Low-cost, High-contrast, and Miniature Optical Imaging Systems for Clinical Applications

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Reducing the cost and size and enhancing the contrast of optical imaging systems improve their potentials for clinical applications. In this dissertation, we describe our endeavors towards development of low-cost and compact photoacoustic microscopy and spatial frequency domain imaging systems as well as improvement of photoacoustic tumor imaging using a specifically designed photoacoustic contrast agent.

Chapters two and three focus on the development of low-cost and compact laser diode based photoacoustic microscopy systems. We first provided an improvement in light delivery of laser diode based photoacoustic microscopy systems that enables imaging biological tissue with high signal to noise ratio. We then developed a laser scanning laser diode based photoacoustic microscopy system that provides substantial improvement of imaging speed and eliminates the need for mechanical scanning of the sample, hence improving the potentials of low-cost and compact laser diode based photoacoustic microscopy for clinical applications.

Chapter four describes synthesis and evaluation of a monomeric porphyrin-based photoacoustic contrast agent for improvement of in vivo tumor imaging. Absorption in near infrared wavelength range, solubility, stability, nontoxicity, and high photoacoustic generation efficiency of the dye were demonstrated. The contrast agent was evaluated for enhancing the photoacoustic images of implanted murine tumors revealing a multi-fold stronger enhancement and a slower washout compared to the benchmark FDA approved indocyanine green (ICG) dye. Favorable filtration and tumor accumulation of the dye further demonstrated its potential as a photoacoustic contrast agent for in vivo tumor imaging.

Finally, chapter 5 describes development of a very low-cost, handheld, and multispectral spatial frequency domain imaging system that incorporates nine different light emitting diodes and all illumination and detection optical components in a small 3D-printed probe. The system performance was evaluated on biological tissue to assess its potentials.
Low-cost, High-contrast, and Miniature Optical Imaging Systems for Clinical Applications

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Doctor of Philosophy Dissertation

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1. Introduction

Optical imaging is the term referred to imaging techniques that utilize light to non-invasively probe the tissue [1]. Upon interaction with biological tissue, optical energy is both scattered and absorbed. Some optical imaging modalities make use of light scattering (e.g. optical coherence tomography (OCT) [2]), some utilize light absorption (e.g. photoacoustic imaging (PAI) [3]), and some consider both scattering and absorption (e.g. spatial frequency domain imaging (SFDI) [4]). Optical imaging can probe shallower targets (mm range depth) with high resolution (e.g. OCT and photoacoustic microscopy (PAM)) or deeper targets (cm range depth) at lower resolution (e.g. photoacoustic tomography (PAT) and diffuse optical tomography (DOT) [5]). Different modalities of optical imaging have been used in various applications including, but not limited to, dermatology [6], ophthalmology [7], and several branches of oncology [8–11].

Photoacoustic microscopy, photoacoustic tomography, and spatial frequency domain imaging are among the rapidly growing modalities in optical imaging with various applications including the diagnosis and characterization of several cancer types including ovarian, colorectal, cervical, and breast cancer. In this dissertation we will discuss our efforts towards improving these imaging modalities and enhancing their potentials for clinical applications. The dissertation is structured as below.

Chapter 1 includes the introduction to the dissertation and general background about photoacoustic imaging and spatial frequency domain imaging.

Chapter 2 discusses our first efforts towards improvement and evaluation of a low-cost laser diode based photoacoustic microscopy system to be capable of imaging ovarian
tissue. Here, using an efficient light delivery method, we managed to increase the signal to noise ratio of photoacoustic imaging with a low-cost laser diode as the excitation source. The system showed capability of imaging vasculature under the surface of ovarian tissue showing the potential for imaging epithelial ovarian cancer. This system, however, still used mechanical scanning of the sample and required multiple averaging in data acquisition. Therefore, as is discussed in chapter 3, we developed a laser scanning laser diode based photoacoustic microscopy system eliminating mechanical scanning of the sample and providing significant improvement in imaging speed.

In chapter 3, we discuss development of the laser scanning laser diode photoacoustic microscopy system. This system provides a significant improvement in the imaging speed and also does not require any mechanical scanning of the sample, therefore improving the potential of the system for clinical applications. Here we show that this system is also capable of imaging thin vasculature under the surface of ovarian tissue and is therefore able to image and characterize epithelial ovarian cancer.

Chapter 4 discusses our collaborative effort with Professor Christian Bruckner’s research laboratory at chemistry department of the University of Connecticut to develop and evaluate the performance of a specifically designed photoacoustic contrast agent in order to improve in vivo photoacoustic tomography of tumor angiogenesis. Here we demonstrate the high photoacoustic generation efficiency of the dye, the multi-fold enhancement of in vivo photoacoustic images of murine tumors upon injection of the contrast agent, and also evaluate the washout and accumulation of the dye.
In chapter 5, we discuss our efforts for developing a very low-cost, handheld, and multispectral spatial frequency domain imaging prototype with potentials for ex vivo cancer characterization.

Finally, chapter 6 provides a summary of the endeavors discussed in this dissertation.

In the continuation of this chapter, we will provide a short background about the optical imaging modalities discussed in this dissertation. First, we will explain photoacoustic imaging which can be divided into two subcategories of photoacoustic microscopy and photoacoustic tomography and then we will explain spatial frequency domain imaging.

### 1.1. Photoacoustic imaging

Photoacoustic imaging, also known as optoacoustic imaging, is a hybrid imaging modality based on the thermoelastic induction of acoustic waves as a result of the absorption of pulsed or modulated optical energy [3,12–14]. In photoacoustic imaging, absorption of the optical energy with a short duration results in a local temperature rise that in turn causes the generation of mechanical pressure which propagates as mechanical waves, i.e. ultrasound wave. The generated photoacoustic signal can be detected by different means of ultrasound transduction, either with conventional contact transducers or in non-contact fashion [15,16]. Figure 1.1 shows a schematic of the generation and detection of photoacoustic signals.
Photoacoustic imaging can be categorized into two sub-modalities: photoacoustic microscopy for imaging in millimeter depth range at micrometer resolution and photoacoustic tomography for imaging in centimeter depth range but with lower resolution compared to photoacoustic microscopy. In PAM, either light or the ultrasound signal are focused, leading to optical resolution PAM (OR-PAM) or acoustic resolution PAM (OR-PAM). OR-PAM provides higher resolution compared to AR-PAM, while AR-PAM can generally image deeper targets compared to OR-PAM. In PAM, ultrasound signal is detected point-by-point via raster scanning the sample or steering the light and/or the ultrasound signal, while in PAT, array ultrasound transducers are used to form two-dimensional images, similar to ultrasound imaging [3,17]. Figure 1.2 shows a schematic comparing photoacoustic microscopy and photoacoustic tomography.
Because photoacoustic signal is generated as a result of light absorption, tissue constituents with higher absorption compared to the background tissue in the excitation wavelength act as the source of contrast in photoacoustic imaging. In the visible and near infrared wavelength ranges hemoglobin acts as the endogenous contrast source of photoacoustic imaging [18], as a result photoacoustic imaging has shown potential in detection and monitoring of various cancer types by mapping tumor angiogenesis [19,20], which is the irregular growth of blood vasculature around the tumor [21].

Three chapters of this dissertation focus on photoacoustic imaging. In chapters 2 and 3, we discuss our efforts in developing laser diode based photoacoustic microscopy systems in order to reduce the cost and size of PAM systems and make them more suitable for clinical settings. Chapter 4 discusses synthesis and evaluation of an exogenous
photoacoustic contrast agent specifically designed to enhance imaging of tumor angiogenesis in vivo.

1.2. Spatial frequency domain imaging

Spatial frequency domain imaging is a large-field-of-view imaging modality that can provide quantitative information about optical absorption and scattering properties of tissue [4,22]. In this method, tissue is illuminated with spatially modulated light (often sinusoidal) and the diffused backscattered light is detected by a camera and the optical properties of the tissue can be estimated using the diffuse reflected light from the target and a calibrated phantom. Figure 1.3 shows a schematic of spatial frequency domain imaging.

![Figure 1.3 Schematic of SFDI. Tissue is illuminated with spatially modulated light and the diffuse reflected light is detected by a camera.](image)

The spatial sinusoidal pattern can be represented as:

\[ I_n = I_0 \sin(2\pi f x + \varphi_n) \]  

(1.1)
where $I_0$ is the source amplitude, $f_x$ is the spatial frequency, and $\varphi_n$ is the illumination phase. Tissue is illuminated with three phase shifted patterns (0, $2\pi/3$, and $4\pi/3$) and the DC and AC components ($f = 0$ and $f_x$) are extracted as:

$$M_{\text{DC}} = \frac{I_1 + I_2 + I_3}{3} \quad (1.2)$$

$$M_{\text{AC}} = \frac{\sqrt{2}}{3} \left[ (I_1 - I_2)^2 + (I_3 - I_2)^2 + (I_3 - I_1)^2 \right]^{\frac{1}{2}} \quad (1.3)$$

The DC and AC components of the diffuse reflected light from the target are compared to the DC and AC components of a calibrated phantom imaged in the same conditions as the target. Moreover, the diffuse reflectance of the calibrated phantom (for $f = 0$ and $f_x$) is calculated from the forward model of spatially modulated light diffusion approximation or Monte Carlo simulations [4]. The calibrated diffuse reflectance of the target is found as:

$$R_d = \frac{M}{M(\text{ref})} R_d(\text{ref}) \quad (1.4)$$

Here, $R_d$ is the diffuse reflectance of the target, $R_d(\text{ref})$ is the calculated diffuse reflectance for the homogenous reference phantom, $M$ is the measured (for $f = 0$ and $f_x$) diffuse reflected light from the target, and $M(\text{ref})$ is the measured (for $f = 0$ and $f_x$) diffuse reflected light from the reference phantom. Therefore two $R_d$ values for DC ($f = 0$) and AC ($f = f_x$) components are measured. Finally, a lookup table that relates different values of $R_d$ with absorption coefficient ($\mu_a$) and reduced scattering coefficient ($\mu_s'$) values is used to reconstruct for $\mu_a$ and $\mu_s'$ of the target at each pixel of the image [4].

Because optical properties of tissue vary in the healthy and diseases stages, spatial frequency domain imaging can be used for disease diagnosis and characterization. This method has been used for characterization of different cancer types including ovarian and breast cancer [9,23]. In chapter 5 of this dissertation, we discuss our efforts in developing...
a very low-cost, handheld, and multispectral spatial frequency domain imaging prototype suitable for \textit{ex vivo} cancer characterization.

\textbf{References}


1. Introduction


2. Improvement and evaluation of a laser diode photoacoustic microscopy system for ovarian tissue imaging

2.1. Introduction

Photoacoustic imaging (PAI) is based on the thermoelastic induction of acoustic waves in a medium due to the optical absorption of pulsed laser or modulated light [1–3]. Photoacoustic imaging is capable of mapping blood vasculature due to the higher optical absorption of hemoglobin in the visible and near infrared (NIR) range in comparison to other tissue chromophores [4]. Angiogenesis is a significant factor for cancer development [5], therefore photoacoustic imaging has been utilized for diagnosis and characterization of various cancer types such as breast and ovarian cancer [6–9]. Photoacoustic microscopy (PAM) can provide high resolution images of microvasculature. Clinical applications of PAM are limited due to the use of expensive and bulky pulsed laser sources [10]. High power pulsed laser diodes (PLD) can be suitable substitute light sources for PAM [10–13]. However, multiple active elements in high power PLDs and intrinsic anisotropy of the PLD beam challenge maintaining low-loss focusing of light [14], hence limiting the applications of laser diode based PAM systems. Here, a laser diode based optical resolution photoacoustic microscopy system that utilizes a near infrared PLD and a low-loss optical setup is presented. Combination of aspherical and cylindrical lenses enables energy efficient collimation and focusing of light. Images of human hairs, polyethylene tubes filled with rat blood, mouse ear ex vivo, and porcine ovary ex vivo are presented to demonstrate the feasibility of the system for imaging ovarian tissue.
2.2. Methods

The configuration of the laser diode based photoacoustic microscopy system is illustrated in Figure 2.1. A 905 nm pulsed laser diode (Laser components, 905D5S2L3J08R) with 650 W maximum output peak power is used as the excitation source. The PLD is placed in a collimation tube where light passes an aspheric lens (Thorlabs, C330TMD-B) for collimation. The optimal choice of the collimating lens and the position of the laser diode with respect to the lens is determined using Zemax simulations (Zemax LLC). The stacked PLD is comprised of 10 active regions placed in two columns, therefore the output beam after collimation is made of two columns each made of five rectangular bars. Each separate bar is collimated by the aspheric lens, however because the active areas are placed in various positions with respect to the optical axis of the lens [14] they will separate from one another in two perpendicular directions after passing the collimating lens. Therefore using a microscope objective for light focusing introduces a significant energy loss [14]. However obtaining reasonable focusing by conventional lenses requires further collimation of the beam. Two perpendicular cylindrical lenses (Thorlabs, LJ1212L1-B and LJ1125L1-B) are used to collimate the light in two perpendicular directions. The collimated beam forms a rectangle of approximately 15 mm by 10 mm 400 millimeters away from the source. The light is then focused on the sample by an aspheric lens (Edmund optics, 66-020) with a numerical aperture of 0.71. The proposed focusing method introduces 6.8 dB less loss in comparison to the previously reported system [10] when using similar PLDs. A function generator provides the PLD driver (Laser components, LSP40) and the data acquisition (DAQ) board with synchronized 1 KHz repetition rate TTL (Transistor-Transistor Logic) pulses. Photoacoustic signal is coupled
from the sample to the ultrasound transducer (Echo, BI933, 3.5 MHz center frequency) by ultrasound (US) gel. The signal is sampled by the data acquisition board with 128 averaging after being amplified by the Panametrics receiver with a gain of 59 dB. No pre-amplification of the signal is required due to the low-loss collimation and focusing of light.

![Schematics of the laser diode based photoacoustic microscopy system](image)

**Figure 2.1 Schematics of the laser diode based photoacoustic microscopy system**

### 2.3. Results and discussion

The resolution of the proposed PAM system was measured to be approximately 40 micrometers by imaging a sharp blade edge. The edge spread function (ESF) was obtained from the one-dimensional cross section profile of the maximum amplitude projection (MAP) image. The best fit of an error function on the ESF was obtained. The full width at half maximum (FWHM) of the line spread function (LSF), which is the first derivative of the edge spread function, represents the resolution of the system [15,16]. The one-dimensional cross section measurement, the corresponding ESF, and the corresponding LSF are presented in Figure 2.2. Furthermore black human hairs were imaged to test the imaging capability of the system. The maximum amplitude projection...
image of crossing hairs is shown in Figure 2.3. The peak to peak photoacoustic signal to noise ratio (SNR) for imaging this phantom was 27.6 dB.

Figure 2.2. The edge response measurement from a sharp blade edge, the fitted edge spread function, and the corresponding line spread function. The resolution is measured to be approximately 40 micrometers.

Figure 2.3. PAM image of crossing human hairs. The peak to peak photoacoustic SNR of 27.6 dB was measured for imaging this phantom.

To further study the feasibility of the system in imaging biological tissues, phantoms made of polyethylene tubing filled with rat blood were imaged. The PAM image of two blood filled tubes placed next to each other is depicted in Figure 2.4A. The tube on the left has
an inner diameter of 0.58 mm and an outer diameter of 0.985 mm. The tube on the right has an inner and outer diameter of 0.38 mm and 1.09 mm, respectively. The normalized one-dimensional cross section profile of the image along the dotted line is shown in Figure 2.4B. The FWHM of the tube images are measured to be approximately 0.625 mm and 0.42 mm for the tube on the left and right, respectively. Possible sources of error could be the generation of ultrasound from the tube wall and non-ideal positioning of the tubes. The peak to peak photoacoustic SNR for this phantom is 32.7 dB. The previously reported system using a microscope objective for focusing the light from a PLD provided a SNR of 12 dB for similar phantoms [10], indicating the reduction of optical loss and a higher signal to noise ratio in the current system.

PAM images of mouse ear *ex vivo* were acquired to demonstrate the feasibility of the system for imaging biological samples. The PAM image and the photograph of the mouse ear are presented in Figure 2.5A and 2.5B, respectively. The peak to peak photoacoustic signal to noise ratio of this image is approximately 22 dB. The signal to noise ratio of this image is enhanced compared to the previously reported NIR laser diode based PAM image of similar biological tissue [10,13].
To investigate the feasibility of the proposed system in imaging ovarian tissue and diagnosis of ovarian cancer, porcine ovary samples were imaged *ex vivo*. The PAM image and the photograph of the porcine ovary are illustrated in Figure 2.6A and 2.6B, respectively. The imaged vasculature was located well below the surface and was not visible for photography until after the sample was dried out. Therefore the proposed PAM system is capable of imaging the vasculature below the surface of the ovary. The peak to peak photoacoustic signal to noise ratio of this image is approximately 25 dB. The maximum laser intensity on the tissue from this system is estimated to be approximately 2 mJ/cm$^2$, which is far below the maximum permissible exposure (MPE) for this wavelength according to the American National Standards Institute (ANSI) safety standard [17]. These results suggest the feasibility of using the proposed laser diode based PAM system for imaging ovarian tissue and potentially diagnosis and characterization of ovarian cancer.
2.4. **Summary**

A laser diode based photoacoustic microscopy system with a low-loss focusing method is presented for imaging biological tissues. The difficulty of providing low-loss focusing of high power PLDs is addressed by collimating the light with an aspheric lens as well as two perpendicular cylindrical lenses and focusing the light with a 0.71 NA aspheric lens. Light loss compared to the previously reported system is decreased by 6.8 dB. Due to the reduction in optical loss the need of a pre-amplifier, which can introduce wideband noise to the signal, is eliminated. The resolution of the system is measured to be around 40 micrometers by using the edge spread function estimation of imaging a sharp blade. Images of human hairs, polyethylene tubing filled with rat blood, mouse ear *ex vivo*, and porcine ovary *ex vivo* are provided to demonstrate the ability of the system in imaging biological samples. The results indicate the feasibility of the proposed low-cost laser diode based PAM system for imaging ovarian tissue and possibly diagnosis and
characterization of ovarian cancer. The low-loss optical system for collimation and focusing brings about the possibility of utilizing much less averaging and therefore faster imaging. Furthermore, the flexibility of having a collimated beam in a long range enables laser scanning photoacoustic microscopy in order to significantly increase the imaging speed. In the next chapter we will explain our efforts towards development of a laser scanning laser diode photoacoustic microscopy system.

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References


2. LDPAM
3. Laser scanning laser diode photoacoustic microscopy system

3.1. Introduction

Photoacoustic imaging (PAI) is a fast emerging imaging modality based on the thermoelastic induction of acoustic waves as a result of the absorption of pulsed or modulated optical energy [1–4]. Optical energy provides the excitation source and the induced acoustic waves are detected via ultrasound transduction [1]. Therefore, the difference in the absorption of tissue constituents provides the optical contrast in PAI.

Photoacoustic imaging is capable of mapping blood vasculature due to the higher absorption of hemoglobin compared to other tissue constituents in the visible and near infrared (NIR) wavelength ranges [1,5]. Angiogenesis, which is the irregular growing of vasculature in the tumor area, is a significant indicator of cancer development [6–10]. Hence, photoacoustic tomography (PAT) and photoacoustic microscopy (PAM) have been utilized in oncology [11,12] for cancer detection and treatment monitoring via mapping tumor angiogenesis in breast [13,14], prostate [15–17], skin [18–20], thyroid [21,22], colorectal [23–25], pancreatic [25], cervical [26], and ovarian cancer [27–32].

Out of the 30 different types of ovarian cancer, almost 90% of the cases are epithelial ovarian cancer, which originates from the cells covering the outer surface of the ovary. Unfortunately only about 30 percent of epithelial ovarian cancer cases are diagnosed at early stages [10,33,34]. Currently, the standard of care is removal of ovaries of high risk patients. This procedure, however, increases the mortality rate of young women [35], and therefore, the development of methods to reduce unnecessary surgeries is critical. Given the significance of angiogenesis in epithelial ovarian cancer [8], Wang et al. demonstrated
the potential of a PAM image feature extraction algorithm for classification of normal and malignant ex vivo ovarian tissues [27].

However, that PAM system included a solid-state laser system (i.e. Ti-Sapphire laser pumped by a second harmonic Nd:YAG laser (LT2211 and LS-2134, Symphotics TII Corp, Camarillo, CA)) and a high precision 3-D linear motor [27]. The system was not only expensive and bulky but also slow on data acquisition. Solid-state lasers can be more compact and fast; however, the cost is still high with prices in the range of $15K to $40K. Low-cost and compact PAM systems will facilitate the transition from laboratory tools to clinical devices. There are many clinical applications that could potentially benefit from low cost, compact and fast photoacoustic microscopy and endoscopy systems such as ovarian cancer [27], skin cancer [18–20], cervical cancer [26,36,37], and colon cancer [23–25] detection and diagnosis. The importance of developing low-cost, compact, and fast photoacoustic imaging systems for enhancing the clinical applicability of photoacoustic imaging in oncology has been emphasized by other researchers as well [11]. Low-cost photoacoustic imaging systems can also encourage biomedical photoacoustic research in developing countries. Additionally, considering the applications of PAI in oncology and the importance of access to affordable medical devices in low-income countries as stated by the World Health Organization (WHO) compendium of innovative health technologies for low-resource settings, low-cost PAI systems can potentially benefit the realization of Universal Health Coverage (UHC) [38].

High-power pulsed laser diodes (PLD) have been used as low-cost and compact substitutes for light sources both in PAM and PAT [39–46]. Wang et al. presented a low-cost laser diode-based PAM system with a 905 nm PLD as the excitation source.
However, the signal-to-noise ratio (SNR) and imaging quality of the system was limited mainly due to the optical energy loss in a 60x microscope objective [41,42]. Moreover, the need for 128 signal averaging limited the imaging speed of the system. Zeng et al. demonstrated a laser diode based PAM system with a 405 nm PLD as the excitation source which required 512 averaging and had a low imaging speed [43]. Li et al. presented a laser diode based PAM system with a Blu-ray DVD pickup head as the excitation source [45]. This system also required 3000 times averaging for imaging biological tissues. We previously reported a method to obtain a low-loss collimation and focusing for a 905 nm high power PLD that enabled obtaining PAM images of porcine ovarian tissue [42]. However, the aforementioned system also suffered from a low imaging speed. Moreover, the laser diode based PAM systems mentioned above require mechanical scanning of the sample which limits potential clinical applications.

Moving toward clinical in vivo PAM applications requires rapid image acquisition. However, rapid image acquisition also benefits ex vivo clinical and research applications. This is because incised ovaries should be fixed in formalin soon after the incision in order to preserve the freshness for histology, thus providing only a short imaging time window after the surgery. Faster ex vivo imaging will hence better preserve the sample freshness and morphological information prior to formalin fixation [47]. Here, for the first time to the best of our knowledge, we present a fast laser scanning laser diode-based photoacoustic microscopy system. No averaging is used for obtaining the images. The imaging speed is 370 A-lines per second. The long-reach and low-loss collimation of the PLD beam needed for laser scanning is achieved using a combination of aspheric and cylindrical lenses. Two galvanometer scanning mirrors perform the two-dimensional scanning of the
beam across an aspheric focusing lens, hence no need for mechanical scanning of the sample. Photoacoustic microscopy images of human hairs, \emph{ex vivo} mouse ear, and \emph{ex vivo} porcine ovary are presented to demonstrate the feasibility of the proposed system for imaging and characterizing biological tissues. The capability of imaging the thin vasculature on porcine ovarian tissue suggests the potential of the proposed system for characterization and classification of ovarian tissue [27] with a low-cost and fast PAM system.

3.2. Methods

3.2.1. Laser scanning laser diode photoacoustic microscopy setup

The schematic of the laser diode based photoacoustic microscopy system is depicted in Figure 3.1A. A 905 nm (± 10 nm), 325 W maximum output peak power pulsed laser diode (Laser components, 905D5S3J08S) is utilized as the excitation source. The laser is pulsed by a PLD driver (D, PicoLas LDP-V 240-100 V3) with 50 ns pulsewidth (Figure 3.1B).
In order to perform laser scanning, the PLD light has to be well collimated for a reasonably large distance. However, the multiple active elements in high power stacked PLDs and the intrinsic anisotropy of their beam [42,48] are challenges for maintaining a low-loss collimation of the light. The PLD used in this system has a 200 × 440 μm emitting area. It consists of five active areas, each 200 μm wide. The active areas are stacked vertically with 110 μm vertical separation between consecutive ones. The PLD is first placed inside a collimation tube (CT) with an aspheric lens (A1, Thorlabs, A230TM-B) in order to improve the collimation. This, however, is not sufficient because of the short focal length of the lens, and the rather large vertical separation between the active elements and beam divergence angle. Therefore, to further improve the collimation, four cylindrical lenses are
utilized. Two of these lenses (C1 and C3, Thorlabs, LJ11878L2-B and LJ1821L1-B, respectively) act as a Keplerian beam expander in the slow axis direction, and the other two (C2 and C4, Thorlabs, LJ1212L1-B and LJ1105L1-B, respectively) act as a Keplerian beam expander in the fast axis direction. Figure 3.1A only shows the changes in the beam in the fast axis direction as the slow axis direction has no vector components in the plane of this two-dimensional illustration. After passing through C4 the entire beam maintains its initial 17 \times 12 \text{ mm} size for more than 30 cm until it reaches the scanning mirrors and the focusing lens. Two galvanometer scanning mirrors (GM, GSI Group, G330) are used to scan the beam across a 1- inch diameter aspheric lens (A2) with a numerical aperture (NA) of 0.71 (Edmund optics, 66-020) which focuses the beam on the sample (S). The generated photoacoustic (PA) signal is detected by an unfocused ultrasound transducer (Tr) with a center frequency of 3.5 MHz and a bandwidth of 60\% (Echo, BI933). Ultrasound gel (G) is used for coupling the PA signal between the sample and the transducer. The transducer and sample are placed on a three-dimensional stage (St) for positioning of the sample.

**3.2.2. Data acquisition and processing**

The PA signal is amplified by an ultrasound pulser and receiver (PR, Panametrics, 5072PR) with 59 dB gain and is digitized by a 65 MHz, 14-bit data acquisition (DAQ) card (Gage-applied, GS8325). The mirrors and the data acquisition card are synchronized by the DAQ PC. A 16-bit multifunction data acquisition card (National Instruments, PCIe 6251) provides a finite number of 1 KHz trigger pulses to a function generator (FG) that feeds the PLD driver. The same trigger signal is sent to the ultrasound receiver. The B-scan signal of the x-axis mirror is a ramp signal from the multifunction DAQ card and it
starts and finishes in synchrony with the beginning and end of the trigger pulse train. A-lines are acquired via a multiple acquisition scheme. A-line data are triggered by the trigger output of the ultrasound receiver, stored in the circular buffer of the Gage DAQ card, and transferred to the PC after a B-scan is finished. The y-axis mirror moves in a step-wise fashion after the B-scan data acquisition and saving is performed. Control, data acquisition, and signal processing to form maximum intensity projection (MIP) images are performed in a single code in MATLAB (MathWorks, Natick, Massachusetts, USA). No averaging is performed on the A-line signals. A MATLAB built-in moving average filter is performed on the raw A-line signals to slightly remove the high frequency noise (higher than 8 MHz) and improve the SNR. The final image undergoes a MATLAB built-in median filter. The final imaging speed is approximately 370 A-lines are per second. Therefore, for instance, a 500 × 200-pixel image can be acquired and displayed in approximately four and a half minutes.

3.3. Results

3.3.1. Lateral resolution

The maximum field of view was measured by imaging targets of known sizes and comparing the size of their image to the field of view. A maximum field of view of approximately 4.6 mm × 3.7 mm ±0.3 mm was obtained. Several measurements were performed and the average value is reported.

The lateral resolution was measured to be approximately 21 µm using edge spread function estimation. The edge of a high contrast target (black tape) was imaged and the one-dimensional cross-sectional profile of the image was fitted by an error function, which is considered as the edge spread function (ESF). The first derivative of the ESF
represents the line spread function (LSF) and the full-width-at-half-maximum (FWHM) of the LSF represents the lateral resolution [49,50]. Figure 3.2A shows the PAM image of the edge of the high contrast target. Figure 3.2B depicts the measured edge response along the dashed line in Figure 3.2A, the fitted ESF, and the corresponding LSF. The pixel size in Figure 3.2A is $0.125 \times 6.7 \, \mu m$ in horizontal and vertical directions, respectively. The pixel sizes are governed by the scanning area and the number of A-lines per B-line for the horizontal direction and the number of B-lines for the vertical direction.

![Figure 3.2 A. PAM image of the edge of a high contrast target. The color bar represents normalized PA amplitude. B. The one-dimensional cross-sectional profile of the image along the dashed line, the ESF, and the LSF. FWHM of the LSF is approximately 21 \, \mu m.](image)

### 3.3.2. Axial resolution

The theoretical axial resolution as derived from the ultrasound transducer is given as $0.88 \, c/B$, where $c$ is the speed of sound in tissue ($\sim 1540 \, m/s$) and $B$ is the bandwidth of the transducer [51]. With a 3.5 MHz central frequency and 60% bandwidth (corresponding to 4.1 MHz), the theoretical acoustic axial resolution is $\sim 331 \, \mu m$. Figure 3.3A shows the
PAM image of a thin human hair. The pixel size in Figure 3.3A is 0.836 × 2.1 μm in horizontal and vertical directions, respectively. As shown in Figure 3.3B, FWHM of the one-dimensional cross-section of the image along the dashed line is approximately 35 μm. Figure 3.3C shows the A-line signal from the thin hair target and its envelope obtained from Hilbert transformation. FWHM of the envelope is ~220 ns, corresponding to an ~339 μm axial resolution [51], which is very close to the theoretical value. Due to the limited axial resolution of the transducer compared to the lateral resolution, only 2D images are presented in this report. It should be noted that with higher pulsewidths, axial resolutions larger than the theoretical value were obtained, and this would further limit the ability to separate targets at different depths.

Figure 3.3 A. PAM image of a thin human hair. The color bar represents normalized PA amplitude. B. One dimensional cross-sectional profile of the image in (A) along the dashed line shows ~35 μm thickness. C. A-line signal from the hair and the corresponding envelope. FWHM of the envelope is ~220 ns, corresponding to ~339 μm axial resolution.

3.3.3. Depth of penetration

In order to assess the penetration depth, a black human hair is inserted approximately two millimeters below the surface of a chicken breast and another black human hair is inserted just below the surface to serve as the reference point for depth measurement. Figure 3.4A and 3.4B show the side and overhead views of the chicken breast piece, respectively. Figure 3.4C shows the maximum intensity projection image of the two hairs and Figure 3.4D shows the B-line image along the dashed line in Figure 3.4C. The 2-mm
axial distance between the hairs is apparent in Figure 3.4D and the SNR, $20 \log \left( \frac{V_{\text{signal}}}{V_{\text{noise}}} \right)$, of both hairs is slightly higher than 6 dB. It should also be noted that the hair close to the surface (on the left side of the image in Figure 3.4C) appears thicker than the deeper hair (on the right side of the image in Figure 3.4C) because the focal spot is set to be closer to the 2-mm deep hair and the hair close to the surface is out of focus considering the 0.71 NA of the focusing lens. The pixel size in Figure 3.4C is $3.1 \times 4.2$ μm in horizontal and vertical directions, respectively. The pixel size in Figure 3.4D is $3.1 \times 23.6$ μm in the horizontal and axial directions, respectively. The axial pixel size is governed by the 65 MHz sampling of the data acquisition card and assuming 1540 m/s speed of sound in the tissue.

![Figure 3.4](image)

Figure 3.4 A. Side view of chicken breast piece used for depth of penetration measurement B. Overhead view of the chicken breast piece C. Maximum intensity projection PAM image of the hairs inside the chicken breast. The hair on the left side of the image is close to the surface and the hair on the right side of the image is about 2 mm deep. D. B-line image along the dashed line in Figure 3.4C. Color bars represents normalized PA amplitude.

### 3.3.4. PAM image of human hairs

PAM image of black human hairs positioned to form multiple branches is depicted in Figure 3.5A in order to demonstrate the imaging of branch-shaped targets. The hairs were measured to be thinner than 100 μm using a standard caliper. The one-dimensional cross-sectional profile of the image along the dashed line is presented in Figure 3.5B.
FWHM of profiles range between 80 µm to 180 µm. Hairs with thicker profiles in the image were positioned slightly out of focus. The pixel size in Figure 3.5A is 6.7 × 18.5 µm in horizontal and vertical directions, respectively.

Figure 3.5 A. PAM image of human hairs. The color bar represents normalized PA amplitude. B. One-dimensional cross-sectional profile of the image in (A) along the dashed line.

To further demonstrate the capability of imaging branch-shaped targets lying deep in the tissue, crossing black human hairs inserted about 1 mm below the surface of a chicken breast were imaged. Figure 3.6A and 3.6B show the side and overhead views of the chicken breast piece and Figure 3.6 C shows the PAM image of the hairs. The hairs are clearly resolved with ~15 dB SNR. The pixel size in Figure 3.6C is 8.3 × 8.4 µm in the horizontal and vertical directions, respectively.
3.3.5. PAM image of mouse ear *ex vivo*

*Ex vivo* PAM image of a mouse ear and its photograph are presented in Figure 3.7A and 3.7B, respectively. Mouse ears were obtained from the University of Connecticut animal facility and the institutional oversight was waived. The thin vessels inside the rectangle are all resolved in the image, even the vessels that have lost blood during the incision and appear less clear in the photograph. The pixel size in Figure 3.7A is $5 \times 12.3 \, \mu m$ in horizontal and vertical directions, respectively. PAM image of another *ex vivo* mouse ear and its photograph are presented in Figure 3.7C and 3.7D, respectively. As it can be seen from the photograph, the ear was imaged from inside rather than outside so that the vessels on the left side of the rectangle in Figure 3.7D lay deeper in the tissue. However, these vessels, as indicated by solid arrows, are clearly resolved in the image. Moreover, the areas where blood is not continuous in the vessel, as indicated by dashed arrows, are
distinguished with details. The pixel size in Figure 3.7C is $4.4 \times 6.9 \, \mu m$ in horizontal and vertical directions, respectively. The SNR for imaging mouse ear *ex vivo* is $\sim 14 \, dB$.

![Figure 3.7](image1)

3.3.6. **PAM image of porcine ovary *ex vivo***

To evaluate the capability of the system for imaging ovarian tissue, a porcine ovary was imaged *ex vivo*. Porcine ovaries were obtained from a local farm and the institutional oversight was waived. PAM image of the *ex vivo* porcine ovary and its photograph are presented in Figure 3.8A and 3.8B, respectively. As can be seen, the system is capable
of resolving thin vessel branches that are positioned under the tissue surface and are not apparent in the photograph. The pixel size in Figure 3.8A is 2.9 × 10.1 μm in horizontal and vertical directions, respectively. The SNR for imaging porcine ovary *ex vivo* is ~ 18 dB.

![Figure 3.8 A. PAM image of the vasculature on a porcine ovary. The color bar represents normalized PA amplitude. B. Photograph of the porcine ovary](image)

FWHM of the vessel branches indicated by the arrows in Figure 3.8A are 50-65 μm. Considering the ability to resolve such vessels on the porcine ovary and the ~21 μm resolution, the system shows potential sensitivity to arterioles, venules, angiogenic sprouting clusters, and micro-vessel clusters, which are present in tumor angiogenesis [6,8,31,52–56]. Also most of the vasculature in the sample PAM images of normal and malignant ovarian tissue presented in Ref. [27], where the authors use feature extraction of PAM images to classify normal and malignant ovarian tissues, are of similar or larger sizes [27]. The FOV of the presented ovarian tissue PAM image is ~ 1.5mm × 3mm. Such
an area can potentially contain part of the vasculature feeding or surrounding a tumor cite. Therefore, when combined with feature extraction algorithms to analyze the configuration and distribution of the vasculature [27], the proposed low-cost and fast laser diode based laser scanning photoacoustic microscopy system has the potential of imaging and classification of ovarian cancer, providing an important step towards the clinical application of PAM for studying ovarian cancer.

### 3.4. Discussion

The proposed PAM system offers significant improvement in the imaging speed compared to previously reported low-cost laser diode-based PAM systems [39–43], eliminates the need for mechanical scanning of the sample, and has demonstrated the potential for imaging and classification of ovarian tissue. Even though laser scanning PAM systems utilizing conventional solid-state lasers with comparable or higher speeds have been reported previously [57,58], this is the first implementation of a fast laser scanning PAM system using a low-cost and small pulsed laser diode. The PLD used in this paper is approximately $540 and the PLD driver is approximately $1350, resulting in a total of $1890, which is significantly lower than the cost of conventional photoacoustic light sources. The proposed system is therefore one step closer to low-cost, compact, and fast PAM devices valuable for clinical use.

A PAT system using a pulsed laser diode as the excitation source (lateral resolution of 180 or 380 µm with a 5 MHz or 2.5 MHz transducer, respectively) has been developed by other researchers [44]. The cost of the PLD used in the PLD-PAT system is ~ $15k [44]. Therefore, given that the system proposed here is much less expansive and yet can provide ~21 µm lateral resolution makes it worthwhile to use. Additionally, to obtain a
lateral resolution of 21 µm with acoustic resolution PAM, the required frequency of the focused ultrasound transducer should be higher than 75 MHz [59]. Such transducers (especially focused ones) are not commercially available and are very expensive if custom designed. For instance, several examples of acoustic resolution PAM using conventional light sources have used a transducer with 50 MHz central frequency and 70% bandwidth with a lab-made acoustic lens of 0.44 numerical aperture and have reported 45 µm lateral resolution [19,20,60,61]. Currently, we are not aware of any acoustic resolution PAM systems utilizing low-cost PLDs.

The theoretical diffraction limited resolution of optical resolution PAM is estimated as 0.5 λ/NA, where λ is the wavelength and NA is the numerical aperture of the lens. With a 905-nm wavelength and 0.71 numerical aperture, the diffraction limited resolution is 0.63 µm, which is much smaller than the resolution obtained in this setup. In order to reach the diffraction limit, the laser light should have a Gaussian profile and it should have a very small divergence angle. Using fast/slow axis microlens collimators can improve the beam profile, collimation, and consequently the focusing [62]. Also, using beam expanders with larger expansion ratios can further decrease the divergence angle and improve the collimation and focusing. However, this will increase the beam size and results in more light energy loss due to the limited aperture of the scanning mirrors and the focusing lens.

As a future work, a single, small, two-dimensional microelectromechanical system (MEMS) based actuator can be used for laser scanning. Such a scanning mirror can be placed after the focusing lens, therefore light will be scanned after the focusing lens rather than inside it. As a result, the beam can be further expanded before reaching the lens, leading to a smaller divergence angle and a better focusing. It should also be noted that
although the focusing lens in this report is a single aspheric lens and introduces less aberrations compared to spherical lenses, it is estimated that the resolution degrades about 20-30 microns near the edges. Using a specific scanning lens (f-theta, etc.) can provide a flatter focal plane for all scanning angles and maintain a fairly similar focal spot even near the edges. However, the relative high cost of such lenses, especially with numerical apertures as high as 0.71, may affect the low-cost nature of the system.

Using visible pulsed laser diodes (e.g. 405 nm) would potentially improve the lateral resolution. However, NIR light demonstrates better penetration depth in optical resolution PAM compared to the visible range [63], which enhances the potential to image vessels lying deeper beneath the surface of the ovarian tissue. Moreover, although the absorption coefficient of hemoglobin is higher in 405 nm compared to 905 nm, available energies of NIR PLDs are higher than those in the visible range [5, 43, 45]. Light emitting diodes (LEDs) have also been reported as photoacoustic light sources [64, 65], however due to the larger divergence of LED beam compared to laser diodes, it is more challenging to obtain low-loss collimation and tight focusing of LED beam [65]. Moreover, PLDs can have shorter pulsewidths compared to LEDs, which leads to better axial resolution [65].

In order to obtain better axial resolution for high resolution 3D imaging, higher frequency ultrasound transducers can be employed. Because in this system PA signals are detected in transmission mode and healthy human ovaries are 1.5-2 cm thick and diseased ones can have larger sizes [66], we have used a relatively low-frequency ultrasound transducer to avoid attenuation of ultrasound waves travelling in the tissue. Considering the current SNR level for ovarian tissue imaging, attenuation caused by higher frequency transducers in the transmission mode will affect the image quality. Higher frequency transducers can
be used in a reflection mode PAM setup that does not require the ultrasound waves to pass through the tissue. It has also been shown that application of Wiener deconvolution on the axial direction can provide ~1.7 times improvement in the axial resolution [51]. Moreover, an unfocused transducer is used in the current system because while light is scanned on the sample, the ultrasound transducer is stationary. If a focused transducer is used, the detection sensitivity decays rapidly away from the focal spot, hence limiting the field of view. Using a focused transducer confocal with the optical excitation, which is common in reflection mode PAM systems, can potentially provide about 20 dB increase in the detected PA signal level [57,67,68]. It should be noted that considering the limited photoacoustic signal level, in order to maintain sufficient SNR without averaging in the reflection mode PAM, efficient coupling of the ultrasound signal to the transducer is necessary. Due to the properties of the PLD, a high numerical aperture lens (1-inch diameter, 17.5 mm focal length, 0.71 NA) is required to reach the desired resolution in the current setup. In the reflection mode laser scanning PAM systems, either the transducer is placed confocal with the optical focus and both light and acoustic wave are steered by the mirror [57], or the transducer is tilted with respect to the sample [58]. In both forms, low numerical aperture lenses (NA ~ 0.1, focal length 50 mm [57] or 60 mm [58]) are used so that either there is enough space for the mirror and beam combining components between the lens and the sample [57] or the transducer has a small degree with respect to the sample (30 mm away, 15 degrees tilted [58]). Therefore, achieving acceptable resolution and efficient ultrasound signal coupling is a challenge that should be addressed in future reflection mode laser scanning laser diode PAM systems. Improving the beam profile and collimation will allow achieving acceptable lateral
resolution with lower numerical aperture lenses, hence paving the way for the reflection mode configuration.

The light energy directly from the PLD (without the optical system) was measured to be approximately 16 µJ and the energy delivered to the tissue was measured to be approximately 13 µJ. Assuming the focal spot to be 0.5-2 mm below the surface of the sample, the maximum surface optical fluence is estimated to be between 1.6-0.1 mJ/cm², which is far below the maximum permissible exposure (MPE) at this wavelength according to the American National Standards Institute (ANSI) safety standard [69].

Imaging and classification of normal and malignant human ovaries using the proposed system is a future task. The imaging speed can also be further increased. The maximum repetition rate of this PLD is governed by its absolute maximum duty cycle of 0.1%. Therefore, at 50 ns or lower pulsewidths the repetition rate can be increased up to 20 KHz. Considering that no averaging is required in this system, using an improved data acquisition scheme and up to 20 KHz repetition rate of the PLD, the imaging speed can be significantly increased. A laser scanning laser diode-based PAM system using a MEMS-based two-dimensional actuator with improved imaging speed is currently under development to obtain better image quality and imaging speed and move towards a reflection-mode setup.

Furthermore, development and application of photoacoustic contrast agents with absorption peaks near 905 nm can enhance the SNR and image quality of this system [70–74]. Studying skin and cervical cancer can be other possible applications of a laser scanning laser diode based PAM system [18–20,26]. With further developments, endoscopic laser diode-based photoacoustic microscopy systems have great potential to
provide angiogenesis distribution of ovarian surface during minimally invasive surgery which can guide surgeons to reduce unnecessary surgeries [27]. Such systems also have great potentials for diagnosis of colorectal [23–25], pancreatic [25], and esophageal [75] cancer.

3.5. Summary

A low-cost and fast laser scanning laser diode based photoacoustic microscopy system is presented. The lateral resolution is approximately 21 µm. PAM images of human hairs, ex vivo mouse ear, and ex vivo porcine ovary have been presented. The imaging speed is significantly higher than previously reported laser diode-based PAM systems and the need for mechanical scanning is eliminated.

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References


[2] P. Beard, Biomedical photoacoustic imaging, Interface Focus. 1 (2011) 602 LP-
3. LSLDPAM


[45] M.-L. Li, P.-H. Wang, Optical resolution photoacoustic microscopy using a Blu-ray DVD pickup head, in: SPIE BiOS, International Society for Optics and Photonics,


[53] P.J. Van Diest, J.P. Zevering, L.C. Zevering, J.P.A. Baak, Prognostic value of microvessel quantitation in cisplatin treated FIGO 3 and 4 ovarian cancer patients,
3. LSLDPAM


[68] NDT resource center, signal-to-noise ratio, (n.d.). https://www.nde-
3. LSDLPM


3. LSLDPAM
4. In vivo photoacoustic tumor tomography using a quinoline-annulated porphyrin as NIR molecular contrast agent

4.1. Introduction

The development of new—or the refinement of existing—imaging techniques of biological processes and tissue is arguably one of the leading driving forces in contemporary biomedical chemistry [1–6]. Photoacoustic imaging (PAI) is a rapid and non-invasive imaging modality that combines optical and ultrasound imaging [7–9]. Photoacoustic signals are optically generated and ultrasonically detected. It thus can take advantage of the optical window of tissue and provides the deep probing depth (multiple cm) and spatial resolution (sub-mm) of ultrasound.

PAI is the consequence of a number of physical effects [9]. The absorption of a light pulse by a chromophore causes it to enter an excited state. Good PAI chromophores relax rapidly primarily along non-radiative pathways, causing a transient rise in temperature (in the mK regime) around the closest vicinity of the absorbing dye, leading to a localized thermal-elastic expansion. Thus, if the light source is a pulsed laser with a short pulse length, light absorption generates a wideband ultrasonic wave. This signal can be acquired with standard ultrasonic transducers known from traditional ultrasound imaging. Furthermore, using wavelengths within the optical window of tissue (~700-1100 nm; the wavelength of maximum penetration of breast tissue is ~725 nm; whole blood has an absorption minimum at ~710 nm) [10,11], dyes many centimeters deep within tissue can be probed. If such a NIR laser beam is scanned across an object, the photoacoustic
data can be used to reconstruct 2D or 3D photoacoustic maps. The laser light energy used for in vivo imaging experiments is generally well below the standard thresholds above which tissue damages can be expected. Variants of PAI are photoacoustic tomography (PAT) [7] for large-scale imaging and photoacoustic microscopy (PAM) [12] for small scale, high-resolution image generation. Endogenous chromophores, primarily hemoglobin, can be used as PAI dyes [13]. This allowed for the imaging of the blood content of the vascular network in rodent brains [12] or cancer in breast [14] and ovary tissues [15,16], the mapping of mesoscopic biological objects, or whole animals [7]. However, particularly cancers in their early stages, cannot be detected by their intrinsic vascular contrast. Therefore, the use of exogenous contrast agents is required to achieve a suitable signal to noise ratio and to allow the PAI of deeply-seated organs or lesions [13]. A number of nano- or micro-scale agents have been introduced as PAI or multimodal contrast agents in recent years, with some showing good in vivo imaging results [17–22]. Among them are metal-based nanoparticles, combined with and without organic dyes, and nanotubes. However, the use of nanoparticles is not without problems with respect to biodistribution, toxicity, or homogeneity [23–25]. Alternative approaches have been the use of molecular dyes assembled into vesicles, most prominent among them porphysomes made from chlorins or bacteriochlorins, such as pyropheophyrin derivative 1 [18,26], microbubbles [19,20], or nanodroplets [21]. Also developed were photoacoustic probes that are generated in tissue [27,28]. Conspicuously rare in the contemporary literature are small molecular contrast agents designed for PAI [13]. One example is 2, shown
to be a suitable contrast agent for imaging dissolved oxygen using photoacoustic lifetime imaging [29]. One of the oldest and best studied PAI contrast agents is the FDA-approved ocular angiographic dye indocyanine green (ICG, 3), and related derivatives [30,31]. ICG possesses NIR absorption ($\lambda_{\text{max}} \sim 800$ nm) properties but is otherwise far from ideal. For example, ICG is, albeit weakly fluorescent. Thus, a portion of the absorbed light is not translated into a photoacoustic signal, diminishing its effectiveness as a PAI contrast agent. ICG is confined to the vasculature space and it clears rapidly ($t_{1/2} < 4$ min) [32,33], complicating longitudinal in vivo studies. Irrespective of these shortcomings, the broad utilization of ICG suggests its use as a benchmark dye. The absence of the development of many molecular contrast agents highlights the fact that reliable structure-function relationships that might guide the rational development of photoacoustic dyes suitable for their use in biological contexts has not been derived.

![Chemical Structures](image)

4a: $X = N$

5a: $X = N^+ \cdot O^-$
The synthesis of *meso*-tetraphenylporphyrin-derived quinoline-annulated porphyrins, such as 4a or 5a [34–36] were recently reported. As a consequence of the extended π-conjugation and their annulation-induced non-planarity of the porphyrinic chromophore, the photophysical properties of quinoline-annulated porphyrins are significantly altered when compared to those of the parent porphyrin. While regular porphyrins generally do not absorb much past 650 nm, quinoline-annulated porphyrins possess unusually red-shifted λ<sub>max</sub> bands in the 750 nm range. It was also shown that the bis-annulated systems and quinoline-annulated chlorins (in which another pyrrole was modified by a non-pyrrole heterocycle) are accessible by step-wise conversion of 5a, further manipulating the optical properties of this family of porphyrinoids [34–36]. Complementary syntheses of some of these derivatives and related chromophores are also known [37,38].

Crucially, quinoline-annulated porphyrins 4a and 5a were shown to be primarily relaxing rapidly non-radiatively, making them very good photoacoustic dyes [31]. We demonstrated a ~2.5-fold PAI contrast enhancement for 4a over pure blood or ICG in phantom tissue experiments [31]. The realization of the combination of a NIR-absorbing chromophore undergoing primarily rapid non-radiating relaxation pathways is difficult, as the direct comparison of the photoacoustic signal generation efficiency of a number of NIR dyes absorbing in the same wavelength range has shown. Despite this promise for 4a or 5a as PAI contrast agents, however, their insolubility in aqueous solutions (in the absence of a formulation vehicle like Cremophore EL®) prevented their *in vivo* assessment.
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We report here firstly the development of a quinoline-annulated porphyrin-based single-molecule, serum-soluble PAI contrast agent and the evaluation of its efficacy as an in vivo PAI contrast agent for the detection of implanted tumors in a mouse model. A multi-fold contrast enhancement when compared to ICG was found, a finding that could be traced to its photophysical properties. Its nontoxicity and renal clearance rates will be demonstrated. We also prepared a water-soluble quinoline-annulated derivative of the quinoline annulated porphyrin carrying a small fluorescent tag to facilitate the biodistribution studies of this intrinsically non-fluorescent chromophore. Alas, it was not fluorescent enough to be of utility. Nonetheless, the experiment demonstrated a facile and general conjugation strategy for these chromophores.

4.2. Synthesis of a freely water-soluble quinoline-annulated porphyrin

A number of strategies were established to render meso-arylporphyrins water-soluble [39–44]. Among them are their functionalization with anionic (carboxylate, sulfonate, phosphonate) or cationic (pyridinium) groups or their decoration with (multiple) polyethylene glycol (PEG) chains. We chose the PEG-strategy for the charge neutrality of the final products and previous reports indicating their suitability for tumor targeting [43,45]. We found that quinoline-annulated porphyrin 4a is chemically stable under the classic acidic methoxy deprotection conditions (BBr₃) but not to, for example, classic basic saponification reaction conditions (NaOH, THF). We thus chose the phenol-protected meso-tetrakis(p-
methoxyphenyl)porphyrin 6b as the basis for the formation of the quinoline-annulated porphyrin chromophore [34], with the intent to deprotect and PEG-ylate the phenolic oxygens using standard Williamson alkylation strategies at the last stages of the dye synthesis pathways. This strategy was successful as demonstrated by the synthesis of the waters-soluble dyes 4d and 4e (Scheme 4.1).

Scheme 4.1. Reaction conditions: (i) 1. 1 equiv. OsO4, CHCl3, r.t.; 2. H2S (ii) 4 equiv. DMP, CH2Cl2, r.t. (iii) 100 equiv. NH2OH·HCl, pyridine, N2 atmosphere, r.t. (iv) DDQ, CH2Cl2, r.t. (v) pyridine, Δ (vi) 1. BBr3, CH2Cl2 (vii) Me(OCH2CH2)nOMs, Cs2CO3, DMF, 90 °C.

p-Methoxy-derivatized quinoline-annulated porphyrin 4b was synthesized from meso-tetrakis(p-methoxyphenyl)porphyrin 6b using the dihydroxylation → oxidation → oximation → annulation route established earlier for this chromophore
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class [34]; all intermediates showed the expected spectroscopic and analytical properties [46]. However, some notable deviations from the established protocols were implemented. The oxidation of diolchlorin 7 required the use of Dess-Martin Periodinane (DMP) for its smooth conversion to the corresponding 2,3-dioxochlorin 8 [47]. The generally used oxidant DDQ failed to produce this dione [48]; instead dehydration to the corresponding enol was observed (the reaction was not further investigated). Oxime 9 formed smoothly; its treatment with p-TSA under forcing conditions (toluene, reflux) produced the quinoline annulated porphyrin 4b in acceptable yields, but this product was contaminated with the corresponding N-oxide 5b. Because their separation was tedious, we treated oxime 9 with DDQ, thus forming N-oxide 5b as the exclusive product in near-quantitative yields. We attribute the electron-rich nature of the p-OMe-substituted oxime to the facile annulation and oxidation. N-Oxide 5b could be readily reduced to quinoline annulated porphyrin 4b heating to reflux in pyridine. Similarly readily losses of the N-oxide were observed previously [34]. Quinoline-annulated porphyrin N-oxide 5b was also susceptible to a BBr3-mediated deprotection of the methoxy groups and concomitant reduction of the N-oxide, without any noticeable degradation of the macrocycle, generating the phenolic quinoline-annulated porphyrin 4c in five linear steps from porphyrin 6b.

Phenol-derivatized quinoline-annulated porphyrin 4c could be PEG-ylated with a methoxy-capped PEG-mesylate using a short PEG chain (n = 4), forming 4d, as well as a longer chain (avg. MW 550, n ~ 12), forming 4e. ESI+ MS and 1H NMR spectra of 4d and 4e confirmed that all four non-equivalent phenolic OH-groups
were PEG-ylated. The mass spectra of 4e reflect the slight chain length inhomogeneity of the longer PEG-chain [46]. Because of the homogeneity of the shorter PEG derivative, we used this derivative for the determination of the photophysical properties of the water-soluble quinoline-annulated porphyrins. Further details about the synthesis steps, materials, and instrumentations are provided in section 4.6.

4.3. Methods

4.3.1. Co-registered pulse-echo-photoacoustic tomography

The details to the 64 channel co-registered ultrasound pulse echo-photoacoustic tomography (PE-PAT) system is described elsewhere [49]. Briefly, the system utilizes a unique field-programmable gate array (FPGA) technology that allows for real-time acquisition of ultrasound and photoacoustic signals. The photoacoustic signal is generated by a 15 Hz, 12 ns pulse width light of a tunable Ti-Sapphire laser (LT-2211, Symphotics TII Corp, Camarillo, CA) pumped by a second harmonic Nd:YAG laser (LS-2134, Symphotics TII Corp). Free space illumination is used for light delivery. The laser light is expanded by a combination of concave and convex lenses and shone on the sample using mirrors. A small portion of the beam is separated by a beam splitter (BSN11, Thorlabs) and is focused on a single element ultrasound transducer for monitoring the fluctuations in laser energy during the experiment [50]. The pulse-echo (PE) ultrasound signal is generated and received by the system in synchrony with the laser pulses. The PE and PAT images are formed by a beamforming algorithm. The data sampling is performed at 40 MHz. A 64 channel ultrasound transducer with a center frequency of 3.5 MHz, a
bandwidth of 80%, and a sector scan type is utilized for acquiring the co-registered PE-PAT images. Ultrasound and PAT images are overlapped to form the co-registered images. The effects of laser energy fluctuations are compensated for in the data analysis. The setup was also used for the phantom photoacoustic signal generation efficiency evaluation of the dyes, as described previously [31].

4.3.2. Animal protocols

All experiments involving mice were performed as approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Connecticut under license #A15-047.

4.3.3. Toxicity test

100 µL of PBS based solution of 4e with 33.3 mM concentration was injected into anesthetized 6 week old BALB/c mice (n = 2). The heart rates of the mice were monitored by a pulse oximeter (MouseStat, Kent Scientific) before injection and during a 3 h period after injection.

4.3.4. Tumor model.

Tumor cell preparation was adopted from the literature [51,52]. Briefly, 4T1 Luc cells were cultured at 37 °C with 5% CO₂ in a T75 flask (BD Biosciences, Bedford, MA); DMEM (Dulbecco's Modified Eagle's medium, Gibco, USA) medium supplemented with 10% FBS and 50 U/mL penicillin/streptomycin. After 3 passes, the cells were suspended in the DMEM and 1 × 10⁵ cells were injected subcutaneously on top of the right leg of female BALB/c mice (6-8 weeks old, body
weight ~20 g). The mice were monitored for approximately two weeks post-inoculation until the tumor size was between 7-10 mm.

4.3.5. In vivo PAT of murine tumor.

Prior to the in vivo imaging, the mice were anaesthetized (1.5 L/min oxygen with 1.5% isoflurane) and the tumor area depilated. The position of the mouse was fixed. Ultrasound gel was applied on the tumor area and a bag of water was placed on top of the tumor for acoustic wave coupling. The pulsed laser at 790 nm of the PE-PAT setup described above (780 nm for ICG) was shone on the tumor through the water bag; surface optical fluence on the tissue was always maintained below the maximum permissible exposure according to the ANSI safety standard [53]. The transducer position was adjusted by a three dimensional mechanical stage to reach a suitable imaging condition. The pre-injection PAT image of the tumor was acquired. Then 100 µL of the PBS based solution of the dye 4e (~33.3 mM concentration) or ICG was injected via retro-orbital injection [54]. Care was taken to avoid any changes in the position of the mouse, transducer, and light beam as a result of the injection. The energy of the laser was monitored and the contribution of laser energy fluctuation on the PAT signal was compensated for in the data analysis [30,50]. The PAT images of the tumor were acquired during the 45 min period following injection. The PAT images and the maximum PA signal levels were compared before and after the injection. The schematic of the in vivo experiment setup is illustrated in Figure 4.1.
4.3.6. **Ex vivo fluorescent imaging**

100 µL of the fluorescent tagged dye (3 mM in PBS) was injected to three BALB/c mice of similar weight and similar tumor size. The mice were sacrificed 15 and 120 min after the injection. Tumor, liver, kidneys, heart, lung, and spleen were harvested, washed twice in PBS, and imaged using an IVIS® Lumina II fluorescent imaging system (Caliper Life Sciences, Hopkinton, MA); \( \lambda_{\text{excitation}} = 465 \text{ nm} \), GFP emission filter.

**4.3.7. Photophysical measurements.**

The photophysical measurements were performed as described previously [31].
4.4. Results and discussion

4.4.1. Photophysical properties and solubility of PEGylated quinoline-annulated porphyrins 4d and 4e.

The presence of the four p-methoxy groups in 4b (or the four p-OH groups in its deprotected derivative 4c) is reflected in a slight red-shift of its optical spectrum ($\lambda_{\text{max}} = 762$ nm for 4b, $\lambda_{\text{max}} = 748$ nm for 4c) when compared to the spectrum of 4a ($\lambda_{\text{max}} = 728$ nm), but the overall characteristics of the quinoline-annulated porphyrins are not altered (Figure 4.2A) [31].

The shorter PEG derivative 4d is highly soluble in CH$_2$Cl$_2$ as well as alcoholic solvents, but is only slightly soluble in pure water. The PEG-ylated quinoline-annulated porphyrin 4e is freely soluble in alcohols, water, serum, and PBS buffer.
As expected, the PEGylated derivatives 4d and 4e possess a $\lambda_{\text{max}}$ value of 764 nm in CH$_2$Cl$_2$. Both compounds exhibit some modest degree of solvatochromism. The spectrum of 4e in water, for example is slightly blue-shifted and overall broadened (Figure 4.2B).

The dissolution of 100 mg 4e/mL PBS was readily possible, forming a 33.3 mM solution. The solubility is therefore at ~ 2 orders of magnitude higher than the reported value for ICG in water [55]. The Lambert-Beer law is maintained for aqueous concentrations up to 0.03 mM (the upper limit we could measure in a 1 mm path length cell) but the addition of the surfactant Triton™ X-100 to dilute solutions slightly blue-shifts and enhances the Soret band [46], suggesting that 4e is somewhat aggregated in aqueous solution. The solutions are stable; no significant changes were observed in the UV-vis spectra of the solutions over many hours, and with only the onset of minor shifts after two weeks (in the dark at 4 °C).

The fluorescence quantum yield $\Phi$ for 4e in CH$_2$Cl$_2$ as well as H$_2$O were determined to be significantly below 0.1%. Transient absorption spectra (in the range from 450 to 900 nm) for 4e in both solvents with pump-probe delay times from 0 to 15000 ps delivered no reliable ISC-quantum yield or S$_1$-lifetime data. Thus, excited time lifetimes for 4e are well below 1 ps. Correspondingly, 4e in CH$_2$Cl$_2$, H$_2$O, as well as H$_2$O-Triton™ X-100 solutions showed no sign for the generation of singlet oxygen (¹O$_2$), as measured by the time-resolved NIR luminescence spectra of solutions of 4e (at O.D. = 0.1 at the excitation $\lambda$ of 532 nm) at 1270 and 1210 nm. Thus, PEGylated quinoline-annulated porphyrin 4e, like its parent compound, absorbs strongly within the spectroscopic window of tissue, is non-emissive, not generating
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$^{1}\text{O}_2$, and characterized by at least 3 order of magnitude faster relaxation kinetics than meso-tetraphenylporphyrin [31]. These are all excellent properties for a photoacoustic imaging agent.

4.4.2. Ex vivo Photoacoustic Signal Generation of Water-soluble Quinoline-annulated Porphyrin 4e

The strength of a photoacoustic (PA) signal generated by an irradiated sample is dependent upon the excitation source, absorption coefficient of the sample, solvent, the Grüneisen parameter, and the thermal efficiency term that represents the efficiency of the photoacoustic signal generation [11]. We previously demonstrated in tissue phantom studies the 2.5-fold increase of the photoacoustic signal resulting from quinoline-annulated porphyrin 4a (dissolved in PBS–1% DMF–1% Cremophore EL®) over the signal resulting from blood at identical absorbance values [31]. Water-soluble quinoline-annulated porphyrin 4e dissolved in water exhibits a similarly excellent performance. In detail, the relative ex vivo photoacoustic signal generation efficiency of the dye 4e placed in a translucent polyethylene tube submerged in a water bath was compared against that of day-old rat blood in the same setup and otherwise identical irradiation and detection conditions. The concentration of 4e was adjusted so as to possess the identical absorbance value of the blood sample at 790 nm, the wavelength of the laser used to excite the dye. The co-registered PE-PAT images clearly show that the tube filled with 4e generated a 4-fold stronger signal, thus generating a much higher contrast image than the blood sample (in water, 1 cm scan depth; Figure 4.3A and 4.3B).
In tissue, light is significantly scattered and absorbed. The fat emulsion Intralipid® provides a strongly scattering medium for light [56]. Thus, at 2.5 cm depth, only a small fraction of the light energy is delivered to the targets [11]. In fact, at the light level used in the experiment, the blood sample could not provide any PA signal higher than the noise level (Figure 4.3C), while the tube filled with 4e still delivers a well-resolved and high-contrast image (Figure 4.3D), demonstrating its potential as a PAI contrast agent.

4.4.3. Toxicity of PEGylated quinoline-annulated porphyrin 4e

Anesthetized 6 week old BALB/c mice were treated with 100 µL of a 33.3 mM PBS solution of 4e via retro-orbital injection and their heart rates were monitored for 3 h.
after injection. No signs of distress of the mice were observed. They also lived for several weeks after the injection with normal weight gain, and showed no abnormal behavior. The majority of the dye is excreted via the renal pathway within the first hour after injection (see also below). These are all promising preliminary indications for the absence of any acute toxicity of the PEG-ylated quinoline-annulated porphyrin 4e.

4.4.4. The use of quinoline-annulated porphyrin 4e as an *in vivo* PAI contrast agent

As a result of the solubility, apparent non-toxicity, and high PA signal generation efficiency of the PEG-ylated dye 4e, we tested the efficacy of this dye as an *in vivo* PAT contrast agent in a mouse model. The dye 4e (100 µL of a 33.3 mM of 4e in PBS) was administered via retro-orbital injection to anesthetized BALB/c mice with tumors (7-10 mm) implanted in their flanks [54], and the PE and co-registered PE-PAT images were recorded, beginning 1 min after injection (Figure 4.4A-B). The PE image shows the outline of the tumor and serves as backdrop to the PA image. Before injection, only few pixels of the PA image possess high enough signal strength to exceed a threshold value (25% of the maximum PA signal after injection). Proximately after injection (1 min), an ~ 4-fold increase in the PAT signal strengths originating from the tumor site are recorded. The enhancement of the PA signal was monitored for 45 min after injection, seeing a gradual loss of the signal strength, but even after 45 min, an at least 2-fold PA signal enhancement was still achieved (Figure 4.5). In comparison, injection of 100 µL ICG solution of identical absorbance at 780 nm as 4e at 790 nm ([ICG] = 1.33 mM) provided a significantly
lower PA signal strength enhancement (~1.6-fold) (Figures 3C-D), with no enhancement after less than 30 min (Figure 4.5). Thus, the rate of which dye 4e is excreted (via renal pathways, see below) is significantly slower than the well-known rapid excretion rate of ICG [32,33]. The effect of PEG-ylation on increasing the blood circulation time of small molecules has also been described [45,57]. The p-values in a student t-test between the results obtained for the dye and ICG are less than 0.005 for all instances, indicating statistical significance.

Figure 4.4. Co-registered PE-PAT images before injection of the contrast agent (A) and ICG (C) and after the systemic injection of 4e (B) and ICG (D). In each image pair (A-B and C-D) the PAT signals were normalized to the same maximum value recorded after the injection; a similar dynamic range of -12 dB is applied to all images (the threshold is 25% of the maximum value). The vertical axis is approximately parallel to the surface of the mouse body and the horizontal axis represents the depth inside the body.
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Figure 4.5. Time-dependence of the relative enhancement of the PAT max value following the injection of 100 µL of the dye 4e (~33.3 mM, λ_{excitation} = 790 nm) and ICG (1.33 mM, λ_{excitation} = 780 nm) at identical absorbance value.

4.4.5. Renal filtration of quinoline-annulated porphyrin 4e

An early indication for the biodistribution of dye 4e was the observation of the change in the color of the urine excreted immediately after the imaging experiments (after ~45 min) to the color of the dye (Figure 4.6). This indicates a very efficient renal filtration of the dye. UV-vis (and HPLC-MS [46]) of the urine were able to show that imaging agent 4e was excreted in an unaltered form.

Figure 4.6. A. PEG-ylated quinoline-annulated dye 4e dissolved in PBS (~33.3 mM) in a microcuvette. B. Mouse urine collected after ~45 min after injection of 4e in a capillary tube. C. UV-vis spectrum (CH₂Cl₂) of mouse (diluted) urine obtained after injection of 4e.
4.4.6. Fluorescent-tagging of quinoline-annulated porphyrin 4e

Quinoline-annulated porphyrin 4e is non-fluorescent. However, the recording of fluorescent images of organs or the measurement of the dye-specific fluorescence of organ or biofluid extracts are convenient methodologies to track the biodistribution of any fluorophore. Thus, we opted to prepare a fluorophore-tagged derivative of quinoline-annulated porphyrin 4e. Recognizing that FRET processes might be particularly efficient of quenching the fluorescence of the tag at the absorption maxima of quinoline-annulated porphyrins, we looked for fluorophores that emit in an area of relative minor absorption of these chromophores (Figure 4.2). BODIPYs are well-established low molecular weight fluorophores of high brightness and multiple options to adjust their optical spectra. Furthermore, a recent report describing the reaction of meso-mercapto-BODIPY derivative 10 with phenols to generate the meso-phenoxy-BODIPY derivative 11 (Scheme 4.2) [58] seemed most suitable for our task of tagging a tetra-phenol-derived quinoline-annulated porphyrin. Moreover, the emission λ_max of 495 nm for 11 lies within the target range, and the attachment of the fluorophore at the most distal position to the porphyrinic chromophore would also reduce the chances for any FRET.

Scheme 4.2. Reaction Conditions: (i) Na_2CO_3, MeCN, 55 °C.

Thus, we chose to attach a single BODIPY moiety to one of the four phenolic oxygens of tetraol 4c, followed by exhaustive PEG-ylation of the remaining three
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phenol functionalities (Scheme 4.3). In anticipation of the copper-catalyzed reaction chosen for the conjugation of the fluorescent tag to quinoline-annulated porphyrin 4c, its central cavity was protected by insertion of zinc(II). A Cu(I) thiophene-carboxylate (CuTC)-mediated reaction of meso-mercapto-BODIPY 10 [58], followed by an acid-mediated removal of the zinc protecting group using a mineral acid wash, generated quinoline-annulated porphyrin 4f in which one of the phenolic oxygens was derivatized with the BODIPY group, while the other three groups remained unmodified (as per ESI+ MS). The lack of regioselectivity of this reaction is reflected in the complex ¹H NMR spectrum of this compound [46].

Scheme 4.3. Reaction conditions: (i) 1. Zn(OAc)₂·2H₂O, CH₂Cl₂/MeOH, Δ; 2. 1 equiv 10, Na₂CO₃, CuTC, CH₃CN, 50 °C; 3. aq. HCl. (ii) Me(OCH₂CH₂)₁₂OMs, Cs₂CO₃, DMF, 90 °C
The three phenolic oxygens of 4f were then PEG-ylated using the long PEG mesylate as described above. Product 4g, a deep yellow oil, shows the expected composition (as per MALDI MS). It is freely water-soluble. The presence of the BODIPY is seen in the (complex) $^1$H NMR spectrum of 4g by the observation of the diagnostic peaks for the α- and β-protons of the BODIPY moiety (at $\delta = 6.5$-7.0 ppm). Its $^{19}$F NMR spectrum indicates the presence of fluorine atoms at $\delta = -146.4$ to -146.6 ppm, the typical range for BODIPY fluorine atoms.

The UV-vis spectrum of non-PEGylated derivative 4f is derived from a linear addition of the spectrum of tetraol 4c and BODIPY 11 (Figure 4.7A), with only some minor shifts and broadening likely derived from the presence of four regioisomers. Upon PEGylation of 4f, the UV-vis spectrum is further broadened, with retention of the overall character of the quinoline-annulated derivatives (Figure 4.7B).

When dyad 4g is excited at the $\lambda_{\text{max}}$ value of the BODIPY moiety (at 441 nm), the molecule emits at 485 nm, this emission can be attributed to the BODIPY portion of 4g [58]. However, the fluorescence yield $\Phi$ for 4g is estimated to lie below 0.3%, i.e., much lower than the fluorescence yield $\Phi$ for 11 [58]. This suggests that some FRET (or other quenching) mechanisms are operative in 4g, but that this quenching is not efficient enough to entirely switch off the fluorescence of the BODIPY moiety. This further questions the possibility whether it might be possible to track dyad 4g in tissue using a fluorescence scanner.
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4.4.7. Biodistribution study of BODIPY-tagged quinoline-annulated porphyrin 4g

The biodistribution of the BODIPY-labeled derivative 4g in the tumor and other organs was studied by ex vivo fluorescent imaging, following the injection of 4g into 4 BALB/c mice as described for 4e. The mice were sacrificed after 15 or 120 min after injection, the organs extracted, and their radiant efficiency fluorescent images recorded ($\lambda_{exc} = 465$ nm; GFP emission filter). Unfortunately, however, the high amount of tissue auto-fluorescence in that wavelength, did not allow the recording of high-contrast images. We note, however, that the tumor was yellow...
stained, suggesting an accumulation of the dye $4g$ in the tumor. Similarly, the tumor was visibly dark brown stained after the injection of $4e$ (Figure 4.8).

![Figure 4.8](image)

**Figure 4.8.** A. A mouse tumor without injection. B. A mouse tumor 48 h after injection of 100 µL of a 33 mM solution of $4e$ in PBS, showing the dark brown-stained tumor site.

### 4.5. Summary

In conclusion, we have demonstrated the *in vivo* efficacy of serum-soluble quinoline-annulated porphyrin derivative $4e$ as a molecular contrast agent for photoacoustic tomography. The solubilization of the quinoline-annulated porphyrin followed a straight-forward PEGylation strategy, with the lengths of the PEG chains determining the solubility of the final product. The product appeared to be low acute toxicity, accumulated in the tumor site, and was rapidly excreted in unaltered fashion via renal pathways. The general solubilisation strategy is conceivably also suitable to other quinoline-annulated porphyrin derivatives. Conjugation of the non-fluorescent PEGylated quinoline-annulated porphyrin to a fluorophore was also demonstrated, but the hopes to utilize this derivative for the tracking of the biodistribution of the contrast agent using fluorescence imaging was not fulfilled. Nonetheless, the flexible derivatization strategy points the way toward the conjugation to other molecules.
4.6. Synthesis: Materials and instrumentation

All solvents (Aldrich, Acros) and reagents MeO-PEG$_4$-OMs (Aldrich) and MeO-PEG$_{12}$-OMs (Creative Peg Works), CuTC (Aldrich) were reagent grade, or better, and were used as received. *meso*-Tetrakis(*p*-methoxyphenyl)-2,3-dioxoporphyrin (7) [59], and BODIPYs 10 and 11 were prepared as described previously [58]. Analytical (aluminum backed, silica gel 60, 250 µm thickness) and preparative (20 × 20 cm, glass backed, silica gel 60, 500 µm thickness) TLC plates, and the flash column silica gel (standard grade, 60 Å, 32-63 µm) used were provided by Sorbent Technologies, Atlanta, GA.

$^1$H and $^{13}$C NMR spectra were recorded on Bruker Avance II 400 and Bruker Avance I 500 instruments in the solvents indicated, and were referenced to residual solvent peaks. High and low resolution ESI mass spectra were provided by the Mass Spectrometry Facilities at the Department of Chemistry, University of Connecticut. MALDI MS Spectra were provided by the Mass Spectrometry & Proteomics Facility at the University of Notre Dame. UV-vis and fluorescence spectra were recorded on Cary 50 and Cary Eclipse photospectrometers, Varian Inc, respectively, and IR spectra on a Bruker Alpha-P FT-IR spectrometer using a diamond ATR unit.

*meso*-Tetrakis(*p*-methoxyphenyl)-2,3-dioxoporphyrin (8). Diol 7 (270 mg, $3.51 \times 10^{-4}$ mol) was dissolved in CH$_2$Cl$_2$ (60.0 mL) in a round-bottom flask equipped with a magnetic stir bar. To the stirring solution was added Dess-Martin periodinane (590 mg, $1.39 \times 10^{-3}$ mol, 4 equiv) in portions at ambient temperature.
When the starting material was consumed (reaction control by UV-vis and TLC), the reaction was quenched by addition of a sat’d aq NaHCO₃ solution. The organic layer was isolated and washed with H₂O (3 × 30 mL). The organic layer was dried over anhyd Na₂SO₄ and evaporated to dryness by rotary evaporation, and the residue purified by column chromatography (silica-CH₂Cl₂) to yield 8 as a dark blue powder in yields ranging from 65 to 90% (243 mg): \( R_f \) (CH₂Cl₂-silica) = 0.52; \(^1\)H NMR (400 MHz, CD₂Cl₂) δ 8.81 (d, \(^3\)J = 4.9 Hz, 1H), 8.65 (d, \(^3\)J = 4.9 Hz, 1H), 8.61 (s, 1H), 8.06 (d, \(^3\)J = 8.6 Hz, 2H), 7.84 (d, \(^3\)J = 8.6 Hz, 2H), 7.31-7.24 (m, 4H), 4.07 (two overlapping s, 6H), -1.90 (br s, 1H, exchangeable with D₂O); \(^13\)C NMR (100 MHz, CD₂Cl₂): δ 188.2, 159.9, 159.6, 155.64, 155.62, 155.59, 140.9, 140.2, 138.4, 135.3, 134.1, 133.8, 133.4, 131.8, 128.4, 128.0, 123.9, 113.4, 112.65, 112.54, 112.47, 55.5, 55.4 ppm; UV-vis (CH₂Cl₂) \( \lambda_{\text{max}} \) (log ε) 410 (5.26), 477 (4.26) nm; FT-IR (neat, diamond ATR) 1730 (νC=O) cm⁻¹; HR-MS (ESI⁺, 100% CH₃CN, TOF) \( m/z \) calcd for C₄₈H₃₇N₄O₆ ([M·H]⁺) 765.2713, found 765.2726.

**meso-Tetrakis(\( p \)-methoxyphenyl)-2-hydroxyimino-3-oxoporphyrin (9).** Dione 8 (94 mg, 1.2 × 10⁻⁴ mol) was dissolved in pyridine (30.0 mL) in a round bottom flask equipped with a magnetic stir bar and N₂ inlet. Hydroxylamine hydrochloride (NH₂OH-HCl, 850 mg, ~100 equiv) was added, and the mixture was stirred for 24 h at ambient temperature. When the starting material was consumed (reaction control by TLC), the reaction mixture was evaporated to dryness by rotary evaporation. The residue was taken up in CH₂Cl₂ and filtered through a glass frit (M). The volume of the filtrate was reduced and submitted to column...
chromatography (silica-CH₂Cl₂/1% MeOH) to afford the olive-green product 9 in 60% yield, (58 mg): $R_f$ (silica-CH₂Cl₂/1% MeOH) = 0.65; $^1$H NMR (400 MHz, CD₂Cl₂) δ 15.78 (br s, 1H, exchangeable with D₂O), 8.81 (d, $^3$J = 5.7 Hz, 2H), 8.62 (t, $^3$J = 8.4 Hz, 4H), 8.03 (dd, $^3$J = 8.4, $^4$J = 2.0 Hz, 4H), 7.85 (dd, $^3$J = 8.4 Hz, 4H), 7.27-7.19 (m, 8H), 4.05 (s, 12H), -2.23 (br s, 1H exchangeable with D₂O), -2.36 (br s, 1H exchangeable with D₂O) ppm; $^{13}$C NMR (100 MHz, CD₂Cl₂) δ 188.2, 159.82, 159.75, 159.6, 156.4, 154.9, 151.9, 145.4, 141.1, 139.8, 138.9, 138.80, 137.98, 135.4, 134.4, 133.6, 133.50, 133.47, 132.1, 128.6, 128.4, 127.63, 127.57, 123.8, 121.8, 115.8, 113.0, 112.7, 112.4, 112.2, 55.5, 55.42, 55.39, 53.8 ppm; UV-vis (CH₂Cl₂) λₘᵡᵢₙ (log ε) 410 (5.44), 465 (sh), 613 (4.04), 670 (sh) nm; FT-IR (neat, diamond ATR): 1732 ($\nu_{C=O}$) cm⁻¹; HR-MS (ESI⁺, 100% CH₃CN, TOF) m/z calcld for C₄₈H₃₈N₅O₆ ([M·H]⁺) 780.2822, found 780.2801.

*meso*-Tris(p-methoxyphenyl)(p-methoxy)quinoline-annulated porphyrin N-Oxide (5b). Oxime 9 (14.7 mg, 1.88 × 10⁻⁵ mol) was dissolved in CH₂Cl₂ (10.0 mL) in a round bottom flask equipped with a magnetic stir bar. To the stirring solution was added 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ, 9 mg, 2 equiv) and the mixture was stirred at ambient temperature for 0.5 h. When the starting material was consumed (reaction control by UV-vis and TLC), the reaction mixture was filtered through a plug of silica gel. The filtrate was washed with water (2 × 10 mL), dried over anhyd Na₂SO₄, and evaporated to dryness by rotary evaporation. The resulting residue was purified by column chromatography (silica-CH₂Cl₂/1% MeOH) to afford the brown product 5b in 97% yield (14 mg): $R_f$ (silica-CH₂Cl₂/2%
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MeOH) = 0.22; ¹H NMR (400 MHz, CDCl₃) δ 8.54 (d, ³J = 4.2 Hz, 1H), 8.41 (d, ³J = 4.9 Hz, 1H), 8.35-8.28 (m, 3H), 8.19 (dd, ³J = 4.3 Hz, 2H), 8.03 (d, ³J = 2.0 Hz, 1H), 7.86-7.80 (m, 4H), 7.68-7.65 (m, 2H), 7.23-7.20 (m, 4H), 7.16-7.10 (m, 3H), 4.08-4.04 (m, 10H), 3.89 (s, 3H), -0.32 (br s, 2H, exchangeable with D₂O) ppm; ¹³C NMR (100 MHz, CDCl₃) δ 185.0, 159.9, 159.6, 159.3, 159.0, 155.9, 153.6, 145.9, 143.7, 143.5, 141.6, 138.2, 135.6, 135.5, 135.2, 134.9, 134.3, 133.7, 133.4, 133.3, 133.10, 132.98, 132.94, 131.0, 129.3, 127.7, 127.4, 127.3, 124.5, 123.8, 122.7, 120.9, 113.2, 113.1, 112.6, 112.5, 102.1, 101.9, 56.0, 55.6, 55.54, 55.47 ppm; UV-vis (CH₂Cl₂) λ_max (log ε) 422 (5.02), 504 (4.27), 543 (4.16), 751 (4.09) nm; FT-IR (neat, diamond ATR): 1686 (υC=O) cm⁻¹; HR-MS (ESI⁺, 100% CH₃CN, TOF) m/z calcd for C₄₈H₃₆N₅O₆ ([M·H]⁺), 778.2666, found 778.2693.

**meso-Tris(p-methoxyphenyl)(p-methoxy)quinoline-annulated porphyrin (4b).** Quinoline N-oxide 5b (23.7 mg, 3.05 × 10⁻⁵ mol) was dissolved in pyridine (25.0 mL) and heated to reflux for 48 h. When the starting material was consumed (reaction control by TLC and UV-vis), the solvent was evaporated and the remaining residue was purified by preparative TLC (silica-CH₂Cl₂/2% MeOH) and solvent exchanged from CH₂Cl₂ to MeOH to afford the brown powder 4b in 44% yield (10 mg): Rf (silica-CH₂Cl₂/2% MeOH) = 0.54; ¹H NMR (400 MHz, CD₂Cl₂): δ 8.97 (two overlapping d, ³J = 7.5 Hz, 2H), 8.37 (dd, ³J = 7.9, ⁴J = 4.9 Hz, 2H), 8.22 (d, ³J = 4.6 Hz, 1H), 8.16 (d, ³J = 4.4 Hz, 2H), 8.00-7.97 (m, 3H), 7.89 (d, ³J = 8.5 Hz, 2H), 7.73 (d, ³J = 8.4 Hz, 2H), 7.54 (dd, ³J = 9.1, ⁴J = 2.7 Hz, 1H), 7.28 (d, ³J = 8.5 Hz, 2H), 7.22 (dt, ³J = 5.3, ⁴J = 3.3 Hz, 4H), 4.09 (s, 3H), 4.06 (two overlapping
singlets, 6H), 4.03 (s, 3H), 1.07 (s, 2H, exchangeable with D₂O); ¹³C NMR (100 MHz, CD₂Cl₂): δ 202.2, 180.4, 160.2, 159.8, 159.4, 159.0, 148.96, 148.85, 148.29, 148.21, 146.8, 146.56, 146.54, 145.7, 135.5, 134.7, 134.3, 133.5, 133.2, 132.9, 132.3, 131.0, 129.9, 127.5, 127.2, 122.9, 120.5, 113.0, 112.9, 112.8, 112.7, 112.6, 112.3, 110.0, 55.8, 55.6, 55.5, 55.4 ppm; UV-vis (CH₂Cl₂) λ<sub>max</sub> (log ε) 418 (5.0), 490 (4.23), 529 (4.23), 701 (sh), 762 (4.12) nm; FT-IR (neat, diamond ATR): 1716 (v<sub>C=O</sub>) cm⁻¹; HR-MS (ESI⁺, 100% CH₃CN, TOF) m/z calcd for C₄₈H₃₆N₅O₅ ([M·H]+) 762.2711, found 762.2738.

**meso-Tris(p-hydroxyphenyl)(p-hydroxy)quinoline-annulated porphyrin (4c).**

Quinoline N-oxide 5b (26 mg, 3.3 × 10⁻⁵ mol) was dissolved in dry CH₂Cl₂ (5.5 mL) in a round bottom flask equipped with a magnetic stir bar and N₂ inlet. A 1.0 M solution of BBr₃ in CH₂Cl₂ (1.35 mL, ~40 equiv) was added drop-wise to the flask and the reaction mixture was stirred for 24-48 hours. When the starting material was consumed (reaction control by TLC), the excess BBr₃ was quenched by the careful addition of the reaction mixture to distilled water (10 mL). The resulting mixture was extracted with EtOAc (3 × 20 mL), washed with sat’d sodium bicarbonate solution and water (2 × 20 mL). The organic layer was then dried over anhydrous Na₂SO₄. The resulting residue was adsorbed onto silica gel and dry-loaded onto a silica gel column and purified by chromatography (silica-CH₂Cl₂/10% MeOH) to afford 4c in 65% yield (18 mg): R<sub>f</sub> (silica-CH₂Cl₂/10% MeOH) = 0.45; ¹H NMR (400 MHz, DMSO-d₆): δ 10.37 (s, 1H, exchangeable with D₂O), 10.09 (s, 1H, exchangeable with D₂O), 9.97 (s, 1H, exchangeable with D₂O), 9.71 (s, 1H,
exchangeable with D$_2$O), 8.81 (s, 1H), 8.61 (s, 1H), 8.34 (s, 1H), 8.20 (s, 1H), 8.12 (d, $^3J = 4.4$ Hz, 1H), 8.03 (d, $^3J = 5.8$ Hz, 1H), 7.75 (d, $^3J = 7.5$, 3H), 7.58 (d, $^3J = 7.8$ Hz, 2H), 7.34 (d, $^3J = 3.9$ Hz, 1H), 7.16 (d, $^3J = 7.9$ Hz, 2H), 7.11 (d, $^3J = 8.2$ Hz, 2H), 7.04 (d, $^3J = 8.4$ Hz, 2H) ppm; $^{13}$C NMR (100 MHz, DMSO-d$_6$): δ 194.6, 158.6, 158.1, 157.5, 157.4, 150.51, 150.49, 150.47, 146.12, 146.10, 142.03, 141.96, 138.92, 138.89, 136.1, 135.8, 135.43, 135.39, 135.2, 134.7, 134.6, 134.03, 133.94, 133.4, 132.5, 131.4, 131.1, 130.6, 130.3, 130.10, 130.09, 129.4, 127.86, 127.83, 127.80, 127.32, 127.30, 127.27, 127.26, 124.59, 124.57, 123.29, 123.26, 123.20, 123.19, 120.98, 120.96, 120.95, 120.26, 120.25, 115.44, 115.41, 114.95, 114.92, 114.82, 114.76, 114.68, 111.9, 109.4, 100.0 ppm; UV-vis (MeOH) $\lambda_{\max}$ (log $\varepsilon$) 417 (5.15), 487 (sh), 531 (4.31), 748 (4.24) nm; FT-IR (neat, diamond ATR): 1705 ($\nu_{\text{C}=\text{O}}$) cm$^{-1}$; HR-MS (ESI$^+$, 100% CH$_3$CN, TOF) $m/z$ calcd for C$_{44}$H$_{28}$N$_5$O$_5$ ([M·H]$^+$) 706.2090, found 706.2090.

**meso-Tris(p-MeOPEG$_4$Ophenyl)(p-MeOPEG$_4$O)quinoline-annulated porphyrin (4d).** Quinoline-annulated porphyrin 4c (10 mg, $1.4 \times 10^{-5}$ mol) was dissolved in dry DMF (4.0 mL) in a two-neck RBF equipped with a magnetic stir bar. MeO-PEG$_4$OMs (25.0 mg, $8.9 \times 10^{-5}$ mol, 6 equiv.) and Cs$_2$CO$_3$ (24.2 mg, 7.4 $\times 10^{-5}$ mol, 5.2 equiv.) were added and the reaction mixture was immersed in a preheated oil bath at ~100 °C. After consumption of the starting material after ~3 h (reaction control by TLC) the solvent was evaporated and the residue was partitioned between EtOAc (10 mL) and H$_2$O (10 mL). The organic layer was washed with H$_2$O (3 $\times$ 10 mL) and dried over Na$_2$SO$_4$. The brown residue was
purified by column chromatography (silica-CH$_2$Cl$_2$/5% MeOH) to afford the tetra-PEGylated product 4d as a yellow-brown oil in 56% yield (12 mg): $R_f$ (silica-CH$_2$Cl$_2$/10% MeOH) = 0.56; $^1$H NMR (500 MHz, CD$_2$Cl$_2$): δ 9.06-9.04 (m, 2H), 8.38 (d, $^3J = 5.0$ Hz, 1H), 8.23 (d, $^3J = 4.5$ Hz, 1H), 8.16 (d, $^3J = 4.5$ Hz, 2H), 7.99 (d, $^3J = 8.5$ Hz, 3H), 7.89 (d, $^3J = 8.5$ Hz, 2H), 7.71 (d, $^3J = 8.5$ Hz, 2H), 7.63 (dd, $^3J = 9.1$, $^4J = 2.5$ Hz, 1H), 7.30 (d, $^3J = 8.6$ Hz, 2H), 7.23 (dd, $^3J = 8.5$, $^4J = 1.8$ Hz, 4H), 4.44 (t, $^3J = 4.5$ Hz, 2H), 4.38 (two overlapping t, $^3J = 4.7$ Hz, 4H), 4.35 (t, $^3J = 4.6$ Hz, 2H), 3.99 (dd, $^3J = 8.8$, $^4J = 4.7$ Hz, 6H), 3.82-3.77, (m, 8H), 3.72-3.57 (m, 34H), 3.55-3.50 (m, 8H), 3.35 (s, 3H), 3.34 (s, 3H), 3.33 (s, 3H), 3.31 (s, 3H) ppm; UV-vis (CH$_2$Cl$_2$) $\lambda_{max}$ (log ε) 419 (5.2), 491 (4.5), 531 (4.4), 764 (4.3) nm; HR-MS (ESI$^+$, 100% CH$_3$CN, TOF) m/z calcd for C$_{80}$H$_{100}$N$_5$O$_{21}$ ([M·H]$^+$), 1466.6905, found 1466.6891.

**meso-Tris(p-MeOPEG$_{12}$Ophenyl)(p-MeOPEG$_{12}$O)quinoline-annulated porphyrin (4e).** Prepared from 4c (22 mg, 3.1 × 10$^{-5}$ mol) in dry DMF (10.0 mL), Cs$_2$CO$_3$ (80 mg, 1.6 × 10$^{-4}$ mol, 5.2 equiv.), and MeO-PEG$_{12}$-OMs (80 mg, 1.2 × 10$^{-4}$ mol, 4 equiv.) as described for the preparation of 4d. Reaction time at 90 °C ~3 h. Purified by column chromatography (silica-CH$_2$Cl$_2$/10% MeOH) to afford the tetra-PEGylated porphyrin 4e as a brown oil in 90-99 % yield (81 mg-89 mg): $^1$H NMR (400 MHz, CD$_2$Cl$_2$): δ 9.04 (s, 1H), 8.38 (two overlapping d, 2H), 8.19 (d, 3H), 7.98 (t, $^3J = 6.6$ Hz, 3H), 7.89 (d, $^3J = 8.3$ Hz, 3H), 7.72 (d, $^3J = 8.4$ Hz, 2H), 7.63 (d, $^3J = 8.3$ Hz, 1H), 7.31 (d, $^3J = 8.5$ Hz, 3H), 7.24 (dd, $^3J = 8.6$, $^4J = 2.2$ Hz, 4H), 3.34 (s, 12H) ppm; UV-vis (CH$_2$Cl$_2$) $\lambda_{max}$ (rel I.) 422 (1.0) 529 (0.20) 763 (0.09) nm; HR-
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MS (ESI+, 100% CH₃CN, TOF) cluster of peaks corresponding to addition of four PEG groups, see ESI.

**[meso-Tris(p-hydroxyphenyl)(p-hydroxy)quinoline-annulated porphyrinato]Zinc(II) (4cZn).** Quinoline-annulated porphyrin 4c (19 mg, 2.7 x 10⁻⁵ mol) was dissolved in CH₂Cl₂/10% MeOH (7.0 mL) in a round bottom flask equipped with a magnetic stir bar. Zn(OAc)₂·2H₂O (18 mg, 8.1 x 10⁻⁵ mol, 3 equiv.) was added and the reaction was gently warmed for 10 min. When the starting material was consumed (reaction control by TLC and UV-vis) the solvents were evaporated, and the residue was taken up in EtOAc and washed with brine and water (3 x 20 mL). The organic layer was dried over anhydrous Na₂SO₄ and evaporated to dryness by rotary evaporation to afford 4cZn as an orange solid in near-quantitative yield (21 mg): Rᵣ (silica-CH₂Cl₂/10% MeOH) = 0.39; ¹H NMR (400 MHz, DMSO-d₆): δ 10.23 (s, 1H, exchangeable with D₂O), 9.94 (s, 1H, exchangeable with D₂O), 9.85 (s, 1H, exchangeable with D₂O), 9.57 (s, 1H, exchangeable with D₂O), 8.90 (t, 3J = 7.9 Hz, 2H), 8.19 (d, 3J = 4.5 Hz, 1H), 8.05 (d, 3J = 4.6 Hz, 1H), 7.99 (dd, 3J = 12.1, 4J = 4.4 Hz, 2H), 7.76 (t, 3J = 6.4 Hz, 4H), 7.70 (d, 3J = 8.2 Hz, 2H), 7.56 (dd, 3J = 9.0, 4J = 2.4 Hz, 1H), 7.47 (d, 3J = 8.2 Hz, 2H), 7.13 (d, 3J = 8.3 Hz, 2H), 7.07 (d, 3J = 8.3 Hz, 2H), 7.00 (d, 3J = 8.2 Hz, 2H) ppm; ¹³C NMR (100 MHz CD₂Cl₂/10% MeOD): δ 195.7, 159.8, 158.1, 157.8, 157.0, 156.6, 152.7, 152.1, 150.9, 150.5, 150.1, 148.0, 146.3, 143.3, 142.7, 135.6, 135.4, 134.8, 134.6, 133.8, 133.7, 133.6, 133.52, 133.47, 133.2, 132.0, 131.2, 131.06, 130.13, 130.0, 126.7, 123.7, 121.0, 115.6, 115.0, 114.7, 114.5, 111.9 ppm; UV-vis (MeOH) λ_max
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(log ε) 420 (5.13), 484 (sh), 518 (sh), 737 (sh), 816 (4.25) nm; FT-IR (neat, diamond ATR): ~1750 (νC=O) cm⁻¹; HR-MS (ESI⁺, 100% CH₃CN, TOF) m/z calcd for C₄₄H₂₆N₅O₅Zn ([M·H]⁺) 768.1225, found 768.1222.

p-(BODIPY)-tris-p-(OH)-quinoline-annulated porphyrin, mixture of regioisomers (4f). meso-thiomethyl-4,4,-difluoro-4-bora-3a,4a-diaza-s-indacene 10 (6 mg, 2.7 × 10⁻⁵ mol) was dissolved in dry CH₃CN (3.0 mL) in a round bottom flask equipped with a magnetic stir bar under N₂ atmosphere. Tetraol 4cZn (21 mg, 2.7 × 10⁻⁵ mol, 1 equiv) was added and the mixture was stirred for 5 min under N₂. Copper(I) thiophene-2-carboxylate (CuTC) (5 mg, 2.7 × 10⁻⁵ mol, 1 equiv.) and Na₂CO₃ (3 mg, 2.7 × 10⁻⁵ mol, 1 equiv.) were added and the reaction mixture was immersed in a pre-heated oil bath at 50 °C and stirred [58]. After 48 h, a saturated aqueous NH₄Cl solution was added and the reaction mixture was stirred for several hours. The resulting biphasic mixture was extracted with EtOAc (3 × 10 mL), washed with H₂O (2 × 10 mL), and dried over Na₂SO₄. The remaining residue was purified by column chromatography (silicia-CH₂Cl₂/10% MeOH) to afford recovered 4cZn (6 mg) and the mono-BODIPY-tagged product 4f as a series of closely running yellow fractions. The combined yellow fractions were dissolved in EtOAc (5.0 mL) and treated with 3M HCl (5.0 mL) until the UV-vis spectrum of a neutralized aliquot indicated full demetallation. The organic layer was then washed with a sat’d aq. NaHCO₃ solution and H₂O, and was dried over anhyd. Na₂SO₄ to provide 4f as a yellow film in 30% yield (7 mg): Rf (silica-CH₂Cl₂/10% MeOH) = 0.21; ¹H-NMR (500 MHz, DMSO-d₆): δ 10.09-10.07 (m, 1H, exchangeable with D₂O), 9.95-9.93 (m,
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1H, exchangeable with D2O), 9.69-9.64 (m, 1H, exchangeable with D2O), 9.10 (br s, 1H), 8.45 (d, 3J = 4.4 Hz, 1H), 8.4-8.37 (m, 1H), 8.18 (d, 3J = 5.2 Hz, 2H), 8.13-8.10 (m, 2H) 8.08-7.95 (m, 3H), 7.92-7.88 (m, 3H), 7.80-7.77 (m, 4H), 7.60-7.59 (m, 1H), 7.31 (s, 1H), 7.19 (d, J = 6.0 Hz, 2H), 7.12 (two overlapping d, 3J = 7.8 Hz, 2H), 7.05-7.03 (m, 2H), 6.81 (d, J = 6.1 Hz, 2H), 6.76-6.72 (m, 1H) ppm; 19F NMR (470 MHz, DMSO-d6): δ -142.1 to -142.2, (m) ppm; UV-vis (MeOH) \( \lambda_{\text{max}} \) (rel I.) 400 (1.0), 443 (0.56), 510 (sh), 784 (0.1) nm; Fl (MeOH, \( \lambda_{\text{excitation}} \) = 441 nm) \( \lambda_{\text{max}} \) 488 nm; HR-MS (ESI+, 100% CH3CN, TOF) m/z calcd for C53H33BF2N7O5 ([M·H]+) 896.2599, found 896.2613.

**p-(BODIPY)-tris-p-(OPEG12)-quinoline-annulated porphyrin, mixture of regioisomers (4g).** Prepared according to the procedure for 4e from 4d (10 mg, 1.1 \times 10^{-5} \text{ mol}), MeO-PEG550-OMs (22 mg, 3.4 \times 10^{-5} \text{ mol, 3 equiv.}), 14 mg of Cs2CO3 (4.4 \times 10^{-5} \text{ mol, 3.9 equiv.}) in DMF (2.0 mL) to afford 4g as a yellow oil in 56% yield (16 mg): \(^1\text{H NMR (400 MHz, CD2Cl2):}\ δ 9.14 (s, 1H), 8.45 (s, 1H), 8.38 (d, 3J = 4.6 Hz, 1H), 8.21 (d, 4H), 8.01 (d, 3J = 6.8 Hz, 3H), 7.91 (d, 3J = 7.6 Hz, 2H), 7.73 (d, 3J = 7.2 Hz, 3H), 7.52 (s, 1H), 7.31 (d, 3J = 8.0 Hz, 4H), 7.25 (d, 3J = 5.0 Hz, 5H), 7.13-7.11 (m, 2H), 6.82 (d, 3J = 5.7 Hz, 1H), 6.46 (s, 1H) ppm; 19F NMR (470 MHz, CD2Cl2): δ -142.1 to -142.2, (m) ppm; UV-vis (CH2Cl2) \( \lambda_{\text{max}} \) (rel I.) 402 (1.0), 473 (sh), 510, (sh), 769 (0.12) nm; Fl (MeOH, \( \lambda_{\text{excitation}} \) = 441 nm) \( \lambda_{\text{max}} \) 485 nm; HR-MS (MALDI, 100% DHBA) cluster of peaks corresponding to addition of three PEG groups [46]
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References


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4. In vivo PAT using QA porphyrin as NIR CA


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[52] F. Zhou, S. Zanganeh, I. Mohammad, C. Dietz, A. Abuteen, M.B. Smith, Q. Zhu, Targeting tumor hypoxia: a third generation 2-nitroimidazole-indocyanine dye-


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5. A very low-cost, handheld, and multispectral spatial frequency domain imaging prototype for \textit{ex vivo} cancer characterization

5.1. Introduction

Optical properties of tissue may vary in healthy and diseased conditions, therefore optical imaging modalities capable of providing quantitative maps of absorption and scattering properties can assist in characterization of healthy versus diseased tissue [1–3]. Spatial frequency domain imaging (SFDI) is a wide-field diffuse optical imaging modality that can quantitatively map various optical properties of tissue and has shown potential in differentiating benign and malignant tissue in several cancer types including breast and ovarian cancer [1–5].

In SFDI, tissue is illuminated with sinusoidal (or square [6]) spatially modulated light and optical properties of the target are reconstructed using the diffusely backscattered light collected by a camera [4,5] and single- or multi-wavelength SFDI systems using single or multiple spatial frequencies have been demonstrated [2,5,7]. Initial reports of SFDI systems utilized general purpose projectors, with built-in digital micro-mirror devices (DMMs), to project the spatially sinusoidal pattern, generated in a computer connected to the projector, on the tissue [2,4,5,8]. External optical filters were used to select the desired wavelength [2,5] and filters could be manually or mechanically switched to use different wavelengths. Later versions of SDFI systems utilized LEDs collimated and co-aligned by multiple collimating lenses, beam splitters, and dichroic mirrors [1,9]. An external DMM controlled by a PC [1], or a printed sinusoidal pattern [9] provides the spatial modulation of light that is then projected on the tissue. Such systems generally utilize four LEDs [1]. Incorporating and co-aligning larger number of LEDs (for example 9 different
wavelengths) in such setups requires many optical components which increases the size, cost, and complexity of the system. Albeit, a system incorporating a 6-wavelengths SFDI head using custom-made fibers, fiber couplers, and a fiber bundle multiplexer to combine all LED lights has been reported previously. However, the light source of this systems is both large and the system overall contains many different components that increases its cost [10]. Applications of digital light projectors (DLP) as the source for SFDI systems have also been demonstrated [11]. In visible DLPs, which are the more common versions, RGB LEDs are collimated and co-aligned using collimator lenses, beam splitters, and dichroic mirrors and the projection pattern is generated by a DMM [11]. Although common (hence, less expensive) DMDs are often sensitive to the visible light, modified DLPs with near infrared (NIR) LEDs or laser diodes are also available [12]. However, the custom-made DLPs are more expensive than the off-the-shelf versions. Moreover, either visible or NIR DLPs only use three fixed wavelengths while utilizing a larger number of wavelengths from visible to NIR enhances SFDI studies [11,12]. A custom-made spatial frequency domain spectroscopy system (analyzing data along a line, rather than forming images) incorporating a broadband source into a DLP has also been reported [13] but to the best of our knowledge we are not aware of a very low-cost, handheld, and multispectral SFDI system with as many as 9 different wavelengths. Here, we report a very low-cost, small, 3D-printed, and handheld SFDI prototype incorporating nine different LEDs (wavelengths from 660 nm – 950 nm) and all illumination and detection components in a handheld probe.
5.2. Methods

5.2.1. SFDI Prototype design

A schematic representation of the system is shown in Figure 5.1. The illumination portion of the prototype consists of a rotational stepper motor (PG20L-D20-HHC0, NMB Technologies), 9 LEDs with center frequencies ranging from 660 nm – 950 nm (660 nm, 740 nm, 770 nm, 810 nm, 830 nm, 850 nm, 890 nm, 935 nm, and 950 nm) placed on a custom-designed printed circuit board (PCB), a light diffuser, an achromatic doublet collimating lens (Thorlabs, AC254-050-B-ML), a printed sinusoidal pattern (sinusoidal pattern is printed on a transparency paper to reduce the cost compared to DMDs or even commercially available printed patterns), a linear stepper motor (19541-12-905, Ametek), and an achromatic doublet projection lens (Thorlabs, AC254-050-B-ML). On the PCB, LEDs are placed on the circumference of a circle with a fixed distance, the rotational motor rotates the PCB in order to switch the LED that is positioned on the optical axis of the lenses. Light from the LED first passes a beam diffuser to homogenize the beam, it is then collimated by the collimating lens. The collimated beam passes the printed pattern and is projected on the sample using the projector lens. Design of the illumination portion of the probe was guided by Zemax simulations (Zemax, LLC). The printed pattern is attached to the linear motor that provides the phase shift between the patterns. Diffuse backscattered light is collected by a CMOC camera (EO-0413M-GL, Edmund Optics). Two polarizer plates are located at the illumination and detection sides in order to reject specular reflection. The illumination area is a circle with diameter of about 13 cm, however the detection field of view is about 5 cm × 4 cm approximately 30 cm away from the probe. The spatial frequency of illumination is about 1 cm\(^{-1}\) at this distance. Control and
synchronization of the parts and detection and data saving are performed in a custom-made LabVIEW code (National Instrument, Austin, TX, USA) combined with Arduino IDE (Arduino, Italy) for controlling the stepper motor drivers and LEDs. The communication with the PC is performed through a serial USB port.

All pieces are fixed and aligned in a 3D-printed probe designed in Solidworks (Solidworks, Waltham, MA, USA). The illumination section of the probe is 17 cm × 6 cm × 6 cm (length × width × height). The camera is held by another piece, designed to fix and align the camera with the illumination light and allow for minor modifications if needed. The camera holder consists of an adaptor 6 cm × 6 cm × 1 cm that is fixed to the illumination probe, a second adaptor 6 cm × 7 cm × 6 cm that is screwed to the first adaptor, and a hollow
cube of 8 cm × 5 cm × 5 cm that holds the camera and is screwed to the second adaptor in order to provide a degree of freedom for adjusting the imaging area. The camera holder part is completely fixed on top of the illumination part and the entire probe can be held by hand or simply fixed on a table. The complete probe is shown in Figure 5.2. Complete data acquisition lasts for approximately 2 minutes.

![Figure 5.2. The SFDI probe with all illumination and detection components.](image)

5.2.2. Processing

The spatial sinusoidal pattern can be represented as:

\[ I_n = I_0 \sin(2\pi f_s x + \varphi_n) \]  

(5.1)

where \( I_0 \) is the source amplitude, \( f_s \) is the spatial frequency, and \( \varphi_n \) is the illumination phase. Tissue is illuminated with three phase shifted patterns (0, \( 2\pi/3 \), and \( 4\pi/3 \)) and the DC and AC components (\( f = 0 \) and \( f_s \)) are extracted as:

\[ M_{DC} = \frac{I_1 + I_2 + I_3}{3} \]  

(5.2)
The DC and AC components of the diffuse reflected light from the target are compared to the DC and AC components of a calibrated phantom and the diffuse reflectance is found using the calculated diffuse reflectance for the calibrated phantom:

\[
M_{AC} = \frac{\sqrt{2}}{3} \left[ (I_1 - I_2)^2 + (I_3 - I_2)^2 + (I_3 - I_1)^2 \right]^{1/2}
\]  

(5.3)

Here, \( R_d \) is the diffuse reflectance of the target and \( R_d(ref) \) is the calculated diffuse reflectance for the homogenous reference phantom. Therefore two \( R_d \) values for DC (\( f = 0 \)) and AC (\( f = f_x \)) components are measured. Finally, a lookup table that relates different values of \( R_d \) with absorption coefficient and reduced scattering coefficient values is used to reconstruct for the absorption coefficient (\( \mu_a \)) and reduced scattering coefficient (\( \mu'_s \)) of the target at each pixel of the image [5].

### 5.2.3. Phantom calibration

In order to evaluate the SFDI system’s capability in reconstructing the absorption and reduced scattering coefficients of turbid media, several liquid phantoms were made with Intralipid as the scattering agent and Indian ink as the absorbing agent. Because the SFDI probe has several different wavelengths, we calibrated the phantoms using separate measurements of scattering and absorption coefficients for each wavelength in a collimated transmission setup [14,15].

The absorption coefficient of a parent Indian ink solution was measured and the parent solution was diluted to different ratios to create different absorption coefficients considering the titration equation [16]. It should be noted that in higher wavelengths, especially for 935 nm and 950 nm, water has considerable absorption and the absorption of water needs to be considered in the calculations [17,18]. Also, it has been shown in
the literature that the absorption of Intralipid solution follows the absorption of water [14], therefore absorption is mainly governed by the concentration of the Indian ink (and water at higher wavelengths).

The scattering coefficient ($\mu_s$) of the parent 20% Intralipid solution was measured using the collimated transmission setup and the Beer-Lambert law with the assumption that the total attenuation coefficient ($\mu_t$) is the addition of absorption coefficient ($\mu_a$) and scattering coefficient ($\mu_s$). Given that for the Intralipid solution $\mu_s \gg \mu_a$ therefore $\mu_t \approx \mu_s$. After careful measurements of the scattering coefficient, the determined scattering coefficient was close to (within the error margin of) the value reported in the literature [15]. Therefore either the measured value or the value from the literature could be used as the $\mu_s$ of the undiluted Intralipid solution. The reduced scattering coefficient, which can be measured in SFDI is $\mu'_s = \mu_s (1-g)$, where $g$ is the anisotropy factor and is wavelength dependent [15]. We used the value of $g$ available in the literature for Intralipid solution similar to the solution used in this study [15]. The Intralipid solution was diluted to different ratios in order to form different reduced scattering coefficient values for different phantoms. Optical properties of the prepared phantoms were reconstructed by the SFDI probe for different wavelengths and were compared to the expected values.

5.3. Results

5.3.1. Phantom evaluation

Figure 5.3 shows an example of the reconstructed and expected values for absorption coefficient (A) and reduced scattering coefficient (B) for a liquid phantom for all wavelengths of the probe.
We evaluated different phantoms to test the performance of the system. Table 5.1 lists the evaluated phantoms and the average error in absorption and reduced scattering coefficients considering all wavelengths for each phantom. Please note that $\mu_a$ and $\mu_s$ are different at each wavelength but in the table we only provide the expected values at 810 nm to distinguish between the phantoms. The absolute average error for reconstruction of absorption coefficient was approximately $9.3 \pm 6 \%$ (error ± std) and the absolute average error for reconstruction of reduced scattering coefficient was approximately $4.5 \pm 3 \%$, considering all wavelengths and all phantoms.

Table 5.1. Phantoms used for SFDI probe evaluation

<table>
<thead>
<tr>
<th>Phantom #</th>
<th>Expected $\mu_a$ @810 nm (cm$^{-1}$)</th>
<th>Average $\mu_a$ error for all $\lambda$s (%)</th>
<th>Expected $\mu_s$ @810 nm (cm$^{-1}$)</th>
<th>Average $\mu_s$ error for all $\lambda$s (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (ref)</td>
<td>0.0745</td>
<td>0.19</td>
<td>4.9056</td>
<td>0.22</td>
</tr>
<tr>
<td>2</td>
<td>0.1293</td>
<td>2.40</td>
<td>4.9056</td>
<td>2.82</td>
</tr>
<tr>
<td>3</td>
<td>0.1844</td>
<td>12.54</td>
<td>4.9056</td>
<td>5.74</td>
</tr>
<tr>
<td>4</td>
<td>0.2387</td>
<td>3.87</td>
<td>4.9056</td>
<td>0.72</td>
</tr>
<tr>
<td>5</td>
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<td>21.62</td>
<td>6.0829</td>
<td>2.86</td>
</tr>
<tr>
<td>6</td>
<td>0.0745</td>
<td>9.82</td>
<td>6.8678</td>
<td>9.77</td>
</tr>
</tbody>
</table>
5.3.2. Human *in vivo* forearm

In order to evaluate the performance of the system for imaging biological tissue, we imaged a volunteer’s forearm *in vivo*. Figure 5.4 shows the reconstructed absorption and reduced scattering coefficient maps for all nine wavelengths in the probe. Regardless of some demodulation noise remaining in the lower wavelengths, absorption and scattering values are within the expected range for skin. Also please note that tissue is not present close to the top right and bottom corner of the images. Figure 5.5 shows the scattering slope and scattering amplitude maps obtained from analyzing the data from all nine wavelengths. The reduced scattering coefficient at a wavelength $\lambda$ can be expressed as

$$\mu'_s(\lambda) = A \mu'_s(\lambda_0) \left( \frac{\lambda}{\lambda_0} \right)^{-B}$$  (5.4)

Here $A$ and $B$ (unit-less) are referred to as the scattering amplitude and scattering power, respectively. $\mu'_s(\lambda)$ is the reduced scattering coefficient at a given wavelength, $\mu'_s(\lambda_0)$ is chosen to be an approximate value of the reduced scattering coefficient of 1% Intralipid solution at 800 nm (10 cm$^{-1}$), $\lambda$ is a given wavelength, and $\lambda_0$ is 800 nm [19]. To form the scattering slope and scattering amplitude maps, we stack the reduced scattering coefficient maps of all wavelengths in a 3 dimensional matrix and fit the reduced scattering coefficient values on each pixel to Equation 5.4 and extract $A$ and $B$ for that pixel. Figure 5.5A shows the scattering amplitude map and Figure 5.5B shows the scattering slope map and the obtained values are within the expected range. Spectral maps can provide valuable information for studying healthy vs diseased tissue.
Figure 5.4. Reconstructed absorption and reduced scattering coefficients of a human forearm *in vivo* for all wavelengths.
5.3.3. Bovine ovarian tissue

Absorption coefficient and reduced scattering coefficient maps in addition to photographs of two ex vivo bovine ovaries are demonstrated in Figure 5.6. The bovine ovaries were obtained from a local farm and the institutional oversight was waived. The images show the capability of reconstructing and differentiating values and heterogeneity of optical properties in biological samples ex vivo. The proposed low-cost and small SFDI system can be used for imaging cancer samples ex vivo and data acquisition from ex vivo cancer samples is an ongoing task.

5.4. Summary

We have developed a very low-cost, handheld, and multispectral SFDI probe that incorporates 9 different LEDs and all illumination and detection components in a small 3D
printed probe. The performance of the probe was evaluated using liquid phantoms made from Intralipid and Indian ink. The system was further evaluated by imaging human forearm \textit{in vivo} and bovine ovarian tissue \textit{ex vivo}. Evaluating the performance of the probe in classification and characterization of \textit{ex vivo} cancer samples is an ongoing task.

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\textbf{References}


5. SFDI
6. Summary

In this dissertation we have described our efforts towards low-cost, high contrast, and miniaturized optical imaging with potential applications in cancer imaging.

In chapters two and three, we explained our efforts in developing laser diode based photoacoustic microscopy systems given that laser diodes are low-cost and compact potential substitutes of conventional light sources used in photoacoustic imaging. In chapter two, we developed a method to be able to have low-loss collimation and focusing of high power pulsed laser diode light for use in photoacoustic microscopy and demonstrated the ability to image vasculature on porcine ovarian tissue \textit{ex vivo}. In chapter three, we developed a laser scanning laser diode based photoacoustic microscopy system that provides fast data acquisition and does not require mechanical sample scanning in addition to being low cost and compact. The capability of this system in imaging thin vasculature on porcine ovarian tissue \textit{ex vivo} also reveals its potential for imaging and characterization of human cancer samples [1–3].

In chapter four, we described the synthesis and evaluation of a porphyrin-based monomeric water soluble photoacoustic contrast agent for \textit{in vivo} tumor imaging. The performance of the dye in improving tumor imaging was evaluated, revealing a strong enhancement of \textit{in vivo} photoacoustic signal from implanted tumors in mice upon injection of the dye. The lack of toxicity, favorable filtering and bioaccumulation, and strong enhancement of the photoacoustic signal suggest the potential of this dye as a suitable photoacoustic contrast agent for improving photoacoustic cancer imaging [4,5].
In chapter five, we reported a very low-cost, handheld, and multispectral SFDI system that incorporates up to nine LEDs and all illumination and detection components in a small 3D printed probe. The system was evaluated by reconstructing absorption and reduced scattering coefficients of phantoms, *in vivo* human forearm, and *ex vivo* bovine ovarian tissue. The results suggest the potential of the system for classification and characterization of *ex vivo* cancer samples. Imaging and classification of human *ex vivo* cancer samples is an ongoing task [6].
7. References


