Platform for quantitative multiscale imaging of tissue composition

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Abstract: Changes in the multi-level physical structure of biological features going from cellular to tissue level composition is a key factor in many major diseases. However, we are only beginning to understand the role of these structural changes because there are few dedicated multiscale imaging platforms with sensitivity at both the cellular and macrostructural spatial scale. A single platform reduces bias and complications from multiple sample preparation methods and can ease image registration. In order to address these needs, we have developed a multiscale imaging system using a range of imaging modalities sensitive to tissue composition: Ultrasound, Second Harmonic Generation Microscopy, Multiphoton Microscopy, Optical Coherence Tomography, and Enhanced Backscattering. This paper details the system design, the calibration for each modality, and a demonstration experiment imaging a rabbit eye.

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1. Introduction

The multi-level structure of tissue, from the arrangement of cells to the composition of organs, is a key factor in many major diseases. For example, cancer mortality is due to the damage the disease does to organ systems yet 90% of cancer deaths are ultimately due to metastasis [1]. Differences in these micro-environments determine how likely a metastasis is to occur and thus how the disease will progress on the tissue-mesoscale and organ-macroscale [2]. The converse can also be true, where macroscale composition affects the disease. A specific example with motivating interest to our lab is the role of collagen in breast cancer. The macroscale alignment of collagen fibers around a tumor boundary increases the risk of metastasis [3], while the macroscale density of collagen is related to risk of developing breast cancer [4]. Collagen thus plays a large role in the disease, but there are still many questions about its multiscale significance because there are no good imaging tools to directly correlate the microscale to macroscale and because we still do not fully understand the mechanism of the macroscale density influencing risk [5]. Many diseases have analogous interactions across spatial scales and, consequently, reports have identified integrating data across multiple scales as a key step forward for improving patient care [6,7].

Imaging is the main way to assess tissue structure, but we are only beginning to understand multiscale structure because most imaging methods image at a single spatial scale. In the
clinic, macroscale modalities such as magnetic resonance imaging, computed tomography, and ultrasound (US) can image whole organs, but they lack the spatial resolution to visualize at the tissue-mesoscale or cellular-microscale. Measurements at the microscale or mesoscale are restricted to near-surface tissues, endoscopic applications, or biopsies due to their small imaging fields-of-view (FOV) and penetration depths. Thus there is significant interest in the emerging field of multiscale imaging [8,9]. Part of this interest arises because some imaging modalities are affected by sub-resolution structures, which however can be quantified by multiscale imaging. For example, US is sensitive to the alignment of collagen fibers and a series of studies showed that it could be used to monitor tissue remodeling during pregnancy [10,11]. However, this type of research requires dedicated procedures to compare between scales or imaging systems that combine multiple modalities.

One difficulty with multiscale-imaging based on separate imaging systems is that it often requires combining images taken at different times and in different conditions. These different conditions complicate the analysis and make it more difficult to accurately spatially register the different images. The aforementioned study combined US images of a whole tissue sample with Second Harmonic Generation (SHG) images of thin slices [10]. The tissue was thus deformed in between imaging and mapping any two images together required image registration based on tissue landmarks. Such a procedure is prone to error and has to be repeated for every comparison. Thus, performing multiscale-imaging on separate systems and in different conditions makes it more difficult and can slow down research.

In order to eliminate these difficulties, we have developed an integrated multiscale-imaging system that can image tissue samples in the same condition for all component modalities: US, Multiphoton Microscopy (MPM), Optical Coherence Tomography (OCT), and Enhanced Backscattering (EBS). These modalities were selected because they are all sensitive to changes in fibrillar collagen organization, which is the initial demonstration for this instrument. The modalities are all inherently multiscale as they derive part of their contrast from scattering off of structures smaller than their resolution. In this work, the MPM modality can acquire SHG and Two Photon Excited Fluorescence (TPEF) signal.

Based on our past published work, SHG can detect collagen fiber organization at sub-micron resolution [12], whereas US is clinically compatible and is sensitive to larger scale collagen architecture [13]. Their combination then allows us to measure how differences in microscale composition and fiber arrangement change in clinical US images. For example, registering and analyzing the images from the combined SHG and US cervical collagen study would be significantly easier and faster because the tissue would be in the same condition for both modalities [10].

While collagen is the predominant tissue component of interest, other components are also important parts of the microenvironment and do not provide SHG contrast. Thus, the inclusion of the other optical modalities makes the system more versatile. For example, TPEF measures fluorescence, allowing label-free visualization of certain tissue components (e.g. elastin) or fluorescence-labeled studies for cells in the microenvironment [14]. OCT is a mesoscale, label-free imaging method, forming a bridge between the microscale SHG and macroscale US [15–18]. Finally, EBS is a non-imaging measurement technique that measures bulk optical scattering properties; these scattering properties are sensitive to changes on the sub-cellular nanoscale, allowing detection of differences in tissues that cannot be seen with optical microscopy [19–21]. Overall, this combination of imaging methods yields sensitivity to structures ranging from nanoscale to macroscale with an array of complementary contrast methods.

This paper describes the multiscale imaging platform and illustrates its imaging capabilities. First, we describe the overall hardware design behind the system, then detail the hardware components of each imaging modality in turn and demonstrate image quality with calibration measurements and example images. This is followed by a discussion on the design considerations
and trade-offs necessary to implement multiple modalities on the same device. We subsequently demonstrate the combined use of the system by performing multiscale imaging of the sclera of the rabbit eye as a biological tissue use case.

2. System design

The optical modalities (MPM, OCT, and EBS) share a central beam path and scanning optics, which are described in an overview below. This is followed by sections dedicated to each of the four main imaging arms (three optical arms plus ultrasound), which elaborate on further design elements and give calibration results and example images using the modalities. Finally, we briefly describe the system software schema, including how the images from each modality are registered and compared.

2.1. Main optical beam path

Figure 1 displays schematic diagrams and photographs for the system. The diagram in Fig. 1(A) displays the overall platform design. The imaging arms for Multiphoton Microscopy are depicted in Fig. 1(B). The diagram for the OCT imaging arm is depicted in Fig. 1(C). A photograph displays the arrangement of optical elements on the table in Fig. 1(D). The light source for MPM and EBS imaging is a 3.5-W femtosecond Titanium:Sapphire laser (Chameleon Ultra II, Coherent, Santa Clara, CA) (L1). The power output of the laser is modulated using a rotating half-waveplate leading into a polarizer (MWPQA2, MGLA12, Karl Lambrecht Corp, Chicago, IL) (λ/2, P). Circular polarization is set using a half- and quarter-waveplate (MWPQA2 and MWPQA4, Karl Lambrecht Corp) (λ/2, λ/4) to obtain uniform SHG signal with fiber angle [22]. The main beam is expanded (BE02, Thorlabs, Newton, NJ) (BE) and shuttered (VS14, Uniblitz, Rochester, NY) (S1) before being integrated with the OCT laser (L2 described in section 2.4) via a flip mirror. The beamline then leads into independent fast/X scanning and slow/Y scanning galvanometer mirrors (62xxH series, Cambridge Technology, Bedform, MA) (G-F, G-S). The galvos direct the beamline through a pair of adjacent achromatic lenses (LBF254, Thorlabs) that act as a scan lens (SL) (effective = 50 mm), placed 50 mm from the galvos (G-F). A tube lens (f = 200 mm) (LA1541, Thorlabs) (TL) is placed 150 mm beyond the scan lenses.

This is followed by a Modular Infinity Microscope (MIM, ASI Imaging) housed on a Rapid Automated Modular Mounting (RAMM, ASI Imaging) frame. The beamsplitter (BS) redirects the beam upwards and functions to direct backscattered light from the sample into the EBS collection path. A beam dump (BT600, Thorlabs) (BD) captures the transmitted light. The illumination light passes through a 4-position dichroic slider (DS) capable of holding a square diagonal mirror and a circular filter at each position. Positions 1 and 2 are configured for SHG and MPM respectively. Both positions contain a long pass dichroic beamsplitter (FF685, Semrock, Rochester, NY) (DB1). The filter slot for Position 1 is fitted with a band pass filter (445/20 nm, Semrock) (BPF). The motorized objective turret is fitted with a 20x magnification 0.75 Numerical Aperture (NA) air objective for SHG and MPM (Plan Apo VC, Nikon, Tokyo, Japan) (O1), a 4x magnification 0.13 NA objective for OCT (N4X-PF, Nikon) (O2), and an empty aperture for EBS. The objective leads to a motorized XY stage and a sample holder insert (Sample/XY Stage) for either slides or glass bottom dishes, where the latter are required for US.

The Forward SHG imaging arm and the US imaging arm are mounted on a manual rail, with stops in either position. Finally, all optical detectors, stages, and lasers are controlled through a computer (C1). The US transducer is controlled through a separate computer (C2).
Fig. 1. Multiscale imaging platform design. (A) Optical diagram of the main beamline, with simplified component boxes for each imaging arm. (B) Schematic diagram of the Multiphoton Microscopy imaging arm. The system switches between the modalities with a filter slider in the backwards position and a filter cube in the forwards. (C) Schematic diagram of the OCT arm. (D) Back photograph of the central beamline, galvos, and OCT imaging arm. The full description of the components is in the text.
2.2. Multiphoton microscopy

While the system is capable of both backward (reflection) and forward (transmission) MPM imaging (Fig. 1(B)), we primarily use the backward path, as samples that are suitable for US are too thick to utilize forward detection. The capability for this detection is included for experiments that use only the optical modalities, as it allows quantitative measurements of the SHG forward/backward ratio [12].

Either SHG or TPEF signals are reflected into the backward path by the dichroic. A focusing lens (f = 50 mm) (LA1102, Thorlabs) (FL) loosely focuses the signal onto a photon-counting photomultiplier tube (H7421-40, Hamamatsu Photonics, Hamamatsu City, Japan) (PMT) through (S2). A short pass filter placed after the shutter (FF680-SP, Semrock) (LPF) further suppresses any remaining laser light.

The elements for detecting forward propagating SHG are mounted on a hanging arm. The arm is affixed to a rail which has stops at two positions, one for forward SHG detection and the other for US. Manual X, Y, and Z stages are used to align the forward arm to the illuminating objective and sample. The arm starts with a 16x magnification 0.8 NA water dipping lens (Achro LWD, Nikon) (O3). Following O3 is a filter cube holder with magnetic cube inserts (DFM1, Thorlabs) (MC). The straight branch continues into a focusing lens (f = 50 mm) (LA1102, Thorlabs) (FL) followed by a short pass filter to block any remaining laser light (FF680-SP, Semrock) (LPF). Finally, a photon-counting photomultiplier tube (H7421-40, Hamamatsu) (PMT) collects the signal, achieving count rates of up to 20 MHz with acceptable pulse pile up. In the photon-starved regime of SHG, this captures the full dynamic range and gives superior sensitivity than analog PMTs [22]. Two-photon excited fluorescence (TPEF) is also collected in this geometry with a change in filters.

One of the main goals of this microscopy arm is to optimize the FOV and resolution under the constraint of the RAMM apertures, so that it is feasible to acquire volumes comparable to those in the US imaging arm while retaining high enough resolution to measure individual collagen fibers. The apertures of the RAMM stage cause vignetting for large FOV, so this task requires correcting for the vignetting (Fig. 2). It should be noted that this vignetting is related to the square of the photon intensity because TPEF and SHG are nonlinear processes [14]. The system can image a circular aperture of approximately 1046 µm diameter (Fig. 2(A)). This corresponds to a maximum 746 µm rectangular FOV, excluding the edges of the circle due to sharp falloff in intensity. The vignetting at this FOV can be corrected through the use of the BaSiC bright-field correction algorithm, which calculates the flat-field illumination and divides the image by this correction factor [23]. We show the correction factor and the impact of this correction on 3x3 tiled images of a mouse mammary gland in TPEF (Fig. 2(B, C, D)) and SHG (Fig. 2(E, F)). These images were taken at a 50 kHz pixel sampling rate.

The multiphoton resolution of the system was measured by imaging six 0.2 µm diameter fluorescent beads (Fluoresbrite, Polysciences) at an excitation wavelength of 890 nm with a 0.75NA air objective. The MetroloJ ImageJ plugin was used to help calculate the point spread function [24]. The PSF from one bead is depicted in Fig. 3. The measured PSF was 0.81 ± 0.14 µm in X, 0.90 ± 0.07 µm in Y, and 8.8 ± 2.3 µm in Z. The theoretical limits of resolution for this objective are 0.6 and 2.5µm, respectively. These resolution limits are within the sub-micron regime necessary to resolve most individual collagen fibers, which range in size from 500 µm to several mm [12]. The large deviation in axial resolution is because the lens was underfilled, due to the limiting apertures in the RAMM frame. Due to these limiting apertures, this was an optimization in the design criteria and a necessary compromise between resolution and FOV. A microscope framework with larger apertures than the RAMM system would obviate the need for this step. We next display an example of the SHG image quality in Fig. 3(D), which is an image of a rat tail tendon extracted from Sprague Dawley rats and placed in a glass bottom dish.
Fig. 2. Field-of-view and vignetting for multiphoton and SHG microscopy. (A) 1044 µm FOV image of a fluorescent slide, displaying vignetting across the usable FOV. The black box in (A) covers the usable FOV. (B) The vignetting can be corrected across the usable FOV using a bright-field correction calculated with the BaSiC algorithm. (C, D) 3x3 TPEF images of a mouse mammary gland before (C) and after (D) BaSiC correction. (E, F) 3x3 second harmonic generation microscopy images of a mouse mammary gland before (E) and after (F) BaSiC correction. All scale bars are in 500 µm. The color bars are in photon counts (A) and bright-field correction factor (B).

2.3. Ultrasound

The US portion of the platform is based on a research ultrasound data acquisition system (HIFU Vantage 256, Verasonics, Kirkland, WA). The platform uses an 18.5 MHz central frequency linear array transducer with 128 elements (L22-14v, Verasonics). The transducer is attached to a custom machined 5-axis positioning system with 2 translational and 3 rotational degrees of freedom. The rotational, transverse (X) and elevation (Y) degrees are locked in place with set screws after calibration procedures to center the transducer over the sample and to orient the transducer face perpendicular to the sample plane. The microscope stage controls sample motion in the transverse and elevation planes during imaging. The positioning system also contains an indicator gauge (DITR-0105, Clockwise Tools, Valencia, CA) to precisely measure the axial (Z) height of the transducer, as the transducer’s height must be adjusted for each imaging session.

The US image quality and echo signal power spectrum is demonstrated in Fig. 4. A mouse mammary gland was embedded between layers of 2% agarose and imaged using a 3D acquisition. The image was windowed to 30 dB and smoothed with a 3-pixel averaging kernel. Figure 4 displays the gland image centered on a lymph node in the lateral/axial plane (Fig. 4(A)), elevation/axial plane (Fig. 4(B)), and lateral/elevation plane (Fig. 4(C)). We measured the power spectrum of the US transducer by recording the echo signal from an optical flat (λ/10 fused silica optical flat, Edmund Optics, Barrington, NJ) submerged in deionized water and calculating the power spectrum in an ROI around the flat surface (Fig. 4(D)). We used the speckle auto-correlation method [25,26] to estimate the resolution (pulse-echo point spread function) of the US system using a phantom that produced only diffuse scattering [27] similar to that reported in [28].
Fig. 3. TPEF and SHG Microscopy image quality. (A, B, C) PSF measurement of a 0.2 µm diameter fluorescent bead (A) in the XY plane, (B) in the YZ plane, and (C) in the XZ plane. (D) SHG image of a Sprague Dawley rat tail tendon. The MetroloJ ImageJ plugin was used to generate a point spread report and n=6 reports were averaged to obtain a measure of the point spread function. The scale bar in (A) is 5µm wide and applies to (A, B, C). The scale bar in (D) is 100µm wide.

Correlation lengths were estimated to be 100 µm in the axial direction (alone the acoustic beam), 110 µm in the lateral direction (along the length of the transducer array length) and 450 µm in the elevation direction (perpendicular to the ultrasound B-scan image plane).

2.4. Common-path optical coherence tomography

Optical coherence tomography (OCT) is an interferometric imaging technique that uses a Fourier transform to obtain depth information for volumetric imaging [15,16,29]. Conventional spectral domain OCT is based on the Michelson interferometer and uses a separate reference arm starting at a beamsplitter that is equal in optical path length to the sample plane within a few hundred micrometers and contains components for physical dispersion compensation. Common-path OCT eliminates the reference arm and instead uses a reflection within the sample arm as its reference [17]. Because our microscope uses an inverted geometry, a strong reflection is always present from the surface of the glass bottom dish. In a conventional OCT system, this would create a strong autocorrelation image artifact. Instead, we turn this reflection to our advantage and use it as the reference for a common-path OCT. Conveniently, common-path OCT has the benefit of eliminating system dispersion [17], which is typically digitally corrected in conventional OCT. This common-path OCT system geometry is compatible with the SHG, TPEF, and US components.

The optical diagram for the OCT arm is displayed in Fig. 1(C). A coherent light source (860 nm SLD, SuperLum) is collimated (F280APC-850, Thorlabs) (L2) and directed into the laser scanning optics (Fig. 1(A)). A low NA objective (Nikon 4x plan fluorite, Thorlabs) (O2) collects backscattered light from the sample and sample-glass reference [18]. This backscattered light
is directed using a beamsplitter (BS-OCT) into a transmission grating (TG) which spectrally separates the interference. The first order diffraction is collected by a 150 mm focal length lens (FL) and focused onto a line-scan camera (AViiVA em4, Teledyne e2v, Essex, England) (S-CAM). The interferogram for each scan position is recorded as a function of wavelength. Interpolation of the interferogram into k-space ($2\pi/\lambda$) followed by Fourier transform recovers the signal in depth (also referred to as an A-line). An A-line is collected for each scan position in one lateral dimension (also referred to as a B-scan) and is used to build up a complete volume image of the sample. Camera exposure is set by the pixel clock time for Nyquist sampling in the lateral dimension and synchronized to the scan by the system line clock for a two-dimensional scan of the object which is reconstructed into a three-dimensional volume.

OCT lateral resolution follows the conventional diffraction limit for a given objective. Objective NA can be artificially lowered by underfilling the back of the objective which increases depth of focus at the cost of lateral resolution. The full NA of the objective was used in this application to maximize the lateral resolution. The galvo size and magnification of the scan and tube lens fix the beam diameter at 12 mm, giving a total NA of 0.13 and lateral resolution of 3.9 $\mu$m. Imaging a United States Airforce Bar Target (1951 USAF Negative Bar Target, Edmund Optics) provides a demonstration of the OCT lateral resolution and FOV (Fig. 5). Lateral resolution as assessed by the slanted-edge method demonstrated 7.5 $\mu$m [30].

OCT axial resolution and axial range of imaging is governed by source bandwidth and spectrometer resolution, respectively [31]. Axial resolution is calculated as $(2 \ln(2) \lambda_0^2)/(\pi \Delta \lambda)$, where $\lambda_0$ is the mean wavelength and $\Delta \lambda$ is the bandwidth measured as the FWHM of the source spectrum [32]. Using our source, we have an expected axial resolution of 10 $\mu$m. Our spectrometer design gave us a spectral sampling of 0.17 nm per pixel, providing an axial range of approximately 1.2 mm and a digital sampling of the axial range of 7.15 $\mu$m. The depth of imaging within a sample in OCT is limited by objective depth of focus, spectrometer-dependent fall-off, and sample-dependent attenuation. The depth of focus of the objective was calculated as
110 μm [32]. The fall-off in depth for OCT intensity also depends on the spectrometer and has been well characterized [33,34]. The signal in our spectrometer decays to 50% of its maximum value at approximately 250 μm. Combining all sources of signal fall-off has been done previously and can be observed to depend on the position of the depth of the objective focal plane [35]. The impact of these factors are discussed further in section 3.3, but can be well-summarized with the 6 dB fall-off depth and was measured as approximately 300 μm when the focus was moved with the specular reflection, which agrees well with the spectrometer-based roll-off. The 6 dB fall-off depth can also be calculated according to [32], which was approximately 340 μm for this system and agrees well with this measurement. Rolled tape is a common OCT phantom which demonstrates axial resolution, axial range, and depth of imaging [36,37]. Figure 5 demonstrates visibility of five or more layers when the sample is mounted on a standard 1 mm microscope slide and with no corrections for signal decay. Images of an air-glass interface had a FWHM of approximately 25 μm in depth while the surface of a strongly scattering material (tape) had a FWHM of 15 μm.

A sample image of the sclera in a rabbit eye in Fig. 6 demonstrates the imaging capabilities in tissue. The OCT system acquires a 3D volume, corresponding to an A-line for each sampling point, and allowing reconstruction of B-scans along the X or Y axes. Full imaging volumes can be processed to reconstruct a surface image of the tissue.

Fig. 5. Left: USAF 1951 Bar Target surface image reconstructed from OCT volume. Pictured is group one, element one. Right: OCT B-scan of rolled Scotch tape (3M, Maplewood, MN). Image demonstrates up to 500 μm of imaging depth in a scattering material. Imaging depth was also well-preserved across the curved surface of the sample.

Fig. 6. Left: OCT B-scans of the sclera. Right: Example OCT surface image data.
2.5. Enhanced backscattering

Enhanced backscattering (EBS) is a non-imaging optical characterization technique used to extract information about a scattering medium. Upon illumination, some light will be scattered and emerge within the illumination spot. The entrance and exit locations of such a scattering path act as sources and interfere because of the time-reversal symmetric nature of light. The net effect of all such possible scattering paths is a peak in the exact backward direction, where the shape (intensity and width) contains information sub-resolution about the medium [20,21,38–40]. See Fig. 7 for a diagram and example data showing the analysis workflow. Briefly, the EBS peak shape is extracted from an image by azimuthal averaging. The illumination impulse response (p(r)) is obtained from that peak shape by Fourier transform.

Combined with Monte-Carlo modeling, EBS data can also yield tissue scattering parameters such as scattering coefficient \(\mu_s\), anisotropy g, as well as more sophisticated metrics such as fractal dimension D [41–43]. Of special note for this work, given that collagen is birefringent, is the sensitivity of EBS to polarization information [21].

The EBS design for this device makes common use of the main beam path, with a few additional components (Fig. 1(A)). The size of the EBS beam can be controlled via an adjustable iris (I) upstream of the galvo mirrors. Since EBS requires a collimated beam instead of a narrowly focused beam, the objective is rotated out and the beam exits an empty aperture. The backscattered light passes through the beamsplitter (BS) into a fold-mirror, through an EBS lens (f = 200 mm) (EL), and finally into a camera (Orca-Flash 4, Hamamatsu) (CAM).

An advantage to having the EBS camera and beam path in the system is that it can be used to guide imaging as well. This custom microscope configuration has no built in eye-pieces or bright-field imaging mode. This makes focusing and use of the instrument challenging. However, with an objective in place, the EBS camera captures an image of the sample rather than angle...
space back-scattering (EBS). This configuration is helpful for navigation of samples, calibration and debugging.

To demonstrate the ability to measure EBS on this platform, we first examine a phantom whose optical properties can be tuned. A convenient substance for this purpose is intralipid, which has been widely used as a scattering phantom or as an ingredient in scattering phantoms [44–46] and has well-characterized scattering properties [47]. We use dilutions of intralipid 20% emulsion (1141-100ML, Sigma-Aldrich, Sweden) to provide a controlled range of scattering coefficients and show the measured EBS peaks and the calculated spatial backscattering impulse response, p(r) (Fig. 8).

EBS measurement demonstrated the expected shift to longer exit distance of path-pairs (shifting the p(r) to the right) associated with decrease in scattering coefficient from cleared compared to uncleared sclera (Fig. 9). Both eyes were fixed overnight in 4% PFA. One eye was stored in PBS while the other was cleared using the ScaleA2 protocol for 6 weeks [48]. The left panel shows the EBS peaks with stray light background removed by subtracting an image acquired with no sample, and then flat field normalized by dividing by an image acquired from very weak scattering sample that provides a narrow EBS peak less than a few pixels wide. The resulting peak is then normalized to the baseline value at 0.75 deg. The right panel shows the spatial backscattering impulse response, p(r), for the two eyes. Differences are especially apparent at length scales of approximately 100 microns which are consistent with decreased scattering from cleared tissue.

### 2.6. Software and registration

The optical imaging modalities are operated by an open-source laser scanning microscopy software known as OpenScan, which runs on the Micro-Manager software framework [49,50]. The software connects to the stage, the galvos, the photomultiplier tubes, and the EBS camera. The OCT camera is operated through MATLAB and synchronized with the galvos using OpenScan. The US data are acquired through MATLAB with the Verasonics Vantage software package. US acquisition is synchronized with OpenScan stage movement through TTL triggers. Once acquired, the US, SHG/TPEF, and OCT image data are stitched into 3D composites by calling the Grid/Collection Stitcher and BigStitcher ImageJ plugins [51,52], then registered using a custom
Fig. 9. EBS data from rabbit eye sclera. Left panel shows EBS profiles (averaged azimuthally around the peak, background subtracted, baseline normalized), comparing sclera and sclera from an eye cleared with ScaleA2. Right panel shows the spatial backscattering impulse response p(r) derived from the EBS peak. Shaded area in all plots are standard deviation from 3 acquisition locations.

Python software package [53]. The software package uses pre-determined transforms and can be used to map any image to any of the imaging coordinate systems.

The US to MPM transform was determined using a registration phantom and registration protocol that is being detailed in a forthcoming publication. Briefly, the registration phantom is made of a hard fluorescent resin that is visible by both US and TPEF. The phantom has fiducial markers in all three axes and so the US and TPEF images can be registered based on those fiducials. The resulting coordinate system transform is robust to subsequent imaging sessions.

The OCT to MPM transform was determined by registering TPEF images of the fiducial phantom through the OCT (O2) and the MPM objective (O1). OCT shares scanning galvo optics with MPM and so the image position of an MPM image using the OCT objective is in the same coordinate system as the OCT image. Thus, registering the MPM image with the O1 to the MPM image with O2 calculates a coordinate transform for OCT to MPM and, therefore, to ultrasound.

3. Design considerations and compromises

Including multiple modalities into this instrument imposed several constraints and required some design compromises, which are detailed below.

3.1. SHG design considerations

The principle constraint on the SHG imaging is the geometry of the RAMM frame which impacts resolution and FOV. The mechanical clear aperture and throw of the RAMM frame constrains the throughput or beam size and range of angles at which that beam can illuminate the objective. A larger beam provides larger illumination NA and higher resolution, but can only be delivered to the objective over a smaller range of angles, reducing field of view. The objective pupil size determines the required beam size to achieve maximum resolution. For this long working-distance objective, the pupil diameter is approximately 15 mm. However, the apertures in the RAMM frame limit the input beam size and cause vignetting on the output, as the beam clips the side of the aperture during scanning. Some correction of vignetting can be performed post-acquisition using flat field correction, but the FOV was limited to less than 500 µm. Under-filling the objective aperture reduces vignetting, but also reduces the NA and severely limits the resolution. To find a compromise between these two undesirable trade-offs, an atypical scan and tube lens design was employed.
A non-telecentric design was used to minimize vignetting due to the limiting apertures in the mechanical RAMM frame while balancing FOV and resolution. The distance between the scan and tube lens was reduced from the sum of their focal lengths of 250 mm to about 150 mm. This design choice allowed a large beam to illuminate a wider range of angles than would be possible in the standard 4f configuration. The shorter path reduces the beam displacement from the optical axis, clearing the RAMM frame input aperture, but comes at the expense of a slightly diverging beam. This impacts the working distance of the (infinity-corrected) objective, shifting the focal plane approximately 200 microns further away than specification (1.06 mm) and reducing the effective NA which reduces the resolution and performance of the objective but balances the vignetting and FOV within the constraints of the RAMM stand. The geometry of this scan and tube lens configuration was optimized using optical design software (OpticStudio, Zemax LLC, Kirkland, WA).

3.2. US design considerations

The main design consideration for the US imaging system is that it should be simple to adjust the size of the transmit and receive apertures. This constraint is due to the glass bottom dish required by the optical modalities. The sample sits on top of a glass coverslip which is highly reflective, where the reflections produce off-axis clutter in the image above the glass itself, thus obscuring parts of the tissue signal. The spatial extent of the clutter is more extreme when the transducer aperture is asymmetric and it grows for larger transmit or receive aperture sizes. This clutter can be compensated by adjusting the size of the transmit and receive apertures, but this comes at the expense of lower tissue signal. A smaller transmit or receive aperture, corresponding to higher F#, reduces the clutter. However, reducing the transmit aperture size lowers acoustic power through the tissue and reducing the receive aperture size reduces the number of elements receiving the signal. Both conditions lower the signal-to-noise ratio and degrade the quality of the image. Thus, adjusting the imaging parameters to account for the clutter lowers the overall quality of the US image but improves the image quality near the glass surface.

The Verasonics research ultrasound system allows for manipulation of the aperture size, as well as many other imaging parameters. For example, custom scripts using the Verasonics system can limit the number of acquired A-lines in the image so that each A-line is acquired with a symmetric aperture. Thus, the flexibility of the Verasonics system was a major advantage for this form of multiscale imaging for dealing with multiscale challenges such as helping mitigate the noise from the optical cover glass in the sample dish used for all imaging.

3.3. OCT design considerations

The OCT design was constrained primarily by the light source, the objective, and the need to conform to the SHG, TPEF, and US geometries. A near-infrared superluminescent diode with a central wavelength of 860 nm (FWHM of 30 nm) was used to minimize noise and to provide adequate axial resolution over a 1 mm of axial range of imaging. OCT axial range is limited by the resolution of the spectrometer and the axial resolution is limited by the bandwidth of the source. Without the use of a telescope between the beamsplitter and grating, the spectrometer resolution is limited by the size of the galvos which in turn limited the number of lines illuminated on the grating. In this case, a 600 lines/mm grating was used for the 3 mm galvo mirrors. The dispersed spectrum is focused onto the camera by a 150 mm lens giving a diffraction limited spot of approximately 40 µm. The line-scan camera is a linear array of 2048 square 14 µm pixels. While this design oversampled the spectrum, the spectrometer resolution was sufficient for a 1 mm axial range. Both the spectrometer resolution (and thus axial range) and the efficiency of the pixel illumination could be improved by using a telescope to expand the beam before illumination of the grating, however the constraints of the objective and microscope geometry imposed greater restrictions, including the confocal fall-off [54].
The OCT objective and its NA were the primary factors governing the imaging depth in samples. The 4x 0.13 NA Nikon objective was chosen to allow the OCT image plane to be parfocal with the other microscopy modalities. The objective was filled completely by the 3 mm galvos with beam expansion to maximize lateral resolution. Due to the full NA of the objective being used, the depth of focus was reduced as described above in 2.4. The combination of spectrometer-based sensitivity fall-off and the confocal PSF reduce the image intensity by half at a depth of 200 µm when the objective is focused at interface between the coverslip and sample. Focusing the objective into the sample will reduce the overall image intensity but provide a more even illumination of the sample. When the focus is placed 300 µm into the sample, the depth at which the image intensity falls to half extends from 200 µm to 375 µm. The maximum depth of imaging in samples was on the order of 500 µm, which was sufficient for this intermediate-scale application.

While the depth of imaging is small compared to other OCT systems, the use of common-path detection and microscope integration was a nontrivial restriction in the OCT design. The depth of imaging was limited by the thickness of the required sample mount for SHG microscopy, where using a 1.5 coverslip glass bottom dish limits the depth to approximately double the optical path of the thickness of the glass bottom dish or approximately 450 µm. The limitation occurs due to duplicate images in the axial range, arising from reflections at the front and back surfaces of the glass bottom dish. These reflections can be segmented out in post-processing provided there is at least some separation between them, as was the case in the system presented here. Future improvements in OCT signal fall-off in depth would need to be balanced with this separation kept in mind. This limitation to the depth of achievable imaging was not considered critical, as the role of OCT was to offer an imaging modality on an intermediate size-scale between SHG and ultrasound.

3.4. EBS design considerations

The main challenge for incorporating EBS into this device is stray light and reflections from optical elements in the acquisition beam path. Since measurement of EBS requires detection of a small signal in the presence of a large background, the relatively strong reflections from components located between the beamsplitter and sample can easily obscure or corrupt the peak. Currently, the biggest source of unwanted reflections in the beam path is the dish itself. When mounted in the translation stage insert, a glass bottom sample dish is perpendicular to the incident beam, and so the surface reflections from the glass coverslip obscure the EBS peak.

The best way to eliminate this problem is to assure such surfaces are sufficiently angled from the optical axis so the reflections fall outside the EBS angular field of view. Although the instrument galvos can tilt the beam (the objective is removed in EBS), the maximum angle was not sufficient to allow effective reflection suppression. The angle was therefore increased to 5 degrees using a 3D-printed wedged ring slipped into the microscope sample table. This allows EBS peaks free of reflections, although the ring must be removed for the other imaging modalities. Given the comparatively large 3-10 mm spatial extent of the EBS beam, registering EBS signal acquisition with other modalities is not a problem, although it necessitates performing EBS before or after all other modalities to avoid disturbing registration.

4. Multiscale imaging of a rabbit eye

This section demonstrates the system’s multiscale imaging capabilities through an example dataset on a common sample for all modalities. Our criteria for the common sample was a tissue that has structure and characteristics of interest for all the instrument modalities.

For this purpose, a rabbit eye is an excellent demonstration sample. The sclera is largely composed of fibrillar collagen and exhibits strong SHG signal [55]. This collagen structure should be reflected in OCT, alongside other tissue components that have no SHG contrast. The eye is
also a subject of numerous ultrasound studies investigating diseases ranging from melanoma to vision loss [56–59], and for demonstration purposes reveals internal structures such as the anterior chamber, lens, and angle. The sclera, usually appearing white, is highly scattering and so has a strong EBS response (see Fig. 9).

4.1. Sample preparation

Juvenile albino rabbit eyes were obtained at the time of sacrifice from an unrelated study using rabbits that was approved by the University of Wisconsin-Madison’s Institutional Animal Care and Use Committee. All eyes were fixed overnight in 4% PFA after extraction.

One eye was placed in a custom glass bottom dish made out of acrylic, with a #1.5 glass coverslip bottom, and fitted to a large petri-dish stage insert (I-3088, ASI Imaging, Eugene, OR). The eye was then embedded in a 2% agarose solution by applying light pressure from the top of the eye to prevent it from floating and then filling the chamber with agarose. Once the agarose congealed, phosphate buffered saline was poured over the eye to give US a water-coupled path. This embedding ensured that the eye would not shift during imaging. The eye was imaged in place for SHG, US, and OCT. However, for EBS imaging the dish had to be removed and re-inserted over the shim.

4.2. Imaging parameters

The agarose embedded rabbit eye was imaged with SHG, US, OCT, and EBS. Due to the disparities in scale and image orientation, each modality imaged the whole accessible volume of the eye. A single SHG image represents only a few US voxels, and so any statistical comparison between SHG and US requires many stitched SHG images to obtain a sufficiently large volume. In addition, the US and images are perpendicularly oriented to the SHG images, and so acquiring images over the SHG volume similarly requires a large number of B-scan US images in order to form a C-scan image in the region of interest.

The SHG image was acquired as a 22×17 grid of 512×512×33 images with a sampling resolution of 1.02×1.02×20 µm. The tiles were stitched into a FOV of 8.46×10.94×0.66 mm in X/Y/Z. The images were taken at a 200 kHz sampling rate, yielding an overall imaging time of approximately five hours.

The US image was acquired as a series of B-mode scans with a transmit and receive focus of 14 mm, a transmit F/# of 6, and a receive F/# of 3.6. Each B-mode scan acquired an image in a 2D X×Z plane of 4×19 mm, corresponding to the X and Z axes of the optical imaging. The acquisition obtained 40 raylines around the central 40 transducer elements, in order to avoid the noise from asymmetry mentioned in section 3.2. There were 6×233 slices, separated by 3733 µm X spacing and 100×Y spacing, for a total FOV of 22.71×23.3×19.05 mm. The US imaging took approximately 15 minutes.

The OCT volumes were acquired using co-registration of the ultrasound and multiphoton scanning grid. A mosaic of OCT volumes provided a 10 mm by 10 mm lateral FOV with approximately 500 µm axial range of imaging. Each OCT volume was acquired at 50 kHz sampling night, which resulted in an overnight acquisition taking approximately 16 hours.

EBS images were obtained as stacks of 10 images for averaging. The sample was manually rotated (EBS measurement was performed first of the modalities) about the center of the beam to suppress speckle. A background image of a black background and a low-scattering scan for flat-fielding were also obtained during the same session. The EBS image acquisition took approximately five minutes, including setup.

The whole imaging procedure took place over two days, starting in the morning and finishing in the late afternoon of the next day.
4.3. Multiscale image

The images from each modality were registered using the procedure described in section 2.6 and the results are depicted in Fig. 10. The multiscale image shows a clear correspondence between the gross curvature of the eye bottom in US, in SHG, and in OCT (Fig. 10(A, B, E)). Further, finer scale anatomical correspondence can be seen in the higher resolution SHG and OCT images (Fig. 10(C, F)). In addition, the EBS peak depicted in panel (D) displays the illumination scattering response of the EBS peak in the graph and an inset of the EBS camera image.

![Fig. 10. Multiscale image of a rabbit eye sclera. (A) A B-mode US image of the eye is overlaid with registered SHG (green) and OCT (magenta) images. (B, C) 3D visualizations of the SHG (B) and OCT (C) from above. (E, F) A close up of the SHG (E) and OCT (F) images from below shows similar biology between the two modalities, with corresponding seams pointed out by white arrows. (D) The EBS image and corresponding illumination scattering response. The curvatures of the SHG image match that of the US image. At higher resolutions, the OCT and SHG images depict corresponding physical structures. The scale bars in A, E, and F are 1000 \( \mu \)m wide, and the tick marks in B and C are 500 \( \mu \)m spacing.]

5. Discussion

This system is a significant advance for multiscale imaging. It demonstrates several new multi-modality combinations and enables a range of multiscale imaging studies. To our knowledge, it is the first system to merge a low-frequency (<20 MHz) linear-array US imaging with SHG or TPEF; this makes the instrument valuable for studies using external US probes and any US parameters available on multi-element (clinical/pre-clinical) imaging systems [60–62]. Several groups have developed endoscopic probes merging high-frequency (>20 MHz) single-element US and optical imaging, but these devices have FOV and speed limitations and cannot perform multi-element acquisitions [63–65]. In addition, this system is the first time that EBS has been incorporated into a microscope or used alongside other modalities. This combination of modalities is sensitive to structures at sub-optical resolutions, and it resolves structures at the
micro, meso, and macro scales. EBS is complementary to the other modalities in this instrument. Having the EBS channel common with the optical imaging modalities allows the opportunity to correlate small structural changes that may present in imaging modalities with EBS signal changes. Both SHG and OCT exploit tissue properties to create contrast and images but do not natively report scattering parameters. As a result, scattering information derived from ultrasound and EBS is valuable to efforts to derive quantitative information in these imaging modalities. For example, scattering information from bulk samples measured with all optical modalities could potentially be mapped to imaging volumes too small to measure with EBS.

We have shown that the system contrast can differentiate between structures in the eye and that it can produce co-registered images across these multiple imaging scales. This example showed clear structural similarities between OCT and SHG, while also showing that OCT detected regions of low-collagen that SHG could not. In addition, we have shown how the EBS peaks can differentiate between regions of tissue and between the same region with different scattering properties. This instrument thus facilitates studies that investigate phenomena link between subcellular composition to macroscale tissue properties.

This system was built to be modular so that more imaging modalities could be added to provide complementary contrasts and additional imaging scales. For example, the system has the components to perform photoacoustic imaging using the Titanium:Sapphire laser and the US transducer [66], which would give contrast for tissue components including hemoglobin, melanin, lipids, and water [9]. In addition, the US system could run with different transducers, allowing either higher resolution with higher frequency transducers, or new imaging modes such as shear wave imaging [67]. The existing imaging modalities will also be enhanced with quantitative imaging capabilities, such as by measuring acoustic scattering properties using the reference phantom method in US [68] or by calculating optical scattering properties using Monte Carlo simulations with EBS [41,42].

The system has several limitations. The main limitation is the area of tissue that can be directly compared between the optical modalities and US. The optical imaging can only penetrate a few hundred micrometers, depending on the scattering properties of the tissue, whereas the US system has an axial resolution of approximately 100 \( \mu \text{m} \). This means that the optical region of interest is only a small segment of the US image, right where the tissue rests above the glass. The full thickness of tissue scanned with ultrasound cannot be directly correlated to the less-penetrating optical modalities. Another limitation is imposed by the glass bottom dish, which affects the US, OCT, and EBS imaging arms. The US clutter noise from the glass bottom limits the image quality near the glass surface. The noise can be reduced at the expense of SNR and resolution by using smaller apertures for transmit and receive. It will be the focus of future work to investigate other approaches for dealing with this noise.

Finally, the use of the RAMM optical platform and stage introduces several apertures that limit the potential FOV and resolution. These apertures caused the 20x objective to be underfilled, and were responsible for the strong vignetting on the system. A design using more custom optical components could improve upon the FOV and resolution, though this would require replacing several of the automated components.

6. Conclusion

This paper describes a multiscale-imaging platform that is sensitive to differences in tissue structure ranging from the organ macroscale to the subcellular nanoscale. It incorporates five modalities onto a single platform: Ultrasound, Second Harmonic Generation microscopy, Multiphoton Microscopy, Optical Coherence Tomography, and Enhanced Backscattering. The platform images with the sample in the same condition for each modality, which simplifies the task of comparing like structures between modalities and facilitates studies into the relation between disease and multiscale tissue composition.
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