests, through clinical evaluation, then launch and monitoring; yet, rarely is this process linear—often requiring iteration between steps before advancing—and demands both foresight and hindsight to move efficiently forward.

As a result, most clinical technologies begin many years (often decades) before clinical trials with humans can ever be performed.\(^1\)\(^-\)\(^4\) The first challenges that must be overcome begin with the inevitable complications of new discoveries in basic research, where certainty or “de-risking” is a desired end goal rather than a feature of the work. Next comes establishing cross-disciplinary collaborations, with difficulties including balancing cultural differences between basic scientists and clinicians, and the different goals and reward mechanisms for each stakeholder, also including patients and payers. Finally, navigating complex regulatory affairs poses its own set of challenges, from materials transfer agreements and intellectual property rights, to clinical trial design and data monitoring. Each of these steps further requires a vast array of resources from workforce, to funding, to infrastructure.

The setting of this thesis is largely in the very first step of the process—within basic science and translational research. The motivation is to enable both preclinical and clinical advances in imaging techniques. Chapter 2 will describe the development of a diagnostic device from the rational assembly of individual optical components into a handheld tool, proof of concept testing in the lab, proof of concept testing in the lab, all the way through initial observations in patients under Institutional Review Board (IRB) approvals between an academic institution and a surgery center. Chapter 3 details a new technique for more efficient preclinical discovery related to studying liver and neurodegenerative diseases, and the validation of a potential biomarker for clinically monitoring
their progression, although not translated so far here. **Chapter 4** begins the story of very early stage discovery, unveiling critical design principles for imaging in a new wavelength window, and lays the groundwork for the results presented in the following chapter. **Chapter 5** builds on this work, showing that one of the only remaining barriers to translating this technique from preclinical to clinical evaluation has been merely a perception and partially a misconception, and demonstrates the immediate clinical readiness of this tool. Each of these chapters builds on decades of prior basic science, preclinical work, and clinical examples, some of the relevant fundamentals of which are introduced below, first as a case study (**Section 1.2**) and then as a literature and field-specific review for our particular technology (**Section 1.3**). With this context, a more technical thesis overview is presented in **Section 1.4**.

### 1.2 A clinical translation case study in biological imaging

Biological imaging is a method of interrogating human, animal, or plant tissue with light. In general, a biological tissue sample is illuminated with a particular wavelength or wavelengths of light, and the diffusely reflected, transmitted, or emitted light is analyzed to determine the two- and sometimes three-dimensional composition and/or function of the sample. At present, most optical imaging uses visible (400–700 nm) and/or near infrared (NIR, 700–1000 nm) light for biological interrogations. In this wavelength region, many different biomolecules absorb, transmit, reflect, and emit light uniquely, enabling the extraction of countless biological parameters from a tissue sample. For example, tissue absorption is primarily due to hemoglobin, melanin, lipids, and water, each with their own unique spectral profile. In the visible and NIR, oxygenated and deoxygenated hemoglobin are the primary physiologically active optical absorbers, and the functional status of tissue can be measured based on their relative concentrations.5–8 Tissue autofluorescence (or emission) is also prevalent at visible wavelengths, reporting the position of molecules such as NADPH, flavins, and collagen.

At this point, many optical imaging techniques are well-established, for both in vitro investigation of cells and in vivo investigations of tissues, and have proven valuable for a variety of diagnostic applications in humans.9–11 In particular, fluorescence imaging, in which the emitted light is observed, has emerged as a powerful tool for preclinical in vivo imaging and as a promising clinical technology, particularly for surgical guidance.12–17 Here described is an example of a biological imaging technique—NIR fluorescence imaging—which building upon decades of prior basic research, has successfully navigated the journey to clinical translation for certain applications, and is still being vigorously evaluated for many more.

However, before NIR fluorescence imaging could be established, several technological requirements had to be met. In order to carry out an optical imaging experiment, one needs a light source of sufficient power and at the right wavelength or wavelengths (e.g. a broadband lamp, an LED, or a laser), the correct optics to deliver the light (e.g. fibers or diffusers) and
subsequently collect the light (e.g. lenses and filters) onto a detector or camera. In fluorescence imaging, often an exogenous light-emitting fluorophore is also introduced into the tissue to target and highlight a specific biological phenomenon that may not naturally send out its own signal. Thus, for these measurements, one also needs a light-emitting and possibly targeted probe (e.g. an organic molecule dye or a nanoparticle, such as quantum dots, rare earth-doped nanoparticles, or metal clusters). Finally, but perhaps foremost, one needs a key biological question or interest motivating the study.

For NIR fluorescence imaging, the motivations behind its development were to expand vision beyond what the eye itself can detect, and also to image deeper into tissue without invasive interventions. In general, there are two main limitations to the effectiveness of optical imaging. Sometimes, an application is “signal-limited,” in which case there is insufficient light reaching the detector to form an image. This can be the result of insufficient light source power, insufficient probe brightness, insufficient detector sensitivity, and/or too much attenuation of the signal by the sample (via absorption or scattering of light). In other cases, an application is “contrast-limited,” in which case the image is too “blurry” to resolve the structures of interest, generally as a result of tissue scattering, high background signal, and/or insufficient signal to be above the detector noise threshold. Both absorption and scattering of light by tissue are high at visible wavelengths of light, thus the eye and other visible light-based technologies are generally restricted to imaging superficial features, and require invasive procedures to expose deeper tissue structures for observation.

The NIR, on the other hand, has been identified as an optimal imaging window for in vivo studies requiring high penetration depths. The absorptive components of tissue share a common absorption minimum between approximately 650 nm and 900 nm, and tissue scattering continually declines from the visible into the NIR, maximizing light transmission. At this point, NIR fluorophores are also widely available (i.e. commercially available) and can be detected on high-performing, inexpensive silicon detectors. Overall, the equipment for these optical measurements is now often simple and portable, interrogations can be carried out without contacting or perturbing the tissue over a wide field of view, and the resulting data is generally easy to interpret, all of which have made NIR fluorescence imaging a powerful technique in clinical settings.

Decades of iterating through research and development on these topics—from tissue optical properties to optical devices to fluorescent probes—has enabled the NIR-based medical devices that are emerging today. The majority of these iterations took place in vitro in cell studies, using ex vivo tissue samples or tissue phantoms, or in vivo in preclinical animal models. These preclinical studies have provided, and are still unveiling, valuable insight into mechanisms of disease and their potential treatments, as in atherosclerosis, Alzheimer’s disease, and many others. However, NIR fluorescence imaging is now being tested in humans in well over 300 clinical trials for applications such as angiography and perfusion...
assessment in reconstructive and bypass surgeries, metastatic lymph node mapping and lymphatic transport measurements in lymphedema, and cancer localization and surgical margin assessment.\textsuperscript{30–36} The FDA has granted approval for only one NIR dye—indocyanine green (ICG). Approved since 1959, ICG has been used clinically for over 50 years for determining cardiac output, hepatic function and liver blood flow, and retinal angiography on the basis of its strong absorption properties (giving it a dark green color).\textsuperscript{37} It is administered at concentrations as high as 2.5 mg/mL (up to 2 mg/kg total in adults), and although this approved form of ICG has no functional group for molecular targeting, it does rapidly associate with plasma proteins like albumin in the blood, forming an excellent, nonspecific vascular agent.\textsuperscript{37–39} Only one other NIR dye, namely IRDye 800CW, has safety and toxicity profiles reported.\textsuperscript{34,38,40,41} Many preclinical imaging studies and clinical trials employ this and other contrast agents with functional groups (e.g. antibodies, antibody fragments, peptides, or sugars) for targeted imaging of cancer, vascular disease, infection, and other targets.

NIR fluorescence imaging is now routinely used in a variety of ophthalmologic indications. For example, fundus autofluorescence imaging is a technique for noninvasively imaging the endogenous ocular fluorophore lipofuscin, present in the retinal pigment epithelium.\textsuperscript{42,43} Over the past decade, fundus autofluorescence imaging has become an essential tool for obtaining earlier diagnoses and better predictions of the progression of age related macular degeneration, macular dystrophies, retinitis pigmentosa, white dot syndromes, retinal drug toxicities, and various other retinal diseases.\textsuperscript{42} Clinicians now have their choice of multiple commercially available imaging systems.\textsuperscript{44} NIR fluorescence imaging using exogenous contrast agents has been slow to become standard of care for a specific indication, however, it is now common to employ ICG in angiography of the eye alongside, or instead of, the conventional visible dye fluorescein. Fluorescein angiography is one of the best ways to examine retinal blood vessels, while ICG is now recognized as a better method of evaluating the deeper choroidal blood vessels that are often difficult to visualize with fluorescein alone.

Building on the clinical utility demonstrated by fundus autofluorescence imaging and ICG angiography of the eye, adoption of NIR fluorescence imaging more broadly among different medical specialties is an anticipated next step, particularly in oncology. For many of these indications, translation through clinical trials is currently as a combinational, investigational product consisting of both a device (the imaging instrument) and a drug (the exogenous contrast agent). While this approval process presents a greater challenge than for imaging endogenous fluorophores inherent to the tissue, the clinically-accessible dye ICG is accelerating adoption in both blood and lymphatic vasculature applications in off-label, investigational studies.\textsuperscript{38}

Thus, NIR fluorescence imaging is an apt example of a technology which has built on decades of fundamental and preclinical research before becoming a routine clinical tool in ophthalmology. This initial clinical adoption has further opened the door for more advanced imaging, now being evaluated in clinical trials within nearly every medical discipline.
1.3 Biological imaging in the shortwave infrared

Building on the same body of scientific results as NIR fluorescence imaging, it has recently been shown that extending optical measurements into shortwave infrared (SWIR, 1000–2000 nm) wavelengths offers several further advantages for in vivo imaging applications. For example, the SWIR regime features absorption from tissue constituents such as water (near 1150, 1450, and 1900 nm), lipids (near 1040, 1200, 1400, and 1700 nm), and collagen (near 1200 and 1500 nm) that are more prominent than corresponding features in the visible and NIR regions. The enhanced sensitivity to these chromophores enables better characterization of changes in their concentration, with recent spectroscopy-based examples in detecting and monitoring cancerous tissues, burns, and intestinal ischemia, distinguishing skin bruises from surrounding tissue and discriminating histologically vulnerable and stable plaques of blood vessels in vivo. In addition, SWIR light offers greater transmission through biological tissue than visible or NIR light, providing sufficient optical penetration depth to noninvasively interrogate changes in subsurface tissue features. The increase in transmission is mostly due to decreased scattering of photons, which is minimized by imaging at the longest wavelengths possible. As scattering misdirects photons from the direct path to the detector, avoiding scattering can also improve spatial resolution. Furthermore, tissue autofluorescence, which creates interfering background signal to an exogenous fluorophore, is primarily emitted at visible wavelengths in tissue in response to ultraviolet or visible radiation; autofluorescence can be mostly avoided using NIR and SWIR excitation light.

Despite these advantages, the SWIR regime has so far been underutilized in optical imaging, and in particular, medical devices have been mostly limited to exploratory or proof-of-principle in nature. One reason for this is the limited availability of technological requirements that are distinct from NIR imaging. Whereas both visible and NIR light can be measured using ubiquitous silicon-based cameras, these sensors cannot detect longer SWIR wavelengths, which require a different sensor material (e.g. indium gallium arsenide, indium antimonide, or mercury cadmium telluride). SWIR detection technology, once cost-prohibitive and restricted by military regulations (e.g. ITAR, the International Traffic in Arms Regulations, in the U.S.), has only recently become readily available for commercial use and research purposes. Manufacturers have made a recent effort to develop cameras which can be classified as dual use, with now over 30 models classified as such by the U.S. Department of State. Simultaneously, advances in detector fabrication are producing high performing sensors with significantly smaller form factors and reduced weight. These technological advances and an increasing supply of SWIR detectors has enabled a price drop of roughly an order of magnitude over the time period from 2010 to 2014.

Furthermore, an absence of high-quality, commercially-available SWIR fluorophores, and the perceived need for a clinically-approved contrast agent has inhibited full realization of
this technique beyond preclinical demonstrations. Within the past decade, a wide variety of biologically-compatible SWIR emitters have emerged, including organic dyes with peak emission in the SWIR,\textsuperscript{62-65} carbon nanotubes,\textsuperscript{47,66} quantum dots,\textsuperscript{67-69} rare-earth-doped nanocomposites,\textsuperscript{70} and gold nanoparticles.\textsuperscript{71} More recently, it was also shown that NIR dyes, which are commercially available and approved for clinical use, can be used for many SWIR imaging applications (expanded on later in this thesis).\textsuperscript{72} Thus, with SWIR detection technology and SWIR fluorophores only recently becoming available, the advantages over conventional methods have yet to be fully demonstrated or realized. With these recent technological developments, SWIR fluorescence imaging is rapidly evolving at present, and we believe, pushing toward clinical utilization. This thesis expands on a series of studies which aim to edge this technology closer to the clinic, answering key fundamental questions on SWIR tissue optical properties and lowering some of the existing barriers to translation.

1.4 Thesis overview

Thus, optical imaging provides advantageous features for both preclinical biological investigations and clinical applications. Building on decades of NIR optical imaging development, SWIR imaging is now emerging as a noninvasive technique for interrogating subsurface tissue features with unique biological contrast. Here, taking advantage of newly available SWIR technology, we demonstrate a number of applications which illustrate the significant preclinical and clinical potential waiting in the SWIR spectral region of the light spectrum.

In \textbf{Chapter 2} and \textbf{Chapter 3}, we explore the use of endogenous SWIR signals of the tissue to provide disease-correlated contrast in an image. The use of endogenous signal in these applications lowers the barrier to clinical translation, as only an imaging device must be approved for human studies, as opposed to both the device and a contrast agent (i.e. drug). Thus, in \textbf{Chapter 2}, we are able to describe the development of a medical otoscope from its proof of principle in a 3D-printed model, all the way through our initial clinical observations using our device to evaluate middle ear pathologies in adult and pediatric populations. We show that SWIR otoscopy has the potential to provide valuable diagnostic information complementary to that provided by conventional visible pneumotoscopy. In addition, use of a SWIR otoscope does not require significant clinician training, as the ergonomics, visual output, and operation are similar to that of a conventional otoscope. In \textbf{Chapter 3}, we begin proof of concept testing of a disease biomarker in animal models, including nonalcoholic fatty liver disease (NAFLD) and cirrhotic liver models, and models of a neurodegenerative disease pathway. While this biomarker has been known for decades, we describe a method for its noninvasive detection in living animals, as opposed to its conventional \textit{ex vivo} detection. This advantage could
significantly reduce the number of animals required per preclinical study, and could potentially enable more rapid and less invasive clinical determinations as well.

In Chapter 4 and Chapter 5, we introduce an exogenous agent into the system to highlight a particular biological feature or process more specifically. While the use of this external agent often delays clinical access due to the need to prove its safety to humans, the advantages can in the long run be well worth it for increased accuracy, sensitivity, and/or specificity. We first show in Chapter 4 a mechanistic understanding of how image contrast in this type of imaging evolves across SWIR wavelengths. Using 3D tissue phantoms, an in vivo animal model, a theoretical model, and ex vivo biological tissue, we obtain results which suggest that water is the dominant optical property contributing to image contrast and penetration depth in the SWIR. This understanding is crucial for rational determination of the optimal imaging window for a given application, or in other words, is critical to understanding which clinical applications could potentially benefit from using SWIR light over conventional techniques, such as NIR imaging.

We further eliminate a major barrier to clinical adoption of SWIR imaging technology in our results described in Chapter 5. Here we show that despite the perceived need for clinically-approved exogenous contrast agents, commercially available NIR dyes, including the FDA-approved contrast agent ICG, exhibit optical properties suitable for in vivo SWIR fluorescence imaging, including intravital microscopy, noninvasive, real-time imaging in blood and lymph vessels, imaging of hepatobiliary clearance, and tumor-targeted SWIR imaging. Our findings suggest that SWIR imaging can be implemented alongside existing imaging modalities in the clinic, simply by switching the detection of conventional NIR fluorescence systems from silicon-based NIR cameras to emerging indium gallium arsenide SWIR cameras.
Chapter 2

Endogenous SWIR reflectance imaging of middle ear pathologies in the clinic

2.1 Introduction: challenges in diagnosing otitis media

Otitis media is one of the most common reasons for pediatrician visits, antibiotic prescriptions, and surgery in the pediatric population.\textsuperscript{73–76} Second in diagnosis frequency only to acute upper respiratory infection, at least 80% of U.S. children will have experienced one or more episodes of otitis media by the age of 3 years.\textsuperscript{77–80} The term otitis media encompasses a variety of inflammatory conditions of the middle ear, such as acute otitis media (AOM), otitis media with effusion (OME), and chronic suppurative otitis media (CSOM).\textsuperscript{81} These conditions are closely related and can overlap, but most importantly they are linked by presence of middle ear fluid or in the case of CSOM, fluid that drains out of the middle ear through a tympanic membrane perforation or a tympanostomy tube.

While CSOM is easily identifiable due to this fluid discharge, diagnosis of OME and AOM is not as straightforward.\textsuperscript{82,83} Successful diagnosis of otitis media is estimated at 51% for U.S. pediatricians, with over-diagnosis of AOM occurring 26% of the time.\textsuperscript{82,84,85} The difficulty in identifying middle ear effusion is largely responsible for both this over-diagnosis of cases of AOM and the frequent under-diagnosis of cases of OME.\textsuperscript{82,86} Over-diagnosis of AOM has made otitis media a primary factor in increased antibiotic resistance.\textsuperscript{87–89} On the other hand, OME is the most common reason for conductive hearing loss in the pediatric population and has been associated with behavioral and learning difficulties.\textsuperscript{90} Recent research on cases of reversible conductive hearing loss has even identified changes that occur in the neurological pathways that persist long after the conductive hearing loss has resolved.\textsuperscript{91} Failure to diagnose AOM or OME can thus lead to long-term hearing impairment, intracranial complications, a delay in language acquisition, or formation of destructive skin growths, known as cholesteatoma, which must be treated by surgical excision.\textsuperscript{76,92–94}

Otoscopy is the most widely used technology for assessing middle ear effusions, and has been the diagnostic workhorse for external auditory canal and middle ear examinations for over a century. Using this tool, physicians assess middle ear pathologies based on the appearance of the tympanic membrane, or ear drum, and its mobility against hand-generated pneumatic pressure. However, otoscopic examinations are known to suffer from subjective
interpretations, especially in the hands of inexperienced practitioners; studies have shown limited accuracy in assessing otitis media, with correct interpretation by only 46% of general practitioners, 51% of pediatricians, and 76% of otolaryngologists. The limitations of otoscopy are due in part to difficulty seeing beyond the tympanic membrane, which despite being semi-translucent, reflects, absorbs, and scatters incident light at the surface, limiting signal penetration to deeper middle ear structures or fluid. A study by Rosenfeld of 135 acute otitis media cases diagnosed by U.S. primary care practitioners found that 40 were false positives, 35 of which had no middle ear effusion.

Here we describe the development of an otoscope sensitive to SWIR light for more objective diagnoses of trans-tympanic middle ear pathologies. The endogenous contrast generated with SWIR light is unique from that observed with traditional visible otoscopy in two key ways. First, deeper tissue penetration is achievable with SWIR light than with visible light, allowing better visualization of middle ear structures through the tympanic membrane. In addition, strong SWIR light absorption by water molecules provides more evident contrast between the presence and absence of middle ear effusions. Meanwhile, a SWIR otoscope maintains the ergonomics, visual output, and small footprint of a conventional otoscope, making it familiar to clinicians and thus readily translated to the clinic. We suggest that a SWIR otoscope could complement conventional otoscopic diagnoses by providing access to the endogenous optical properties of the middle ear across an expanded wavelength range.

2.2 Optical characterization of middle ear tissue

We first aimed to characterize the interaction of SWIR light with the tissues of the middle ear. We obtained both human tympanic membrane tissue and human middle ear fluid samples ranging in consistency from serous to mucoid and measured their optical properties. Human tympanic membrane tissue samples (approximately 1 mm sections) were collected intraoperatively from pediatric patients (ages 0–18 years) during a typical tympanoplasty procedure. Measuring the attenuation spectrum, we find that light attenuation decreases with increasing wavelength (Fig. 2.1A), consistent with the inverse power law relationship between wavelength and scattering of photons measured previously for skin. Absorption beyond 1300 nm is also evident in the tympanic membrane attenuation spectrum due to water in the tissue, however, the overall transparency of the tympanic membrane is greater at SWIR wavelengths than in the visible.

The enhanced transmission of SWIR light through the tympanic membrane should benefit visualization of the middle ear structures, such as the highly reflective ossicles and the promontory, underlying this tissue. In particular, reduction in scattering at SWIR wavelengths should benefit image contrast and resolution, as reflected light travels more directly through the tympanic membrane to the detector after reflecting off of the structures of interest (Fig. 2.1B,C).
We investigated this idea further using a sector star resolution target and a liquid Intralipid® tissue phantom. The phantom was prepared by diluting 20% Intralipid® (Baxter Healthcare Corporation, Deerfield, IL, USA) to 2% Intralipid® in water. This was added to a glass-bottom microwell dish (MatTek Corporation, Ashland, MA, USA) between 0.5-2 mm deep. The dish was placed on top of the sector star resolution target and images were acquired in reflection geometry (illumination and detection from above the phantom). We observed that adding Intralipid® to the top of the resolution target had no effect on the resolution of the target below, up to 2 mm thickness of phantom (Fig. 2.1E). This was true for both broadband SWIR detection, which had a resolution of 21 lp/mm with and without phantom, and 1300 nm long-pass detection, which had a resolution of 20 lp/mm with no phantom and 19 lp/mm with 2 mm phantom. For the visible imaging system, on the other hand, the resolution was strongly dependent on the thickness of the phantom. Because the pixel size of the CMOS camera is much smaller (3.6 μm) the imaging resolution is initially higher, even through the phantom, until at some point the transmission of the phantom is too low to observe the resolution target below (Fig. 2.1D). Resolution values were 85 lp/mm for no phantom, 53 lp/mm with approximately 0.5 mm of phantom, 38 lp/mm with approximately 1 mm of phantom, and at 2 mm of phantom the target could not be resolved. Based on these results, we expect that using SWIR light for otoscopy will improve visualization of anatomy behind the thin tympanic membrane.
We next characterized human middle ear fluid samples for their light attenuation properties. Human middle ear fluid samples (30–200 microliter volumes) were collected intraoperatively from pediatric patients during myringotomy and placement of pressure-equalizing tubes for standard treatment of recurrent otitis media or persistent middle ear effusion. See Sec. 2.7 for sample collection, storage, and preparation details. Spectroscopic characterization confirms strong attenuation of specific spectral bands of SWIR light (Fig. 2.2A). Water in the fluid gives rise to absorption observed in small features around 970 nm and 1180 nm, and strong features around 1440 nm and beyond 1800 nm which correspond to the vibrational overtone of the O-H bond, and the first overtone of the O-H stretching respectively. Thicker-consistency mucoid middle ear fluid samples contain less water relative to thin samples, but water absorption is still the dominant cause of attenuation (Fig. 2.2B). Two peaks are also distinguishable at 540 nm and 575 nm from the absorption of oxygenated hemoglobin, but middle ear fluid generally appears translucent by eye due to minimal absorption of chromophores at visible wavelengths. Light scattering processes that occur within the viscous mucous also cause attenuation, particularly at visible wavelengths of light (400–700 nm). The strength of this wavelength dependence is a complicated function of the geometry of the

![Figure 2.2: Optical properties of human middle ear fluid.](image)

The attenuation of light through a sample of middle ear fluid shows strong absorption between 1400 and 1550 nm due to water content (A). This absorption of SWIR light causes the fluid to appear black in a SWIR image, whereas it is translucent with visible imaging or by eye. Serous, or thin, middle ear fluid (B, thin solid line) shows strong absorption between 1400 and 1550 nm and beyond 1800 nm from water present in the fluid. Centrifuging such a sample isolates this absorptive component from the scattering particles (e.g. cells), and attenuation of the supernatant solution (B, bold solid line) shows the strong water absorption isolated from the majority of the attenuation due to scattering. Absorption between 1400 and 1550 nm is less for a thicker, or mucoid, fluid sample (B, dashed line) due to less water; however, water absorption is still the dominant cause of attenuation.
scattering particles, which vary with the composition of individual fluid samples; however, in general, the attenuation steadily rises with decreasing wavelength. In the thickest samples, attenuation is as strong at visible wavelengths due to scattering, as it is in the SWIR due to water absorption. We therefore expect SWIR wavelengths to provide better optical contrast than visible and NIR imaging in the detection of middle ear fluid, due to the strong absorption endogenous to water molecules in the fluid, regardless of fluid consistency.

2.3 Proof of concept imaging in a 3D-printed model

To take advantage of decreased SWIR light scattering, we designed a SWIR otoscope prototype capable of imaging the middle ear with 900–1700 nm light (Fig. 2.3). Using a 5 mm speculum, the device images an approximately 10.5 mm diameter circular field of view with 45 μm maximum resolution. The SWIR otoscope is comprised of a fiber-coupled broadband halogen light source, a medical speculum that guides the device into the ear canal (Welch Allyn, Skaneateles Falls, NY, USA), and a lens system to focus the diffusely reflected light onto an Indium Gallium Arsenide (InGaAs) array detector. The lenses used in these experiments were Edmund Optics (Barrington, NJ) near-infrared achromatic doublet lenses with 75 mm and 100 mm effective focal lengths. The detector is a Xenics (Leuven, Belgium) XS Trigger InGaAs detector with a 320 x 256 array of 30 μm pixels and 14 bit analog to digital conversion. A filter holder in front of the sensor allows easy adaptation with various shortpass, longpass, and band-pass filters, enabling optimization of the device sensitivity for a variety of applications.

Initially, we tested the performance of the SWIR otoscope in a 3D-printed middle ear model (Fig. 2.4A). The middle ear phantom was 3D-printed using computer-assisted design software Solid Edge ST7 (Siemens, Plano). The shapes and angles were modeled to resemble the normal middle ear anatomical configuration based on previously described anatomical measurements. A Makerbot Replicator Desktop 3D Printer (Makerbot Industries, LLC) was used to fabricate the model.

Figure 2.3: Schematic and characterization of the SWIR otoscope prototype. The SWIR otoscope prototype is composed of a compact InGaAs SWIR detector, a filter holder, a pair of achromatic doublet lenses, a fiber-coupled light source, and a disposable medical speculum (A). Analysis with a concentric square calibration target (B) and a sector star resolution target with 36 bars over 360° (C) indicates that the imaging system can achieve a maximum resolution of 22 lp/mm or 45 μm and de-magnifies the object by a factor of 0.76 onto the sensor with minimal aberrations.
used with polylactic acid (PLA) as the printing filament. To model the semi-rigid and angled nature of the external auditory canal, a 3D-printed PLA cast was made and high performance platinum silicone (Dragon Skin FX-Pro, Smooth On, Inc. Macungie, PA) was used for the canal castings. The silicone was also cast into a thin, translucent sheet approximately 0.5 mm thick for the tympanic membrane and lining of the 3D-printed middle ear cavity, representing the mucosa. A small hole in the back of the middle ear cavity was used to add fluid via a syringe into the phantom behind the silicone tympanic membrane while recording video using the SWIR or a visible otoscope inserted in the model canal.

We then evaluated the effectiveness of the SWIR otoscope at detecting fluid in the 3D-printed middle ear model. In our model, orange juice was selected as a phantom for middle ear fluid.

![Figure 2.4: Fluid visualization in a 3D-printed middle ear model.](image-url)

A model middle ear cavity and ossicle were 3D-printed from polylactic acid, covered with a thin tympanic membrane phantom, a thick silicone ear canal, and cased with a model outer ear and lobe (A). Orange juice was selected as a phantom for middle ear fluid, as the attenuation (B, dashed line) includes both water absorption (B, thin line) and scattering of visible light exhibited by middle ear fluid (B, thick line). As the model middle ear is filled with fluid phantom, a subtle intensity change behind the membrane can be observed using visible otoscopy (C, top). The SWIR otoscope provides a more striking contrast between presence and absence of fluid, particularly with a 1300 nm longpass filter which selectively passes the wavelengths of maximum fluid absorption (C, bottom). Monitoring the Weber contrast of the model ossicle shows that the contrast of this feature is poorer for visible otoscopy (D) compared with SWIR otoscopy (E). With SWIR otoscopy, the contrast increases four-fold to 1.2 compared with contrast in the absence of fluid of 0.30; this is a significant improvement over the two-fold contrast increase from 0.36 to 0.72 observed using visible otoscopy (F).
fluid, as it has representative spectroscopic properties, particularly the dominant water absorption features and scattering of visible wavelengths of light (Fig. 2.4B). Using visible otoscopy, slow addition of fluid into the ear model is barely perceptible; reflected light intensity from the middle ear space decreases by only 29% (Fig. 2.4C, top). The malleus, which is a superficial anatomical structure, experiences relatively less change in intensity as fluid is added. As a result, the Weber contrast of this feature, defined as the difference between the feature intensity and the background intensity all divided by the background intensity, is doubled from 0.36 to 0.72 when the malleus is surrounded with fluid (Fig. 2.4D, F).

On the other hand, using a 1300 nm longpass filter and SWIR detection, the SWIR otoscope can selectively image between 1300–1700 nm where absorption of middle ear fluid is maximal. Addition of fluid to the model obstructs any features lying behind the superficial structures at the tympanic membrane, and reduces the overall reflected light intensity by 73% (Fig. 2.4C, bottom). This notable intensity reduction causes contrast of the malleus to increase four-fold from 0.30 to 1.2 when surrounded by fluid (Fig. 2.4E, F), a significant improvement over the visible case.

Based on these models and spectroscopic characterization of the middle ear tissues, we therefore expect the imaging contrast of middle ear anatomy and fluid in vivo to be significantly enhanced with SWIR light compared to what is currently achievable with visible otoscopy. The relative level of SWIR absorption of middle ear fluid, which provides a striking contrast, could facilitate a clinician’s determination of the absence or presence of fluid in the middle ear.

2.4 Imaging middle ear anatomy in adults

We tested in vivo performance of the SWIR otoscope on ten adults (18 ears) and show that the increased penetration of SWIR light through the tympanic membrane enables the SWIR otoscope to image middle ear anatomy with exceptional detail compared to visible otoscopy (Fig. 2.5). The external auditory canal was used as the optical access for visible and SWIR imaging of middle ear structures. The speculum was inserted into the canal within approximately 2 cm of the tympanic membrane and the middle ear was illuminated with the light source. Reflected light was collected by the optical system either broadband or filtered through a bandpass, longpass, or shortpass filter.

Using traditional visible otoscopy, the only clearly identifiable features besides the tympanic membrane are typically those that are large or superficial, such as the cochlear promontory (observed in 15/18 cases), which is formed by the outward projection of the first turn of the cochlea against the posterior wall of the middle ear cavity, and the malleus (observed in 18/18 cases) which lies directly under the tympanic membrane. In a few cases, the tympanic membrane is thinner, and light reflection off of smaller or deeper anatomy such as the incus (9/18 cases), stapes (2/18 cases), and stapedial tendon (2/18) is identifiable.
We show that in a typical human middle ear, the SWIR otoscope images landmarks of the entire ossicular chain (middle ear bones), including the incus and stapes (16/18 and 11/18 cases respectively), in addition to the malleus (18/18 cases). In 12 out of 18 cases it was also possible to image the supporting stapedial tendon. Besides the ossicular chain, the SWIR otoscope can also clearly image the cochlear promontory (18/18 cases), and visualization of the round window niche—one of the two openings from the middle ear to the inner ear—was also achieved (16/18 cases). Furthermore, the SWIR otoscope could identify the chorda tympani (9/18 cases), a branch of the facial nerve that carries taste sensation from the anterior two-thirds of the tongue. The location of the chorda tympani is generally obstructed by a thicker region of the tympanic membrane with visible imaging (visible in 4/18 cases). See Table 2.1 for a summary of anatomy visualization.

Table 2.1: Number of ears in which each middle ear anatomical structure was identifiable using visible versus SWIR otoscopy. A two-proportion z-test was used to assess whether the difference between the visible anatomy visualization and SWIR anatomy visualization is significant. At a significance level of 0.05, the proportion of anatomy that could be visualized using SWIR otoscopy was significant for the incus, stapes, stapedial tendon, cochlear promontory, round window, and chorda tympani. The difference was insignificant for the malleus which was easily visualized by both techniques.
Thus, SWIR otoscopy enables visualization of anatomical features that would normally be undetectable due to poor transmission of visible light through the tympanic membrane. We also find that for middle ear structures already detectable by visible otoscopy, SWIR otoscopy further improves their contrast. We quantified contrast using Weber contrast, defined as

\[
\frac{I_f - I_b}{I_b}
\]

where \( I_f \) is the average intensity of a middle ear feature region of interest and \( I_b \) is the average intensity of a background region of interest. The absolute value was taken for round window contrast calculations, since the round window is given by negative contrast relative to the reflected light from the promontory.

This analysis was carried out for the incus and round window of all imaged ears in which the anatomy was visible using both SWIR and visible otoscopy (Fig. 2.6). The average contrast value for the incus was 0.21 using visible otoscopy and 0.61 using SWIR otoscopy—an increase by a factor of 4. The average contrast value for the round window was 0.26 using visible otoscopy and 0.52 using SWIR otoscopy—an increase by a factor of 2. The incus and round window contrast value for each volunteer is plotted and shown in Fig. 2.6D,E.

![Figure 2.6: Quantification of contrast in SWIR and visible otoscopy of adult human subjects.](image)

The intensity profile was plotted across middle ear features as imaged using SWIR otoscopy (column 1, orange trace) and visible otoscopy (column 2, blue trace) in order to calculate the relative contrast of each method. The round window of one subject is shown in (A) with Weber contrast values inset in the intensity plot (0.57 for SWIR and 0.27 for visible). The incus of a separate subject is shown in (B) with SWIR contrast 0.87 and visible contrast 0.63, and the incus of a third subject is shown in (C) with SWIR contrast 0.43 and visible contrast 0.12. The contrast was likewise plotted for 8 volunteers in whom the incus was identifiable by both methods (D) and 7 volunteers in whom the round window was identifiable (E). In all cases, SWIR otoscopy provides the greatest contrast of the middle ear feature.
2.5 Clinical observations in a pediatric population

The utility of the SWIR otoscope for middle ear diagnostics was evaluated in a pediatric patient population. First, the design of the SWIR otoscope was optimized based on the findings in Section 2.3 and Section 2.4 to improve ergonomics and adaptability to a wide range of patient ear geometries (Fig. 2.7). Achromatic lenses were replaced with three 250 focal length biconvex lenses each (Thorlabs, LB1056-C) to greatly reduce spherical aberration in the images. A right angle cage system with a silver mirror (Thorlabs, PF10-03-P01) was implemented between the lenses for a more ergonomic arrangement, and the focusing lenses were placed in a translating lens tube to enable adjustable focus for ear canals of different sizes and geometries. The sensor was also replaced with a higher resolution, smaller form factor, and lower weight InGaAs sensor, the OWL 640 Mini VIS-SWIR (Raptor Photonics, Northern Ireland).

Patients 3 years of age and older seen in the otolaryngology clinic with an audiogram and tympanogram obtained within a week of the visit were recruited broadly for the study. Ears with a tympanic membrane perforation or history of cholesteatoma surgery were excluded from the study. Otoscopic evaluation was performed by two pediatric otolaryngologists using video otoscopy during the office visit. Consecutive videos were obtained for visible otoscopy and SWIR otoscopy.

A total of 74 ear video recordings were obtained in the study. Images of eleven ears were excluded from analysis due to poor image quality for reasons including patient movement, inadequate camera integration time, or presence of cerumen limiting view of the tympanic membrane. Of the excluded videos, 7 were from SWIR imaging and 4 were from visible light otoscopy. Images were deemed adequate for interpretation in 63/74 (85.1%) of videos examined. There was no statistical significance between ability to perform SWIR otoscopy versus white light video otoscopy as indicated by a p-value of 0.376.

To determine differences between visible light otoscopy and SWIR otoscopy in the ability to identify the promontory, the ossicular chain, and middle ear effusion, videos were obtained with both modalities. All videos were de-identified and displayed on a computer for review. For each video, the otolaryngologist was asked to rate the quality of the video, ability to identify the
promontor, ability to identify the ossicular chain, and presence or absence of middle ear fluid. Criteria to document absence of middle ear effusion were adequate movement of the ear drum during the office examination with pneumatic otoscopy and identifiable promontory and ossicles behind the tympanic membrane in visible and SWIR otoscopy. Criteria to document the presence of middle ear fluid included visible fluid in visible otoscopy and decreased light intensity from absorption of SWIR light by fluid in SWIR otoscopy. Results of the audiograms and tympanograms and ear examination were made available to the pediatric otolaryngologists at the time of the evaluation. Inter-rater agreement was performed using Cohen’s kappa, and Chi square analysis was used for our nominal data using SPSS 22.0 (SPSS Inc. Chicago, Illinois). Differences were considered significant for a p-value < 0.05.

In 8 cases, the ossicular chain was visible when using the SWIR otoscope compared to 1 case using the visible otoscope (Fig. 2.8). Thus, there was statistical significance between SWIR otoscopy and visible otoscopy in the ability to image the promontory (p=0.012) and the ossicular chain (p=0.010). There was high inter-rater agreement for identification of both the

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Figure 2.8: Evaluation of healthy middle ear anatomy in pediatric patients. A normal type A tympanogram from a clinically normal patient is shown (A). The incus (B) and promontory (C) of this patient is shown with greater clarity in SWIR otoscopy images compared to the visible otoscopy images. Likewise a second clinically normal patient tympanogram (D), incus images (E), and promontory images (F) are shown.
promontory and the ossicular chain with kappa values of 0.81 and 0.92 respectively. In addition, three patients were determined to have presence of middle ear effusion, confirmed by pneumatic otoscopy during the otolaryngology visit. The patients were all identified using both visible and SWIR otoscopy. In these patients, contrast was also quantified with ImageJ software by calculating the standard deviation in signal intensity divided by the mean signal intensity for a defined region of interest within the image. The region of interest encompassed areas both with and without suspected fluid accumulation. This calculation was carried out for four video frames and averaged. An improvement in contrast using SWIR otoscopy was observed in some areas where effusion was present in the middle ear (Fig. 2.9). The average contrast for visible otoscopy was 0.097 and for SWIR was 0.29.

Additional pathologies were also observed in individual patients and differences between SWIR and visible otoscopy noted (Fig. 2.10). For example, in tympanic membranes with myringosclerosis, neither technique was able to see through areas of myringosclerosis. However, we observed that the SWIR otoscope was able to see through dried blood, dried
secretions and thin dry areas of cerumen overlying the tympanic membrane. In one particular case, this enabled visualization of an underlying tympanostomy tube that could otherwise not be evaluated. The SWIR otoscope in general aided evaluation of tympanostomy tube flanges to assess the tube’s placement across the tympanic membrane and fluid draining from the tube, confirming no blockage.

Thus, we have demonstrated the feasibility of using SWIR otoscopy in a pediatric population. Furthermore, we show that expanding otoscopy into the SWIR can offer advantages over traditional visible otoscopy, such as better visualization of the middle ear structures, seeing through opaque media, and increased contrast of middle ear effusion. SWIR otoscopy could provide an adjuvant method for identification of middle ear pathologies in the clinic.

2.6 Discussion and conclusions

We have developed an otoscope sensitive to SWIR light for imaging the middle ear and its pathologies. We show that deeper tissue penetration of SWIR light enhances contrast and enables better visualization of middle ear anatomy through the thin tissue of the tympanic membrane. While the middle ear anatomy is obfuscated by the tympanic membrane during visible light examinations, SWIR otoscopy can be used to examine the ossicular chain, cochlear...
promontory, round window niche, and chorda tympani. The ability to inspect the ossicular chain in greater detail could provide valuable diagnostic information in cases of conductive hearing loss such as in ossicular discontinuity or otosclerosis, a disorder characterized by abnormal bone growth. Imaging deeper within the middle ear can also allow evaluation of cholesteatoma extension within the middle ear, especially in cases where the ossicular chain is suspected to be involved. The round window is used as an insertion site for cochlear implant electrodes, and has also recently been used as an implantation site for hearing aid transducers. Clear visualization of the round window using a SWIR otoscope could provide an alternative to radiographic imaging for evaluation of such surgical implants. Thus, a SWIR otoscope has diagnostic potential in evaluating the cause of a variety of middle ear complications and in informing surgical procedures.

Furthermore, we predict that a SWIR otoscope can indicate middle ear effusions based on the strong light absorption of middle ear fluid beyond 1300 nm. Integrating SWIR light into otoscopy extends the available wavelengths to a regime in which endogenous contrast of middle ear fluid is much greater than at visible wavelengths. It should therefore provide a more objective determination of the presence or absence of middle ear effusion. Bringing objectivity to this diagnosis which has long been plagued by diagnostic and therapeutic inconsistency has the potential to reduce over-prescription of antibiotics and unnecessary tympanostomy tube surgeries, which is the most common surgical procedure performed in US children. Potentially, a SWIR otoscope could also be used for differential detection of mucoid versus serous middle ear effusion, which is also of clinical interest. Measuring the attenuation of the middle ear at different wavelength regimes (e.g. 1100 nm versus 1450 nm) simultaneously should provide a measure of the volumetric percentage of water in the fluid, enabling distinction of thin versus thick ear fluid.

The conventional pneumatic otoscope has long served as an initial means of identifying tympanic membrane perforations, causes of conductive hearing loss, and most commonly, otitis media. However, to compensate for the limitations of pneumatic otoscopy, other diagnostic tools such as tympanometry and acoustic reflectometry have emerged to assist clinicians with the diagnosis of middle ear effusions. The Current American Academy of Otolaryngology-Head and Neck Surgery (AAO-HNS) guidelines for management of OME recommend use of tympanometry to assist with the diagnosis of middle ear effusion when pneumatic otoscopy examination is not conclusive. Tymanometry is an audiometric method shown to raise diagnostic success of otitis media to 83%; however, this method is not widely used in primary care settings. Studies show that primary care providers find the use of tympanometry easier than pneumatic otoscopy, yet utilization is low, due to the need for additional training, increased time during the visit, and the cost of the device. Specificity of this method is also low, between 50-75%. Emerging technologies such as optical coherence tomography, spectral gradient acoustic reflectometry, and sonography have likewise shown potential for
improving the diagnosis of otitis media, but so far have not been widely adopted in general practice due to the difficulty of data interpretation or unfamiliarity of physicians with their use.\textsuperscript{111–116}

We therefore underscore the importance of having a photonic method for direct visualization of the functional status of biological tissue. Using SWIR technology does not add time or complexity to a diagnosis, and requires little additional training of medical practitioners who have already been trained in otoscopy. It is thus easily integrated into the clinic. The general architecture of the SWIR otoscope, which provides immediate functional information, could also be extended to the development of a variety of other medical devices to assist in a wide range of surgical procedures throughout the airway and gastrointestinal tract. For example, other disease conditions characterized by the build-up of fluid could be characterized by the enhanced contrast due to endogenous SWIR absorption. The SWIR otoscope is therefore only an initial example of how extending optical measurements into the SWIR can provide complimentary visual data to conventional visible light-based technologies, addressing some of their existing limitations.

2.7 Additional experimental details

All biospecimen sample collection and transfer procedures were approved by the Connecticut Children’s Medical Center Institutional Review Board and were from volunteers who provided informed consent. Samples were used and characterized following the procedures approved by the Massachusetts Institute of Technology Committee on the Assessment of Biohazards and Embryonic Stem Cell Research Oversight (CAB/ESCRO) and Biosafety Program. All participants in the imaging experiments provided informed consent and consent to publish images, and methods were carried out in accordance with the procedures approved by the Massachusetts Institute of Technology Institutional Review Board and Committee on the Use of Humans as Experimental Subjects, and Connecticut Children’s Medical Center Institutional Review Board, and all procedures were in accordance with the Declaration of Helsinki guidelines.

Tympamic membrane tissue samples (1 mm solid sample) were cut from the tympanic membrane via tympanic membrane perforation during a typical tympanoplasty procedure of pediatric patients (ages 0–18 years). The tissue was flash frozen with liquid nitrogen on Tefla and stored in a specimen cup at -80°C for up to one week before being transferred over approximately 3 hours in a Styrofoam container of dry ice. Tissue was stored in the second location at -80°C for one week before being thawed to room temperature and mounted intact on a glass slide. Human middle ear fluid samples were collected intraoperatively from pediatric patients (ages 0–18 years) during myringotomy and placement of pressure-equalizing tubes for standard treatment of recurrent otitis media or persistent middle ear effusion. Following incision
on the tympanic membrane with a Beaver blade, a Juhn Tym-Tap (Medtronic Xomed Inc., Jacksonville, FL) middle ear fluid aspirator was used to collect 30–200 microliter samples from either the left or the right ear into Eppendorf tubes. The collected fluid ranged from serous to mucoid in consistency, designated as such based on visual inspection and ability to suction the fluid. One set of samples was centrifuged at 500 rpm for 5 minutes to separate cells from middle ear fluid and subsequently stored at −80 °C. The other set was not centrifuged and was frozen directly at −80 °C. After five days, the middle ear fluid samples were transported over approximately 3 hours in a Styrofoam container of dry ice to a new location where they were again stored at −80 °C. Immediately prior to spectroscopic characterization, samples were thawed to room temperature and transferred to 1 mm or 0.2 mm path-length, demountable quartz cuvettes. Attenuation spectra of human tympanic membrane tissue and middle ear fluid were measured using a Cary 5000 UV-VIS-NIR spectrophotometer (Varian/Agilent, Santa Clara, CA, USA).

Otoscopic measurements were made on comparable SWIR and visible otoscope devices. The SWIR otoscope was designed as described in Section 2.3. The visible otoscope analogue was assembled using a Thorlabs, Inc. 1280 x 1024 pixel CMOS color sensor, and a mounted achromatic lens pair (Edmund Optics) with 75 and 100 EFL achromats (MgF₂-coated for visible wavelengths). The visible camera and lens were adapted to the otoscope head and broadband illumination from the otoscope was used to illuminate the object of interest. Resolution measurements of both systems were determined using Thorlabs, Inc. (Newton, New Jersey, USA) positive sector star test targets with 36 bars and 72 bars and a concentric square calibration target. Depending on the resolution of the optical system, the sector star bars will appear to merge at some radial distance from the center of the target; by measuring this distance, \( r \), the thickness of a line pair can be calculated using the formula for the chord length:

\[
c = 2r \times \sin \frac{\theta}{2}
\]

where the angle theta is the number of degrees covered by one pair of light and dark bars. The resolution in lp/mm is thus given by \( 1/c \). Using this method, the maximum resolution for the optical system was determined to be 22 lp/mm, or 45 μm. Analysis with a concentric square calibration target indicated that the lenses de-magnify the object onto the sensor by a factor of 0.76, resulting in an approximately 10.5 mm diameter circular field of view while using a 5.0 mm speculum.

Light intensities output from the SWIR otoscope were also measured. At a distance of 2 cm away from the end of the otoscope speculum, the broadband illumination between 900-1700 nm is 10–30 mW/cm² depending on the output power of the lamp. Illumination specifically between 1300-1700 nm was measured to be roughly 1–3 mW/cm².

Ten adults were imaged in total in both left and right ears. In eighteen out of twenty ears, clear visualization of the middle ear was achieved; two out of twenty ears were excluded due to
cerumen in the external auditory canal blocking access to the middle ear. For the eighteen ears with a clear view of the middle ear, the individual anatomical structures were identified by the otolaryngologist performing the examination (Table 2.1). A two-proportion one-tailed z-test was used to assess whether the difference between the visible anatomy visualization and SWIR anatomy visualization is significant. We hypothesized that the proportion of volunteers in which the middle ear anatomy could be visualized would be greater using SWIR otoscopy than using visible otoscopy. Using a one-tailed test, and at a significance level of 0.05, the proportion of anatomy that could be visualized using SWIR otoscopy was significantly greater for the incus, stapes, stapedial tendon, cochlear promontory, round window, and chorda tympani. The difference was insignificant for the malleus which was easily visualized by both techniques. At a significance level of 0.01, the difference was significant for visualization of the incus, stapes, stapedial tendon, and round window and insignificant for the malleus, promontory, and chorda tympani.

2.8 Chapter-specific acknowledgements

The results described in this chapter are reported in Carr, et al.\textsuperscript{117} and an additional manuscript in preparation.

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Chapter 3

Endogenous SWIR autofluorescence imaging as a biomarker for disease

3.1 Introduction: autofluorescence as a biomarker for disease

When biological tissue is irradiated with light, some biological structures within the tissue can absorb the light and re-emit light at a lower energy wavelength, a phenomenon known as autofluorescence. Autofluorescent molecules in cells, e.g. aromatic amino acids, lipopigments, NAD(P)H, and flavin coenzymes, can act as endogenous reporters of the morphological and metabolic properties of cells and tissues, providing a means for living cell analysis in basic research studies, and also as a disease biomarker. As the signal is inherent to tissue, autofluorescence imaging is part of a wider range of analytical procedures known as optical biopsy, which promise diagnostic information in a noninvasive or minimally-invasive manner, without removal of tissue specimens or the injection of exogenous markers. Moreover, unlike histochemical procedures on biopsies, which are the standard method in the diagnosis of many disorders, optical biopsies can be performed in real-time and in a manner that is nondestructive to the sample, requiring no pre-treatment steps (e.g. fixing or staining).

The diagnostic potential of autofluorescence was first recognized over a century ago, yet difficulties detecting low-intensity signals or interpreting complex, overlapping signals redirected most research focus to the use of exogenous fluorophores. The relatively recent availability of high-sensitivity, low-noise cameras has improved detection of autofluorescence signals, however, and the development of more compact, powerful light sources and fiber optics is renewing interest in autofluorescence both in basic research and as a diagnostic technique. Moreover, a variety of methods have now been developed to help uncouple autofluorescence signal from various other signals within the tissue (e.g. autofluorescence lifetime imaging). The autofluorescence of biomolecules and their combinations have now been investigated as biomarkers for various cancers, liver functionality, gastrointestinal disorders, and other conditions. Over the past decade, fundus autofluorescence imaging, which measures the ocular fluorophore lipofuscin in retinal pigment epithelia, has become an essential clinical tool for obtaining earlier diagnoses and better predictions of the progression of age related macular degeneration, macular dystrophies, retinitis pigmentosa, white dot syndromes, retinal drug toxicities, and other retinal diseases.
Nonetheless, most autofluorescence imaging to date uses ultraviolet radiation (UVA, 315-400 nm) or high energy visible wavelengths to excite endogenous fluorophores. However, it is known that UVA exposure induces oxidative stress in cells and photo-oxidation processes, resulting in acute and persistent cellular injury or even cell death.\textsuperscript{145} High energy visible light can also create an uncomfortable patient experience, as has been documented in fundus autofluorescence imaging.\textsuperscript{146} Furthermore, UVA and visible light are limited in their tissue penetration depth, and can only image superficial (e.g. skin or retinal) or surgically/endoscopically-accessible structures.\textsuperscript{45} Another major problem in conventional autofluorescence microscopy techniques is photobleaching, resulting in diminished autofluorescence signal. Thus, there remains a need for a noninvasive method to detect autofluorescence that is repeatable and nondestructive for cells and tissue.

Here, we make use of NIR excitation and high-sensitivity, low-noise cameras (silicon-based and InGaAs-based) to detect endogenous NIR and SWIR autofluorescence. The choice of NIR excitation enables nondestructive, noninvasive, \textit{in vivo} detection, as it has lower energy and greater tissue penetration depths than UVA or visible light wavelengths.\textsuperscript{19,22} Detection of longer NIR/SWIR autofluorescence emission further facilitates deep-tissue measurements, due to greater tissue transmittivity, less scattering of light, and lower background autofluorescence compared with visible imaging.\textsuperscript{19,45,47} Most healthy tissue has very little autofluorescence of NIR and SWIR light, but NIR excitation can give rise to elevated NIR/SWIR autofluorescence signals under certain disease conditions, providing disease-correlated contrast in an image, as has already been shown clinically feasible in the eye.\textsuperscript{19,146,60,147,148}

We show that lipopigments in particular can serve as NIR- and SWIR-autofluorescent pigments, and demonstrate the potential for their use as disease biomarkers. For example, we find strong NIR/SWIR autofluorescence in the liver of mouse models of cirrhosis and of nonalcoholic fatty liver disease (NAFLD). In the latter, we show further that the increase of NIR/SWIR signal parallels the progression of disease. We have also detected NIR/SWIR autofluorescence in the brain and liver of 12/15-lipoxygenase knockout mice with consequential up-regulated autophagy, a major intracellular degradation pathway that may be linked to an increasing number of neurodegenerative and other diseases.\textsuperscript{149–151} We show here the noninvasive detection of these disease-correlated autofluorescence signals \textit{in vivo} in preclinical animal models and discuss the potential relevance of this technique to human clinical samples.

3.2 Fluorescence imaging apparatuses for whole animal down to sub-cellular imaging

We designed an optical system to detect NIR and SWIR fluorescence of tissues at both macroscopic and microscopic scales (Fig. 3.1). These systems were used for the studies presented in this chapter, and were also adapted for many of the following studies described in
Figure 3.1: Optical set-up for NIR/SWIR fluorescence imaging. The optical system uses a near infrared excitation unit, a transmission unit configured to illuminate a region of interest with incident excitation light, and a detection unit configured to sense the resulting fluorescence. For macro- and mesoscopic imaging, the transmission unit is composed of an optical fiber which guides light from the excitation unit, an excitation filter to block undesirable excitation wavelengths, and a diffuser to spread the excitation light across the biological sample (A). The detection unit contains a set of filters to block reflected excitation light and to select the desired fluorescence wavelengths, a lens system to image the fluorescence onto the sensor, and a sensor sensitive to NIR/SWIR light. For microscopy, the transmission unit contains a dichroic mirror which reflects the desired excitation wavelength from the excitation unit through an objective which focuses the excitation light onto a biological sample; autofluorescence from the sample is focused through the objective, passed by the dichroic mirror, and detected by a unit composed of a set of filters to block reflected excitation light and a sensor sensitive to NIR/SWIR light (B). In both set-ups, NIR excitation light is used (e.g. 808 nm laser light), and NIR and/or SWIR emission wavelengths are measured (C).
Chapter 4 and Chapter 5. The systems include an excitation source for illuminating a sample with incident light, a series of filters and optics to focus select excitation onto the sample and to focus specific reflected or emitted light onto a detector.

For macro- and mesoscopic imaging, the output of a 10 W 808 nm laser (Opto Engine; MLL-N-808) was coupled into a fiber (Thorlabs; MHP910L02) and passed through a ground-glass plate (Thorlabs; DG10-220-MD) directed over the working area. The final laser intensity at the working surface was approximately 50 mW/cm², well below the maximum permissible exposure limit (330 mW/cm² for 808 nm continuous wave light). Sample fluorescence was directed from the imaging stage to the camera using a four-inch square first-surface silver mirror (Edmund Optics; Part No. 84448). The laser light was blocked with two colored glass 2” 850 nm longpass filters (Thorlabs; FGL850S) and an 850 nm longpass dielectric filter (Thorlabs; FELH0850) in front of the objective (Navitar; F/2.8, 50 mm EFL, MVL50M23) before collecting the fluorescence on an InGaAs camera (Princeton Instruments; NIRvana, 640x512 pixel array) or a back-illuminated, deep depletion silicon CCD camera (Princeton Instruments; PIXIS 1024BR, 1024x1024 pixel array). Fig. 3.2A,B shows an example of in vivo mouse imaging and ex vivo tissue imaging using this set-up.

For microscopy imaging, the 808 nm laser output of the multimode fiber was directed through the diffuser of a laser speckle reducer (Optotune; LSR-3005) and deflected at a silver mirror (Newport; 10D20ER.2) after which it entered an inverted microscope (Nikon; Eclipse Ti) through its backport unit. A longpass 850 nm dichroic mirror (Thorlabs; DMLP850R) was used.

Figure 3.2: NIR/SWIR autofluorescence imaging from macroscopic in vivo imaging to ex vivo tissue imaging and tissue microscopy. An example of macroscopic, whole-animal, in vivo NIR/SWIR autofluorescence imaging is shown in which signal is detected in the liver (A, yellow arrow) of a NAFLD mouse model (described in greater detail in Section 3.5) and in the genitourinary anatomy (A, white arrows). NIR/SWIR autofluorescence detected in ex vivo tissue is shown in (B) of the excised liver tissue of the animal shown in A. NIR/SWIR autofluorescence microscopy of cells is also shown in (C) in 5 μm sections of formaldehyde-fixed, paraffin-embedded liver tissue from a cirrhosis mouse model (described further in Section 3.4).
to guide the laser beam onto the back aperture of the objective (Nikon; CFI Plan Apochromat 4x or 10x) which focused the laser onto the sample placed on a motorized stage (Ludl Electronic Products Ltd.; MAC 6000 Systems). Light emitted by the sample was collected by the same objective and passed through the longpass 850 nm dichroic mirror and a hard-coated 850 nm longpass filter (Thorlabs; FELH0850). The light in some cases was magnified by an additional 1.5x using a built-in microscope function and was directed to a microscope output port onto the array of the InGaAs or silicon camera. Fig. 3.2C shows an example of microscopic autofluorescent granules imaged using this set-up.

In some studies, visible light microscopy was carried out for comparison. The above-described microscopy set-up was adapted for this purpose. Samples were illuminated with either a halogen lamp (Nikon; Halogen 12 V 100 W, LHS-H100C-1) for reflectance imaging or a Xenon lamp (Nikon; Intensilight C-HGFI) for fluorescence excitation. Excitation and emission light was filtered through one of the filter cubes described in Table 3.1, and imaged onto either the grey-scale CCD camera (PIXIS 1024BR) or a color CMOS camera (Thorlabs; DCC1645C).

For all imaging, the NIRvana 640 camera was cooled to –80 °C, the analog to digital (AD) conversion rate set to 2 MHz or 10 MHz, and the gain set to high. All images were background- and blemish-corrected within the LightField imaging software. The PIXIS 1024BR camera was cooled to –70 °C, the AD conversion rate set to 2 MHz, and the gain set to high. Detailed acquisition settings (e.g. integration time, filters) for each figure are detailed in Appendix A, Table A.1. ImageJ (1.48v, NIH) was used for all image measurements (frame averaging, pixel intensity average, standard deviation, etc.).

Table 3.1: Filter cube descriptions for visible light microscopy. The following table details the excitation and emission wavelengths selected by the filter cubes used in the following microscopy studies.

<table>
<thead>
<tr>
<th>Filter cube label</th>
<th>Excitation filter wavelengths</th>
<th>Emission filter wavelengths</th>
</tr>
</thead>
<tbody>
<tr>
<td>DAPI/BFP</td>
<td>372–408 nm</td>
<td>425–520 nm</td>
</tr>
<tr>
<td>GFP</td>
<td>452–486 nm</td>
<td>505–545 nm</td>
</tr>
<tr>
<td>Cy3/TRITC/DSRed</td>
<td>532–552 nm</td>
<td>594–646 nm</td>
</tr>
<tr>
<td>Cy5</td>
<td>590–650 nm</td>
<td>662–738 nm</td>
</tr>
<tr>
<td>NIR/SWIR</td>
<td>&lt; 850 nm</td>
<td>&gt; 850 nm</td>
</tr>
</tbody>
</table>
3.3 Identification and optical characterization of a NIR/SWIR-autofluorescent pigment

Most cell autofluorescence originates from molecules within the mitochondria and lysosomes, such as aromatic amino acids, lipopigments, NAD(P)H, and flavin coenzymes. Collagen, elastin, and melanin are also common fluorophores in the extracellular matrix, autofluorescent porphyrins and eosinophil granulocytes can be found in blood, and the autofluorescent biomolecules biliverdin and bilirubin are present in bile. Many of these molecules are readily detected in the visible spectrum using UVA or short-wave visible excitation, but few absorb significant amounts of NIR light and even fewer emit sufficient autofluorescence to be detected by NIR or SWIR detection. We show here the identification of lipopigments, namely lipofuscin/ceroid, as unique NIR-excit able and NIR/SWIR-detectable autofluorescent pigments, demonstrated primarily in tissue from mouse models of liver cirrhosis.

Previous studies have shown that ceroid-laden macrophages are present at high densities in cirrhotic liver tissue relative to healthy liver tissue, and are often accompanied by granules of autofluorescent lipopigments. We therefore investigated the possibility that NIR/SWIR autofluorescence in liver tissue could originate from these or similar granules. Male C3Hf/Sed mice were administered 20% carbon tetrachloride (CCl\textsubscript{4}) in olive oil via oral gavage (200 µL) three times per week, causing hepatotoxic cirrhosis. Ethanol was also added to the drinking water at a concentration of 5% to intensify the fibrogenesis. Mice were sacrificed after 8 weeks or after 12 weeks of their treatment, and excised liver samples were fixed in 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS) for 24h, washed in PBS and further processed to paraffin embedding. Paraffin-embedded tissue was sectioned into 5 µm thick slices onto glass microscope slides and were subsequently deparaffinized, stained, and/or mounted in a mounting medium.

Under 808 nm excitation, in the microscope described in Section 3.2, we indeed detect autofluorescent granules throughout the paraffin-embedded cirrhotic liver tissue. We detect using 850 nm longpass filters on both a silicon NIR camera (i.e. 850–1050 nm detection) (Fig. 3.3A), and an InGaAs SWIR camera (i.e. 900–1620 nm detection) (Fig. 3.3B). We also find that the signal survives deparaffinization (Fig. 3.3C) and mounting procedures using a variety of mounting media types (Fig. 3.3D-F) without substantial quenching. Furthermore, we find that the signal can be detected on deparaffinized, stained, and mounted slides (Fig. 3.3G,H). We show here representative images of 5 µm slices of cirrhotic liver tissue that were deparaffinized, immunohistochemically stained for P62 or F4/80 and α-smooth muscle Actin (α-SMA), and mounted with Faramount medium (DAKO North America Inc.; S3025). The NIR/SWIR autofluorescence signal did not overlay with P62 or LC3A/B stain (not shown), indicating that the signal may not correlate with autophagy in this model. The NIR/SWIR signal also did not correlate with α-SMA, which indicates activated stellate cells that are involved in fibrogenesis.
Figure 3.3: Tissue treatments for microscopy of mouse cirrhotic liver tissue. Paraffin-embedded mouse cirrhotic liver tissue was sectioned into 5 μm thick slices onto glass microscope slides and were subsequently deparaffinized, stained, and/or mounted in a mounting medium. Under 808 nm excitation, in the microscopy set-up described in Section 3.2, autofluorescence could be detected using either 850 nm longpass detection on a silicon camera (i.e. 850–1050 nm) (A) or using 850 nm longpass detection on an InGaAs camera (i.e. 900–1620 nm) (B). The autofluorescence was also detectable on deparaffinized tissue (C) and deparaffinized tissue mounted with Faramount (D), Permunt® (E), and ProLong® Gold (F), shown here using with 850 nm longpass detection on the silicon camera. Furthermore, we detected NIR/SWIR autofluorescence in slides stained for P62 (G) and F4/80 with α-SMA (H). We show here visible light color camera images on the left and 850 nm longpass images on the right for each stain. All scale bars indicate 100 μm.
However, in these same slides, the NIR/SWIR signal did seem to correlate with cells having F4/80-positive membranes. The NIR/SWIR signal did not directly overlay with F4/80, but rather with the cytoplasm of the F4/80-positive cells, which indicate Kupffer cells or tissue macrophages. The light brown appearance of the cytoplasm of these cells in this stain is reminiscent of ceroid macrophages.\textsuperscript{160}

We subsequently explored the correlation of the NIR/SWIR autofluorescent pigment with three known stains for lipofuscin/ceroid lipopigments: Nile Blue A Sulphate (NBS), periodic acid schiff reaction after diastase (PAS-D), and Sudan Black B (SBB).\textsuperscript{165-170} NBS is a fatty acid and acidic phospholipid stain, often used for the differentiation of melanin and lipofuscin/ceroid.\textsuperscript{170} PAS-D is a polysaccharide stain that is also positive for lipofuscin/ceroid granules. SBB is a highly lipophilic dye that binds to lipofuscin/ceroid granules, staining them a brown-black color, and as a consequence, quenching their fluorescence at visible light wavelengths. SBB is therefore frequently used to remove undesired lipofuscin/ceroid background autofluorescence during immunofluorescent microscopy.\textsuperscript{171,172}

For the NBS stain, deparaffinized sections (using xylenes and ethanol) were stained for 20 minutes in NBS at room temperature, prepared with 0.05% Nile Blue A (Acros Organics; pure, certified, 415690100; Lot number A0377281) in 1% sulfuric acid. Sections were then rinsed in running water for 15 minutes and no counterstain was performed. We find that the NIR/SWIR autofluorescence correlates nearly perfectly with NBS-positive regions of the tissue (Fig. 3.4A). A subset of sections were alternatively rinsed in 1% sulfuric acid followed by four changes of absolute acetone to remove most of the NBS, and hence destain lipofuscin/ceroid in the slide (Fig. 3.4B). We found that the NIR/SWIR autofluorescence intensity does not change significantly after destaining (average pixel intensities were 11,020 counts per second for NBS-stained slides and 10,955 counts per second for destained slides), confirming that the fluorescence detected in these slides is primarily due to the endogenous pigment and not Nile Blue A itself, which in 1% sulfuric acid has peak absorption at 572 nm and peak emission at 673 nm (Fig. 3.4C). This method further enables differentiation of lipofuscin/ceroid from melanin, as melanin does not destain via acetone wash.

Other deparaffinized sections were stained with PAS-D or SBB. For PAS-D, slides were incubated in 0.2% malt diastase (MP Biomedicals; ICN10153925) in a phosphate buffer for 1 hour at 37°C. After washing, they were subsequently oxidized with 1% periodic acid for 5 minutes and incubated with Schiffs reagent (Electron Microscopy Sciences; 26052-05) for 20 minutes at room temperature. Counterstain was performed with Gills haematoxylin #2. For the SBB stain, sections were incubated in 0.7% SBB (Sigma-Aldrich; certified by the Biological Stain Commission, 199664; Lot number MKBX9860V) in 70% ethanol for 6 minutes, then rinsed quickly in 50% ethanol solution and subsequently counterstained with nuclear fast red.\textsuperscript{167} We find that the NIR/SWIR autofluorescence correlates well with both PAS-D-positive (Fig. 3.4D) and SBB-positive tissue (Fig. 3.4E).
Figure 3.4: Histological stains for lipofuscin/ceroid and their correlation with NIR/SWIR autofluorescence. Paraffin-embedded liver tissue from mouse models of cirrhosis was cut into 5 μm slices, deparaffinized, stained, and mounted with Faramount on glass microscope slides. Nile Blue Sulfate (NBS) stain was used to indicate lipofuscin/ceroid granules in the tissue in dark blue under visible illumination and color detection (A, left). The NIR/SWIR autofluorescence image of the same slide using 808 nm excitation and 850 nm longpass silicon detection (A, right) shows nearly perfect correlation between autofluorescent and NBS-positive regions. The NIR/SWIR autofluorescence has the same intensity after destaining the slide with acetone (B), indicating that the detected fluorescence is likely not from the NBS stain itself which has an emission peak at 673 nm in a solution of 1% sulfuric acid as was used to stain the slides (C). Emission is shown here for a 0.005% solution measured on a Fluoromax-3 (HORIBA Jobin Yvon, Inc.); absorption is shown as measured on a Cary 5000 UV-Vis-NIR spectrometer (Varian) for a 0.05% solution as was used to stain the slides. There is further agreement between the NIR/SWIR autofluorescent pigment and the PAS-D stain (D), which indicates areas of lipofuscin/ceroid with a dark magenta color under visible illumination, as well as the SBB stain (E), which indicates areas of lipofuscin/ceroid with grey-black pigment under visible illumination. All scale bars indicate 100 μm.
Since lipofuscin/ceroid is known to have broad excitation and emission spectra, we also investigated the visible autofluorescence of the lipopigment in this cirrhotic liver tissue (using the excitation/emission parameters described in **Section 3.2, Table 3.1**, and depicted in **Fig. 3.5C**). We find that fluorescence is detectable in many visible channels, consistent with the behavior of lipofuscin/ceroid and lipofuscin-like pigments (**Fig. 3.5A**,171,173) Moreover, we find that the autofluorescent pigments in the cirrhotic liver tissue are indeed quenched at visible wavelengths after SBB staining (**Fig. 3.5B**), but are still detectable at NIR/SWIR wavelengths beyond the strong absorption maximum of the SBB, measured here for a 0.37 mg/mL solution of SBB in 70% ethanol in 1 mm path-length quartz cuvettes (Spectrocell) on a Cary 5000 UV-Vis-NIR infrared spectrometer (Varian) (**Fig. 3.5D**). It is theoretically possible that SBB could itself fluoresce in the NIR/SWIR channel (due to residual absorption at 808 nm), making it difficult to distinguish SBB fluorescence from the endogenous autofluorescent pigment. However, we note the nearly perfect co-localization of the fluorescent signal with SBB-positive regions of the tissue (there is no endogenous autofluorescence in SBB-negative tissue regions), and due to the minimal absorption of SBB at the 808 nm excitation wavelength, believe that any SBB fluorescence would be negligible relative to the endogenous autofluorescent pigment. Notably, this method of staining tissues with SBB extends the wavelength span for immunofluorescence imaging, as the lipopigments can be imaged in the NIR/SWIR wavelengths while traditional immunofluorescent stains can still be used in visible channels in the absence of interfering lipopigment autofluorescence.

Thus, as NBS, PAS-D, and SBB staining of cirrhotic liver slices all show a positive correlation between stain and autofluorescence, and the behavior at visible wavelengths is further consistent, we attribute the autofluorescence detected in these tissues to lipopigments, and specifically to lipofuscin/ceroid. Lipofuscin has been characterized previously as having an absorption maximum between 320–480 nm and an emission maximum in the range of 460–630 nm; however, the peak wavelengths can vary widely depending on the tissue (as even within a given tissue the composition of lipofuscin/ceroid is quite heterogeneous), and in general, both excitation and emission spectra are very broad.173 The larger than expected Stokes-shift suggests that several intramolecular energy-transfers occur between different electron systems before the absorbed energy is emitted as a “multi-Stokes-shifted” fluorescence photon, resulting in broad spectra.44 We are thus able to excite the lipofuscin/ceroid in tissue at 808 nm even though this is far from their absorption maxima and detect fluorescence from the tail of their emission in the NIR/SWIR.

We characterized the autofluorescence emission spectrum on the cameras used subsequently for **ex vivo** and **in vivo** imaging, and show that indeed the detected autofluorescence is a decreasing tail in this region (**Fig. 3.6**). The emission intensity was measured in 20 nm spectral bands across the NIR and SWIR by exciting an **ex vivo** liver sample with approximately 70–90 mW/cm² of diffuse 808 nm excitation light and filtering the resulting
Figure 3.5: Autofluorescence microscopy using visible through SWIR wavelength channels. We show here autofluorescence microscopy of mouse cirrhotic liver tissue, deparaffinized and mounted with Faramount, either with or without Sudan Black B (SBB) stain which quenches visible wavelength emission of lipofuscin. Autofluorescence is detectable in Cy3-TRITC, Cy5, and NIR/SWIR channels for unstained sections (A), but is only detectable in the NIR/SWIR channel for SBB stained sections (B), indicating that the signal detected in unstained sections is likely lipofuscin. The excitation and emission wavelengths for each filter cube setting are depicted in (C) and shows separation of the NIR/SWIR channel from the other channels. The absorption spectrum of Sudan Black B shows that the dye absorbs visible light up to approximately 800 nm, which is the mechanism of lipofuscin emission quenching at visible wavelengths, but transmits NIR/SWIR light, allowing signal to be detected in this channel even for Sudan Black B stained sections (D). All images have the same scale as the last panel of A, which indicates 100 μm.
autofluorescence through an 850 nm longpass dielectric filter and a liquid crystal tunable filter (PerkinElmer VarSpec LNIR, 20 nm bandwidth). The autofluorescence was collected using two achromatic doublet lenses (Thorlabs; AC254-075-C, AC254-500-C) mounted to either the InGaAs SWIR camera or the silicon NIR camera. In the case of the silicon camera, two colored glass 850 nm longpass filters (Thorlabs; FGL850S) were also used to block the excitation light due to its increased sensitivity at this wavelength. The liver tissue intensities were normalized using a reference lead sulfide film sample with peak emission around 900 nm. The film sample was imaged on the same imaging set-up and characterized on a spectrometer to obtain a set of wavelength-dependent correction factors, which were applied to the measurements of the liver.

Figure 3.6: Emission characterization of liver tissue. Mouse liver tissue was illuminated with 808 nm light, and the autofluorescence detected on a silicon camera (PIXIS-808, blue outline) or on an InGaAs camera (NIRvana-808, red outline) which were used subsequently for in vivo imaging. The autofluorescence was filtered through a liquid crystal tunable filter with 20 nm bandwidth. The intensities were then normalized using a reference lead sulfide film sample with peak emission around 900 nm. The film sample was imaged on the same imaging set-up and characterized on a spectrometer to obtain a set of wavelength-dependent correction factors, which were applied to the measurements of the liver.

3.4 NIR/SWIR autofluorescence imaging ex vivo and in vivo in cirrhotic liver models

As methods of lipopigment detection have long been established using visible light techniques, particularly in histological sections, our goal here is to show the potential for in vivo imaging in a fully noninvasive manner enabled by the use of longer wavelength excitation and emission. We demonstrate the ability to detect autofluorescence in vivo through the intact skin of mice models of cirrhosis in this section, and even at earlier stages of liver disease in the following section, Section 3.5. We investigated two cirrhosis models to probe the influence of two distinct disease mechanisms: one model is a hepatotoxin-induced liver fibrosis in which administered carbon
Tetrachloride (CCl₄) is metabolized into free radicals and reactive oxygen species, while the other is based on obstructive cholestasis inducing portal fibrosis.

All animal procedures were carried out following the Public Health Service Policy on Humane Care of Laboratory Animals and approved by the Institutional Animal Care and Use Committee of Massachusetts General Hospital and the MIT Committee on Animal Care. Both liver cirrhosis models were investigated using male C3Hf/Sed mice. For the first experimental group, mice were administered 20% CCl₄ in olive oil via oral gavage (200 µL) three times per week, causing hepatotoxic cirrhosis, or were administered just olive oil in the case of the respective controls. Ethanol was added to the drinking water of both groups at a concentration of 5% to intensify the fibrogenesis. Mice were sacrificed after 24 hours (acute intoxication; n=3/group), 3 weeks (n=4–5/group), or 6 weeks (n=5/group), of their respective treatments. Prior to imaging, mice were anesthetized via intraperitoneal injection of ketamine/xylazine (100 mg/kg ketamine and 10 mg/kg xylazine in saline) and sufficient depth of anesthesia was maintained, re-dosing with one third the original dose in ketamine only as necessary. Mice were shaved with electric clippers and residual hair was removed using Nair™, applied for approximately 3 minutes. Animals were then imaged in vivo. Whole blood samples were subsequently obtained by intra-cardiac puncture using EDTA-flushed needles. Next, the liver was extirpated, weighed, and after ex vivo NIR/SWIR imaging, further processed. The left liver lobe was taken for histology, while the remaining liver and the spleen were snap-frozen in liquid nitrogen and stored at −80°C until further processing.

In the acute model (i.e. those sacrificed 24 hours after a single high dose of CCl₄), we noted that mice administered CCl₄ treatment did manifest a detectable signal in vivo 6 hours and 24 hours post-administration, but not fully anatomically attributable to the liver. Upon dissection, it was apparent that the administration of CCl₄ induces an acute gastritis, resulting in gastroparesis wherein the food causes fluorescence of the stomach in these mice, but not in mice receiving only vehicle. Intensities of the ex vivo livers, on the other hand, were not significantly different between mice administered CCl₄ and those administered only vehicle (22 ± 4 counts per millisecond, and 20 ± 5 counts per millisecond respectively). This result is expected, as lipofuscin/ceroid cannot be formed within 24 hours.

When CCl₄ is administered chronically, we find that the livers become highly autofluorescent in this model. Autofluorescence in ex vivo tissue of the CCl₄-administered group was significantly higher than the control group—over two times greater after 6 weeks of administration—and was also significantly higher in vivo even after only three weeks of treatment (Fig. 3.7, Table 3.2, Appendix A.1). Both in vivo and ex vivo intensities increase with increasing weeks of CCl₄-administration. Furthermore, the difference in in vivo liver intensities between CCl₄ and control groups was detectable in awake and freely behaving mice at real-time imaging speeds of 9.17 frames per second, a testament to the brightness of the signal.
Table 3.2: Summary of *in vivo* and *ex vivo* mouse liver autofluorescence intensities for cirrhosis models. Shown here are the autofluorescence intensities of the liver both *in vivo* and *ex vivo* given in counts per millisecond (cpms). Results are shown for 3 and 6 weeks of oral administration of CCl₄ in olive oil (OO) and 5% ethanol (EtOH) in the drinking water, for 3 and 6 weeks of control mice which received only OO orally and 5% EtOH in the drinking water, for mice sacrificed 4 weeks after a CBDL procedure, and for age-matched control mice having no procedure.

<table>
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<tr>
<th>Weeks of treatment</th>
<th>Treatment</th>
<th>n</th>
<th><em>in vivo</em> intensity (cpms)</th>
<th><em>ex vivo</em> intensity (cpms)</th>
</tr>
</thead>
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<tr>
<td>3</td>
<td>OO + EtOH</td>
<td>4</td>
<td>7 ± 1</td>
<td>17 ± 1</td>
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<tr>
<td>6</td>
<td>OO + EtOH</td>
<td>5</td>
<td>10 ± 1</td>
<td>20 ± 1</td>
</tr>
<tr>
<td>3</td>
<td>CCl₄/OO + EtOH</td>
<td>5</td>
<td>14 ± 2</td>
<td>31 ± 3</td>
</tr>
<tr>
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<td>CCl₄/OO + EtOH</td>
<td>5</td>
<td>16.8 ± 0.4</td>
<td>44 ± 4</td>
</tr>
<tr>
<td>4</td>
<td>control</td>
<td>8</td>
<td>7 ± 1</td>
<td>17 ± 2</td>
</tr>
<tr>
<td>4</td>
<td>CBDL</td>
<td>5</td>
<td>10 ± 1</td>
<td>22 ± 2</td>
</tr>
</tbody>
</table>

The results of these experiments indicate that the liver signal manifests from chronic administration of CCl₄, but fluorescence from the stomach and gastrointestinal tract may impact the *in vivo* results due to intrinsic autofluorescence of chow. In most cases, the position of the liver is anterior to that of the stomach, which aids quantification of liver signal (see Appendix A.1 for full image sets). Nevertheless, we excised the stomachs of the mice administered CCl₄ for 3 weeks and find that the intensity of the stomach is on average 1.5 times brighter in treated mice versus controls, whereas the *ex vivo* liver is 1.8 times brighter. Meanwhile, the *in vivo* intensity differs between the two groups by a factor of 2. For mice administered CCl₄ for 6 weeks, this difference in the stomach intensity went away (average intensities were not significantly different), whereas the *ex vivo* liver became 2.2 times brighter than controls. Potentially the CCl₄-treated mice increased tolerance to the treatment over time, resulting in less gastritis, while still progressing in severity of cirrhosis. Thus, the measured *in vivo* intensities may have contributions from both stomach and liver, but we expect the measurement to be dominated by the liver intensity, especially when disease pathology is further advanced.

A separate experimental group of this model was studied over longer time scales of treatment. Mice were sacrificed after 2-4 weeks (n=12), 8 weeks (n=8), and 12 weeks (n=9) of CCl₄ and ethanol treatment, and their livers fixed in 4% PFA in PBS and embedded in paraffin. We quantified the paraffin-embedded liver tissue autofluorescence in the mesoscopic imaging set-up described in Section 3.2 with 850 nm longpass detection on the silicon camera (Fig. 3.8). The average autofluorescence intensity of the tissue nearly doubled between 2-4 weeks and 8 weeks of treatment, but remained approximately the same between 8 weeks and 12 weeks of treatment. Standard deviations of the signal intensities increased significantly with increasing weeks of treatment. It should be noted that different thicknesses of tissue embedded in the paraffin blocks could give rise to variability in the detected intensities; however, this
Figure 3.7: NIR/SWIR autofluorescence in mouse models of cirrhosis. We observed NIR/SWIR autofluorescence in mouse models of cirrhosis based on oral administration of CCl₄ in olive oil (OO) and 5% ethanol (EtOH) drinking water versus control mice which received only OO orally and 5% EtOH drinking water. Using 808 nm excitation and 850 nm longpass detection on a silicon camera, the intensity of the liver was high enough to be detected in vivo after only 3 weeks of treatment (A), although difficult to distinguish from stomach fluorescence here (bright field image on the left and fluorescence on the right). Ex vivo livers were clearly distinguishable (visible image on the left, fluorescence on the right). By 6 weeks of treatment, the CCl₄-treated liver is clearly distinguishable from the control both in vivo and ex vivo (B). Quantification of the intensities (in counts per millisecond, or cpms) shows clear separation of the two groups (C, D). The CBDL model of cirrhosis, on the other hand, shows a more subtle increase in liver intensity over age-matched control mice. All intensity values and their standard deviations are detailed in Table 3.2.

should contribute approximately the same amount of variability to each time point. The increasing variability in fluorescence intensity with weeks of treatment could more likely be the result of varying degrees of disease which progresses at different rates in each animal.¹⁷⁹
The second model of liver cirrhosis investigated in this study was the common bile duct ligation (CBDL) model. CBDL surgery was performed in mice to double ligate the common bile duct, causing a secondary biliary cirrhosis. Mice (n=5) were sacrificed 4 weeks after the procedure. We find that the liver autofluorescence of these mice is slightly elevated compared to age-matched control mice which did not have a CBDL procedure (n=8), but not as significantly as mice with CCl₄-induced cirrhosis (Fig. 3.7C,D, Table 3.2). In contrast to CCl₄, CBDL cirrhosis relies less on oxidative stress mechanisms, which are known to play a role in the formation of lipofuscin/ceroid. These results confirm that the oxidation damage pathway is, as expected, an important factor in the enhancement of autofluorescence with disease progression (originating from lipofuscin/ceroid). We further noted that in CBDL mice, the bile duct and gall bladder, while significantly enlarged compared to controls, was not autofluorescent, ruling out a component of the bile (e.g. biliverdin/bilirubin) itself as the origin of the autofluorescent signal.

Taken together, our results indicate that NIR/SWIR autofluorescence is capable of in vivo and ex vivo detection of liver damage. Models of cirrhosis based on CCl₄ administration are clearly distinguishable from controls, even in awake, freely behaving animals without administration of anesthesia and without excision of tissue. However, cirrhosis models based on CBDL were only subtly more autofluorescent than controls, indicating that this method is not necessarily a measure of cirrhosis, but perhaps rather a measure of the experienced oxidative stress of the tissue.
3.5 Detection of liver disease progression in NAFLD models

As we have demonstrated the ability of our NIR/SWIR autofluorescence technique to detect the evolution of severe liver disease in vivo in cirrhosis mouse models, we investigate here the sensitivity of our technique to earlier stages of liver disease and their progression in a mouse model of nonalcoholic fatty liver disease (NAFLD).

Male C3Hf/Sed mice were fed a control diet (CD) or a choline-deficient, L-amino acid-defined, high-fat diet (CDAHFD) (A06071314i and A06071302i; Research Diets, New Brunswick, NJ, USA) for indicated time points (3, 6, 9, 12 weeks of diet; n=6/group). After 9-12 weeks of diet, the CDAHFD diet induces NASH and fibrosis. Animals were imaged in vivo as described above. The mice were also injected with 60 mg/kg body weight of pimonidazole (for hypoxia studies) at a concentration of 6 mg/mL in PBS 1 hour before sacrifice. Whole blood samples were subsequently obtained by intra-cardiac puncture using EDTA-flushed needles. Next, the liver was extirpated, weighed, and after ex vivo SWIR imaging (taking approximately five minutes), further processed. The left liver lobe was taken for histology, while the remaining was snap-frozen in liquid nitrogen and stored at –80˚C until further processing. The heart, kidneys and spleen were extirpated, weighed and snap-frozen as well. Finally, the inter-scapular brown adipose tissue, the gonadal white adipose tissue (as an indicator for visceral fat mass) and the inguinal white adipose tissue (as an indicator for subcutaneous fat mass) were identified, dissected and snap-frozen.

We find that NIR/SWIR autofluorescence is indeed detectable at higher levels both in vivo and ex vivo in the CDAHFD mice compared with CD mice. After 9 weeks of diet, and especially after 12 weeks of diet, CDAHFD mice livers were sufficiently autofluorescent even for in vivo detection (Fig. 3.9). However, just under 30% of the mice had to be excluded from in vivo quantification, due to interfering autofluorescence signal in the intestines near the liver. We discuss the origin of this signal below in greater detail. Quantification of ex vivo liver signal intensities confirms increasing average intensity with increasing weeks of CDAHFD, while CD mice show no significant intensity change over the course of the experiment (Fig. 3.10). The complete set of ex vivo and in vivo images for all mice is shown in Appendix A.1, and all quantification data is summarized below in Table 3.3. Histology of liver sections (see Section 3.8 for histology methods) confirms that CD mice have normal livers, while CDAHFD mice have severe steatosis from 3 weeks of diet and longer, and begin signs of inflammation and fibrosis (based on H&E and Sirius Red stains) by 12 weeks of diet.

In another group of mice, the effect of the angiotensin receptor type 1 blocker telmisartan was investigated. After 8 weeks of CDAHFD or CD, mice were fed their respective diet for an additional 4 weeks while also being treated daily with 3 mg/kg body weight telmisartan or with an equal volume of vehicle (4% DMSO in PBS) via intraperitoneal injection. After 12 weeks of diet total, 4 of which included treatment, the mice (n=6/group) were sacrificed.
Figure 3.9: NIR/SWIR autofluorescence detection in vivo in a NAFLD mouse model. Mice fed 12 weeks of CDAHFD to induce NAFLD are clearly distinguishable from mice fed a control diet (CD) which does not induce NAFLD, based on the intensity of signal in the liver (white arrows). The livers of mice on the CD show an average of approximately 7 cpm of NIR/SWIR autofluorescence whereas mice on the CDAHFD show an average of approximately 10 cpm (averages and standard deviations are detailed in Table 3.3). Note, there is also presence of autofluorescence of the male genitourinary tract in both CD and CDAHFD mice. All images were taken using the same integration time and scaled to the same maximum and minimum intensities.

We find no difference in the in vivo nor the ex vivo liver intensities between telmisartan- and vehicle-treated mice (Table 3.3). This result suggests that the autofluorescence measurement may not be sensitive to disease regression. This is not surprising, given that lipofuscin/ceroid is known to be difficult to dissolve (hence its persistence through many tissue processing conditions, as noted in Section 3.3) even after disease severity has regressed, which has been noted by others for the behavior of lipofuscin/ceroid in liver disease.\textsuperscript{162,183,184} We therefore conclude that the NIR/SWIR autofluorescence measurement may not be effective as a real-time representation of the current disease state, but rather as an indicator of cumulative liver injury.

We noted during these experiments that the CDAHFD is itself autofluorescent at NIR/SWIR wavelengths, while CD is not and this may have affected the above observations. Thus, to investigate the effect of fluorescent chow on the liver, mice were fed standard chow (SC; LabDiet, 5066), which is also autofluorescent (at an intermediate intensity compared to the experimental diets), and were sacrificed at time points corresponding with 0, 6, and approximately 30 weeks of diet to provide adequate controls (n=8, 7, and 4 respectively). We found that there is no significant increase in the in vivo nor the ex vivo liver intensities between 0
Figure 3.10: NIR/SWIR autofluorescence detection in ex vivo liver tissue shows correlation between signal intensity and disease progression in NAFLD mouse models. Mice were fed up to 12 weeks of choline-deficient, L-amino acid-defined, high-fat diet (CDAHFD) to induce NAFLD or were fed a control diet (CD). Representative images of the ex vivo livers of mice are shown for mice on their respective diet for 3 weeks, 6 weeks, 9 weeks, and 12 weeks, taken with 808 nm excitation and 850 nm longpass detection on a silicon camera (A). The images are displayed using a color map to represent their relative intensities, given by the color bar on the right. A region of interest in the livers of mice on the CD (n=24, blue points) show on average 12.5 counts per millisecond (cpms) of NIR/SWIR autofluorescence whereas mice on the CDAHFD show on average approximately 18, 20, 24, and 29 cpms for 3 weeks, 6 weeks, 9 weeks, and 12 weeks of CDAHFD respectively (n=6 for each time point, orange points), as plotted in (B). The position of the average is indicated by the black bar and all averages and their standard deviations are detailed in Table 3.3. We show also the same quantification of ex vivo liver intensity represented by the percent increase in signal versus the average intensity of the controls at the respective time point (C).
and approximately 30 weeks, indicating that diet-induced autofluorescence and aging-induced lipofuscin/ceroid does not accumulate at a detectable level over the time-frame of our experiments. When compared with the experimental diets, all SC mice had liver autofluorescence at a similar level to the 3 and 6 week CDAHFD mice (Table 3.3, Appendix A.1). These results suggest that autofluorescent chow (i.e. the dietary composition) may cause a base level of autofluorescence, in this case at a level higher than mice with non-fluorescent chow (i.e. the CD mice). However, since this signal does not increase over time, we conclude that the increasing signal observed in CDAHFD mice is an effect of the investigated disease.

For all mice, we further investigated if the measured *in vivo* NIR/SWIR autofluorescence intensities are representative of the actual *ex vivo* liver intensities, given that the signal could be scattered, attenuated, or obscured to different extents by the skin or surrounding tissues. We plotted the *in vivo* liver intensities versus the respective *ex vivo* liver intensities (for the same mouse) for each of the CCl₄-treated mice and their respective controls, CBDL, SC, CD, CDAHFD, CD/CDAHFD with vehicle, and CD/CDAHFD with telmisartan mice, and indeed observed a linear trend (see Section 3.8, Fig. 3.15).

Table 3.3: Summary of *in vivo* and *ex vivo* mouse liver autofluorescence intensities for NAFLD models and SC mice. Shown here are the autofluorescence intensities of the liver *in vivo* and *ex vivo* given in counts per millisecond (cpms). Results are shown for 3-12 weeks of CD and CDAHFD in NAFLD mouse models, for 12 weeks of CD and CDAHFD in NAFLD mouse models which received 4 weeks of vehicle (VEH) or telmisartan (TELMI) treatment, and for mice receiving only standard chow (SC). The table also includes the number of animals excluded from *in vivo* quantification due to an interfering autofluorescent gastrointestinal artifact. The *ex vivo* liver intensities of special diet and/or treated mice are also shown as the percent of the average control liver intensity; in the case of treated mice, the CDAHFD / VEH mice are compared to CD / VEH and CDAHFD / TELMI mice are compared to CD / TELMI.

<table>
<thead>
<tr>
<th>Weeks of diet/treatment</th>
<th>Diet/treatment</th>
<th><em>in vivo</em> intensity (cpms)</th>
<th>excluded</th>
<th><em>ex vivo</em> intensity (cpms)</th>
<th><em>ex vivo</em> intensity (% of controls)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>CD</td>
<td>7 ± 1</td>
<td>2 / 6</td>
<td>12 ± 1</td>
<td>–</td>
</tr>
<tr>
<td>6</td>
<td>CD</td>
<td>7 ± 1</td>
<td>3 / 6</td>
<td>11 ± 1</td>
<td>–</td>
</tr>
<tr>
<td>9</td>
<td>CD</td>
<td>8 ± 1</td>
<td>3 / 6</td>
<td>13 ± 1</td>
<td>–</td>
</tr>
<tr>
<td>12</td>
<td>CD</td>
<td>6.6 ± 0.4</td>
<td>1 / 6</td>
<td>11.0 ± 0.4</td>
<td>–</td>
</tr>
<tr>
<td>3</td>
<td>CDAHFD</td>
<td>8 ± 1</td>
<td>1 / 6</td>
<td>16 ± 1</td>
<td>141 %</td>
</tr>
<tr>
<td>6</td>
<td>CDAHFD</td>
<td>7.6 ± 0.4</td>
<td>2 / 6</td>
<td>17 ± 2</td>
<td>156 %</td>
</tr>
<tr>
<td>9</td>
<td>CDAHFD</td>
<td>10 ± 1</td>
<td>1 / 6</td>
<td>21 ± 2</td>
<td>165 %</td>
</tr>
<tr>
<td>12</td>
<td>CDAHFD</td>
<td>10 ± 1</td>
<td>0 / 6</td>
<td>24 ± 3</td>
<td>219 %</td>
</tr>
<tr>
<td>12 / 4</td>
<td>CD / VEH</td>
<td>7 ± 1</td>
<td>0 / 6</td>
<td>10 ± 1</td>
<td>–</td>
</tr>
<tr>
<td>12 / 4</td>
<td>CD / TELMI</td>
<td>7.3 ± 0.4</td>
<td>0 / 6</td>
<td>11 ± 1</td>
<td>–</td>
</tr>
<tr>
<td>12 / 4</td>
<td>CDAHFD / VEH</td>
<td>9 ± 1</td>
<td>0 / 6</td>
<td>24 ± 1</td>
<td>236 %</td>
</tr>
<tr>
<td>12 / 4</td>
<td>CDAHFD / TELMI</td>
<td>9 ± 1</td>
<td>0 / 6</td>
<td>24 ± 3</td>
<td>208 %</td>
</tr>
<tr>
<td>0</td>
<td>SC</td>
<td>7 ± 1</td>
<td>2 / 8</td>
<td>17 ± 2</td>
<td>–</td>
</tr>
<tr>
<td>6</td>
<td>SC</td>
<td>8 ± 1</td>
<td>0 / 7</td>
<td>18 ± 2</td>
<td>–</td>
</tr>
<tr>
<td>~30</td>
<td>SC</td>
<td>8 ± 1</td>
<td>0 / 4</td>
<td>18 ± 2</td>
<td>–</td>
</tr>
</tbody>
</table>
We further investigated the gastrointestinal tract as a potential source of interfering signal in addition to the above-discussed role of the stomach, as we noted autofluorescence in the intestines of several mice regardless of diet (Fig. 3.11). We dissected the gastrointestinal tract of these mice and found that areas of fluorescence correlate with regions of the intestines that contained food or feces for CDAHFD and SC mice. For CD mice, the stomach and feces were not as fluorescent as CDAHFD and SC mice. Given that the diet of CDAHFD and SC mice is itself autofluorescent, it is not surprising that the diet remains fluorescent throughout the digestion process. However, it is surprising that fluorescence in the intestines was also observed for some CD mice whose diet is not significantly autofluorescent. Autofluorescence in the intestines is a common problem reported in *in vivo* fluorescence studies using NIR light, and

![Figure 3.11: NIR/SWIR autofluorescence of the gastrointestinal tract in SC, CD, and CDAHFD mice.](image)

We show an example of a SC mouse with significant autofluorescence in the gastrointestinal (GI) tract detectable during *in vivo* imaging and in the excised tissue in the stomach, intestines, and feces. We also show two examples of CD mice—one without autofluorescence in the GI tract, despite feces present, and one with autofluorescence present. We also show an example of a CDAHFD mouse with some autofluorescence detectable in the stomach but none elsewhere; however, no feces were present in this example, which in general were autofluorescent in CDAHFD mice. Note the autofluorescence present in all mice in the inferior region of the abdomen during *in vivo* imaging is not from the gastrointestinal tract, but rather from the genitourinary anatomy, discussed in greater detail below (Fig. 3.12).
strategies for its avoidance have been proposed (mostly via changing diet composition).\textsuperscript{178,185,186} These observations suggest that the digestion process itself creates an autofluorescent pigment, perhaps through oxidation of fat and/or complex polysaccharides in the diet (creating crosslinked proteins, lipids, and carbohydrates like the lipopigments which autofluoresce in the liver), although the exact molecular origin of the signal was not further investigated here.

We further note that the genitourinary anatomy of the mice frequently exhibited measurable autofluorescence, detectable with \textit{in vivo} imaging. We dissected the genitourinary anatomy of SC mice and find that the testis, epididymis, and preputial gland give rise to this signal (\textbf{Fig. 3.12A}). It is known that the Sertoli and interstitial cells of Leydig in mice testis and the Leydig cells of human testis contain high levels of lipofuscin (increasing with age) as a consequence of moderate lipid droplet content in these cells.\textsuperscript{187–190} Therefore, we speculate that the signal that we observe in the mice genitourinary anatomy may also originate from lipofuscin/ceroid. We also note that the blood and gall bladders of the mice did not contain measurable autofluorescence (\textbf{Fig. 3.12B,C}), regardless of diet, suggesting that porphyrins and biliverdin/bilirubin do not have significant NIR/SWIR autofluorescence and do not contribute to

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure3.12}
\caption{Genitourinary anatomy NIR/SWIR autofluorescence and other potential competing signals. In nearly all mice (SC, CD, and CDAHFD) NIR/SWIR autofluorescence was detectable in the inferior region of the abdomen during \textit{in vivo} imaging (A, top). By excising the genitourinary anatomy, we identify this signal to be from the testes (white arrows), the epididymis, and the preputial gland (yellow arrow) (A, bottom). We further imaged the blood of CD and CDAHFD mice (B) and the gall bladders (C) and find that they are negative for NIR/SWIR autofluorescence, suggesting that porphyrins and biliverdin/bilirubin likely do not play a significant role in our NIR/SWIR autofluorescence quantification.}
\end{figure}
our measured disease-correlated signal. Potential signal of biliverdin/bilirubin was unlikely based on our results of CBDL cirrhotic liver models, which did not exhibit increased NIR/SWIR signal despite having high levels of accumulated bilirubin as a characteristic of the model.

3.6 NIR/SWIR autofluorescence in mouse models with dysregulated autophagy

Having identified lipofuscin/ceroid as the origin of NIR/SWIR autofluorescence in liver disease models, we can predict extensions of this work to other diseases characterized by changes in intracellular lipopigments. For one, an increasing number of neurodegenerative diseases are thought to feature accumulation of intracellular pigments like lipofuscin. These pigments often accumulate as a result of dysregulated macro-autophagy, which is a major intracellular degradation pathway. Intracellular autophago-lysosomes contain a mixture of cellular contents in various stages of degradation, including mitochondria and protein aggregates, and their steady state levels are determined by a balance of vesicle formation versus vesicle clearance. Among the neurodegenerative diseases that in at least some animal models feature enhanced levels of autophagic vacuoles are Alzheimer’s, Huntington’s, and Parkinson’s Disease, as well as neuronal ceroid lipofuscinosis (NCL).

One major limitation in studying autophagy and its either beneficial or pathologic effects has been the difficulty of analyzing autophagic vacuoles, which typically involves either laborious ultrastructural techniques such as electron microscopy, or introducing a fluorescently labeled transgene into cells and organisms to be probed. Probing the marker protein LC3-II can be a good indicator, but needs to be properly interpreted. Moreover, all these methods give a snapshot of a highly dynamic process, obscuring clear interpretation between increased formation and/or decreased breakdown. Developing a quick and straightforward imaging method for autophagic vacuoles would be a major advance, and may accelerate investigations into autophagic mechanisms contributing to brain pathology in neurodegenerative diseases, but also in other fields wherein autophagy is pathophysiologically involved. We investigate here the potential of our NIR/SWIR autofluorescence imaging technique to provide such an advance.

We investigate the NIR/SWIR autofluorescence of 12/15-lipoxygenase knockout mice as models of increased autophagy. The enzyme 12/15-lipoxygenase (12/15-LOX) has been studied for many years for its causative role in stroke-related brain injury, and recent studies have discovered that mice in which the enzyme is genetically deleted (12/15-LOX KO) exhibit increased autophagy, also in brain and liver, even under non-ischemic conditions. These knockout mice are otherwise reported to be phenotypically normal, although they have reduced breeding success and some of the mice may develop murine leukemia. The increased autophagy could result in accumulation of pigments like lipofuscin/ceroid in these mice, and thus we predict an increase in NIR/SWIR autofluorescence.
We imaged male 12/15-LOX KO mice and their age-matched C57BL/6 controls (n=10/group) in the imaging set-up described in Section 3.2 with 1000 nm longpass detection on the InGaAs camera (i.e. 1000–1620 nm detection). Mice were imaged in vivo after shaving and the liver and brain were subsequently extracted and imaged ex vivo. We find that the liver of 12/15-LOX KO is significantly more autofluorescent both in vivo and ex vivo than wild type controls (Fig. 3.13A,B). The brain was further found to be more autofluorescent in 12/15-LOX KO mice than wild type controls, however, the difference was much less than in the liver, and was not significant enough to detect a difference in vivo.

**Figure 3.13:** Autofluorescence in the brain and liver of 12/15-LOX KO mice versus wild type mice. Mice were imaged with 808 nm excitation and 1000 nm longpass detection on an InGaAs SWIR camera (1000–1620 nm detection). The liver both in vivo and ex vivo, and the ex vivo brain of 12/15-LOX KO mice were measured to be significantly greater than wild type control mice (A). Representative images of the brain (B, left) and the liver (B, right) of a 12/15-LOX KO and a wild type mouse are shown. We also find that old 12/15-LOX KO mice (9.5 months old, orange bar) have significantly greater autofluorescence signal than young 12/15-LOX KO mice (2 months old, purple bar) for ex vivo tissue samples (C).
Given that these mice are characterized by an accumulation of autophagic vacuoles over time, we further investigated the evolution of NIR/SWIR autofluorescence signal with mice age. We compared 12/15-LOX KO with age-matched wild type controls at approximately 38 weeks of age and at approximately 8 weeks of age (n=6 and n=3 respectively for old mice; n=2/group for young mice). We find that old mice have greater autofluorescence than young mice both in the liver and brain ex vivo tissues (Fig. 3.13C), in line with the expected accelerated age-related lipofuscin formation resulting from increased autophagy.

We further analyzed the 12/15-LOX KO mice tissue through immunohistochemistry to identify which neural cells exhibit SWIR autofluorescence. PFA-fixed brain tissue slices (20 μm thick) from 60 week old 12/15-LOX KO mice were stained with GFAP (for astrocytes), CD-31 (for endothelial cells), or Iba-1 (for microglia) antibodies conjugated to Alexa 647, or NeuN (for neurons) antibody conjugated to Alexa 488. We imaged the slides in the microscope (described in Section 3.2) using 900 nm longpass detection on the silicon camera (900–1050 nm detection) to detect the NIR/SWIR autofluorescent pigment, or using the Cy5 channel (Table 3.1) to detect Alexa 647 or the GFP channel to detect Alexa 488 on the same camera. We found no co-localization of the NIR/SWIR signal with the astrocyte and endothelial cell markers. However, we did find partial co-localization with NeuN and Iba1 cell markers, suggesting that the signal clusters intracellularly within neurons and microglia (Fig. 3.14A,B). This is consistent with the idea of NIR/SWIR autofluorescent intracellular deposits, possibly those containing lipofuscin/ceroid, which is characteristic of some neurodegenerative diseases. While these studies are ongoing, we suggest that NIR/SWIR autofluorescence in these models or others could be used to study the mechanisms of increased autophagy and its relevance to disease.

![Image of Immunofluorescence staining of 12/15-LOX KO brain tissue.](image-url)

**Figure 3.14: Immunofluorescence staining of 12/15-LOX KO brain tissue.** Brain tissue from 12/15-LOX KO mice were sliced 20 μm thick and stained with Iba-1 (A, for microglia) or NeuN (B, for neurons) conjugated to Alexa 647 and Alexa 488 respectively. The slides were imaged in the microscope described in Section 3.2 using 900 nm longpass detection on the silicon camera (i.e. 900–1050 nm detection) to detect the autofluorescent pigment (red image) or using the Cy5 or GFP channels (Table 3.1) and the same camera to detect Alexa 647 and Alexa 488 respectively (green image). The overlay of the two images is shown in the third column, with co-localized signal indicated by white arrows. Scale bars.
3.7 Discussion and conclusions

Liver cirrhosis is a severe health condition with high rates of morbidity and mortality. Over 600,000 adults in the U.S. are affected by cirrhosis, yet nearly 70% percent report being unaware of having liver disease, suggesting that many cases are undiagnosed.\textsuperscript{206} Non-alcoholic fatty liver disease (NAFLD) is the most common liver disorder in Western industrialized countries, and prevalence worldwide is steadily increasing due to increasing incidence of several common risk factors, such as metabolic syndrome, obesity, and type II diabetes.\textsuperscript{207,208} An estimated 30% of adults in the U.S. have NAFLD, with 2–3% of the general population estimated to have advanced to non-alcoholic steatohepatitis (NASH), which can progress to advanced fibrosis, liver cirrhosis, and hepatocarcinoma.\textsuperscript{209}

We show here enhanced NIR/SWIR autofluorescence signal in a variety of animal models for liver disease, sufficiently bright to detect noninvasively \textit{in vivo} and with sufficient contrast to delineate between abnormal and healthy biological structures. We find strong NIR/SWIR autofluorescence in the livers of mice with CCl\textsubscript{4}-induced cirrhosis (based on free radical induction), bright enough for \textit{in vivo} detection in awake, and freely-behaving mice at near-video rate speeds. We further show detection of very early-stage and less severe liver pathologies in NAFLD mouse models. The signal intensity in these models correlated with disease progression, promising a method of detecting cumulative liver disease-related injury in these animals, and potentially having broader implications as a diagnostic technique for patients in the clinic. We have further detected NIR/SWIR autofluorescence in the brains and livers of 12/15 lipoxygenase knockout mice with consequential up-regulated autophagy, a major intracellular degradation pathway that may be linked to an increasing number of neurodegenerative diseases including Huntington's, Parkinson's, and Alzheimer's disease.

We attribute the NIR/SWIR autofluorescence signal in these models to the lipopigment lipofuscin/ceroid. Lipopigments are granules consisting of an autofluorescent pigment and lipid components enclosed by a common, continuous membrane.\textsuperscript{210} They appear to be the product of lipid peroxidation, and may be symptomatic of membrane, mitochondrial, or lysosomal damage, or alterations of the intracellular degradation pathway, macro-autophagy.\textsuperscript{119} Ceroid and lipofuscin—terms that are closely related and often used interchangeably—are two types of lipopigments. Although ceroid and lipofuscin have the same cellular substance with similar physiochemical and histochemical properties, some distinguish the two based on the underlying pathophysiological mechanism of accumulation.\textsuperscript{180,211} Lipofuscin is often referred to as the “aging pigment” or “wear-and-tear pigment,” as it has long been associated with senescence, and can be found in post-mitotic cells undergoing regressive changes, \textit{e.g.} in the liver, neurons, cardiac myocytes, and retinal pigment epithelial cells.\textsuperscript{43,212} The term ceroid is generally used for the same lipopigment in all but aging, and is thus a broader term used to reflect pathological lipopigment accumulation in a variety of cells/tissues, including, but not limited to the brain, liver, pancreas, and testis; ceroid presence has been used as a diagnostic parameter in a variety of
pathological conditions such as tumors, atherosclerosis, malnutrition, toxic injury, and retinal degeneration. The intracellular accumulation of lipofuscin-like pigments can also be found as a consequence of oxidative stress, as a response to pathological conditions (e.g. the neuronal ceroid lipofuscinosis or Batten disease), or to toxic compounds. As we show here the use of NIR/SWIR autofluorescence to monitor lipofuscin/ceroid accumulation, we suggest that this technique could aid investigations into mechanisms of disease, mechanisms of senescence, or serve as a diagnostic/prognostic medical device.

Previous autofluorescence techniques have suffered from difficulties in detecting low-intensity signals, interpreting overlapping signals due to their spectral complexity, and/or the use of high-energy excitation sources. Our technique can detect low-quantum yield autofluorescence signals through the use of high-sensitivity, low noise cameras (both silicon-based and InGaAs-based for NIR/SWIR detection), and lower energy NIR excitation, which is less destructive to and penetrates deeper through biological tissue than UVA or visible light wavelengths. Furthermore, detecting longer NIR/SWIR wavelengths could enable greater specificity to lipofuscin/ceroid autofluorescence, as there is less autofluorescent background from other biomolecules at these wavelengths. These factors combined—nondestructive and deeper-penetrating excitation, and higher specificity due to suppressed background autofluorescence—enable our method to monitor disease-correlated autofluorescence noninvasively.

The possibility to monitor samples directly, in real-time is one of the most attractive features of autofluorescence-based techniques. At present, histochemical methods are the standard procedures in diagnosis of many disorders. However, these diagnostic procedures require the surgical removal of tissue samples (biopsies) followed by laboratory processing and staining, which can be expensive and time consuming. The invasiveness of repeated biopsies can further be considered unethical and hinder accurate longitudinal follow-up of disease. On the contrary, autofluorescence techniques do not require sample processing, as the detected signal is an intrinsic property of the tissue, and is a nondestructive method of analyzing cells and tissue in real-time with high spatial resolution. Potentially, in vivo analysis could be carried out without the need for sample excision (or sacrifice of the animal, in the case of longitudinal animal studies), especially as the development of fiber-optic instrumentation has made it possible to perform analyses in any area accessible to an endoscope.

We show here that indeed we can detect the state of disease in animal models in real time, and suggest that the NIR/SWIR wavelengths could open a new window for autofluorescence imaging at all size scales, from in vivo imaging down to microscopy of cellular and sub-cellular structures.
3.8 Additional experimental details

We validated the above-presented animal models of disease through histology on the liver tissue of each model, and describe the methods here in detail. H&E and picrosirius red (Electron Microscopic Sciences; 26357-02) sections of liver tissue of mice fed a CD and CDAHFD-diet were blindly scored according to the SAF-score, that provides a semi-quantitative evaluation of the severity of NAFLD. The amount of steatosis was scored using the percentage of the hepatic parenchyma occupied by fat-laden hepatocytes: a score of 0 indicated no fat; 1 indicates <33%; 2 indicates 34–66%; 3 indicates >67%. The activity is the compound score of hepatocellular ballooning and inflammation, whereas ballooning is graded: 0: none; 1: presence of clusters of ballooned cells; and 2: same as 1 but with some enlarged hepatocytes (≥2 times). Inflammation was graded as: 0: none; 1: ≤2 foci per 20x; and 2: >2 foci per 20x. Fibrosis was scored as F0: no fibrosis; F1: mild pericellular fibrosis; F2: pericellular and (peri)portal fibrosis; F3: bridging fibrosis; F4: cirrhosis. NASH is defined as the presence of steatosis accompanied by both inflammation and ballooning.

We also investigated the relationship between the in vivo and the ex vivo NIR/SWIR autofluorescence intensities measured for all mice of Section 3.5 (CCl₄-treated mice and their respective controls, CBDL, SC, CD, CDAHFD, CD/CDAHFD with vehicle, and CD/CDAHFD with telmisartan mice). We plotted the in vivo liver intensities versus the respective ex vivo liver intensities (for the same mouse) and observed a linear trend (Fig. 3.15). Fitting a linear regression to the data resulted in an R² value of 0.702.

![Figure 3.15: Correlation between in vivo and ex vivo liver intensities for mouse models of liver disease. This plot shows the NIR/SWIR autofluorescence intensities measured for all mice of Section 3.5, in which each point (black circles) represents the in vivo and ex vivo intensity for a given mouse. A linear regression was fit to the data (dotted line) which had an R² value of 0.702.](image-url)
3.9 Chapter-specific acknowledgements

The results presented here are based on a manuscript in preparation.

Many individuals contributed to the conception and design of these studies, and discussed their results and implications. Dr. Oliver Bruns and Dr. Matthias Pinter are acknowledged for their initial observation of SWIR autofluorescence in the liver of a CCl₄ cirrhotic mouse model. Matthias, Ivy Chen, and Dr. Wilhelmus (Wilco) Kwanten provided all liver disease mouse models for these studies. Wilco, in particular, provided CBDL mice, NAFLD mice, and 3/6 week CCl₄ mice, and all of their tissue processing (slices, staining procedures, slides, histology). Jessica prepared all imaging set-ups with prior guidance from Oliver, and Wilco, Ivy, and Juanye Zhang were also present for and aided in animal imaging. Jessica and Wilco performed microscopy of tissue slides. Professor Rakesh Jain, Professor Mounig Bawendi, Wilco, and Ivy participated with Jessica in essential discussions and helped plan these experiments. We further thank Julia Kahn and Sam Chatterjee for excellent technical support. Assistant Professor Klaus van Leyen and Dr. Yi Zheng provided all of the 12/15-lipoxygenase knock-out mice, aided in their imaging and tissue harvesting, and processed the tissues for all staining procedures. Professor Bawendi, Klaus, Yi, Franziska Lieschke and Oliver all provided many helpful suggestions and discussions in these experiments. Jessica processed and analyzed all imaging data.

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Chapter 4

Tuning fluorescence imaging contrast in the SWIR

4.1 Introduction: the role of tissue optical properties in fluorescence imaging

Fluorescence imaging has emerged as a powerful tool for preclinical in vivo imaging and as a promising clinical technology, particularly for surgical guidance.\textsuperscript{12–17} The strength of this technique has largely derived from the development of exogenous contrast agents that are capable of specifically highlighting particular anatomical features or biological processes, down to the single-cell level in some applications.\textsuperscript{219–221} The agent is designed to target a biomolecule of interest, is injected or ingested, and imaged either noninvasively through the skin or accessed during surgery. Fluorescence imaging has become the standard of care in a variety of ophthalmologic diagnostics, and is emerging as a diagnostic and/or surgical aid in nearly every other medicinal practice, including tumor resection and metastatic lymph node mapping in oncology, bypass surgeries in gastroenterology and cardiology, and perfusion assessments in reconstructive and plastic surgeries.\textsuperscript{30–36}

Most fluorescence imaging approaches use widely available visible (400–700 nm) and near infrared (NIR, 700–1000 nm) light emitting fluorophores, which can be detected on high-performing, inexpensive silicon detectors. However, recent advances in longer wavelength detection and synthesis of infrared emitters have enabled fluorescence imaging to also expand into the shortwave infrared (SWIR, 1000–2000 nm) region. Recent progress in indium gallium arsenide (InGaAs) sensor fabrication has led to the steady decline in the cost of SWIR imaging cameras, while camera sensitivity and resolution have increased. Moreover, a wide variety of biologically-compatible SWIR emitters have emerged, including organic dyes with peak emission in the SWIR,\textsuperscript{62–65} carbon nanotubes,\textsuperscript{47,66} quantum dots,\textsuperscript{67–69} rare-earth-doped nanocomposites,\textsuperscript{70} and gold nanoparticles.\textsuperscript{71} It has also been shown that NIR dyes, which are commercially available and approved for clinical use, can be used for many SWIR imaging applications.\textsuperscript{72,222}

Selecting an appropriate imaging wavelength is critical to enabling a given application in biological fluorescence imaging. Imaging sensitivity, penetration depth, and spatial resolution depend on the optical properties of biological tissue, and these tissue properties, including
autofluorescence, absorption, and scattering, are each wavelength-dependent. Tissue autofluorescence creates interfering background signal, decreasing sensitivity to an exogenous fluorophore. Autofluorescent molecules, such as NADPH, flavins, and collagen, emit the majority of light at visible wavelengths and can be avoided by moving into the NIR and SWIR. Tissue absorption, which reduces overall signal intensity, is primarily due to hemoglobin, melanin, lipids, and water. These tissue components each have unique spectral profiles, but share a common absorption minimum between approximately 650–900 nm, thus, the NIR has long been identified as an optimal imaging window for in vivo studies requiring high penetration depths. Finally, scattering, caused by the refractive index inhomogeneities within most tissues, misdirects photons from the direct path to the detector, degrading spatial resolution. Tissue scattering is dominated by Mie scattering, which has a complicated wavelength-dependency, but in general can be minimized by imaging at longer wavelengths.

Considering the combined effects of tissue autofluorescence, absorption, and scattering, the SWIR regime promises greater fluorophore sensitivity, higher tissue penetration depths, and greater spatial resolution than visible and NIR imaging. For many applications, these improvements are related to an overall increase in image contrast. Studies in two-dimensional tissue-mimicking phantoms predict fluorescent contrast to be the highest between 700 nm and 1400 nm and between 1500 nm and 1600 nm, corresponding to the minima in the transmission spectra of biological tissues. Furthermore, recent demonstrations in vivo, with fluorescence originating from a three dimensional network of structures, show particularly high contrast between 1300 nm and 1400 nm and an additional contrast increase between 1500 and 1700 nm. However, in vivo image contrast has only been probed using wide wavelength windows in the SWIR and has not been investigated with high wavelength resolution.

By probing image contrast with high wavelength resolution, we show that SWIR fluorescence contrast is primarily governed by water absorption in tissue. We first show the wavelength-dependence of contrast in three-dimensional tissue phantoms, disentangling the individual contributions of tissue absorption and tissue scattering toward image contrast. We then corroborate our phantom results with studies in vivo in mice. We further propose a theoretical model to investigate how contrast and penetration depth vary with wavelength in the SWIR, and confirm the results with experimental data from ex vivo biological tissue. We find in each case that the highest contrast occurs at wavelengths with the highest water absorption. While these wavelengths have conventionally been avoided to obtain greater signal throughput, we show that in contrast-limited applications, such as tissue with high fluorescent label density, the improvement in contrast from water absorption enables resolution of deeper structures, resulting in a greater imaging penetration depth. The trade-off between contrast, penetration depth, and fluorescence signal intensity can thus be balanced for a given application by selectively imaging at wavelengths with a particular level of tissue absorption.
4.2 The effects of absorption and scattering on fluorescence contrast in three-dimensional tissue phantoms

Here, we aim to probe the relationship between tissue scattering, tissue absorption, and fluorescence contrast at SWIR wavelengths. The combined role of tissue scattering and water absorption determines optimal contrast, however, their individual influence are important to understand to be able to predict the expected behavior of contrast with wavelength. It is expected that contrast should continuously increase with wavelength due to scattering alone, as tissue scattering decreases largely in accordance with Mie theory. However, biological tissue absorption, dominated by water in the SWIR, has a more dynamic wavelength-dependency; water exhibits three local absorption peaks near 970 nm, 1200 nm, and 1450 nm with increasing respective intensity. A significant difference in the behavior of scattering and absorption occurs at wavelengths longer than 1450 nm: scattering decreases beyond these wavelengths, suggesting an increase in contrast, while water absorption decreases past its strong peak near 1450 nm. Thus, the dominant mechanism of contrast improvement can be identified if the contrast is probed with sufficient wavelength resolution.

We probed the individual influence of absorption and scattering on fluorescence contrast through three-dimensional tissue phantoms (Fig. 4.1). Liquid tissue phantoms were designed based on previous literature which confirmed similarity to biological tissue optical properties. Intralipid® 20% (Baxter Healthcare Corporation), as a scattering medium, was diluted to 1% in water (H₂O) to mimic the attenuation (both scattering and absorption) of tissue, or in deuterium oxide (D₂O, Aldrich; 99.9 atom % D) which exhibits approximately equal scattering properties but has no absorption in the SWIR. Thus, the D₂O phantom was used to measure the influence of scattering alone on contrast, while the H₂O phantom revealed the influence of scattering in the presence of absorption. Attenuation spectra of the phantom materials were recorded on a Cary 5000 UV-Vis-NIR infrared spectrometer from Varian (Fig. 4.1A).

Glass capillaries were filled with a mixture of fluorescent quantum dots (QDs) and submerged in the phantom materials. We chose QDs as the fluorescent probe in our study in order to obtain bright emission across all SWIR wavelengths. Lead sulfide (PbS) QDs were prepared according to the procedure of Hines and Scholes, and solutions with emission maxima near 1020 nm, 1200 nm, and 1430 nm were mixed to obtain a broadband SWIR emitter. Photoluminescence spectra of the QD mixture was measured by exciting the samples with a 532 nm diode laser, and collecting the emission using a pair of gold-coated off-axis parabolic mirrors directing to a single-grating spectrometer (Acton; Spectra Pro 300i). The output of the spectrometer was measured using a liquid nitrogen cooled InGaAs line camera (Princeton Instruments; OMA V, 512 x 1 pixel array) (Fig. 4.1B). The QD mixture was then loaded into glass capillaries (1 mm diameter, VWR) and submerged in the liquid phantoms individually or in two, perpendicular layers (Fig. 4.1C). Submersion depths of 2 mm and 4 mm were chosen for the two-layer capillaries to mimic overlapping vessels beneath a layer of tissue; the depth
roughly corresponds to that reported previously for brain vessels underlying the scalp tissue of a mouse.\textsuperscript{47} The submerged QD capillaries were excited with diffuse 808 nm light and their fluorescence was imaged in 50 nm wavelength bands across the SWIR (Fig. 4.1D, see Section 4.7, Fig. 4.13 for full image sets).

We then quantified the contrast of each image of the fluorescent QD capillaries. We used the coefficient of variation as our contrast metric, which is defined as the ratio of the standard deviation in signal intensities, $\sigma$, to the mean signal intensity, $\mu$.

Figure 4.1: Water absorption enhances contrast in a three-dimensional tissue phantom through background signal suppression. The tissue phantom is composed of Intralipid\textsuperscript{®} diluted to 1\% in either water or deuterium oxide. The attenuation spectra of the tissue phantom materials show scattering of light for 1\% Intralipid\textsuperscript{®} in deuterium oxide ($D_2O$, orange line) and both scattering and absorption for 1\% Intralipid\textsuperscript{®} in water ($H_2O$, blue line) (A). We filled two capillaries with a mixture of SWIR-emitting lead sulfide (PbS) quantum dots (QDs), the photoluminescence (PL) spectrum of which shows broad SWIR emission (B). We then submerged the capillaries 2 mm and 4 mm deep in the liquid tissue phantoms, illuminated with diffuse 808 nm light, and imaged from the top with an InGaAs SWIR camera (C). Images of the capillaries in both $D_2O$-based (top) and $H_2O$-based (bottom) phantoms were taken, filtering the emission through 50 nm bandwidth bandpass (BP) filters centered across SWIR wavelengths (D, complete image set shown in Fig. 4.13). All images are scaled to fill the maximum displayable intensities and scale bars represent 1 mm. The contrast, $c_V$ (Eq. 4.1) of the images varies strongly with wavelength in the $H_2O$-based phantom and is overall greater than in the $D_2O$-based phantom (E).
\[ c_V = \frac{\sigma}{\mu}. \quad \text{Equation 4.1} \]

In a high contrast image, pixel intensities are high for regions of signal and are low for regions of background, resulting in a high \( \sigma \) and thus in a high value of the contrast metric. Low contrast images, on the other hand, have little variability in pixel intensity, and thus a low \( \sigma \) and contrast metric. We divide \( \sigma \) by \( \mu \) to account for the fact that a small \( \sigma \) is negligible if the average intensity is high, while the same small \( \sigma \) matters if the average intensity is low. This metric is applicable when comparing different images of the same object, and can be applied to an entire image with multiple features, unlike the signal to background ratio contrast metric, which can only be applied to a single image feature, and also requires known boundaries between the signal-generating feature and the background.\(^\text{235}\)

We find that only in the presence of absorption does contrast significantly change as a function of wavelength between 900-1600 nm (Fig. 4.1E). In the D\(_2\)O-based phantom, contrast increased slightly across SWIR wavelengths. However, when D\(_2\)O in the phantom is replaced with H\(_2\)O, contrast was overall greater, and increased up to 1450 nm before decreasing beyond 1450, to the edge of our detection range at 1600 nm. The decreasing contrast beyond 1450 nm reveals the important role of absorption in the enhancement of contrast, as the decline in scattering beyond these wavelengths should, in its absence, favor a contrast increase. This result is reproducible and perhaps even more evident in a separate tissue phantom based on silica beads as a scattering medium, which is more weakly-scattering, approximating the scattering of tissue such as skull (see Section 4.7).\(^\text{236–238}\)

The increase in contrast that we observe at wavelengths of strong water absorption can in part be attributed to the suppression of background signal from the deeper submerged capillary. According to the Beer-Lambert law, the signal attenuation depends exponentially on the depth of the emitting structure and the absorption strength of the medium. By varying the absorption strength, which is achieved by tuning the imaging wavelength, we can tune the depth at which the signal falls below the noise level of the system. Thus, the relative intensities of structures at different depths is controlled by the choice of wavelengths.

In addition, we find that water absorption also improves SWIR contrast by suppressing scattered light from the emitting object itself. We immersed a single capillary into the tissue phantoms at a depth of 2 mm and imaged with the same set of 50 nm bandpass filters (Fig. 4.2). Analyzing the intensity profile of the capillary cross-section reveals a narrowing of the intensity profile for wavelengths with strong water absorption. Narrowing is also observed in the D\(_2\)O phantom, but continuously with wavelength and overall to a lesser extent. We quantified this effect by calculating the inverse of the capillary intensity profile peak widths at ten percent maximum height and find that the metric is indeed the greatest at 1450 nm, with broadening of the width observed for wavelengths longer than 1450 nm. This indicates that water absorption is
responsible for suppressing the scattering around the capillary, thus improving capillary contrast and resolution.

Therefore, our results suggest that water absorption is the primary optical property which enhances fluorescence contrast across SWIR wavelengths. We expect that highly scattered photons from deeper lying structures, as well as scattered photons from an object itself, are more likely to be absorbed than ballistic photons of the same object, as scattered photons travel a longer path length through the tissue. This difference in absorption probability becomes more pronounced for wavelengths that exhibit stronger water absorption. As a result, fluorescence contrast is greatest at those wavelengths with the highest absorption.

![Figure 4.2: Water absorption enhances contrast and resolution through scattering suppression. A SWIR-emissive capillary was submerged 2 mm deep in 1% Intralipid® tissue phantom diluted in D₂O (A, top) or H₂O (A, bottom), was illuminated with 808 nm light, and the emission filtered through 50 nm bandwidth bandpass filters centered at the indicated wavelengths. Scale bars represent 1 mm and images are scaled to fill the maximum displayable intensities. The intensity profiles (B, C) and the inverse of the full width at 10% maximum height (FW10%M) of the intensity profiles (D) shows that the scattering pedestal of capillaries in the H₂O-based phantom narrows at wavelengths of stronger water absorption, and is overall narrower than that of capillaries immersed in the D₂O-based phantom, which narrows continuously.](image)
4.3 The wavelength-dependence of contrast *in vivo*

To investigate the wavelength-dependence of contrast *in vivo*, we labeled the brain vasculature of a mouse using a mixture of QDs, and imaged the fluorescence through the mouse’s skin and skull in 50 nm wavelength bands centered across SWIR wavelengths. For this application, we used indium arsenide (InAs) QDs, as their ability to be protected with multiple over-coated “shell” layers (e.g. cadmium selenide (CdSe) and/or cadmium sulfide (CdS)) compared to PbS QDs better protects the core and thus the fluorescence quantum yield when transferred into aqueous solutions. InAsCdSeCdS and InAsCdSeZnS core-shell-shell QDs were synthesized\textsuperscript{67} and transferred into aqueous solution via phospholipid micelles\textsuperscript{239} as previously described. To obtain broad SWIR emission, aqueous QD samples emitting at 970 nm, 1110 nm, and 1300 nm were mixed. A C57BL/6J mouse (34 g, male, 22 weeks, Jackson Lab) was anaesthetized via intraperitoneal injection of ketamine/xylazine, shaved, and placed in the imaging setup. It was irradiated with 808 nm laser light at 50–70 mW/cm\(^2\) and 300 $\mu$g of aqueous QD-phospholipid micelles were injected via the tail vein. The brain vasculature was imaged using 50 nm bandwidth bandpass filters centered across SWIR wavelengths, refocusing the optics to maximize the resolution of the central brain feature after each filter change (Fig. 4.3).

![Figure 4.3: Mouse brain vasculature imaged across SWIR wavelengths.](image)

The brain vasculature of a mouse was fluorescently labeled with a broadly-emitting InAs QD mixture and imaged through intact skin and skull. The emission was filtered with 50 nm bandwidth bandpass filters centered in 50 nm spacing between 950 nm and 1600 nm. Each image is scaled to fill the maximum displayable intensities.
The contrast $c_V$ (Eq. 4.1) was evaluated for each image, and plotted as a function of bandpass center wavelength (Fig. 4.4). In accordance with our phantom studies, we find that the wavelength-dependence of contrast for the in vivo vasculature follows the same trend as the water absorptance spectrum, suggesting that water absorption plays a key role in improving in vivo fluorescence image contrast at SWIR wavelengths. The contrast generally increases with wavelength until reaching a peak at 1450 nm. In particular, the decrease of contrast in vivo beyond 1450 nm supports the dominant influence of water absorption on contrast over scattering, as the continuous decrease of scattering would cause an increase in contrast beyond these wavelengths.

We reproduced our findings with multiple regions of interest, and further verified our findings with a secondary contrast metric (Fig. 4.5). The contrast metric $c_V$ (Eq. 4.1) was assessed for regions of interest across three distinct vessels in the brain, and for all pixels of the image. Furthermore, we compared the trend reflected by our contrast metric $c_V$ (Eq. 4.1) to that of the signal to background ratio. The signal to background ratio metric requires a region of interest with a clearly defined object of interest (in our case, a particular brain vessel). The mean signal intensity of the object of interest is then divided by the mean intensity of all other values in the region of interest which are background signal. Using this contrast metric likewise shows good agreement with the water absorptance spectrum and with the contrast metric, $c_V$. 

Figure 4.4: Correlation of in vivo fluorescence contrast with water absorptance. Brain vasculature of a mouse was fluorescently labeled with a broadly-emitting InAs quantum dot mixture and imaged with 50 nm bandwidth bandpass filters centered in 50 nm spacing between 950 nm and 1600 nm (Fig. 4.3). We show here images taken with 1000 nm, 1200 nm, 1450 nm, and 1600 nm filters and their respective intensity profiles across a line of interest (yellow line in image inset) used to calculate the contrast (standard deviation/mean) of a vessel (A). Images are scaled to fill the maximum number of displayable intensities. The contrast, $c_V$ (Eq. 4.1), of the vessel plotted against bandpass center wavelength (black solid line) shows correlation with water absorptance (blue dotted line) (B).
Figure 4.5: The wavelength-dependence of contrast for different regions of interest and contrast metrics. The contrast $c_V$ (Eq. 4.1) was calculated for each of the images in Fig. 4.3 across the entire image (A, B) and across three different brain vessels (C-H). Regions of interest are shown in yellow on the images, which are taken with a 1600 nm bandpass filter. The contrast was plotted against wavelength for each region of interest (black solid line) and overlaid with the water absorptance spectrum (blue dashed line) and shows good agreement. In I-J, the same region of interest depicted in E was used to calculate contrast using the signal to background ratio, defined as the average signal intensity of the vessel divided by the average signal intensity of all other lines of interest values. The plot also shows good agreement with the water absorptance spectrum and with $c_V$ (Eq. 4.1).
4.4 The attenuation-dependence of contrast and penetration depth in a theoretical contrast model

Conventionally, wavelengths of high tissue absorption have been avoided in fluorescence imaging, due to the belief that penetration depth is limited by signal intensity. Indeed, if tissue attenuation is too high, fluorescence emission from submerged structures will never reach the detector. However, we show here the existence of two regimes—one in which penetration depth is signal-limited and one in which penetration depth is contrast-limited. We demonstrate an example of the latter case, and show that tissue absorption can enhance imaging penetration depth in the SWIR for such applications.

We first quantify the relationship between contrast and attenuation through an illustrative theoretical model. In the model, tissue is described by a semi-infinite slab with emitting cells homogenously distributed throughout the slab. The signal of interest, $S$, arises from the focal plane at depth $D$, and a background signal, $BG$, arises from all planes elsewhere (Fig. 4.6A). The background signal $BG$ can result from autofluorescence, non-specific labeling, or a high label density, for example. The signal intensities are modeled according to Beer-Lambert’s law:

$$ S = S_0 e^{-D\mu_{ex}} e^{-D\mu_{em}} $$

Here, $S_0$ describes the in-focus signal intensity at zero depth, and $\mu_{ex}$ and $\mu_{em}$ are the attenuation coefficients of the excitation and emission wavelengths, respectively. We consider the attenuation coefficient as a lumped parameter containing all sources of attenuation (e.g. absorption and scattering). The first exponential term accounts for the attenuation of the excitation light on the way to the focal plane and the second exponential term denotes the decay of the emitted light traveling from the focal plane to the detector.

Similarly, we calculate the background intensity, assuming that the emission is uniform through all planes of the slab:

$$ BG = BG_0' \int_0^\infty e^{-z(\mu_{ex}+\mu_{em})} \, dz $$

$$ = BG_0' \frac{1}{\mu_{ex}+\mu_{em}} $$

where $BG_0'$ is the out-of-focus background intensity per millimeter slab at zero depth. We then approximate the contrast as the ratio of the signal intensity to the background intensity:

$$ C = \frac{S_0}{BG_0'} e^{-D(\mu_{ex}+\mu_{em})} (\mu_{ex} + \mu_{em}) $$

Equation 4.2

We then use Eq. 4.2 to plot contrast as a function of the total attenuation at a fixed depth of 1 mm. Our results show that initially, contrast increases with attenuation, until reaching a maximum, after which stronger attenuation reduces the contrast (Fig. 4.6B). For a fixed
excitation wavelength, this trend can be explained by the roles of the emission originating from behind the focal plane and the emission originating from in front of the focal plane. Initially, the stronger the attenuation, the less emission originates from background signal behind the focal plane, reducing background signal and increasing contrast (Fig. 4.6C). A maximum is reached when the attenuation length (namely the inverse of the overall attenuation coefficient) equals the imaging depth (Fig. 4.6D). If the attenuation is further increased, the signal from the focal plane is attenuated and therefore the contrast decreases (Fig. 4.6E).

With this model, we further estimated the penetration depth trend across SWIR wavelengths. The penetration depth can be thought of as the maximum imaging depth at which one can still resolve the structures of interest; this depth is reached when the image contrast drops below some threshold contrast value. In our model, we required $C$ to be at least 3, such that $S_0$ is significantly greater than $BG_0'$ at zero depth, and therefore distinguishable from background. We first calculated the contrast as a function of imaging depth ($D$) and background ($BG_0$) using literature values$^{238}$ for the tissue attenuation coefficient at 808 nm for the excitation wavelength and at wavelengths of different SWIR bandpass filters for the emission (Fig. 4.7A). Using the established threshold for the minimum resolvable contrast, $C$, we extracted the penetration depth for each wavelength (Fig. 4.7B,C).
Figure 4.7: Dependence of contrast on imaging depth and inherent background intensity. Contrast is plotted as a function of imaging depth and inherent background intensity for a fixed level of signal (A). Contrast is scaled according to the color bar shown, with contrast values greater than 5 displayed at the maximum intensity color. The white dotted line indicates the threshold contrast value of three, considered to be the minimal resolvable contrast; values to the right of this line are considered unresolvable. Thus, this line indicates the maximum penetration depth for a given level of background signal in the system as plotted in Fig. 4.8. The penetration depth shows opposing trends with SWIR wavelength for applications with low levels of background (B), versus applications with high levels of background (C) (schematized in Fig. 4.8).
The results show the existence of two regimes which have opposite relationships between penetration depth and imaging wavelength (Fig. 4.8). In the first regime, wavelengths with low tissue absorption favor contrast and therefore also penetration depth. This “signal-limited” regime applies in the case when $\frac{s_0}{BG_0}$ is inherently high, i.e. systems with inherently strong contrast, such as tissue with a large, isolated emitting structure of strong signal or tissue with sparse label density and low background signal. In the second regime, wavelengths with high absorption enable the greatest contrast and simultaneously enable the greatest imaging penetration depth. The system is thus “contrast-limited” which occurs in the case when $\frac{s_0}{BG_0}$ is small, as in a highly-labelled tissue with significant background emission, or for small, weakly-emitting objects with inherently low signal to background. We note that while our discussion above centered on a fixed excitation and a varying emission wavelength, Eq. 4.2 implies that the same findings should apply when varying the excitation wavelength while keeping a fixed emission window.

Figure 4.8: Theoretical model of penetration depth versus inherent background signal shows two regimes with opposing wavelength relationships. By selecting a threshold contrast in our model to define minimally resolvable structures (Fig. 4.7), we extracted the penetration depth for each wavelength, plotted here against background signal for select wavelengths. We find that for a fixed signal intensity at small $BG_0$, wavelengths of minimal absorption have the greatest penetration depth, defining a signal-limited regime, schematized as a large, single-emitting structure, whereas at large $BG_0$ the opposite trend prevails, defining a contrast-limited regime, schematized as many small, distributed, labeled structures.
4.5 Contrast and penetration depth enhancement in \textit{ex vivo} tissue microscopy

In support of the findings of our theoretical contrast model, we demonstrate an example of a contrast-limited \textit{ex vivo} tissue sample in which fluorescence image contrast and imaging penetration depth are both improved by imaging at SWIR wavelengths with strong tissue absorption. We labeled liver tissue with a broadly emitting QD emulsion and acquired z-stacks of the extracted, labeled tissue, filtering the emission with 50 nm bandpass filters centered across SWIR wavelengths to obtain both wavelength and depth resolution.

The QD emulsion was prepared by adding 1 mg of InAs quantum dots\textsuperscript{67} with peak emission near 1150 nm and 1420 nm (\textbf{Fig. 4.9A}) dispersed in chloroform to a mixture of 28 \(\mu\)L lecithin (phosphatidylcholine, 25 mg/mL in chloroform), 100 \(\mu\)L of phospholipid-PEG2000 (25 mg/mL in chloroform, Avanti Polar Lipids; Cat. No 880130), and 400 \(\mu\)L of soybean oil (25 mg/mL in chloroform). The mixture was sonicated for 30 seconds, and afterwards the remaining solvent was evaporated by pressurized air flow. Particles were re-dispersed in 2 mL of 0.9% sodium chloride (USB; Bacteriostatic 0.9% Sodium Chloride Injection) and the mixture was sonicated until a homogenous emulsion was formed. The final solution was filtered through a 0.45 \(\mu\)m pore filter after sonication and again immediately before injection.

Two hundred microliters of the QD emulsion was injected intravenously via the tail vein into a C57BL/6J mouse (The Jackson Laboratory, male, 9 weeks old). Approximately 20 minutes after administration, a perfusion fixation was performed using 2\% paraformaldehyde (PFA, Electron Microscopy Sciences, EM grade, 20\% solution) in phosphate-buffered saline (PBS, Corning, PBS 1X), and the liver was dissected. For imaging, the liver sample was kept in a glass-bottom micro-well dish (MatTek Corporation) containing water (Corning; Cell Culture Grade Water) to avoid dehydration. The micro-well dish was placed on the stage of a

\textbf{Figure 4.9: Ex vivo liver microscopy.} The photoluminescence spectrum of an Indium Arsenide (InAs) quantum dot emulsion mixture shows broad SWIR emission (A). The quantum dots accumulate in individual cells in the liver of a mouse, shown here imaged with a 50 nm bandpass filter centered at 1450 nm (B). The scale bar represents 100 \(\mu\)m. The liver is imaged via fluorescence microscopy using 808 nm laser light for excitation and the emission filtered through 850 nm long-pass filters and a 50 nm bandwidth SWIR bandpass (BP) filter (C).
microscope (described previously in detail in Section 3.2) to acquire z-stacks of images for different wavelengths (Fig. 4.9B, C). Light emitted by the sample was collected by the objective (Nikon; CFI Plan Apochromat λ 10x) and passed through a longpass 900nm dichroic mirror, a hard-coated 850 nm longpass filter (Thorlabs; FELH0850), and a 50 nm bandpass filter (Edmund Optics) for wavelength-selective imaging. The light was directed to an output port of the microscope onto the array of the InGaAs camera. Acquisition settings (e.g. integration time, filters) for each figure are detailed in Appendix A, Table A.2.

For each image, we determined the contrast, \( c_V \) (Eq. 4.1), over the entire image for each wavelength, and the penetration depth of the z-stacks (Fig. 4.10). The penetration depth was determined using an algorithm adapted from Rowlands et al.\(^{240}\) This algorithm determines the deepest tissue position at which an image with a given spatial resolution can be formed (see Section 4.7 for algorithm details).

Figure 4.10: Contrast and penetration depth enhancement in microscopy of \textit{ex vivo} liver tissue. A mouse liver labeled with a QD emulsion was imaged via microscopy at 10x magnification with 50 nm bandpass (BP) emission filters centered across SWIR wavelengths, a subset of which are shown here at imaging depths of 40 µm, 70 µm, and 100 µm for 1000 nm, 1200 nm, 1450, and 1600 nm filters (A). All images are set to fill the maximum displayable intensities and the scale bar represents 200 µm. The contrast, \( c_V \) (Eq. 4.1) was calculated and plotted as a function of wavelength (black line) for images taken at a depth of 80 µm (B). The contrast shows a similar trend to the water absorptance spectrum (blue line). Furthermore, penetration depth was calculated using an algorithm adapted from Rowlands et al. and plotted as a function of wavelength, which exhibits a similar trend (C).\(^{240}\) The dashed lines mark the depths at which the images in (A) were taken, and the crosses display the wavelength positions. Green boxes and crosses indicate images considered to be resolvable within the contrast threshold.
Consistent with our macroscopic tissue phantom and in vivo data, we find that the liver microscopy images have the greatest contrast at wavelengths of high water absorption. Furthermore, as predicted by our theoretical model, we observe that the penetration depth likewise increases with water absorption. At shallow depths, e.g. 40 μm, an image of the labeled liver features is formed at all SWIR wavelengths, but imaging much deeper, e.g. 100 μm, causes significant loss of image contrast for most SWIR wavelengths except for those immediately around the water absorption peak at 1450 nm. While signal decreases when imaging at 1450 nm versus 1000 nm due to an almost 20 times greater optical density (OD) of the tissue, contrast and penetration depth more than double.

Our data also demonstrate that in this application, imaging at maximum absorption around 1450 nm enhances the penetration depth by almost 50% compared to imaging at 1600 nm which was previously believed to be the optimum for SWIR imaging. This observation shows that the contrast, and not the signal, limits penetration depth in this case. Although deep structures have sufficient signal to be detected, they are unable to be resolved due to overlapping signal from structures in the background, foreground, or scattered light from the object itself (Fig. 4.11). Imaging at wavelengths of strong tissue absorption enhances image contrast, and therefore enables resolution of deeper structures, resulting in a higher penetration depth.

### 4.6 Discussion and conclusions

Our findings provide a rationale for tuning image contrast in biological SWIR fluorescence imaging. We show in a three-dimensional tissue phantom, in in vivo brain vasculature imaging of a mouse, and in ex vivo microscopy of liver cells that image contrast has a wavelength-dependence governed by the absorption of the tissue, which is dominated by water absorption.
in the SWIR. We observe in these experiments that the greatest image contrast is obtained around 1450 nm where absorption from water is the greatest. We demonstrate the correlation between absorption of water and the selective suppression of light having a longer path length through tissue, such as multiply-scattered photons and fluorescence from deep background structures. This interplay between absorption and scattering enables fine-tuning of fluorescence imaging contrast along the water absorption spectrum. Suppression of this light, however, comes at the cost of decreasing the overall signal intensity, which requires longer integration times to achieve a sufficient signal to noise ratio. In our study, the bright indium arsenide and lead sulfide quantum dots used for contrast had sufficient signal to be imaged in 50 nm wavelength bands across the SWIR, eliminating signal limitations. However, for contrast agents or applications that radiate less fluorescence signal, it may be necessary to choose an imaging wavelength that balances signal requirements with optimal image contrast, such as wavelengths near 1300–1350 nm or using a 1300 nm longpass filter on an InGaAs camera (Fig. 4.12).

Furthermore, we predict with a theoretical model and demonstrate in ex vivo liver tissue microscopy that penetration depth is likewise tunable by selection of the SWIR wavelength (either the emission and/or the excitation wavelength). In our study, the greatest penetration depth was achieved at wavelengths of greatest water absorption, around 1450 nm, which had the greatest image contrast. We expect any contrast-limited tissues such as those with a high label density, strong autofluorescence, or other strong background signal, to show a similar

![Figure 4.12: Opposing wavelength dependencies of signal and contrast.](image)
effect. However, our theoretical model suggests that the penetration depth for other applications, such as those with low fluorescent labeling density, low background signal, or large, easily resolvable structures (e.g. whole organs), may be limited by signal rather than contrast. For these applications, wavelengths with lower absorption lead to the highest penetration depths. Furthermore, we expect imaging with higher numerical aperture, and consequently a smaller depth of field, as in microscopy, will benefit more from this absorption effect than imaging with lower numerical aperture. We conclude that it is therefore important to understand whether a given imaging application is contrast-limited or signal-limited in order to predict the behavior of the penetration depth with wavelength.

These results suggest an approach for improving both fluorescence image contrast and penetration depth in tissue by deliberate selection of specific SWIR wavelengths. The variance of the magnitude of water absorption within the SWIR spectral window distinguishes this wavelength regime from the NIR, which was previously proposed as an ideal optical window for fluorescence imaging. Given these results, which show that contrast and penetration depth increase with increasing tissue attenuation, the SWIR wavelength regime may be preferable for imaging over the NIR in contrast-limited applications (e.g. resolution of fine, highly labelled structures, as in angiography). This work further motivates the continued development of bright and biocompatible fluorescent probes for the longest SWIR wavelengths.

4.7 Additional experimental details

All animal experiments were conducted in accordance with approved institutional protocols of the MIT Committee on Animal Care.

The fluorescence imaging set-up in these experiments was assembled as previously described in detail (Section 3.2). The InGaAs camera (Princeton Instruments; NIRvana, 640 x 512 pixel array) was equipped with achromatic doublet lenses (Thorlabs; AC254-150-C and AC254-75-C). The laser light was blocked with two colored glass 2" 850 nm longpass filters (Thorlabs; FGL850S) in front of the lenses and an 850 nm longpass dielectric filter (Thorlabs; FELH0850) in front of the sensor. Emission light was selected using various 50 nm bandpass filters (Edmund Optics) between the lenses. All images were background-corrected using LightField software and analyzed using ImageJ or MATLAB R2016a. Acquisition settings (e.g. integration time, filters) for each figure are detailed in Appendix A, Table A.2.

We expand here on the three-dimensional tissue phantoms used to probe the individual influence of absorption and scattering on fluorescence contrast described in Section 4.2. The QD capillaries submerged in D₂O- and H₂O-Intralipid® liquid tissue phantoms were excited with diffuse 808 nm light and their fluorescence was imaged in 50 nm wavelength bands across the SWIR. The full set of images are shown here for both phantoms (Fig. 4.13) and were used to derive the plot shown in Fig. 4.1.
Figure 4.13: Complete set of two-layer capillary phantom images in 1% Intralipid®. Two SWIR-emitting quantum dot-filled capillaries were submerged in a perpendicular arrangement 2 mm and 4 mm deep in a (A) water-based or (B) deuterium oxide-based Intralipid® tissue phantom. The quantum dots were excited with 808 nm light and the emission imaged, filtering through 50 nm bandwidth bandpass filters centered across SWIR wavelengths. All images are scaled to fill the maximum number of displayable intensities, and scale bars represent 1 mm.
Figure 4.14: Contrast trend in a weakly-scattering silica bead-based liquid tissue phantom. The attenuation spectra of the H$_2$O-based silica phantom (blue) shows strong absorption and weak scattering while the D$_2$O-based phantom (orange) exhibits weak absorption and weak scattering (A). Images of two SWIR-emitting capillaries submerged 1 mm and 3 mm deep in a D$_2$O-based (top row) and H$_2$O-based (bottom row) tissue phantom were taken with 50 nm bandwidth bandpass (BP) filters centered across the SWIR (B). Images are scaled to fill the maximum number of displayable intensities and the scale bar represents 1 mm. Plotting the contrast versus wavelength for the capillary images shows that contrast improves sharply with phantom attenuation in the H$_2$O-based phantom (blue), while little change in the contrast is observed in the D$_2$O-based phantom (orange) (C).

We further reproduced the contrast trend observed in our Intralipid®-based phantoms in a separate tissue phantom based on silica beads as a scattering medium (Fig. 4.14). The polydisperse silica beads (U.S. Silica; MIN-U-SIL® 40) were dispersed at a concentration of 5 mg/mL in water to mimic the absorption of tissue, or dispersed in deuterium oxide which has no absorption in the SWIR. MIN-U-SIL® 40 contains ground silica beads with a median diameter of 11 μm and a maximum diameter of 40 μm. This size regime mimics the size range of refractive objects in tissue, such as sub-cellular organelles (<10 μm), microvasculature (5–10 μm), and entire cells (10–50 μm). The resulting phantom was weakly-scattering, approximating the scattering of tissue such as skull. The D$_2$O phantom was used to measure the influence of scattering alone (without any absorption), while the H$_2$O phantom revealed the influence of scattering in the presence of absorption. The medium was stirred with a magnetic stir bar to prevent sedimentation of the silica beads while images were acquired across SWIR wavelengths.

We evaluated the contrast $c_V$ (Eq. 4.1) between the top capillary, which represents the object of interest, and the bottom capillary, which introduces a background signal. The contrast was observed to be relatively constant across SWIR wavelengths in the D$_2$O phantom. However, when D$_2$O in the phantom is replaced by water, contrast increases through 1450 nm and then decreases again up to 1600 nm, indicating an important role of absorption in the enhancement of contrast, as observed in our Intralipid® phantoms.

The penetration depth evaluation of ex vivo liver tissue (described in Section 4.5) was evaluated with an adapted algorithm from Rowlands et al. in MATLAB R2016a. Each image
of the z-stack was transformed to Fourier space applying a two-dimensional fast Fourier transform. The amplitude spectrum was calculated and divided by the total number of pixels for image size normalization. The quadrants were shifted such that low frequencies were displayed in the center and high frequencies at the edges of the Fourier space image. A radial averaging was performed over a ring of one pixel thickness centered at the DC frequency. This averaging process is repeated for all ring radii ranging from the DC frequency (center pixel) to the Nyquist frequency (edge pixel). The radial averaging resulted in the intensity of all spatial frequencies.

A threshold that determines the highest visible frequency in a single image was found by fitting the logarithm of the frequency intensity versus spatial frequency curve with an exponential function of the form \( f(x) = a \cdot e^{-bx} + c \). The median of the parameter \( c \), which represents the noise baseline of the camera, was determined over all images of one z-stack and averaged over all z-stacks. The highest visible frequency was set at the highest frequency that had greater intensity than twice the overall noise baseline, corresponding to a signal to noise ratio of 2. It was found that a signal to noise ratio of 2 yields penetration depths that agree best with a viewer's perception when looking at a random choice of z-stacks. The frequency thresholding procedure was repeated for all images of one z-stack, resulting in a threshold frequency versus imaging depth curve. A five-point moving average was applied on this curve to reduce the influence of outliers, and create a continuous threshold frequency curve.

To extract the penetration depth, the threshold frequency for a given imaging depth was compared to the spatial frequency of the cells, which was approximated by 100 lp/mm as cells have an average size of roughly 10 \( \mu \)m and are homogeneously distributed. The penetration depth was defined at the imaging depth where the threshold frequency drops below the spatial frequency of the cells. Performing this procedure for z-stacks acquired at wavelengths regime of the SWIR, the penetration depth as a function of wavelength was determined.

4.8 Chapter-specific acknowledgements

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Chapter 5

SWIR fluorescence imaging with the clinically-approved near infrared dye indocyanine green

5.1 Introduction: from preclinical animal imaging to image-guided surgery in humans

For diagnostic imaging techniques, the journey towards clinical translation starts with building a proof of concept device around a preclinical model. Access to the necessary optics, detectors, and light sources are key, as well as access to the model itself, be it animal or otherwise. Many imaging technologies additionally require the administration of an exogenous contrast agent to highlight a particular anatomical feature or biological process; this agent must then also be designed as biocompatible or clinically approved, and effective (sensitive, selective, or both) at its targeting. Most techniques require years of design iterations at this phase before achieving sufficient validation to enter the next risky and costly phase of clinical testing.

Near infrared (NIR, 700-1000 nm) fluorescence imaging, while at first limited to preclinical applications in small animals, is an example of a technology that has emerged as a versatile tool, currently being evaluated in many clinical trials for a variety of image-guided surgery applications in humans.\textsuperscript{34,36,241,242,19,243} Compared with conventional diagnostic imaging such as computed tomography, positron emission tomography, or magnetic resonance imaging, NIR fluorescence imaging provides a lower cost, high sensitivity method for real-time molecular imaging.\textsuperscript{34,242,19} A variety of NIR contrast agents are now commercially available that exhibit high brightness and the ability to target a range of biological substrates. Moreover, one of these dyes—indocyanine green (ICG)—has been approved by the FDA for clinical use since 1959, and has been the standard of care for several applications in ophthalmology for decades.\textsuperscript{34,40} ICG and other NIR dyes such as IRDye 800CW are the subject of over 300 clinical trials for emerging applications such as fluorescence angiography and perfusion assessment in reconstructive and bypass surgeries, metastatic lymph node mapping and lymphatic transport in lymphedema, cancer localization and surgical margin assessment, and many others.\textsuperscript{30–36}

Recent research has shown that extending fluorescence imaging into shortwave infrared wavelengths (SWIR, 1000–2000 nm) can further enhance the advantages of NIR imaging, as discussed in previous sections in detail (Section 1.3, Section 4.1). In particular, low levels of background tissue autofluorescence in the SWIR increase imaging sensitivity to a target
fluorophore, and the different tissue absorption and scattering properties increase contrast of structures at greater penetration depths compared to fluorescence imaging in the NIR. However, the translation of SWIR fluorescence imaging from preclinical to clinical settings has been largely unaccomplished. The limited availability of SWIR detection technology has historically prevented the necessary research for this translation (see Section 1.3), but as detector availability has increased in the past 5 years, clinical translation has not immediately followed. Clinical translation has most recently been prevented by the perceived need for clinically-approved fluorophores with peak emission in the SWIR. Several examples of inorganic nanomaterials and hydrophobic organic molecules exist with peak emission in the SWIR; however, increasing the quantum yield, functionality, and biocompatibility of SWIR fluorophores is still an active focus of emerging research studies.

Here we show that two commercially available dyes with peak emission in the NIR spectral region, including the FDA-approved contrast agent ICG, can function sufficiently as SWIR emitters. Our findings are based on the observation that even though the emission spectra of NIR dyes such as ICG or IRDye 800CW peak outside of the SWIR spectral region, these spectra exhibit broad shoulders with a spectral tail extending well into the SWIR, that can be easily detected by modern SWIR cameras, and even outperforms commercial SWIR fluorophores with peak emission in the SWIR. Demonstrating both functional and targeted in vivo SWIR imaging, we suggest that NIR dyes could bridge the gap between current shortcomings of fluorescent SWIR probes and applications in clinical setting, and highlight advantages of imaging ICG using SWIR detection over conventional NIR detection. Our findings further suggest that new SWIR-fluorescent contrast agents should be benchmarked against the SWIR emission of ICG.

5.2 Characterization of ICG emission through SWIR wavelengths

The visible and NIR emission properties of fluorescent materials are conventionally characterized using ubiquitous silicon-based detection technology and spectrometers. However, the detection efficiency of these detectors sharply declines beyond 900 nm, making calibration beyond this wavelength challenging, and often limiting detection of the full spectrum of materials with NIR emission (Fig. 5.1). Many of approximately 3000 papers published on the NIR dye ICG, for example, under-detect the extent of its NIR and SWIR emission. According to the Franck-Condon principle (or mirror image rule), most fluorescent organic dye emission spectra should approximate the mirror image of their absorption spectra. However, spectra of NIR dyes reported in the literature and by dye manufacturers often appear to disobey this rule: while the absorption spectra of the dyes exhibit a clear shoulder to the blue of the absorption maximum, the reported emission spectra lack this shoulder. We reproduce this disparity using an example commercial silicon-based spectrometer system (Fig. 5.2A).
Recording the emission spectrum on a more suitable system, such as an InGaAs-based system with visible through SWIR light sensitivity, and applying appropriate spectral corrections, shows that ICG emission indeed follows the Franck-Condon principle, and that the tail of its emission spectrum extends well into the SWIR (Fig. 5.2A, see Section 5.7 for spectral correction details). It is even possible to detect emission from an aqueous solution of ICG on an InGaAs camera beyond 1500 nm, even though the emission of ICG peaks at 820 nm (Fig. 5.2B, see Section 5.7 for methods). The same principle applies not only to ICG, but also to other NIR dyes, such as IRDye 800CW (Fig. 5.2C), which is currently in multiple phase II clinical trials and promises improved stability over ICG. This finding is significant in the context of recent studies showing the improvement in contrast, sensitivity, and penetration that can be gained by performing fluorescence imaging at the longest SWIR wavelengths.

Figure 5.1: Representative detector efficiencies and responsivities. Silicon-based spectrometer systems have high quantum efficiency at visible and some NIR wavelengths, but decline sharply beyond 900 nm, shown here for the Ocean Optics QE650000 spectrometer (blue line). InGaAs-based detectors have high responsivity at SWIR wavelengths (yellow line), and some thinned InGaAs detectors can span visible, NIR, and SWIR wavelengths (red line).

Figure 5.2: Optical properties of ICG and IRDye 800CW. Measuring the emission of ICG on a thinned InGaAs detector (A, red line) shows that the full emission spectrum mirrors the absorption spectrum of ICG (A, black line), as predicted by the Franck-Condon principle, whereas measuring the spectrum on the Ocean Optics QE650000 (A, blue line) artificially truncates the spectrum. The emission intensity of a 0.027 mg/mL aqueous ICG solution as detected in 20 nm spectral bands on an InGaAs camera shows that emission from an aqueous solution of ICG is detectable even up to 1575 nm (inset image of vial) (B). The lower intensity observed between 1400 nm and 1500 nm is due to absorption of water in that region. We also show the absorption and emission spectra of aqueous IRDye 800CW-PEG (C). The absorption spectrum (black) exhibits a peak at 776 nm and the emission spectrum (red), measured using an InGaAs-based spectrometer, mirrors the absorption spectrum, exhibiting an emission peak at 801 nm and a lower energy shoulder extending into the SWIR.
In fact, the SWIR emission from NIR dyes such as ICG and IRDye 800CW exceeds the brightness of a commercially available organic fluorophore developed specifically for in vivo SWIR imaging applications (IR-E1050). We compared the optical properties of aqueous ICG and IRDye 800CW to IR-E1050, which has its emission peak in the SWIR (Fig. 5.3). We define brightness of the fluorescent probes as the product of the fluorescence quantum yield with the probe’s absorption cross-section at the excitation wavelength. ICG and IRDye 800CW exhibit both higher quantum yields in aqueous solutions (0.9% and 3.3% respectively), and higher peak absorption cross-sections (15x10^4 M^-1 cm^-1 and 24x10^4 M^-1 cm^-1) than IR-E1050 (quantum yield 0.2%, peak absorption cross section 0.80 x10^4 M^-1 cm^-1). Consequently, when normalized to equimolar concentrations, we measure the emission intensity of ICG between 1000 nm and 1300 nm to be 8.7 times higher than IR-E1050. Indeed, multiplying the absorption cross-section, quantum yield, and ratio of the fluorescence signal between 1000 nm and 1300 nm to the total fluorescence signal predicts that ICG should be 9.1 times brighter than IR-E1050, consistent with our measurements (Table 5.1). The superior brightness of ICG
Table 5.1: SWIR brightness comparison of ICG to IR-E1050. The following table shows our independently measured quantum yields, the maximum absorption cross-sections, and the ratio of fluorescence signal between 1000 nm and 1300 nm to the total fluorescence signal of ICG and IR-E1050. The SWIR brightness is the multiplication of these three parameters.

<table>
<thead>
<tr>
<th></th>
<th>Absorption cross section</th>
<th>Quantum yield</th>
<th>SWIR photons</th>
<th>SWIR brightness</th>
</tr>
</thead>
<tbody>
<tr>
<td>ICG</td>
<td>$1.5 \times 10^5$ M$^{-1}$ cm$^{-1}$</td>
<td>0.9 %</td>
<td>5 %</td>
<td>68 M$^{-1}$ cm$^{-1}$</td>
</tr>
<tr>
<td>IR-E1050</td>
<td>$0.08 \times 10^5$ M$^{-1}$ cm$^{-1}$</td>
<td>0.2 %</td>
<td>47 %</td>
<td>8 M$^{-1}$ cm$^{-1}$</td>
</tr>
</tbody>
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compared to IR-E1050 in the SWIR is further apparent when comparing images of vials of the two dyes on the InGaAs camera that we subsequently used for in vivo imaging (Fig. 5.3E,F).

We further compare the emission intensity of ICG, IRDye 800CW, and IR-E1050 in bovine blood on an InGaAs camera to best estimate the in vivo brightness of the probes (Fig. 5.4). It is important that a full characterization includes a comparison in blood, as characterization in water under-represents the in vivo brightness of ICG due to (1) formation of H-dimer species at low concentrations in water that causes fluorescence quenching, and (2) the association of ICG with albumin and other proteins in blood that stabilize the dye and increases its quantum yield.\textsuperscript{35,264} To quantify the molar brightness of the probes on the camera, the intensity of each vial containing equal masses of the respective dye was measured individually, averaged, normalized to integration time, and multiplied with the molecular mass of the respective dye. In blood, equimolar ICG and IRDye 800CW were 16 and 1.3 times brighter, respectively, than IR-E1050 in the wavelength range between 1300–1620 nm when excited with 808 nm light. We further confirm these results with in vivo SWIR imaging which shows that ICG is at least one order of magnitude brighter than IR-E1050 when imaged beyond 1000 nm, in good agreement with the in vitro comparison.

Figure 5.4: Comparison of ICG and IR-E1050 in blood and in vivo. (A) We further compared the fluorescence intensity of IR-E1050, (0.01 mg/mL), with IRDye 800CW PEG (0.01 mg/mL, not accounting for PEG shell of 25–60 kDa), and ICG (0.01 mg/mL) in bovine blood on a SWIR camera. (B) On a molar basis, the NIR dyes outperform IR-E1050 beyond 1300 nm. Similarly, we found that the in vivo fluorescence intensity of the recommended bolus of (C) IR-E1050 (67 nmol) and (D) ICG (64 nmol) injected via the tail vein of a mouse was approximately one order of magnitude greater for ICG beyond 1000 nm. Shown here is the relative fluorescence intensity of ICG in the vasculature and in the liver imaged noninvasively through intact skin approximately one minute after ICG injection.
5.3 High contrast SWIR fluorescence imaging \textit{in vivo} using ICG

The SWIR emission of commercially available and FDA-approved ICG enables straightforward application to \textit{in vivo} fluorescence imaging in the SWIR. We present here a selection of preclinical \textit{in vivo} imaging applications in mice using the clinically approved dose of ICG, and highlight advantages of imaging ICG using SWIR detection over conventional NIR detection.

We first show that imaging ICG in the SWIR enables high-contrast mesoscopic imaging of brain and hind limb vasculature in mice through intact skin (Fig. 5.5), as has been previously demonstrated with carbon nanotubes.\textsuperscript{47} For this, two C3H/HeJ mice (21.7 g and 20.5 g, female, 10 weeks old, The Jackson Laboratory) were anesthetized, and the hair on the head was removed with electric clippers and hair removal cream. We injected an aqueous solution of ICG into the tail veins at a dose of 0.2 mg/kg, which is within the recommended dose for humans (0.2–0.5 mg/kg recommended, 5 mg/kg maximum).\textsuperscript{35} We illuminated the mice with 50–70 mW/cm\textsuperscript{2} of 808 nm excitation light, staying below the maximum permissible exposure limit (330 mW/cm\textsuperscript{2} for 808 nm continuous wave light).\textsuperscript{152} We noninvasively imaged the resulting fluorescence on a silicon camera at NIR wavelengths, and on an InGaAs camera at SWIR wavelengths between 1300 nm and 1620 nm, the detection cutoff of the cooled SWIR camera. We chose the 1300–1620 nm wavelength range, since as we show here and others have previously demonstrated, contrast and resolution are maximized above 1300 nm.\textsuperscript{45,47,244}

\begin{figure}[h]
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\includegraphics[width=\textwidth]{figure5.5.png}
\caption{High contrast \textit{in vivo} SWIR fluorescence imaging using ICG. (A) We noninvasively imaged the brain vasculature of a mouse using ICG contrast and find that the vessels are difficult to resolve through skin and skull using 850 nm longpass NIR detection on a silicon camera. (B) Switching to 1300 nm longpass SWIR detection on an InGaAs camera greatly improves vessel contrast (see Fig. 5.6 for contrast quantification). (C) Similarly, only large hind limb vessels are imaged with good contrast through the skin using NIR detection. The intensity across a line of interest (red line) shows insufficient contrast to resolve smaller vessels from background signal. (D) Using 1300 nm longpass SWIR detection greatly improves image contrast and resolution of vessels. All images were scaled to the maximum displayable intensities.}
\end{figure}
Our images show that ICG is successfully imaged noninvasively in vivo at SWIR wavelengths, and that imaging ICG in the SWIR enables greater contrast than imaging ICG in the NIR. We quantified the contrast within a region of interest containing the brain vasculature in the NIR image and the SWIR image by calculating the coefficient of variation, defined as the standard deviation of pixel intensity normalized to the mean pixel intensity (Fig. 5.6A). We find that the SWIR image contrast for brain vasculature is nearly 50% greater at a value of 0.29, compared to the NIR image with a contrast value of 0.20, and it is more than 58% greater for hind limb vasculature at a value of 0.19 for SWIR imaging compared to 0.12 for NIR imaging.

Furthermore, we calculated the apparent vessel width for a brain vessel by measuring the full width at half maximum of a two-Gaussian fit to the intensity profile across a brain vessel of interest (Fig. 5.6B). We find that the apparent vessel width in this specific example is over twice as wide in the NIR image as in the SWIR image with values of 430 μm and 210 μm, respectively. These findings are in good agreement with previous studies employing other fluorophores, e.g. the contrast and resolution of brain vessels originally demonstrated through noninvasive imaging using SWIR-emissive carbon nanotubes. Thus, contrast and resolution of fine vasculature structures can be greatly improved while using FDA-approved ICG contrast by

![Figure 5.6: Contrast and resolution quantification of brain and hind limb vasculature in a mouse.](image)

Two-hundred microliters of aqueous ICG (0.025 mg/mL) were injected into the tail vein of two mice and the fluorescent vasculature was imaged noninvasively through intact skin. (A) The contrast, defined as the standard deviation (σ) divided by the mean (μ) pixel intensity, was quantified for a region of interest (yellow box) to compare NIR and SWIR imaging of the brain vasculature and hind limb vasculature. The contrast was found to be 0.20 for NIR-imaged brain vasculature and 0.29 for SWIR-imaged brain vasculature. The contrast was 0.12 and 0.19 for NIR- and SWIR-imaged hind limb vasculature respectively. (B) Apparent vessel width was also calculated by calculating the full width at half maximum of a two-term Gaussian fit to the intensity profile of a brain vessel. Gaussian-fitting was performed in MATLAB using the Curve Fitting Toolbox™. The apparent vessel width was 430 μm and 210 μm for NIR- and SWIR-imaged brain vasculature, respectively.
simply switching the detection wavelength from traditional NIR imaging using a silicon camera to detection beyond 1300 nm on an InGaAs SWIR camera.

We further show that the contrast improvement of SWIR detection over NIR detection can be enabling for intravital microscopic imaging (Fig. 5.7). We incorporated ICG into polyethylene glycol-phospholipids\textsuperscript{39} to increase its blood half-life, which is typically limited to 3–4 min (see Section 5.7 for experimental details.\textsuperscript{272,273} We injected an aqueous solution of these micelles at a dose of 5 mg ICG/kg mouse into the tail vein of a NU/NU nude mouse (female, 15 weeks old, Massachusetts General Hospital colony) with implanted cranial window. The window was implanted as previously described.\textsuperscript{274,275} The cranial window of the mouse was then fixed to the stage of a Nikon Ti-E inverted microscope (described in Section 3.2) and the fluorescence of the ICG-phospholipid micelles in the brain vasculature was imaged with both NIR and 1300 nm longpass SWIR detection. Images of the entire cranial window at 2x magnification show the ability to resolve nearly all the same vessels using either NIR or SWIR imaging. The overall contrast, however, was 1.4 times greater for the SWIR image (standard deviation/mean was 0.24 for NIR versus 0.33 for SWIR). Higher magnification (6x) reveals that this contrast improvement using SWIR imaging enables the resolution of vessels which, due to the high label density of surrounding vessels, are difficult to distinguish from background signal in the NIR image.

Figure 5.7: High contrast SWIR intravital microscopy of mouse brain vasculature. An aqueous ICG-phospholipid mixture was injected via the tail vein of a mouse, and the fluorescence in the brain vasculature was imaged through a cranial window. (A) Microscopy of the brain vasculature through the cranial window shows poor contrast with 850 nm longpass NIR detection. (B) The intensity across a line of interest (red dotted line) shows insufficient contrast to resolve overlapping vessels from background signal. (C) Using 1300 nm longpass SWIR detection greatly improves image contrast and (D) resolution of the vessels. Scale bars represent 1 mm in 2x images and 100 µm in 6x images.
5.4 Real-time SWIR fluorescence imaging in vivo using ICG

An essential component of fluorescence-guided surgery is the ability to perform real-time imaging. Image acquisition speed should be faster than motion in the surgical field to avoid blurring in the image and also to avoid impeding the surgical work flow. We show that the SWIR emission of ICG is sufficiently bright for real-time imaging at high frame rates using clinically approved doses of ICG in several preclinical in vivo examples.

In one example, we performed SWIR fluorescence angiography in the heart blood vessels of mice using ICG contrast. Three Friend Virus B mice (FVB/NJ NIH Jackson, male, 18 weeks old, The Jackson Laboratory) were anaesthetized and surgery performed to expose the heart (as for perfusion\textsuperscript{276}). One or two 200 μL boluses of 0.25 mg/mL ICG in water were injected via the tail vein while concurrently imaging the emission. Diffuse 808 nm excitation light was used for excitation of ICG and the fluorescence was imaged with 1300 nm longpass emission detection at 9.17 frames per second. The fluorescence of the ICG bolus was tracked as it reached the heart, lungs, peripheral veins, and finally the liver (Fig. 5.8). The acquisition speed was sufficiently fast to resolve fine vessels on the exterior heart surface against the underlying

![Figure 5.8: Temporal resolution in intravital ICG angiography.](image)

SWIR fluorescence angiography was performed intravitaly in three mice at 9.17 frames per second using ICG for contrast, diffuse 808 nm excitation, and a 1300 nm longpass emission filter on an InGaAs SWIR camera. The heart of the mouse was exposed via surgery\textsuperscript{276} and two-hundred microliters of aqueous ICG (0.25 mg/mL) injected into a mouse via the tail vein was tracked as fluorescence first appears in the heart, followed by the lungs, the peripheral veins, and finally the liver (A). The fluorescence intensity of the heart atrium (B, orange), heart ventricle (B, red), lung (B, blue), liver (B, green), and peripheral vasculature (B, purple) are plotted to show a single injection in one representative mouse as it arrives first at the heart and the lungs (C), and at the peripheral vasculature before beginning to be cleared through the liver, which is known to be the main metabolic clearance site for ICG (D).\textsuperscript{35,291} Images in A have the same scale.
contrast while in motion and to capture the anesthetized mouse heart rate of 207 beats per minute, determined by tracking the intensity fluctuations of the heart (Fig. 5.9).

In a second example, we show real-time SWIR imaging of ICG in the liver and small intestine of a mouse, and further demonstrate in vivo imaging of ICG fluorescence beyond 1500 nm (Fig. 5.10A). An NCr nude mouse (male, 14 weeks old, Taconic Biosciences) was anaesthetized, placed in the imaging setup, and injected via a tail vein catheter with 200 μL of ICG at a concentration of 0.25 mg/mL in water. Hepatobiliary clearance of ICG from the liver into the intestines was then monitored and imaged noninvasively through intact skin over approximately 2 hours using an 850 nm dielectric longpass filter and a silicon detector, or an additional 1200 nm longpass or 1500 nm longpass filter with InGaAs detection. ICG emitted sufficient SWIR signal for near video rate imaging (19.7 frames per second, 1200 nm longpass filter), enabling capture of the peristaltic movements of the small intestine. Although at the cost of speed (2.0 frames per second), it was even feasible to image the ICG clearance using a 1500 nm longpass filter, capturing wavelengths between roughly 1500 and 1620 nm. Images shown were taken approximately 1.5–2 hours after the initial ICG injection.

In a third example, we demonstrate noninvasive imaging of lymphatic flow in mice. Four C57BL/6 mice (male, 12–15 weeks old, The Jackson Laboratory) were anaesthetized and the hair was removed. We injected 10–100 μL of 0.18 mg/mL aqueous solution of ICG subcutaneously in the hind feet and subcutaneously in the tail. The fluorescence from the dorsal
lymph vessels and nodes was immediately imaged at 9.17 frames per second noninvasively through the intact skin using 850 nm, 1000 nm, 1200 nm, 1300 nm, 1400 nm longpass dielectric filters (Thorlabs). We find that lymph vessels and nodes are visible with ICG contrast up to approximately 1400 nm, at which point only the vessels and superficial nodes are visible and the signal of deeper lymph nodes becomes attenuated (Fig. 5.10B). Thus, for applications which are not contrast-limited, such as those with low background signal and/or high label specificity, it may be preferable to image ICG in the NIR or the shorter wavelengths of the SWIR where signal attenuation is minimized.
5.5 Targeted SWIR fluorescence imaging *in vivo* using IRDye 800CW

The ease of molecular targeting is one of the major strengths of fluorescence imaging over other imaging modalities. We show here that straightforward conjugation chemistry can be used to perform targeted imaging in the SWIR using the NIR dye IRDye 800CW. We used a commercially-available labeling kit (Li-Cor, P/N 928-38040) to conjugate IRDye 800CW to the tumor-targeting antibody trastuzumab (Genentech). Briefly, 1 mg of trastuzumab was dissolved in 900 μL PBS buffer and 100 μL 1M potassium phosphate buffer (pH 9) to obtain an 8.5 pH solution. Twenty-five microliters of ultra-pure water were added to the NHS-IRDye 800CW and the solution was shaken for 2 minutes until the dye completely dissolved. Eight microliters of the resulting solution were added to the trastuzumab solution and the mixture was incubated for 2 hours at room temperature protected from light. Subsequently, the trastuzumab-dye conjugate was separated from free residual dye using Pierce Zeba desalting spin columns.

We injected 330 μL of IRDye 800CW-trastuzumab (15 mg/kg dose) intraperitoneally into two nude mice (female, 11 weeks old) implanted with human BT474 breast cancer cells in the brain (see *Section 5.7* for experimental details of tumor implantation). Two additional tumor-bearing nude mice were not injected and served as controls. After three days, the mice were anaesthetized and noninvasively imaged through intact skin and skull using diffuse 808 nm excitation, and an 1150 nm longpass emission filter on an InGaAs camera. Subsequently, all mice were injected via the tail vein with up to 300 μL (3 nmoles) of IRDye 800CW conjugated to polyethylene glycol (IRDye 800CW-PEG, Li-Cor, P/N 926-50401) to highlight the brain vasculature surrounding the tumor. The mice were then either illuminated with 808 nm excitation and imaged on the SWIR camera, or illuminated with 780 nm excitation (Thorlabs, M780L3) and imaged with an 800 nm longpass filter on a NIR camera. A multi-color functional image of the brain was generated by temporally resolving the two labels, i.e. by assigning different colors before and after the addition of IRDye 800CW-PEG (*Fig. 5.11*).

5.6 Discussion and conclusions

We show that commercially available and clinically-approved NIR fluorophores have significant SWIR emission, eliminating one of the key barriers to adoption of SWIR fluorescence imaging in both basic research and clinical applications. These results also emphasize that the candidates for SWIR fluorescence contrast agents are not limited to probes with peak emission in the SWIR. As conventional SWIR fluorophores have low quantum yields and low absorption cross sections, we find the brightest probe to be one with only tail emission in the SWIR. New SWIR contrast agents should therefore be benchmarked against the SWIR emission of ICG in blood, as the latter is sufficiently bright for *in vivo* imaging, and is already FDA-approved and clinically used.
These findings could improve clinical use of ICG in fluorescence angiography. Clinical imaging of ICG in the NIR has already shown value for angiography in ophthalmology, intraoperative assessment of blood vessel patency in tissue grafts, bypass surgeries, and intracranial aneurism surgeries.\(^{34,241,279–282}\) Noninvasive imaging of lymphatic vasculature using ICG has also been described for surgical mapping and intraoperative identification of sentinel nodes, following surgical excision of nodes, and evaluation and monitoring of lymphedema.\(^{34,241,283,284}\) Increasingly, ICG fluorescence imaging is also being used in robot-assisted surgery, in which the surgeon cannot rely on tactile feedback.\(^{33,36,284}\) However, full clinical implementation has been partially limited by insufficient image quality in deep operating fields. The higher contrast of ICG SWIR fluorescence imaging over NIR imaging could benefit these applications and enable the resolution of finer vessels, especially in systems with high label density or interfering background signal. Importantly, the implementation of this contrast improvement would be straightforward, requiring only a switch from cameras with NIR detection to those with SWIR detection while continuing the familiar surgical set-up and use of ICG.
Our real-time in vivo imaging examples demonstrate the applicability of ICG SWIR imaging to fluorescence guided surgery. ICG is bright enough to image in the SWIR at speeds sufficiently high for intraoperative imaging of dynamic or moving features, shown here in the heart, intestine, and lymphatic system of mice. We show that the required speed for a given application can be balanced with the desired contrast by selecting the imaging wavelength; using the full SWIR regime enables the highest frame rates due to maximized signal from ICG, while imaging at the longest SWIR wavelengths can be used to improve contrast at the cost of speed. Thus, using a SWIR camera for detecting ICG provides a tunable platform for optimizing both contrast and speed in fluorescence guided surgery.

While targeted SWIR imaging has previously been hindered by either the challenging preparation of targeted nanomaterials, size-dependent delivery effects, or the unavailability of commercial solutions, the use of small NIR dye labelling kits overcomes these barriers. NIR imaging of these readily available, targeted dyes has already shown promise for aiding cancer localization and intraoperative surgical margin assessment. Our results suggest that SWIR imaging of these NIR dyes could further benefit these applications by increasing the resolution of both fine and large structures which may be overlapping, as this is a common occurrence in highly vascular malignant lesions.

We show that established, commercially-available NIR dyes, including the FDA-approved dye ICG, can be used to perform state-of-the-art SWIR imaging including intravital microscopy, noninvasive, real-time imaging in blood and lymph vessels, imaging of hepatobiliary clearance, and molecularly-targeted in vivo imaging. The advantages of SWIR imaging over NIR techniques, such as increased sensitivity, contrast, and resolution of fine anatomical structures are therefore more readily available for increased adoption in pre-clinical and clinical imaging systems, simply by switching the detection from conventional silicon-based NIR cameras to emerging, high-performance InGaAs SWIR cameras. While no FDA-approved fluorophores with peak emission in the SWIR yet exist, we show here that detecting the off-peak fluorescence of clinically accessible NIR dyes on SWIR detectors bears the potential for rapid translation of SWIR fluorescence imaging to humans in clinical applications.

5.7 Additional experimental details

Absorption and emission characterization measurements are presented here in detail. An aqueous solution of ICG (Pfaltz & Bauer), IRDye 800CW PEG (LI-COR Biosciences), or IR-E1050 (Nirmidas Biotech) was prepared in quartz cuvettes. Absorption spectra were recorded on a Cary 5000 UV-Vis-NIR infrared spectrometer (Varian). To collect the emission spectra, samples were excited with a 635 nm diode laser (Thorlabs), and emission was collected using a pair of off-axis parabolic mirrors, and directed to a single-grating spectrometer (Acton, Spectra Pro 300i). An InGaAs calibrated photodiode (Thorlabs, DET10N) was used to detect the
The intensity of the emission. As the determination of the spectral responsivity is critical to accurately measuring a fluorescence spectrum, we carefully calibrated the fluorescence setup across visible and SWIR wavelengths. Using a 200W Quartz Tungsten Halogen Lamp (Newport Instruments), we acquired an absolute spectrum which we compared to the known spectral irradiance of the lamp to arrive at a spectral responsivity curve as depicted in Fig. 5.12A. We further corrected the fluorescence spectra for solvent reabsorption as described in our previous work, and for plots in energy, we apply the Jacobian correction. The effects of these corrections on a raw spectrum are shown in Fig. 5.12B. The emission spectrum of ICG was also measured using excitation from a 532 nm diode laser, and the resulting emission detected on a silicon-based spectrometer (OceanOptics, QE65000) as shown in Fig. 5.2A.

The emission intensity of ICG in water (0.027 mg/mL) was measured in 20 nm spectral bands using an InGaAs camera (Princeton Instruments NIRvana 640) as shown in Fig. 5.2B. A glass vial containing the ICG solution was placed in the imaging set up previously described in detail (Section 3.2). The vial was illuminated with 50–70 mW/cm² of 808 nm light, and the fluorescence of the ICG was filtered through two colored glass 850 nm longpass filters (Thorlabs FGL850S) and through a liquid crystal tunable filter (PerkinElmer VariSpec LNIR, 20 nm bandwidth), and focused onto the SWIR camera with a C-mount objective (Navitar SWIR-35). The tunable filter was scanned from 900 to 1650 nm in 25 nm steps. A final image at 950 nm was acquired to ensure that the intensity matched the first measurement, verifying that the ICG solution was not bleached throughout the measurement.

Quantum yield measurements were obtained using an integrating sphere (Labsphere RTC-060-SF). The sample was illuminated using a 785 nm diode laser with an excitation power of 25 mW that was modulated at 260 Hz. The output was collected using a calibrated
germanium detector (Newport: 818-IR) through a Stanford Research Systems lock-in amplifying system. Colored glass longpass filters (2x Schott Glass RG800 or 1x RG850) were used to block the excitation beam. The sample was placed in a PTFE-capped quartz cuvette and a solvent blank was used to ensure a consistent environment inside the integrating sphere. The measured photocurrent was adjusted to account for the external quantum efficiency of the detector when calculating the quantum yield. Finally, the measured quantum yield was corrected to account for leakage of the excitation light and the transmittance of the filter.

While measuring the absolute quantum yield of IR-E1050 using an integrating sphere, we found a lower value (0.2%) compared to the one reported by the manufacturer (2%), which used the dye IR-26 as reference for relative quantum yield measurements. Their relative quantum yield measurements rely on a quantum yield of 0.5% for IR-26 that was reported by Drexhage and coworkers in 1982. More recently however, two independent publications by Beard and coworkers (0.05%) and Resch-Genger and coworkers (0.07%) have found the original value to be ~ten times too large. Measuring the absolute quantum yield of IR-26 in our laboratory yielded a value of 0.05%, which is in line with the more recent publications. We therefore believe that 0.05% is the correct value of the quantum yield of IR-26.

To compare the brightness of ICG and IR-E1050 in aqueous solutions (Fig. 5.3), ICG (Pfaltz & Bauer) and IR-E1050 (Nirmidas Biotech) were diluted to a concentration of 0.01 mg/mL in cell culture grade water and in PBS, respectively. The solutions were imaged in the imaging set-up (described in Section 3.2) with 808 nm excitation, and the emission filtered through two colored glass 1000 nm longpass filters and an additional dielectric 1300 nm longpass filter. In the resulting background-corrected images, the average emission intensity was calculated from a region of interest within the vials and normalized to the integration time.

To compare brightness in blood (Fig. 5.4), ICG, IR-E1050, and IRDye 800CW PEG (LICOR Biosciences) were diluted in bovine blood (Rockland Immunochemicals, sodium citrate-conjugated) to a concentration of 0.01 mg/mL. The solutions were imaged individually, and side-by-side with 808 nm excitation, and the emission filtered through either two colored glass 1000 nm longpass filters or through an additional dielectric 1300 nm longpass filter. In the resulting background-corrected images, the average emission intensity was calculated from a region of interest within the vials and normalized to the integration time.

To compare the brightness of the SWIR emitters in vivo, ICG and IR-E1050 were injected at the recommended concentrations (ICG: 0.25 mg/mL ≈ 322 nmoles/mL in cell culture grade water, IR-E1050: 1 mg/mL ≈ 333 nmoles/mL in PBS), which yielded roughly equimolar concentrations. 200 µL of the respective solution (64 nmoles of ICG, 66 nmoles of IR-E1050) was injected into the tail vain of a NU/NU nude mouse (12 weeks old, male, Taconic Biosciences) and excited with diffuse 808 nm excitation (50 mW/cm²). The fluorescence was collected with a commercial SWIR lens (Navitar, 35 mm), filtered through two colored glass
1000 nm longpass filters, and detected on an InGaAs camera (Princeton Instruments, NIRvana 640, cooled to –80 °C) using an integration time of 1 ms. To estimate the *in vivo* brightness, the intensity in the heart was measured one minute post-injection (100 frame average).

All animal procedures were carried out in accordance with approved institutional protocols of the MIT Committee on Animal Care. Mice were anesthetized via intraperitoneal injection of a ketamine/xylazine and shaved prior to imaging. The *in vivo* imaging set-up was as described in Section 3.2 with diffuse 808 nm excitation (50–70 mW/cm²), and an exchangeable filter holder for incorporating various bandpass and longpass emission filters. Different objectives and filters were used depending on the requirements of each application as detailed in Appendix A, Table A.3. The InGaAs camera (Princeton Instruments, NIRvana 640) was cooled to –80 °C, the analog to digital (AD) conversion rate set to 2 MHz or 10 MHz, the gain set to high, and different exposure times used to achieve sufficient signal and/or frame rates (Appendix A, Table A.3). All images were background- and blemish-corrected within the LightField imaging software. The silicon camera (Princeton Instruments PIXIS 1024BR) was cooled to –70 °C, the AD conversion rate set to 2 MHz, the gain set to high, and the exposure time adjusted to achieve sufficient signal and/or frame rates (Appendix A, Table A.3). ImageJ was used to average 10 frames for NIRvana and PIXIS images. Frame averaging was not used in videos unless otherwise noted. ImageJ was also used for all image measurements as well (pixel intensity average, standard deviation, etc.).

We validated our imaging set-up using tissue phantoms, and support our key finding that SWIR imaging of ICG improves image contrast and resolution compared with NIR imaging of ICG. Tissue phantom images were taken by submerging a 0.8–1.1 mm wide (inner diameter) capillary filled with 0.0175 mg/mL aqueous ICG (Pfaltz & Baur) in 20% Intralipid® (Baxter Healthcare Corporation, Deerfield, IL, USA) that was diluted to 2% Intralipid® in water. Images were taken in the NIR and the SWIR of the capillary before and after being submerged 3 mm below the surface of the 2% Intralipid® solution (Fig. 5.13A). Importantly, the resolution of the capillary is high on the NIR imaging set-up without tissue phantom present, confirming that the resolution and the optics of the NIR imaging set-up are not responsible for the poorer contrast observed compared to SWIR imaging when the phantom is present or in our *in vivo* imaging.

We further verified that the poor contrast observed in NIR imaging relative to SWIR imaging is not due to excitation leak through being detected with higher sensitivity on the NIR camera (Fig. 5.13B,C). The two C3H/HeJ mice described previously for noninvasive, through-skull brain vasculature imaging were used for this control experiment. The first mouse was imaged before and after ICG injection with 850 nm longpass NIR detection. There was minimal detectable signal prior to ICG injection, thus we conclude that the scattering of ICG signal observed after injection is not attributed to leak-through of 808 nm excitation light being imaged on the NIR camera, but must instead be caused by the tissue. Similarly, there is minimal leak-
through of excitation light imaged on the SWIR camera using a 1300 nm longpass filter, and the majority of detectable signal comes from ICG after being injected.

Additional experimental details related to the in vivo imaging presented are expanded on here. Preparation of ICG-phospholipid micelles were prepared by dissolving 4.8 mg ICG in a 2:1 mixture of chloroform and methanol, agitating with 23 mg polyethylene glycol-2000 phospholipids in chloroform (1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (ammonium salt), Avanti Polar Lipids, #880130C), drying off the solvent under nitrogen flow, and re-suspending the dried phospholipids in 2 mL cell grade water, resulting in a final concentration of 13.9 mg/mL ICG-phospholipid solution, or 2.4
mg/mL concentration of ICG. The aqueous ICG-phospholipid mixture was passed through a 0.22 μm syringe filter before injecting approximately 50 μL via the tail vein into the anaesthetized mouse, and the fluorescence was immediately imaged.

For in vivo targeted imaging with IRDye 800CW, female nude mice (8 to 9 weeks of age) were implanted with a 0.36- or 0.72-mg 17b-estradiol pellet (Innovative Research of America) the day before implantation of tumor cells. Injection of BT474-Gluc cells in the brain parenchyma was performed as previously described. Briefly, the head of the mouse was fixed with a stereotactic apparatus and the skull was exposed via skin incision. Using a high-speed air-turbine drill (CH4201S; Champion Dental Products) with a burr tip size of 0.5 mm in diameter, three sides of a square (~2.5 mm in length, each side) were drilled through the skull over the left hemisphere of the brain until a bone flap became loose. The bone flap was pulled back, exposing the brain parenchyma. 100,000 BT474-Gluc cells diluted in 1 μL PBS were stereotactically injected in the left frontal lobe of the mouse brain using an insulin syringe. Subsequently, the bone flap was placed back into position in the skull and sealed using histo-compatible cyanoacrylate glue, and the skin atop the skull was sutured closed. All animal procedures were performed according to the guidelines of the Public Health Service Policy on Human Care of Laboratory Animals and in accordance with a protocol approved by the Institutional Animal Care and Use Committee of Massachusetts General Hospital.

Tumor growth was determined by measuring the Gluc activity in the blood as previously described. Blood Gluc activity was measured with a Promega Glomax 96 microplate luminometer (Fisher Scientific, Waltham, MA). The correlation between Gluc activity and tumor size was previously determined and established using in vivo ultrasonography imaging performed through a cranial window. At the time of imaging, we estimate the tumors to have a size of 50–70 mm³.

5.8 Chapter-specific acknowledgements

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Appendix A

Raw quantification data and detailed image acquisition settings

A.1 Full image sets used for quantifications in Chapter 3

In Chapter 3, much of the data presented was the result of analyzing image intensities and their numerical quantifications in the form of averages. In an effort toward transparency, we show here the individual images used for these analyses. The images themselves are also rich with information in terms of the morphological distribution of signal intensities and their anatomical relationships beyond numerically averaged results.
Figure A.1: NIR/SWIR autofluorescence detected *in vivo* and *ex vivo* in liver tissue of cirrhosis mouse models. *In vivo* images were taken using 1000 ms of integration time and *ex vivo* images were taken using 500 ms. Intensities are given in counts per second as indicated by the color bar.
Figure A.2: NIR/SWIR autofluorescence detected in \textit{ex vivo} tissue of NAFLD mouse models on CDAHF diet versus mice on a control diet for 3, 6, 9, and 12 weeks. Intensities are given in counts per second as indicated by the color bar at the bottom. Most images were taken with 1000 ms integration, but some taken with 500 ms were scaled to 1000 ms in post processing (intensities doubled) for comparison here. One liver had to be excluded from the quantification due to contamination on the surface.
Figure A.3: NIR/SWIR autofluorescence detected \textit{in vivo} in NAFLD mouse models on CDAHF diet versus mice on a control diet for 3, 6, 9, and 12 weeks. Intensities are given in counts per second as indicated by the color bar at the bottom. Most images were taken with 1000 ms integration, but some taken with 500 ms were scaled to 1000 ms in post processing (intensities doubled) for comparison here. Several mice were excluded from the quantification due to interfering signals in the GI tract (red boxes).
Figure A.4: NIR/SWIR autofluorescence detected *ex vivo* in NAFLD mouse models on CDAHF diet versus mice on a control diet for 12 weeks, 4 of which included treatment with telmisartan. Images were taken with 1000 ms integration. Intensities are given in counts per second as indicated by the color bar at the bottom.
Figure A.5: NIR/SWIR autofluorescence detected in control mice receiving standard chow for the equivalent to 0, 6, and approximately 30 weeks, with no other treatment. All images were taken with 1000 ms integration. Intensities are given in counts per second as indicated by the color bar.
A.2 Tables of integration times and optics for each image

The following tables detail the exact optical components and camera settings used for the images presented in this thesis. In these tables:

- The “Figure” column indicates the figure number and panel referred to within the thesis.
- The “Camera” column refers to the Princeton Instruments PIXIS 1024BR camera, which is a deep depletion silicon CCD for NIR imaging, the Princeton Instruments NIRvana 640, which is an InGaAs camera for SWIR imaging, or the Thorlabs color CMOS camera (part number DCC1645C).
- The "Filters" column lists Thorlabs part numbers of the filters used except for 50 nm bandwidth bandpass (BP) filters which are from Edmund Optics (EO). In general, the first letters of the part number indicate the filter type (FGL: Longpass Colored Glass Filters, FEL: Longpass Edgepass Filters, FELH: Premium Hard-Coated Longpass Edgepass Filters, DML: Longpass Dichroic Mirrors). The following numbers indicating the wavelength, and an "S" at the end of the part number refers to a square filter, whereas the absence of an "S" denotes circular filters. Part number FGL1000S for example denotes a colored glass 1000 nm longpass filter with square dimensions.
- The “Exposure time” column indicates the camera setting used for exposure time in each image, given in milliseconds (ms) of integration.
- The “Objective” column indicates whether a commercial lens was used (Navitar SWIR-35 (F1.4/35mm EFL), Navitar MVL50M23 (F2.8/50 mm EFL), or Nikon CFI Plan Apochromat λ. 4x or 10x on the Nikon Eclipse Ti-E microscope) or lists the individual optical components assembled using Thorlabs part numbers. A part number of the form AC254-###-C indicates an achronatic doublet lens, 25.4 cm in diameter, with a ### effective focal length, and a C-coating (C antireflection coating is for 1050–1700 nm). In some images, biconvex lenses were used, with part number LB1945-C (200 mm focal-length biconvex lenses, 25.4 mm, C-coated) or LB1909-C (500 mm focal-length biconvex lenses, 50.8 mm, C-coated).
Table A.1: Camera, emission filters, exposure times, and objective used for each of the images presented in Chapter 3.

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<td>Fig. 3.12</td>
<td>A, B, C (right column)</td>
<td>PIXIS</td>
<td>2x FGL850S, FELH0850</td>
<td>1000/Navitar F2.8/50</td>
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<tr>
<td>Fig. 3.12</td>
<td>B</td>
<td>NIRvana</td>
<td>2x FGL850S, 2x FGL1000S</td>
<td>500/Navitar SWIR-35</td>
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<tr>
<td>Fig. 3.13</td>
<td>column 1</td>
<td>PIXIS</td>
<td>DMLP900R, FELH0900</td>
<td>1000/Nikon 10x</td>
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<td>Fig. 3.13</td>
<td>column 2</td>
<td>PIXIS</td>
<td>Cy5 channel (Table 3.1)</td>
<td>500/Nikon 10x</td>
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Table A.2: Emission filters and exposure times used for each of the images presented in Chapter 4. All images were taken with the NIRvana SWIR camera unless otherwise noted in the figure captions. The objective used was as noted in the Chapter 4 text.

<table>
<thead>
<tr>
<th>Figure</th>
<th>Filters</th>
<th>Exposure time (ms)</th>
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<tbody>
<tr>
<td><strong>Fig. 4.1</strong></td>
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<tr>
<td>D (1000 nm)</td>
<td>2xFGL1000S, FELH0850, 1000 nm BP (EO)</td>
<td>D$_2$O: 50  H$_2$O: 50</td>
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<tr>
<td>D (1200 nm)</td>
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<td>D$_2$O: 50  H$_2$O: 50</td>
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<tr>
<td>D (1450 nm)</td>
<td>2xFGL1000S, FELH0850, 1450 nm BP (EO)</td>
<td>D$_2$O: 50  H$_2$O: 2500</td>
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<td>D (1600 nm)</td>
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<td>D$_2$O: 50  H$_2$O: 50</td>
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<td>A (1200 nm)</td>
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<td>D$_2$O: 50  H$_2$O: 50</td>
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<td>2xFGL1000S, FELH0850, 1450 nm BP (EO)</td>
<td>D$_2$O: 25  H$_2$O: 2500</td>
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<td>A (1600 nm)</td>
<td>2xFGL1000S, FELH0850, 1600 nm BP (EO)</td>
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<td>1000 nm</td>
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<td>1300 nm</td>
<td>2xFGL1000S plus noted bandpass filter</td>
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<td>A (1200 nm)</td>
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<td>2xFGL1000S, 1450 nm BP (EO)</td>
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<td>A (1600 nm)</td>
<td>2xFGL1000S, 1600 nm BP (EO)</td>
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<td>A, C, E, G, I</td>
<td>2xFGL1000S, 1600 nm BP (EO)</td>
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**Fig. 4.14**

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Table A.3: Camera, emission filters, exposure times, and objective used for each of the images presented in Chapter 5.

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<th>Figure</th>
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<th>Filters</th>
<th>Exposure time (ms)</th>
<th>Objective</th>
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<td>E</td>
<td>NIRvana</td>
<td>2x FGL1000S, FELH1300</td>
<td>1000</td>
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<td>Fig. 5.4</td>
<td>A</td>
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<td>2x FGL1000S, FELH1300</td>
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<td>A, C</td>
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<td>PIXIS</td>
<td>FELH0850</td>
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<td>Fig. 5.7</td>
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<tr>
<td>Fig. 5.8</td>
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<td>FELH1000, FELH1300</td>
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<td>Fig. 5.9</td>
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<td>FELH1000, FELH1300</td>
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<td>Fig. 5.10</td>
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<td>NIRvana</td>
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<td>Fig. 5.11</td>
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<td>NIRvana</td>
<td>FELH0850, FELH1000, FELH1150</td>
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<td>FELH1000, FELH1400</td>
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<tr>
<td>Fig. 5.11</td>
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<td>FELH0850, FELH1150</td>
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<td>FELH0850</td>
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<td>NIRvana</td>
<td>FELH0850, FELH1000, FELH1300</td>
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