Bio-Inspired Multi-Spectral and Polarization Imaging Sensors for Image-Guided Surgery

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Bio-Inspired Multi-Spectral and Polarization Imaging Sensors for Image-Guided Surgery

by

Nimrod Missael Garcia Hernandez

A dissertation presented to
The Graduate School
of Washington University in
partial fulfillment of the
requirements for the degree
of Doctor of Philosophy

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December 2017
I dedicate this to the pursuit of knowledge and to those who aspire to greatness
ABSTRACT OF THE DISSERTATION

Bio-Inspired Multi-Spectral and Polarization Imaging Sensors for Image-Guided Surgery

By

Nimrod Missael Garcia Hernandez

Doctor of Philosophy in Computer Engineering

Washington University in St. Louis, 2017

Professor Roger Chamberlain, Chair

Image-guided surgery (IGS) can enhance cancer treatment by decreasing, and ideally eliminating, positive tumor margins and iatrogenic damage to healthy tissue. Current state-of-the-art near-infrared fluorescence imaging systems are bulky, costly, lack sensitivity under surgical illumination, and lack co-registration accuracy between multimodal images. As a result, an overwhelming majority of physicians still rely on their unaided eyes and palpation as the primary sensing modalities to distinguish cancerous from healthy tissue. In my thesis, I have addressed these challenges in IGC by mimicking the visual systems of several animals to construct low power, compact and highly sensitive multi-spectral and color-polarization sensors. I have realized single-chip multi-spectral imagers with 1000-fold higher sensitivity and 7-fold better spatial co-registration accuracy compared to clinical imaging systems in current use by monolithically integrating spectral tapetal and polarization filters with an array of vertically stacked photodetectors. These imaging sensors yield the unique capabilities of imaging simultaneously color, polarization, and multiple fluorophores for near-infrared fluorescence imaging. Preclinical and clinical data demonstrate seamless integration of this technologies in the surgical work flow while providing surgeons with real-time information on the location of cancerous tissue and sentinel lymph nodes, respectively. Due to its low cost, the bio-inspired
sensors will provide resource-limited hospitals with much-needed technology to enable more accurate value-based health care.
Chapter 1: Introduction

Surgery is the primary curative option for patients with cancer, with the overall objective of complete resection of all cancerous tissue while avoiding iatrogenic damage to healthy tissue. In addition, sentinel lymph node (SLN) mapping and resection is an essential step in staging and managing the disease [1]. Even with the latest advancements in imaging technology, incomplete tumor resection in patients with breast cancer is at an alarming rate of 20–25%, with recurrence rates of up to 27% [2]. The clinical need for imaging instruments that provide real-time feedback in the operating room is unmet, largely due to the use of imaging systems based on contemporary technological advances in the semiconductor and optical fields, which have bulky and costly designs with suboptimal sensitivity and co-registration accuracy between multimodal images [3-7].

In this thesis, I demonstrate that image-guided surgery can be dramatically improved by shifting the design paradigm away from conventional advancements in the semiconductor and optical technology fields and instead adapting the elegant designs of two arthropods’ compound eyes: the mantis shrimp and the Morpho butterfly [8-11] – condensed biological systems optimized for high-acuity detection of polarization and multi-spectral information. Nature has served as inspiration for many engineering sensory designs with performances exceeding state-of-the-art sensory technology and enabling new engineering paradigms, such as achromatic circular polarization sensors [12], artificial vision sensors [13-15], silicon cochlea [16, 17] and silicon neurons [18]. The artificial compound eyes, inspired by the Morpho butterfly’s photonic crystals and the mantis shrimp’s vertically stacked photoreceptors with orthogonal microvilli, monolithically integrate pixelated polarization and spectral filters with a three-dimensional array of silicon-based photodetectors. The bio-inspired color-polarization imaging sensor has the
advantage over state-of-the-art polarimeters of capturing both color and polarization information with high co-registration accuracy. The bio-inspired near-infrared imaging sensors for image-guided surgery that I have developed have the prominent advantage of capturing both color and multiple near-infrared fluorescence (NIRF) spectra, translating to the identification and differentiation of multiple fluorophores, with high co-registration accuracy and high sensitivity under surgical light illumination, which allows simultaneous identification of anatomical features and multiple tumor-targeted molecular markers. Both architectures shared the benefits of streamlined design – at 20 g including optics, the bio-inspired image sensor does not impede surgical work flow; and low cost, which will provide resource-limited hospitals with much-needed technology to enable more accurate value-based health care.

1.1 Mantis Shrimp-Inspired Design

The mantis shrimp visual system has evolved to be arguably one of the most sophisticated sensory systems in the animal kingdom [10, 11]. With its two apposition compound eyes, the mantis shrimp perceives the world by sensing 16 different spectral channels, 4 equally spaced linear polarization orientations, and 2 circularly polarized states, making the animal one of the best-adapted predators in shallow waters. This sophisticated and compact visual system combines three fundamental principles within each individual ommatidium: spectral tuning, enabled by crystalline cones [19, 20]; multi-spectral sensitivity, enabled by vertically stacked rhabdomeres [21]; and polarization detection, enabled by structurally organized microvilli [22]. In this thesis, I demonstrate that, by mimicking the space- and energy-efficient implementation of the mantis shrimp visual system, I have designed a compact, single-chip, low-power color- and polarization-sensitive imager. The bio-inspired imager is realized by combining an array of vertically stacked photodetectors capable of discerning three different broadband spectral
channels, with pixelated nanowire filters for polarization sensitivity. Testing results reveal that this sensor captures co-registered color and polarization information with high accuracy, sensitivity, and resolution and can enable a wide range of applications, including remote sensing, cancer imaging, label-free neural imaging, and the study of new underwater phenomena and marine life behavior [23-25]. This bio-inspired color-polarization imaging sensor, with its low-power, compact design and high sensitivity, enables a glimpse of the underwater world where animals actively exploit color and polarization information for both camouflaging and covert signaling channels.

1.2 Morpho Butterfly-Inspired Design
Light has imposed significant selection pressure for perfecting, optimizing, and miniaturizing animal visual systems since the Cambrian period some 500 million years ago [26]. Sophisticated visual systems emerged in a tight race with prey coloration during that time, resulting in a proliferation of photonic crystals in the animal kingdom used for both signaling and sensing [27, 28]. For example, not only are the tree-shaped photonic crystals of the Morpho butterfly the source of its wings’ magnificent iridescent colors, which can be sensed by conspecifics from a mile away, but these crystals also can sense vapors [29] and infrared photons [30] with sensitivity that surpasses state-of-the-art man-made sensors. Similar photonic crystals are also present in the compound eye of the Morpho butterfly. These photonic crystals, known as tapetal filters, are realized by stacks of alternating layers of air and cytoplasm, which act as interference filters at the proximal end of the rhabdom within each ommatidium. The light that enters an individual ommatidium and is not absorbed by the visual and screening pigments in the rhabdom will be selectively reflected by the tapetal filters and will have another chance of being absorbed before exiting the eye. The spectral responses of the
tapetal filters together with screening and visual pigments in the rhabdom determine the eye shine of the ommatidia [8] and the inherent multi-spectral sensitivity of the butterfly’s visual system. Individual ommatidia have different combinations of visual pigments and tapetal filter stacks, enabling selective spectral sensitivity across the ultraviolet, visible, and near-infrared (NIR) spectra.

By imitating the compound eye of the Morpho butterfly using dielectric materials and silicon-based photosensitive elements, I developed a multi-spectral imaging sensor that operates radically differently from the current state-of-the-art multi-spectral imaging technology. The tapetal spectral filters are constructed using alternating nanometric layers of SiO$_2$ and TiO$_2$, which are pixelated with a 7.8-µm pitch and deposited on the surface of a three-dimensional imaging array, i.e. vertically stacked photodetectors capable of trichromatic vision at each pixel location. The alternating stack of dielectrics acts as an interference filter, allowing certain light spectra to be transmitted while reflecting others. Two distinct pixelated spectral filters are replicated throughout the imaging sensor in a chessboard pattern by modulating the thickness and periodicity of the dielectric layers in individual pixels. One of the two pixels is designed to sense the visible spectrum, i.e. designed as a shortpass filter, and the other pixel captures NIR photons with wavelengths greater than 680 nm, i.e. designed as a longpass filter. The inherit trichromatic vision capabilities of the pixels allows for color and NIR-shade reconstruction, respectively. The proximity of the imaging array’s two base pixels inherently co-registers the captured multi-spectral information, similar to its biological counterpart.
My contributions over the course of my doctoral studies include:

- Investigated the realization of a high resolution, real-time, bio-inspired color-polarization imaging sensor by integrating vertically stacked photodetectors with aluminum nanowires. To realize this imaging system and deployed in the field, I was involved in:
  - Developed, designed and tested a high speed printed circuit board for operating a bio-inspired imager with a field programmable array and transmit real-time data via UCB 3.0 protocol to the PC.
  - Developed and tested various finite state machines for reading out data in real-time from a bio-inspired imager with vertically stacked photodetectors and transmitting data to the PC via a USB3.0 interface.
  - Investigated monolithic integration of pixelated polarization filters with vertically-stacked imaging sensor to realize a single chip color-polarization imager.
  - Designed, tested and filed deployed underwater imaging platform for real-time data recording of underwater color-polarization information.
  - Developing Matlab and C++ libraries and graphical user interfaces (GUIs) for processing polarization and multi-spectral data and performed quantitative analysis of spectral-polarization data captured from various filed experiments.
  - Investigated the realization of an algorithm for 3-d surface normal reconstruction using single view, polarization image.
  - Investigated polarization-based calibration methods for endoscopes to enable the use of polarization sensors in minimally invasive surgeries.
• Investigated snap-shot, bio-inspired, multi-spectral imaging instruments for image guided surgery. My contributions in this research trusts are:
  − Investigated various pixelated spectral filters realizations using stacked dielectrics and optimize their performance for near infrared image guided surgery.
  − Investigated fabrication and integration of pixelated spectral filters with different imaging sensors, including a bio-inspired imager with vertically stacked photodetectors.
  − Developed real-time imaging and image processing algorithms for assisting surgeons during oncology procedures.
  − Investigated the use of my bio-inspired multi-spectral imaging sensor for evaluating critical limb ischemia in a diabetic murine model.
  − Investigated various algorithm to correctly segment and differentiate multiple near infrared fluorescent markers for tumor guided surgery.

The rest of the thesis document is organized as follows. Chapter 2 introduces a mantis shrimp-inspired color-polarization imager, its characterization and performance evaluation, and its use in animal underwater imaging. Chapter 3 introduces a polarization-based surface normal reconstruction algorithm. Chapter 4 introduces polarization calibration schemes for endoscopes and its uses. Chapter 5 introduces the first iteration of a Morpho butterfly-inspired multi-spectral imager, its characterization and performance evaluation, and its use in preclinical and clinical trials. Chapter 6 introduces an angiography technique using the imager described in Chapter 5 for critical limb ischemia in a diabetic murine model. Chapter 7 introduces a bio-inspired hexachromatic imager for NIR IGS, and shows the molecular probe differentiation capabilities of the system.
1.3 Dissemination of Work

The work presented on this dissertation has appeared in peer reviewed conference and journal articles, each of which I was the first author. For each manuscript, co-authors helped with the text. The following lists the chapters and the associated publications.

- Chapter 5: “A Bio-Inspired Imager Improves Sensitivity for Near-Infrared Fluorescence Image-Guided Surgery” is a journal paper that has been submitted to the Journal of Biomedical Optics and it is currently under review. The paper is co-authored with C. Edmiston, T. York, R. Marinov, S. Mondal, N. Zhu, G. Sudlow, W. Akers, J. Margenthaler, S. Achilefu, R. Liang, M. Zayed, M. Pepino and V. Gruev.
- Chapter 7: “A 1280 by 720 by 3, 250 mW, 24 fps Hexachromatic Imager for Near-Infrared Fluorescence Image-Guided Surgery” is a conference paper that will be
presented in the International Symposium on Circuits and Systems 2018 conference. The paper is co-authored with K. Kauffman, T. Davis, R. Marinov, and V. Gruev.
Chapter 2: Bio-inspired Color-Polarization Imager for Real-Time in Situ Imaging [31]

Nature has a large repertoire of animals that take advantage of naturally abundant polarization phenomena. Among them, the mantis shrimp possesses one of the most advanced and elegant visual systems nature has developed, capable of high polarization sensitivity and hyperspectral imaging. Here, I demonstrate that, by shifting the design paradigm away from the conventional paths adopted in the imaging and vision sensor fields and instead functionally mimicking the visual system of the mantis shrimp, I have developed a single-chip, low-power, high-resolution color-polarization imaging system. This bio-inspired imager captures co-registered color and polarization information in real time with high resolution by monolithically integrating nanowire polarization filters with vertically stacked photodetectors. These photodetectors capture three different spectral channels per pixel by exploiting wavelength-dependent depth absorption of photons. The bio-inspired imager is comprised of 1280 by 720 pixels with a dynamic range of 62 dB and maximum signal-to-noise ratio of 48 dB. The quantum efficiency is above 30% over the entire visible spectrum, while achieving high polarization extinction ratios of ~40 on each spectral channel. This technology is enabling underwater imaging studies of marine species, which exploit both color and polarization information, as well as applications in biomedical fields.

2.1 Motivation for a Biologically Inspired Polarimeter

The mantis shrimp visual system has evolved to be arguably one of the most sophisticated sensory systems in the animal kingdom [10, 11]. With its two apposition compound eyes, the mantis shrimp perceives the world by sensing 16 different spectral channels, 4 equally spaced linear polarization orientations, and 2 circularly polarized states, making the animal one of the
best-adapted predators in shallow waters. This sophisticated and compact visual system combines three fundamental principles within each individual ommatidium: spectral tuning, enabled by crystalline cones [19, 20]; multi-spectral sensitivity, enabled by vertically stacked rhabdomeres [21]; and polarization detection, enabled by structurally organized microvilli [22]. Here, I demonstrate that, by mimicking the space- and energy-efficient implementation of the mantis shrimp visual system, I have designed a compact, single-chip, low-power color- and polarization-sensitive imager (Figure 2.1). The bio-inspired imager is realized by combining an array of vertically stacked photodetectors capable of discerning three different broadband spectral channels, with pixelated nanowire filters for polarization sensitivity. Testing results reveal that this sensor captures co-registered color and polarization information with high accuracy, sensitivity, and resolution and can enable a wide range of applications, including remote sensing, cancer imaging, label-free neural imaging, and the study of new underwater phenomena and marine life behavior [23-25].

Polarization of light is caused by the scattering of light in air or water media and by reflection or refraction from objects or live organisms [32]. Polarization contains valuable information about the imaged environment, such as material and tissue properties, surface roughness, structural composition, and three-dimensional shape [33-36]. This information is orthogonal to the information captured by the other two fundamental properties of light: intensity and color. Many animals utilize polarization for both sensing and signaling purposes [10, 37-40]. Animals have evolved extraordinary visual systems that can detect subtle differences in polarization states, as well as photonic structures along their bodies, enabling both color and polarization camouflage [38-41]. Polarization also can serve as a covert communication channel between
conspecifics, which often is visible over longer distances than color or intensity in highly scattered environments [42].

Figure 2.1 The mantis shrimp’s compound eye (left) is divided into three morphological parts: two hemispheres and a midband section. The rhabdons in the peripheral hemispheres are sensitive to two orthogonal orientations of linearly polarized light by alternating stacks of bidirectional microvilli, while the midband section utilizes vertically stacked photosensitive cells for spectral discrimination. Similar to its biological counterpart, the bio-inspired imaging sensor (right) utilizes a combination of vertically stacked photodetectors for spectral sensitivity and pixelated metallic nanowire for polarization sensitivity.

a) The stomatopod crustacean *Odontodactylus latirostris*. b) Close up view of the ommatidia (inset in (a)). The following abbreviations are used: midband (MB), dorsal hemisphere (DH), and ventral hemisphere (VH). c) Diagrammatic representation of a sagittal section (line in (b)) of a generalized stomatopod ommatidia. d) Photograph of the bio-inspired color-polarization imager. e) Diagrammatic representation of (d). a) and b) photographs by Michael Bok.
Capturing both color and polarization properties of light has been of great interest for many biomedical applications. Among its many applications, polarization information has been used to study tissue biomechanics, detect early cancer formation, and record label-free neural activity [25, 43-45]. One of the key challenges in these and other applications is the correct and temperature-invariant superposition of the color and polarization information. For example, to utilize polarization in distinguishing cancerous from healthy tissue, polarization information must be accurately superimposed to the correct anatomical features captured by color cameras. Unfortunately, most state-of-the-art multi-spectral and polarization-sensitive imaging devices suffer from temperature-dependent co-registration errors, in the order of tens of pixels, due to the use of multiple optical elements, such as beam splitters, relay lenses, and polarization filters [3, 46, 47].

Today’s state-of-the-art color-polarization imaging sensory technology is realized either by computational approaches, such as computed tomography imaging spectrometry, Fourier transform hyperspectral spectrometry, compressive sensing, or by direct measurements through the combination of traditional panchromatic imaging arrays with spectral-polarization optics that are modulated in either time, light amplitude, or focal plane [48-54]. These technologies are based on advances in conventional signal processing, 2-D planar imaging arrays, and optics, and have yielded to complex, bulky, and expensive systems with intolerably low polarization performance and limited translation to field experiments for the study of naturally occurring phenomena. For example, although ocean water covers ~70% of Earth’s surface and is home to more than ~20% of all animal life, we have very little knowledge of this hidden world; less than 10% of marine animal species are catalogued due in part to shortcomings of the dominant imaging technology—the digital color camera, which is an incomplete imaging sensor [55]. The
visual systems of various animals provided the blueprint for designing artificial vision imagers whose performance exceeds that of current state-of-the-art imaging technology [12-15, 44, 56-58]. In a similar fashion, the bio-inspired color-polarization imaging sensor, with its low-power, compact design and high sensitivity, enables a glimpse of the underwater world where animals actively exploit color and polarization information for both camouflaging and covert signaling channels.

2.2 Methodology

2.2.1 Fabrication of nanowire polarization filters

The nanofabrication of the pixelated nanowire polarization filter is achieved using a series of optimized nanofabrication steps:

1. A 250-nm-thick aluminum layer is deposited on a substrate via e-beam deposition.
2. A 75-nm-thick SiO$_2$ layer is deposited on top of the aluminum layer via chemical vapor deposition. This layer will act as a hard mask for etching the underlying aluminum.
3. A 140-nm-thick S-1805 photoresist layer is spin-coated at 3000 rpm.
4. The sample is baked at 120 °C for 90 s, followed by 30 s cooling at 70 °C to avoid cracks in the photoresist.
5. Quartz mask is brought in contact with the sample. The mask is composed of a layer of chromium to block light except at selected pixels where the chromium is removed. Every even-numbered pixel in every even-numbered row has the chromium removed and will be exposed to an interference pattern.
6. An interference pattern is generated using a 532-nm continuous-wave neodymium-doped yttrium aluminum garnet (Nd:YAG) laser coupled with a frequency doubler. Two laser beams are aligned to intersect at ~110° and generate a 140-nm periodic interference pattern on the surface of the exposed photoresist. The photoresist is exposed for 40 s.
7. The chromium mask is shifted one pixel over in the horizontal direction and brought in contact with the substrate. Both mask and substrate are rotated 45° with respect to the interference pattern. The photoresist is exposed for 40 s.

8. Step 7 is repeated two more times to generate 90° and 135° pixelated nanowire filters.

9. After the four consecutive exposures, the photoresist is developed for 60 s while stirring the sample.

10. Inductively coupled plasma reactive-ion etching (ICP RIE) is used to first etch the SiO$_2$. A standard recipe is used to etch the SiO$_2$. At the end of this step, the 75 nm nanowire pattern is transferred from the photoresist to the underlying SiO$_2$ layer.

11. The aluminum layer is etched next using a standard ICP RIE recipe. The SiO$_2$ layer acts as a hard mask for etching aluminum and enables forming deep trenches, i.e. 250 nm tall and 75 nm wide aluminum nanowires. (One cannot achieve high-aspect-ratio aluminum structures using photoresist because the higher etching rate of photoresist compared to aluminum would necessitate forming photoresist structures with an aspect ratio of 100 or higher, which is impossible.) The fabrication steps were performed on individual dies in the cleanroom facility at Washington University.

2.2.2 CMOS imager with vertically stacked photodiodes
The bio-inspired imaging sensor is fabricated in a 180nm feature process with 1 poly and 3 metal layers. The substrate for fabricating the sensor is a custom wafer with three epitaxially layers. The imager is fabricated on a positively doped silicon wafer (10$^{15}$ boron atoms per cm$^3$). The first epitaxial layer is grown on top of the silicon wafer with ~1.5 μm thickness. This epitaxial layer is positively doped with ~10$^{16}$ boron atoms per cm$^3$. Next, the negative terminal of the red photodiode is implemented by doping a selective region in the epitaxial layer with 10$^{17}$ phosphorus atoms per cm$^3$ at 75 keV energy followed by rapid thermal annealing. A negatively
doped isolation region between neighboring pixels is created next to prevent lateral flow of photoinduced electron-hole pairs. This isolation region is formed by using three different doping concentrations at three different energy levels. The first doping is at 1500 keV with $10^{18}$ phosphorus atoms per cm$^3$, the second at 1000 keV with $0.5 \times 10^{18}$ phosphorus atoms per cm$^3$, and the third at 750 keV with $10^{17}$ phosphorus atoms per cm$^3$. Rapid thermal annealing is performed next. The impedance of this isolation region is not critical as it only serves to remove optical cross talk between neighboring pixels.

The second, positively-doped epitaxial layer ($10^{16}$ boron atoms per cm$^3$) is grown on top of the first epitaxial layer with a thickness of ~2 μm. The next step is to form: 1) the negative terminal of the green photodiode, 2) connection to the negative terminal of the red photodiode and 3) connection to the negatively doped isolation region between pixels. The latter two connections are implemented first by using three different doping concentrations at three different energy levels. The first doping is at 1500 keV with $10^{18}$ phosphorus atoms per cm$^3$, the second at 1000 keV with $0.5 \times 10^{18}$ phosphorus atoms per cm$^3$, and the third at 750 keV with $10^{17}$ phosphorus atoms per cm$^3$. The last doping step is to form the negative terminal of the green photodiode with $10^{17}$ phosphorus atoms per cm$^3$ at 75 keV energy followed by rapid thermal annealing.

The third, positively-doped epitaxial layer ($10^{16}$ boron atoms per cm$^3$) is grown on top of the second epitaxial layer with a thickness of ~0.8 μm. The next step is to form: 1) the negative terminal for the blue photodiode, 2) connections to the negative terminals of the green and red photodiodes and 3) connection to the negatively doped isolation region between pixels. The latter two connections are implemented first by using two different doping concentrations at three different energy levels. The first doping is at 1000 keV with $0.5 \times 10^{18}$ phosphorus atoms per cm$^3$ and the second is at 750 keV with $10^{17}$ phosphorus atoms per cm$^3$. The last step is to form the
negative terminal of the blue photodiode with $10^{17}$ phosphorus atoms per $\text{cm}^3$ at 75 keV energy followed by rapid thermal annealing. The positive terminals of all photodiodes are connected to ground potential. The negative terminals of the individual photodiodes are connected to individual readout circuits in the pixel.

The readout circuit comprises three transistors: reset transistor, source follower, and access transistor. The reset transistor controls the integration (or exposure) time of the photodiode such that when the gate voltage is low the photon-generated electron-hole pairs are integrated on the photodiode intrinsic capacitance. The source follower buffers the integrated photodiode voltage before outputting it on the column bus. The access transistor controls access to the readout bus, such that all pixels in a column share the same readout bus. The gates of the reset and access transistors for the red, green, and blue photodiodes are connected together; hence, all three photodiodes have the same exposure control and are accessed in parallel at the same time. This minimizes both the number of metallic lines per pixel and the pixel pitch. Difference double sampling is performed on the readout, eliminating mismatches between pixels’ voltage thresholds.

### 2.2.3 Experimental optical setups

A variety of optical setups were employed to obtain the optoelectrical characterization measurements of the color-polarization imaging system. For the response to Malus’s law, fixed-pattern noise histograms, and estimation error for DoLP and AoP as a function of the input light’s AoP, an optical setup that produced broadband, collimated, and fully linearly polarized light was constructed. Three current-controlled and narrowband LED sources at 460 nm, 515 nm, and 625 nm were connected to the input ports of an integrating sphere (819D-SF-4, Newport). The output port of the integrating sphere was aligned to an adjustable iris (SM2D25, Thorlabs), an aspheric collimating lens (ACL7560, Thorlabs), a linear polarizer (20LP-VIS-B, Newport),
and the imaging sensor under test, in that order. The linear polarizer was mounted on a nanorotator stage (NR360S, Newport) to modulate the input light’s AoP.

To evaluate the estimation accuracy of the degree of linear polarization, a monochromator (Acton SP2150, Princeton Instruments), outputting light at 532 nm, was instead connected to the integrating sphere’s input. A zero-order quarter-wave retarder at 532 nm (20RP34-532, Newport) was added to the optical path between the linear polarizer and the sensor. The retarder was mounted on a nanorotator stage to modulate the input light’s DoLP. A calibrated photodiode (S130C, Thorlabs) driven by a power meter (PM100D, Thorlabs) in conjunction with a rotating linear polarizer was utilized to calculate the true DoLP. For the diattenuation ratio as a function of the input light’s wavelength measurement, the previously described optical setup was utilized without the quarter-wave retarder, and the monochromator produced narrowband light from 400 nm to 650 nm in steps of 10 nm. For the quantum efficiency measurement, the previously described optical setup was utilized without any polarization optics. A calibrated photodiode driven by a power meter was utilized to calculate the total photon flux.

2.2.4 Data interpolation
A spatial interpolation algorithm was used to recover full-resolution polarization frames and to minimize instantaneous field-of-view artifacts. To reconstruct polarization information at each pixel location, the full-frame bicubic spline interpolation method was utilized per color channel [59]. This method has the advantage of yielding a higher modulation transfer function gain and wider validation frequency bandwidth than bilinear-based interpolation methods.

2.2.5 Color and polarization calibration
To color correct the trichromatic intensity images produced by the imaging system and to closely replicate the color perception of the human eye, a linear regression algorithm was used. A Macbeth color calibration target was used as the training data to produce a color-mixing 4 by 3
elements matrix. This matrix then can be used to color-calibrate images taken under similar illumination conditions.

To correct for imperfections in the nanofabrication process of the nanowire polarization filters, a division-of-focal-plane polarimeter calibration method is employed [60]. The nanowires can have spatial variations across the filter array, which can cause fixed-pattern noise and deviation in the filters’ transmission ratios and desired AoP. To minimize the errors caused by these filter artifacts, the calibration scheme characterizes the polarization filters by computing an array of analysis matrices and dark offsets using a linear regression algorithm and Mueller theory. These parameters are used in real time to calibrate newly acquired polarization data.

### 2.2.6 Underwater imaging

To acquire the underwater videos of animals, an underwater imaging setup was utilized. The bio-inspired color-polarization imaging system was enclosed in an underwater housing (Bluefin VX2000, Light and Motion). A single-board computer (QM67PC-2715QE, ADL) was placed inside of the underwater housing to control the imaging system and record the data to a solid-state drive. A microcontroller board (Teensy 3.2 ARM, PJRC) was programmed to control a Canon EF lens and process the user commands from the underwater housing integrated control buttons. The system included an external HDMI monitor for the user to see the data in real time. The underwater system was powered by a lithium-ion polymer battery (Li-Ion 18650 14.8V 6600mAh, Tenergy). The data for the marine animals were acquired in the proximity bays of Lizard Island, Queensland, Australia, with the help of the Lizard Island Research Station facilities.
2.3 Results
A block diagram of the bio-inspired imaging sensor and its functional similarities to the mantis shrimp visual system is shown in Figure 2.1. The mantis shrimp’s compound eye is divided into three morphological parts: two hemispheres and a midband section. The rhabdoms in the peripheral hemispheres are sensitive to two orthogonal orientations of linearly polarized light by alternating stacks of bidirectional microvilli. The rhabdoms are rotated 45° between hemispheres, giving the mantis shrimp the capability to fully reconstruct partially linearly polarized light. The midband section in the mantis shrimp’s eye is where most of the spectral discrimination takes place. Unlike traditional color sensors in which color is sensed over four spatially distributed pixels, the mantis shrimp’s visual system is capable of sensing multiple spectra within a single pixel location or ommatidium. This is accomplished by vertically stacked photosensitive cells and spectral filters in a single ommatidium. As light enters the ommatidium, its spectrum is initially filtered by the crystalline cone on top of the rhabdom, followed by wavelength-dependent absorption in the vertically stacked rhabdomeres. Within the ommatidium, deeper and larger photosensitive cells are most sensitive to longer wavelengths, while the shallower and shorter photosensitive cells are sensitive predominantly to shorter wavelengths. Neighboring ommatidia are separated by black pigments, which absorb scattered light and prevent optical cross talk between neighbors [61].

The bio-inspired imaging sensor mimics the visual system of the mantis shrimp by utilizing pixelated linear polarization filters deposited on an array of silicon-based vertically stacked photodetectors. Similarly to its biological counterpart, silicon’s absorption coefficient is wavelength dependent and monotonically decreases from the blue to red wavelengths [62]. The absorption coefficient for red wavelengths (650 nm) is about 30-fold lower than that for blue wavelengths (400 nm), leading to 99% of blue and red photons being absorbed within the first
~500 nm and ~15 μm, respectively. The initial concept of color imaging with vertically stacked photodetectors was proposed and patented by Kodak in the early 1980s [63]. However, it took more than a decade to overcome the technological challenges for fabricating vertically stacked photodetectors in CMOS technology and was first achieved by researchers at Foveon [64]. Today’s advanced CMOS processes allow for implementation of vertically stacked photodiodes, although its spectral responsivity is poor due to an unoptimized fabrication process for the photodiodes [65-67].

![Cross sectional diagram of the pixel’s circuitry with its vertically stacked photodiodes. Three transistors per photodiode are utilized in the pixel. The first positively doped epitaxial layer is grown on top of the silicon wafer with 1.5 μm thickness, followed by selective negative doping to realize the red photodiode. The second epitaxial layer is grown on top of the first epitaxial layer with a thickness of 2 μm to realize the green photodiode, and the third epitaxial layer for the blue photodiode, with ~0.8 μm thickness, is grown last.]

The bio-inspired imager is fabricated in Foveon’s 180 nm vertically stacked process. Using an epitaxial growth of three separate layers on a positively doped silicon wafer and alternating positive and negative doping of six junctions, three broadband vertically stacked photodiodes within each individual pixel are realized (see Methodology). The thicknesses of the doped junctions are optimized for peak quantum efficiencies in the blue, green, and red spectra for the
three vertically stacked photodiodes. Figure 2.2 depicts the cross-sectional profile of the pixel. The top photodiode is ~400 nm beneath the silicon surface, the middle photodiode extends up to ~1 μm, and the bottom photodiode extends up to ~3 μm.

The full bio-inspired sensor comprises 1280 by 720 pixels, where each pixel contains three vertically stacked photodiodes (see Methodology). The pitch of each pixel is 7.8 μm, with 48% fill factor and well capacity of ~70 ke−. Negatively doped isolation regions are implanted between neighboring pixels to minimize electrical cross talk. These regions, resembling the black pigments between ommatidia, prevent photon-generated electron-hole pairs in one pixel from diffusing to neighboring pixels and corrupting information stored in these pixels. The integrated photodiode voltages from the three photodiodes are accessed simultaneously in a column-parallel fashion and stored in a bank of capacitors placed at the periphery of the imaging array (see Methodology).

When designing the imaging sensor’s pixel, tradeoffs between pixel pitch, number of transistors per pixel, fill factor, and readout noise performance were carefully evaluated. Today’s state-of-the-art imagers utilize four transistors per pixel with pinned photodiode to achieve sub-electron readout noise by utilizing correlated double sampling techniques (CDS) [68, 69]. However, the complex layout in implementing pinned photodiodes, floating diffusion nodes, and individual transfer transistors for each of the three vertically stacked photodiodes, would result in a pixel with prohibitively large pitch and small fill factor. Therefore, three transistors per individual photodiode are implemented to reduce the pixel pitch of this imager (Figure 2.2). Each pixel has a total of nine transistors, where the gates of the access and reset transistors are connected respectively to reduce the number of metal lines utilized within each pixel.
Difference double sampling (DDS) is implemented on chip by first sampling the integrated photodiode charges followed by sampling of the reset value on two banks of column parallel capacitors respectively. The difference between the two banks of capacitors is computed as individual elements are sequentially read out via a programmable gain differential amplifier. The final output is digitized by a 14-bit analog-to-digital converter. The dynamic range of the imager is 62 dB, with a root-mean-square readout noise of 70 e⁻ and power consumption of ~250 mW. The DDS operation reduces the voltage threshold variations between the individual pixel’s source followers and improves the spatial uniformity across the image. However, the DDS operation doubles the readout noise (~70 e⁻ in our implementation) and limits the operation of the sensor in low light illumination settings. Therefore, small pixel pitch is achieved at the expense of higher readout noise. As this imaging technology matures and feature sizes of transistors continue to decrease with advanced fabrication techniques, four transistors per pixel together with pinned photodiodes and correlated double sampling technique will be feasible and will reduce the readout noise in imaging sensors with vertically stacked photodiodes.

Figure 2.3(b) shows that the measured peak quantum efficiency (QE) for the top, middle, and bottom layers of photodiodes are 33.27% at 430 nm, 25.46% at 550 nm, and 26.23% at 620 nm, respectively. These three photodiode layers represent the three-color channels, named by their peak QE as blue, green, and red channels. The aggregated response of the color channels yields a maximum QE of 63.46% at 570 nm.

Polarization sensitivity is added to the imager with vertically stacked photodiodes by spatially modulating two 45°-shifted pairs of orthogonal pixelated polarization filters arranged in a checkerboard pattern that repeats across the imaging array. The 2 by 2 pattern has four pixelated polarization filters oriented nominally at 0°, 45°, 90°, and 135°. Figure 2.3 shows the combined
polarization filters and photodiode raw response to Malus’s law, fixed-pattern noise histograms, QE plots for the color channels, and scanning electron micrographs of the filter features and orientations. The polarization filters are realized by depositing aluminum nanowires after CMOS fabrication using optimized interference lithography and reactive ion etching (see Methodology). The aluminum nanowires are 75 nm wide and 250 nm high (Figure 2.3(c)) and have a 50% duty cycle. This high aspect ratio of the aluminum nanowires is crucial to achieve the high diattenuation ratios.

![Figure 2.3 Optical and spectral characteristics of the bio-inspired color-polarization imaging system.](image)
a) Sinusoidal response of nanowire polarization filters to Malus’s law, with fixed-pattern noise histograms at data points close to full pixel well-depth capacity. b) Quantum efficiency over the visible spectrum. c) Scanning electron micrograph of the aluminum nanowires deposited on top of the imager. d) Scanning electron micrograph showing the four pixelated filter orientations (scale bar, 2 μm). The high overall quantum efficiency is due to the combination of both shallow and deep photodetectors, which are individually optimized for maximum conversion rate at different wavelengths across the visible spectrum.
The plot in Figure 2.3(a) shows that the pixels in the imaging system exhibit sinusoidal response in accordance with Malus’s law. More specifically, the means and standard deviations of the filters’ orientations across the imaging array are -0.66±0.02°, 45.13±0.03°, 89.98±0.02°, and 135.65±0.01°, with diattenuation ratios of ~95%, which correspond to extinction ratios of ~40. These high extinction ratios are comparable to those for state-of-the-art panchromatic or monochromatic polarimeters [51, 54, 70, 71], which range between ~5 and ~50. Furthermore, it has been shown that extinction ratios as low as 3 are sufficient for reliable polarimetry, although extinction ratios above 10 are preferred for accurate polarization reconstruction [72]. Figure 2.4(d) shows that the high extinction ratio is kept across the visible spectrum of interest for each color channel. The normalized fixed-pattern noises (Figure 2.3(a) histograms) for the 0°, 45°, 90°, and 135° filters with signals close to the full dynamic range of the pixels are 1.90%, 2.10%, 2.11%, and 1.80%, respectively. State-of-the-art polarization imaging sensors have fixed pattern noise of 5% or higher [51, 54, 71] mainly due to large variations in the fabricated pixelated polarization filters. The highly optimized nanofabrication process for constructing pixelated polarization filters coupled with the difference double sampling circuitry enables the lowest fixed pattern noise for pixelated polarization cameras reported in the literature to date.

Two figures of merit are typically used to describe the polarization responsivity and sensitivity of polarization-sensitive imaging sensors: the degree of linear polarization (DoLP) and the angle of polarization (AoP) [71]. These metrics can be derived from the Stokes parameters, which themselves can be calculated from the filter-modulated light-intensity measurements. The different optoelectronic characteristics of an imaging system, such as extinction ratio, QE, and spatial noise, ultimately translate into how accurately and precisely the system can detect the polarization figures of merit across its imaging array.
Since the polarimeter is a trichromatic imaging system, the polarization metrics are computed per each color channel. To improve polarization sensitivity, a calibration scheme is utilized (see Methodology). Figure 2.4(a) and Figure 2.4(b) show the AoP and DoLP errors, respectively, when the imaging system is exposed to fully linearly polarized light with the input light’s AoP ranging from 0° to 180°. The DoLP and AoP errors are less than 1.2% and 0.18%, respectively, across the whole AoP domain for all color channels. Similarly, Figure 2.4(c) shows the DoLP error when the imaging system is exposed to partially polarized light, with the input light DoLP ranging from 0 to 1, where 0 represents unpolarized and 1 represents linearly polarized light. As the input DoLP decreases—hence, as the signal-to-noise ratio decreases—the DoLP error increases but remains below 5% even for weak polarization signatures. These results show that the color-polarization imaging system can effectively measure polarization signatures with low errors across the visible spectrum. This high polarization sensitivity of the bio-inspired sensor is essential for applications that require detecting slight changes of polarization states in biological tissue, such as label-free neural recording or cancer detection.
Figure 2.4 Optoelectronic characterization of the imaging system per color channel. a) AoP and b) DoLP errors as a function of the fully polarized input light’s AoP. c) DoLP error as a function of the partially polarized input light’s DoLP. d) Diattenuation ratio as a function of the input light’s wavelength over the visible spectrum. This high polarization sensitivity of the bio-inspired sensor is a result of the high aspect ratio of the aluminum nanowires and high quantum efficiency of the vertically stacked detectors.

Figure 2.5 shows a sample image of the co-registered color and polarization information acquired by the bio-inspired imaging system. Figure 2.5(b) shows the DoLP of the scene in a
linear false-color map, where red and blue indicate fully polarized and unpolarized light, respectively. Figure 2.5(c) shows the AoP of the scene in a circular false-color map, where red and light blue indicate horizontally (0° or 180°) and vertically (90°) polarized light, respectively. The objects included in the scene are a Macbeth color-calibration target, an orange toy race car, a silicon conical ingot, a polarization target composed of six polarization filters offset by 60°, a black plastic horse, and a few beach rocks. The Macbeth color chart shows the color reconstruction accuracy of the imaging system (Figure 2.5(a)), yielding a root-mean-square error of less than 4%. The polarization filters show high DoLP and very homogeneous AoP, as expected, due to their intrinsic properties for each of the orientations in the target. The ingot, the toys, and the rocks demonstrate the AoP dependence on shape and the DoLP dependence on both shape and material properties of the imaged targets.

Most neuroethology and sensory ecology polarization studies in marine animals have to be done outside of the animal’s natural environment: the animal of interest has to be captured and brought into a fish tank in a laboratory setting. The lack of a color-polarization camera that yields meaningful real-time, high-resolution data and is sufficiently compact to be integrated with an underwater system prohibits the study of marine animals with polarization capabilities in their natural habitat. Here, I show that the color-polarization imaging system is sufficiently compact, robust, and low power to be placed inside of an underwater camera housing to capture high-frame-rate color-polarization videos of marine life. Figure 2.6 depicts—for the first time reported in the literature—still frames captured from video of four marine animals imaged in their natural habitat. These animals exhibit polarization signatures along their bodies, and in some cases both color and polarization information is actively controlled by the animal.
Depicted are the color and polarization signatures on the antenna scales of the mantis shrimp *Odontodactylus scyllarus* (Figure 2.5 (a)), the blue maxillipeds of the mantis shrimp *Haptosquilla trispinosa* (Figure 2.5 (b)), the polarized stripes of the cuttlefish *Sepia latimanus* in two distinct color states (Figure 2.5 (c) and Figure 2.5 (d)), and the polarized stripes of the squid *Sepioteuthis lessoniana* (Figure 2.5 (e)). Observations during experiments in a laboratory setting indicate that the polarization signatures displayed by marine animals are wavelength and orientation dependent [73-75].

This phenomenon is not detectable by panchromatic or monochromatic polarimeters used in field experiments. I recorded wavelength-dependent polarization signatures from several marine animals in their natural habitat with the bio-inspired sensor. The difference between the DoLPs of the blue and red channels is ~10% for the *H. trispinosa* and ~6% for the *S. latimanus*, and these real-time measurements agree with data taken in the laboratory setting (see Supplementary
Video S2). The bio-inspired sensor can be used in the natural habitat, which will enable future studies of the hidden color-polarization marine world.

Here, I have shown that, by mimicking the mantis shrimp visual system, I have designed a compact, low-power, and highly sensitive imaging system capable of capturing co-registered color and polarization information in real time. Due to its size, low power, and ease of use, the bio-inspired sensor enables many versatile and challenging applications, such as underwater imaging, remote sensing, and various bio-medical applications, where polarization yields a body of information orthogonal to that of the color channels.

![Figure 2.6 Still frames captured from video of four marine animals underwater. Left: color images. Right: DoLP represented in a false-color maps, where red and blue indicate highly polarized and unpolarized light, respectively (see scales at right). a) O. scyllarus, with polarized antenna scales. b) H. trispinosa, with polarized blue maxillipeds. c) S. latimanus, swimming away from the diver, with polarized stripes. d) S. latimanus, in a stationary state with polarized stripes (same specimen as in c). e) S. lessoniana, with polarized stripes.](image-url)
Chapter 3: Surface normal reconstruction using circularly polarized light [33]

The polarization properties of reflected light capture important information about the object’s inherent properties: material composition, i.e. index of refraction and scattering properties, and shape of the object, i.e. surface normal. Polarization information therefore has been used for surface reconstruction using a single-view camera with unpolarized incident light. However, this surface normal reconstruction technique suffers from a zenith angle ambiguity. Here, I have utilized circularly polarized light to solve for the zenith ambiguity by developing a detailed model using Mueller matrix formulism and division of focal plane polarization imaging technology. Experiment results validate the model for accurate surface reconstruction.

3.1 Motivation for the Use of Circularly Polarized Light

The topic of surface reconstruction from polarization has seen much interest from the computer vision community [32, 36, 54, 76-83]. Light reflected from a surface changes its polarization state based on the angle of the reflection, so observing the polarization state allows for the recovery of the surface normal. The difficulty in the reconstruction problem lies in the fact that it is under constrained; the information acquired from using unpolarized light illumination from a single view is not sufficient to successfully resolve for the zenith and azimuth ambiguities. Rahmann and Canterakis [76] constrained the problem by taking images from multiple viewpoints and applied their method to specular surfaces using an iterative optimization routine. Miyazaki et al. [82] took two images and tilted the target object at a small angle in their second image to constrain the zenith angle, and propagated information at the boundary of the object to the entire image to resolve the azimuth angle. Atkinson and Hancock [80] combined polarization and shading information from stereo images to recover the surface normals from diffuse
reflections. Morel et al. [77] applied polarization images to metallic surfaces. They exploited the large index of refraction of these materials to constrain the zenith angle and developed an active lighting system to disambiguate the azimuth angle. Stolz et al. [83] use a multi-spectral system, relying on two different degrees of polarization for two different wavelengths, to solve the zenith ambiguity. Other methods [36, 78] simultaneously recover the index of refraction and surface normal. However, both methods require numerous images to solve for the various ambiguities and are computationally expensive.

All the existing methods for shape reconstructions from polarization only analyze the linear component of polarization. The contribution is to exploit the phase information of light, i.e. circular polarization properties of light, to determine shape. The phase difference between the two orthogonal components of the light wave determines the elliptical component of the polarization state. Using the elliptical component of polarization, the ambiguity of the zenith angle in Fresnel’s reflections can be resolved from a single viewpoint using a single imaging sensor. In addition, I have applied the powerful concept of Stokes vectors and Mueller matrices to analyze more complex input light sources and derive a closed-form solution for both the zenith and azimuth angles. To solve for the azimuth ambiguity, I have used a reconstruction method based on the one presented in [82]. Furthermore, I have employed a division of focal plane polarimeter capable of acquiring polarization information at every imaged frame with high spatial resolution [23]. The imaging sensor is realized by monolithically integrating pixelated nanowire polarization filters with an array of pixels that use CCD technology.

3.2 Polarization Properties of Light and Stokes Parameters
Light is a transverse wave that is fully characterized by its intensity, wavelength, and polarization [79]. Polarization of light, which defines the orientation of light waves as they
propagate through space and time, is undetectable with the unaided eye or with typical color or monochromatic imaging sensors. Nevertheless, polarization imaging is of great interest to various research communities, ranging from remote sensing \cite{79, 84, 85}, to marine biology \cite{86-90} and medical imaging \cite{35, 91-93}. The wide research interest in polarization imaging is in part because the polarization state of light changes when it interacts with a tissue, whether through reflection, transmission, or scattering \cite{87}.

Sir George Stokes was the first to propose a mathematical model representing both the polarized and unpolarized states of light in terms of observable quantities \cite{94}. He found that the properties of light can be described in terms of four quantities, termed the Stokes parameters, and are modeled via Equation (3.1):

\[ I(\alpha, \phi) = \frac{1}{2} (S_0 + S_1 \cos 2\alpha + S_2 \sin 2\alpha \cos \phi - S_3 \sin 2\alpha \sin \phi) \]  

(3.1)

This equation describes the intensity of a light beam observed through a linear polarizer rotated by an angle \( \alpha \) with respect to a reference x-axis and a retarder with a phase shift \( \phi \) and its fast axis aligned with the reference x-axis, as is shown in Figure 3.1.

![Figure 3.1 Typical setup of a polarizer rotated \( \alpha \) degrees followed by a retarder with a phase shift of \( \phi \) degrees.](image)

In Equation (3.1), \( S_0 \) describes the total intensity of the light; \( S_1 \) describes the light that is horizontally or vertically polarized; \( S_2 \) describes the light that is linearly polarized at 45\(^\circ\) or 135\(^\circ\), and \( S_3 \) describes the light that is right- or left-handed circularly polarized.
Together these four parameters form the Stokes vector \( S = [S_0 \ S_1 \ S_2 \ S_3]^T \), which can describe the polarized, partially polarized, or unpolarized state of light. From Equation (3.1), we can conclude that a minimum of four measurements must be conducted with linear polarization filters and a retarder to determine the Stokes vector. If the retardance \( \varphi \) is set to either 0º (i.e. there is no retarder in the optical path) or 90º and the angle of linear polarization, \( \alpha \), is set to 0º, 45º, 90º, and 135º, the Stokes vector can be uniquely computed as presented by Equations (3.2) through (3.5).

\[
S_0 = \frac{1}{2} (I(0º, \varphi) + I(45º, \varphi) + I(90º, \varphi) + I(135º, \varphi)), \quad \varphi \in [0º, 180º] \tag{3.2}
\]

\[
S_1 = I(0º, 0º) - I(90º, 0º) \tag{3.3}
\]

\[
S_2 = I(45º, 0º) - I(135º, 0º) \tag{3.4}
\]

\[
S_3 = I(135º, 90º) - I(45º, 90º) \tag{3.5}
\]

Three additional quantities are computed from the Stokes vector: the Degree of Linear Polarization (DoLP), the Degree of Circular Polarization (DoCP), and the Angle of Polarization (AoP), which are defined by Equations (3.6) through (3.8).

\[
DoLP = \frac{\sqrt{S_1^2 + S_2^2}}{S_0}, \quad DoLP \in [0, 1] \tag{3.6}
\]

\[
DoCP = \frac{S_3}{S_0}, \quad DoCP \in [-1, 1] \tag{3.7}
\]

\[
AoP = \frac{1}{2} \arctan \left( \frac{S_3}{S_2} \right), \quad AoP \in [0º, 180º] \tag{3.8}
\]

The DoLP describes the fraction of the light which is linearly polarized, where 0 indicates unpolarized light; 1 represents linearly polarized light and any number between 0 and 1 represents partially linearly polarized light. The DoCP describes the fraction of the light that is
circularly polarized: left-handed circularly polarized light has negative values, while right-handed circularly polarized light has positive value. For values between -1 and 1, light is elliptically polarized with an exception of DoCP = 0 indicating no circular or elliptical component in the light. The AoP describes the orientation of the major axis of the polarization ellipse, formed by linearly and circularly polarized light, relative to the reference axis.

3.3 Shape from Polarization

3.3.1 Geometrical model
Light reflected from the surface of an object changes its polarization state based on the angle of the reflection and the index of refraction of the object [32]. We are interested in uniquely determining the surface normal orientation with respect to the camera position by analyzing the polarization state of the reflected light from an object. The surface normal orientation is given by the zenith angle, \( \theta_i \), which is equivalent to the angle of incidence, and the azimuth angle \( \varphi \) as shown in Figure 3.2.

![Figure 3.2 Light reflection on a specular surface.](image)
The Snell-Descarte’s law, presented by Equation (3.9), describes the relation between the ratio of
the angles of incidence \( \theta_i \) and refraction \( \theta_r \) and the ratio of the indices of refraction of the object
\( n_2 \) and the incident medium \( n_1 \). If medium one is air, then \( n_1 \) is approximately 1.

\[
\frac{\sin \theta_i}{\sin \theta_r} = \frac{n_2}{n_1}
\]  

(3.9)

### 3.3.2 Mueller matrices

When a light beam interacts with an object or medium, the polarization state of the output light
(reflected or refracted) is always changed. The polarization signature can change in the
amplitude of the orthogonal components of the electromagnetic wave, as well as their relative
phases and directions. This interaction can also change the total amount of light that is polarized,
either linearly or circularly, by transferring energy from a polarized state to an unpolarized state
and the other way around. This interaction can be modeled by a set of linear equations
represented by a 4-by-4 matrix called the Mueller matrix [32]. This means that Mueller matrices
are designed to represent any changes in the polarization signature caused by a physical
interaction or a geometrical transformation, such as coordinate system rotations. Equation (3.10)
shows this interaction, where \( \mathbf{M} \) is the Mueller matrix, \( \mathbf{S}_{in} \) is the Stokes vector of the incident
light, and \( \mathbf{S}_{out} \) is the Stokes vector of the resulting light.

\[
\mathbf{S}_{out} = \mathbf{M} \cdot \mathbf{S}_{in}
\]  

(3.10)

The Mueller matrix for reflection at an air-object interface is given by Equation. (11). Detailed
discussion on Muller matrix for light reflections and refraction can be found elsewhere [23, 32].

\[
\mathbf{M}_r(\theta_i, \theta) = \frac{1}{2} \left( \frac{\tan \theta_i}{\sin \theta} \right)^2 \begin{pmatrix}
\cos^2 \theta_i + \cos^2 \theta & \cos \theta_i \cos \theta & 0 & 0

\cos \theta_i \cos \theta & \cos^2 \theta_i - \cos^2 \theta & 0 & 0

0 & 0 & -2 \cos \theta_i \cos \theta & 0

0 & 0 & 0 & -2 \cos \theta_i \cos \theta
\end{pmatrix}
\]  

(3.11)

Where \( \theta = \theta - \theta_i \), \( \theta = \theta + \theta_i \).
To compute the Stokes vector of the reflected light from an object at any orientation, it is necessary to first rotate the input Stokes vector to the same coordinate system as the object tangent plane by an angle $\theta$. This rotation is achieved via the Muller matrix presented by Equation (3.12) [32]. When light is reflected this rotation is represented by the azimuth angle, $\varphi$, of the surface.

\[
M_{rot}(\theta) = \begin{pmatrix} 1 & 0 & 0 & 0 \\ 0 & \cos 2\theta & \sin 2\theta & 0 \\ 0 & -\sin 2\theta & \cos 2\theta & 0 \\ 0 & 0 & 0 & 1 \end{pmatrix}
\]  

Using Equations (3.10) through (3.12) we can write the relation between a light beam with a Stokes vector $S_{in}$ that is reflected on a rotated tangent surface by an angle of $\theta$ with an incident angle $\theta_i$ and refraction angle $\theta_r$ and the output Stokes vector captured by the camera $S_{out}$ as shown in Equation (3.13).

\[
S_{out} = [M_{rot}(-2\theta)M_R(\theta, \theta_i)M_{rot}(2\theta)]S_{in}
\]  

The use of Mueller matrices and Stokes vectors to represent the change in polarization from the light beam coming from a light source that is reflected on an object and then captured by the polarimeter allows us to have a simple closed form representation of how the geometrical object model interacts with the metrics of polarization of light. Furthermore, this approach allows analysis of polarization of light using more complex light sources rather than completely unpolarized light as has been the case in previous works [36, 76-78, 80, 82, 83].

### 3.3.3 Zenith ambiguity

If the input Stokes vector, $S_{in}$, happens to be unpolarized, $S_{in} = [1,0,0,0]$, then using Equations (3.6) through (3.8) we can find the DoLP and AoP of the output Stokes vector, $S_{out}$, as a function...
of $\theta$, $\theta_i$, and the object index of refraction as shown in Equations (3.14) and (3.15). However, the DoCP metric for $S_{out}$ will always be zero regardless of the object properties.

\[ AoP = \theta \]  \hspace{1cm} (3.14)

\[ DoLP = \frac{2 \sin \theta \tan \theta_i \sqrt{n^2 - \sin^2 \theta}}{n^2 - 2 \sin^2 \theta_i + \tan^2 \theta_i} \]  \hspace{1cm} (3.15)

The first problem with using unpolarized light as the input light source is that the zenith angle cannot be mapped to a DoLP function, since there is a double ambiguity on $\theta_i$ everywhere in the zenith domain but the Brewster angle (BA), which is defined as the point where the reflected light beam has a DoLP of 1, as shown in Figure 3.3. For this reason, previous works have been restricted to work with objects with a high index of refraction to move the Brewster angle closer to 90° [95], reconstruct convex objects with small zenith angles in order to stay on the left side of the ambiguity [96], or use more than one scene view [80, 82].

![Figure 3.3 In dashed red DoLP vs zenith angle $\theta_i$ showing the ambiguity. In solid blue DoCP vs DoLP with the light source having an $S_3$ component of -1. Brewster angle is located at 57.99° for an object with an index of refraction of 1.60.](image)
In Figure 3.3 there is an ambiguity in solving for the zenith angle, \( \theta_i \), using the DoLP (dashed red line) but none with the DoCP (solid blue line). The Brewster angle is located where the DoCP crosses zero at 57.99°.

If the input Stokes vector, \( S_{in} \), is partially circularly polarized, \( S_{in} = [1,0,0,p] \) where \( p \) is the normalized \( S_3 \) component of the light source, the equations for the AoP and DoLP metrics remain the same as in Equations (3.14) and (3.15), when we use completely unpolarized light. However, the DoCP has an interesting curve over the zenith angle with no ambiguity present in the figure. The DoCP expression as a function of the zenith angle and a particular index of refraction is shown in Equation (3.16). For Equation (3.16) the DoCP has a range from \(-p\) to \(p\) with \( p \in [-1,1] \), and it is zero at the Brewster Angle.

\[
S_{in} = \begin{bmatrix} 1 & 0 & 0 & p \end{bmatrix}^T \rightarrow \text{DoCP} = -\frac{2p \cos \theta \cos \theta_i}{\cos^2 \theta + \cos^2 \theta_i}
\]

Where \( \theta_\perp = \theta_i - \theta_r \), \( \theta_r = \theta_i + \theta_r \)

\[
\theta_i = \arcsin \left( \frac{\sin \theta_i}{n} \right) \quad \text{for an air-object medium}
\]

Equation (3.16) gives a closed-form solution for the zenith angle without any ambiguity as long as the index of refraction of the object material is known and the light source has a known polarization signature.

### 3.3.4 Azimuth ambiguity

The azimuth angle \( \varphi \) represents the slant on the normal surface, or the orientation of the plane of incidence. That orientation has a direct relation to the angle that the plane has been rotated as expressed by Equation (3.17).

\[
\varphi = \theta \pm 90^\circ
\]

If light is partially circularly polarized or completely unpolarized then using Equation (3.14) we can express the azimuth angle as an expression of the AoP as shown in Equation (3.18).
\[ \varphi = \text{AoP} \pm 90^\circ \]  
\hspace{1cm} (3.18)

We can notice that the ambiguity is symmetrical by 180º and that if we have a continuous surface the change in ambiguity should happen when the AoP is 90º or the azimuth angle is 0º or 180º. In order to solve for this ambiguity a similar approach to the one in [82] is taken; one pixel in each column of the image is initialized to a known solution of the ambiguity. This pixel serves as a seed pixel, and the algorithm propagates across the rest of the pixels in the same column in the following manner: the azimuth angle \( \varphi \) is calculated in its vertical neighbors using both hypotheses, adding 90º or subtracting 90º, and it’s compared to the previous known \( \varphi \) value, where the algorithm starts at the seed point. Since we assume that the surface of interest is smooth and continuous, the azimuth angle with the smaller square difference gets selected and the algorithm continues to the next neighbor. The reason we are choosing to initialize one pixel in each column rather than a pixel in each row is that the camera x-axis should be parallel to the imager rows, therefore making the change of ambiguity across the vertical direction. Certainly, there could be some noise in the image that could result in a wrong estimation of \( \varphi \) but this can be compensated for by initializing more than one contiguous vertical pixel in each column to compute the difference in a neighborhood tail rather than a single pixel and accurately specifying the border or discontinuities of the object, which can be done with a grow region algorithm in the intensity domain.

### 3.3.5 From azimuth and zenith angles to surface reconstruction

To reconstruct the object surface we have to make the assumption that the surface is a Cartesian one, defined by Equation (3.19).

\[ z = f(x, y) \]  
\hspace{1cm} (3.19)
If a transformation from spherical to Cartesian coordinates is performed, using the azimuth and zenith angles, the surface normal can be expressed via Equation (3.20).

\[
\mathbf{n} = \begin{bmatrix}
\frac{\partial f(x,y)}{\partial x} & \frac{\partial f(x,y)}{\partial y} & 1
\end{bmatrix} = \begin{bmatrix}
\tan \theta \cos \varphi \\
\tan \theta \sin \varphi \\
1
\end{bmatrix}
\]  

(3.20)

The goal is to reconstruct the objective function defined by Equation (3.19) by integrating the surface normals at each pixel location. To perform this step, surface is assumed to be continuous and derivable over the x and y domain. We also must consider the noise induced by the polarimeter measurements, where if we assume that the noise is Gaussian, we need to minimize the least-square cost function, shown in Equation (3.21) in its continuous form:

\[
\epsilon = \iint_{\mathcal{D}} \left( \hat{z}_x(x,y) - z_x(x,y) \right)^2 + \left( \hat{z}_y(x,y) - z_y(x,y) \right)^2 \, dx \, dy
\]

Where \( \hat{z}_x \) and \( \hat{z}_y \) represent the measured gradient

Equation (3.21) represents the volume of the squared differences of the functions, therefore the discrete form over a rectangular grid, such as the one represented by the imager grid is shown in Equation (3.22):

\[
\epsilon = \| \hat{Z}_x - Z_x \|_F + \| \hat{Z}_y - Z_y \|_F
\]

(3.22)

Where the subscript “F” denotes the Frobenius norm. The algorithm to minimize the least-square cost function is explained in [97]. There are several other algorithms that can solve the surface normals problem to output a reconstructed surface, the most common being the Frankot-Chellapa algorithm [98]. The Frankot-Chellapa algorithm is computationally fast since it relays on computationally efficient Fast Fourier transforms; however it suffers from being restricted to surfaces with a zero mean slant [77], while the solution proposed in [97] does not make this
assumption. In addition, this algorithm minimizes the error induced by the noise by the polarimeter, which leads to aberrations that propagate over the whole surface.

3.4 Polarimeter Technology and Experimental Setup

A typical polarization imaging setup is composed of rotating linear polarization filters and/or rotating quarter-wave retarders. This imaging apparatus, known as a division of time polarimeter [46], provides very accurate polarization information at the cost of reduced frame rate. Furthermore, if there is motion in the scene, the object/target of interest will move across the several different sample images with different polarization filters. Hence, the polarization information will be inaccurate due to pixel misregistration between frames. Division of focal plane (DoFP) imaging sensors combine pixelated polarization filters monolithically with an array of imaging elements [54]. This imaging sensor has the benefit of capturing all polarization information in a single frame and does not suffer from motion artifacts as do division of time polarimeters. The DoFP image sensor works by grouping its pixels into 2x2 super-pixels and covering the four pixels with linear polarizers at orientations of 0° (upper left), 45° (upper right), 90° (lower right), and 135° (lower left) as shown in Figure 3.4. Loss in spatial resolution caused by the DoFP filter pattern can be recovered through interpolation and denoising algorithms, where the inherent noise of the sensor is included in the model for improved reconstruction results [99, 100]. Another DoFP approach is to include a pixelated quarter wave retarder (QWR) within the super-pixel configuration in order to extract all four Stokes parameters in a single snapshot [101].
Figure 3.4 DoFP CCD polarimeter with an overlaid pixelated polarization filter.

For the surface reconstruction work in here, a partially complete Stokes vector DoFP imaging sensor was utilized. The sensor is achieved by monolithic integration of pixelated aluminum nanowire linear polarization filters with an array of CCD imaging elements. The pixel pitch of the aluminum nanowire filters is matched to the pixel pitch of the CCD camera, which is 7.4 microns. The aluminum nanowires are 70 nm wide with 70 nm air gap between adjacent wires and they are 140nm tall. The nanowires are ~6.4 microns long, leaving 1 micron “dead” space between adjacent pixels. The CCD polarization imager is 1900 by 1600 pixels and operates at 40 frames per second. Detailed characterization of the opto-electronic performance of the imager is presented in [71]. The sensor is calibrated using a Muller matrix approach as described in [60].
To capture the circular polarization properties of light, the fourth Stokes parameter $S_3$, a QWR is mounted in front of the imaging sensor with its fast axis aligned to the camera’s $x$-axis as is shown in Figure 3.5. To avoid problems in intensity normalization between measurements caused by the non-perfect transmission ratio of the QWR, $S_3$ is normalized using the computed total intensity $S_0$ when $\phi$ is $90^\circ$, as presented by Equation (3.2).

The object of interest is placed inside of a cylindrical structure made of achromatic circular polarizer sheets. The cylinder has a small aperture for the camera to look inside. The cylinder is inside of a diffusing cloth which also has an opening from one side for the camera to image inside it. The setup is designed in such a way that the light illuminating the object passes first.
through the diffusing cloth and then through a circular polarizer sheet. To avoid artifacts caused by shadows or reflections the whole setup is suspended in the air and white fluorescent light illuminates the setup from different perspectives. The DoFP polarimeter uses Canon EF lenses, with focal lengths ranging from 18 mm to 100 mm.

Figure 3.6 presents a monochromatic image, angle of polarization (AoP), degree of linear polarization (DoLP), and degree of circular polarization (DoCP) obtained from the polarization sensor. The polarization information is calibrated using a Mueller matrix calibration procedure described in [60]. The scene shows a PET plastic bottle, with an index of refraction of 1.64. The angle of polarization image is presented using a false color scheme, where red represents horizontally polarized light, i.e. 0 degree of the angle of light oscillations and blue color represents vertically polarized light, i.e. 90-degree angle of light oscillations. The angle of polarization maps directly to the azimuth angle after providing the seeding pixels in the image. The degree of linear polarization image is presented using false color scheme, where blue depicts low degree of linear polarization and red depicts high degree of linear polarization. The degree of linear polarization has the highest value at and around the Brewster angle of the object. Figure 3.7 depicts a profile plot of the DoLP across a single line traversing on the bottle as shown in Figure 3.6(c). The peak of the DoLP can be observed as well as the ambiguity of uniquely solving for the zenith angle from the DoLP.

The degree of circular polarization image is depicted in false color as well, where blue color represents left circularly polarized light and red color represents right circularly polarized light. The Brewster angle of the object is depicted by a black contour plot around the bottle by examining where the DoCP is zero. Figure 3.7 shows a profile plot of the DoCP on the same line.
traversing the bottle, shown in Figure 3.6(d), demonstrating the unique mapping of zenith angle from the DoCP measurement.

Two regions, A and B, shown in Figure 3.6(b) and Figure 3.6(d) are selected for performing surface reconstruction. Figure 3.8(a) and Figure 3.9(a) show the computed azimuth angle in regions B and A respectively, where region B crosses the ambiguity region. Figure 3.8(b) and Figure 3.9(b) show the computed zenith angle in regions B and A respectively, with a black contour plot showing the location of the Brewster angle. Figure 3.8(c) and Figure 3.9(c) show the 3-D reconstruction in regions B and A respectively from of the plastic bottle. The smooth variations of the reconstructed surface are indicative that the surface normal computed from the polarization information is accurate.

Figure 3.10 presents surface reconstruction of a spherical HDPE object with an index of refraction of 1.54 using the method described here. Spherical objects have continuous variations in AoP, DoLP, and DoCP, covering most of the function domain described by Equation (3.19). The region C selected to reconstruct is shown in Figure 3.10(b) and Figure 3.10(d). Figure 3.11(a) shows the computed azimuth angle, where the algorithm previously described successfully resolves the ambiguity. Figure 3.11(b) shows the computed zenith angle where again the slant angle is correctly characterized. Figure 3.11(c) shows the reconstructed sphere on the region C selected.
Figure 3.6 Scene showing a PET plastic bottle. (a) Monochromatic picture of the reconstructed scene. (b) AoP in false color. (c) DoLP in false color. (d) DoCP in false color with a black contour plot pointing to the location of the Brewster angle. (b) & (d) Black rectangles pointing to the regions A and B to be reconstructed. (c) & (d) Dotted line showing the location of the profile plot in Figure 3.6.
Figure 3.7 (a) DoLP vs zenith angle, $\theta_i$, cross profile plot of scene shown in Figure 3.6. (b) DoCP vs zenith angle, $\theta_i$, cross profile plot of scene shown in Figure 3.6. Dotted black line shows the location of the Brewster angle. Equation (3.16) was used to compute the zenith angles. Each data point represents the information computed on a single pixel.

Figure 3.8 (a) Azimuth angle map in false color in reconstructed region B. (b) Zenith angle map in false color with a black contour plot pointing to the location of the Brewster angle in reconstructed region B. (c) Orthographic projection of the reconstructed surface in region B.
Figure 3.9 (a) Azimuth angle map in false color in reconstructed region A. (b) Zenith angle map in false color with a black contour plot pointing to the location of the Brewster angle in reconstructed region A. (c) Orthographic projection of the reconstructed surface in region A.

Figure 3.10 Scene created by a HDPE blue sphere. (a) Monochromatic picture of the reconstructed scene. (b) AoP in false color. (c) DoLP in false color. (d) DoCP in false color. (b) & (d) Black rectangles pointing to the regions A and B to be reconstructed.
The algorithm used to disambiguate the azimuth angle depends on the surface being continuous (with the same index of refraction) and having a seed pixel on each column where the ambiguity is known. A figure showing all disambiguated azimuth angles is not possible unless the surface to be reconstructed is larger than the camera field of view. For Figure 3.6, the PET plastic bottle, the scene contains a bottle cap (with a different index of refraction) in the middle, which makes the surface not continuous.

### 3.5 Error Calculation

To evaluate the accuracy of the method combined with the polarimeter technology used I designed an experiment to measure the root-mean-square errors (RMSE) of the DoCP and zenith angle estimations. The experiment consisted of mounting a flat black plastic surface, with an index of refraction of approximately 1.4, on a rotational stage (Thorlabs NR360S) inside of the experimental setup as shown on Figure 3.12. The stage’s rotational axis was parallel to the camera’s x-axis, and the plastic plane was orthogonal to the stage’s plane. The stage and plastic plane were aligned such that the plastic plane would be parallel to the camera’s sensor plane at the initial stage position (zero degrees).
The experiment consisted on rotating the stage, via computer, to change the incident angle on the plastic plane viewed by the camera and estimating the DoCP and zenith angle at each rotation using the chapter’s method. The stage rotated from 0º to 85º in steps of 5º. Figure 3.13(a) shows the DoCP vs true incident angle response with an RMSE of 0.0005 while Figure 3.13(b) shows the zenith angle measured error with an RMSE of 0.1150º.

3.6 Conclusion
I have introduced a method for estimating surface normals from a single camera view using circular polarization information if the index of refraction of the imaged object is known.
Conversely, the imaging method can estimate the index of refraction of an object is the shape is known a priori. Future work will address the limitations of reconstructing surface normal reconstruction for objects with known index of refraction and include objects with multiple indices of refractions or unknown index of refraction. Also detailed modeling of the scattering properties of the object can further expand this method to broader class of objects.

The main contributions are twofold: a mathematical framework for polarization based surface normal reconstruction and utilizing circular polarization to uniquely solve the zenith angle of the surface normal. The mathematical framework presented here utilizes Mueller matrices and Stokes vectors to model polarization properties of the incident light source, the object’s inherent properties (surface and index of refraction), and the polarization state of the reflected light.

Utilizing circularly polarized incident light and capturing the circular polarization properties of the reflected light, a unique solution for the zenith angle of the surface normals is computed.

Experimental images are obtained using a unique imaging sensor composed of a CCD pixel array with aluminum nanowires for polarization sensitivity.
Polarization imaging can reveal orthogonal information with respect to color about the structural composition of biological tissue, and with the advance of superior polarimeters its use for biomedical applications has proliferated in the last decade. Polarimetry can be used in pre-clinical and clinical settings for the early detection of cancerous tissue. Polarization-based endoscopy with the complementary near-infrared fluorescence imaging modality improves the early diagnosis of flat cancerous lesions in colorectal tumor models. With the development of new polarization sensors, the need to use standard laboratory optics to create custom imaging systems increases. These additional optics can behave as polarization filters effectively degrading and modifying the original tissue’s polarization signatures leading to erroneous judgments. Here, I present a framework to characterize the spectral and polarization properties of rigid endoscopes for polarization-based endoscopic imaging. I describe and evaluate two calibration schemes based on Mueller calculus to reconstruct the original polarization information. Optical limitations of the endoscopes and minimum polarimeter requirements are discussed that may be of interest to other researchers working with custom polarization-based imaging systems.

4.1 Motivation for endoscopic calibration
The polarization state of light has been demonstrated to carry paramount information about the intrinsic properties of objects, tissues, and media with which light interacts [32]. The polarization properties of light are essentially a fingerprint of its past optical interactions. These properties can reveal the structural composition and shape of the object that reflected or refracted the light
beam, and the composition of the medium through which the light propagated. Polarization signatures hold orthogonal information with respect to the other fundamental properties of light, i.e. intensity and wavelength. Although the unaided human eye lacks the ability to differentiate polarization states, a vast range of polarization-sensitive imagers or polarimeters have been developed by combining polarization filters with polarization-blind photodetectors. To realize these sensors, polarization filters are modulated either in time, light amplitude, or focal plane [46, 54, 103].

Polarization imaging sensors have enabled a wide range of applications, such as fingerprint detection, shape reconstruction, underwater target recognition, contrast enhancement in hazy conditions, material detection, and many others [33, 34, 36, 104-107]. In the biomedical arena, polarization imaging has been used to discriminate cancerous vs. healthy cells and colorectal cancer detection, to identify stress in ligaments and tendons, to extend the capability of optical coherence tomography, and more [23, 35, 48, 108-110]. For example, due to the differences in nuclei size in healthy vs. cancerous tissue, circularly polarized light is utilized for non-invasive diagnosis of lung cancerous tissue [43].

Minimally invasive surgeries have the benefit of faster recovery, less post-operative pain and discomfort, shorter hospital stay, and lower morbidity compared to regular surgeries. Incorporating spectral and polarization imaging in endoscopes can greatly enhance the surgical outcome. Charanya et al. reported an endoscopic imaging technique which combines three imaging modalities for the detection of flat lesions in colitis-associated cancer [25]. Colitis-associated cancer arises from premalignant flat lesions in the colon. These flat lesions, which blend seamlessly with healthy tissue, are often misdiagnosed during colonoscopy utilizing intensity and color-based endoscope systems. To mitigate these false negatives, a color-
polarization-fluorescence endoscope system was reported, which improved the overall sensitivity and specificity in identifying and differentiating pre-cancerous and cancerous from healthy tissue. Figure 4.1 shows a gray scale image (left) and a polarization image (right) in a linear false-color map for an in vivo polarization endoscopy of an adenomatous tumor in a mouse. Red areas indicate a higher degree of polarization while dark blue areas indicate a lower degree of polarization. A higher polarization is associated with healthy tissue while lower polarization signatures indicate possible tumor locations. Polarization-based endoscopic imaging has also enabled biomechanics studies by evaluating stress in tendons and ligaments under circular polarized light [23], multi-spectral studies by detecting micrometer sized particles using elastic light scattering spectroscopy [111], and more. Analogously, near-infrared fluorescence image guided surgery for colonoscopy with tissue biomarkers has demonstrated diagnostic yield superior to that of white-light colonoscopy [112-114].

As more biomedical applications emerge which utilize the polarization imaging modality, the need increases for compact, flexible, and versatile polarimeters, i.e. polarization cameras that are ready to be integrated into a custom optical setup similarly to other industrial-grade scientific instruments. In response to this demand, both research groups and industry have developed adaptable and precise polarimeters with standards similar to those of the traditional color sensor industry. These off-the-shelf polarization-sensitive camera systems offer the ability to couple with existing laboratory optics, such as camera lenses or endoscopes, to create tailored imaging setups for specific applications. Nevertheless, this versatility can jeopardize the integrity of the polarization states of interest due to the use of non-inert polarization optics that behave as polarization filters. Optical elements within endoscopes can modify or degrade the original polarization signatures, and even create unreadable or unrecoverable polarization states for linear
polarimeters. It is crucial to perform optical evaluation of each additional optical component used in the polarization imaging modality and extract its Mueller matrix, which models the polarization behavior of an individual element. Although some work has been reported in the literature to characterize rigid endoscopes, little effort has been made to outline and evaluate calibration schemes that thoroughly correct for the induced polarization effects caused by the endoscopes’ optical elements [115, 116]. Comprehensive calibration schemes are needed, and their performance has to be properly evaluated to assess their polarization reconstruction capabilities. As a result of this shortcoming, modified endoscopes have been utilized to mitigate the polarization effects of their optics instead of employing polarization calibration methods [111]. These custom endoscopes are still far from behaving as ideal inert polarization elements and sacrifice the ability of choosing off-the-shelf commercial and clinically approved endoscopes.

Here, a framework is presented to evaluate, characterize, and calibrate polarization and spectral properties of rigid endoscopes, which are widely used in endoscopic pituitary surgeries, laparoscopic surgeries, orthopedic surgeries, endoscopic biopsies, brain tumor resections, and more [117-121]. I describe two polarization calibration schemes based on Mueller matrix theory and machine learning algorithms and provide experimental data demonstrating the benefits of these methods in polarization imaging. In addition, I provide spectral response, optical resolution, depth of field (DOF), and modulation transfer function (MTF) for two commercially available rigid endoscopes: (1) 5 mm x 30cm (WA50372B, Olympus Germany) and (2) 10 mm x 33 cm (WA53000A, Olympus Germany). These rigid endoscopes include an input light port which directs the illumination to the imaged object through optical fibers and a series of relay lenses which transmit the image captured by the objective lens at the distal tip to the eyepiece at
the proximal end. While the endoscopes are intended for use primarily in reflection-based imaging, the calibration methods presented here work in either transmission- or reflection-based imaging since no assumptions about the input light are made. These endoscopes are the bedrock for many small and medium animal studies and are used in minimally invasive surgeries in human patients. Hence, correct polarization signatures and in-depth understanding of the optical performance of these endoscopes will help scientists and physicians to choose the endoscope for their applications. Furthermore, the polarization calibration schemes presented here can be applied to any endoscope or other instrument utilized for polarization imaging, which will enable correct collection and interpretation of polarization information.

![Figure 4.1](image.png)

Figure 4.1 Gray scale image (left) and a polarization image (right) in a linear false-color map for an in vivo polarization endoscopy of an adenomatous tumor in a mouse. Red areas indicate a higher degree of polarization while dark blue areas indicate a lower degree of polarization. A higher polarization is associated with healthy tissue while lower polarization signatures indicate possible tumor locations.

4.2 Experimental Setup and Theory

4.2.1 Polarization Theory Overview

Light as an electromagnetic wave can be described with three fundamental properties: (1) intensity, (2) wavelength, and (3) polarization. Intensity and wavelength can be interpreted as brightness and color respectively, while polarization describes the phase between the orthogonal components of the electric field as light propagates through time and space. A random phase
indicates that the light is completely unpolarized, while a phase ranging from $-\pi/2$ to $+\pi/2$ indicates that the light is elliptically polarized with the special cases of being circularly polarized at the extremes and linearly polarized at a null phase. Polarization refers to the aggregated photon result, with partial polarization being the most common case in nature. The Stokes vector is commonly used to describe the polarization state of the light wave and it is composed of four parameters: (1) $S_0$ describes the light intensity, (2) $S_1$ describes the portion of the light that is either horizontally or vertically polarized, (3) $S_2$ describes the portion of the light that is diagonally polarized, and (4) $S_3$ describes the portion of the light that is circularly polarized. The Poincaré sphere serves as a visualization tool for the Stokes vector in a three-dimensional Cartesian coordinate system. The x, y, and z axes are represented by the $S_1$, $S_2$, and $S_3$ parameters respectively. Any possible polarization state can be placed as a data point inside or on the surface of the Poincaré sphere with center at the origin and radius $S_0$. Data points on the sphere’s surface are fully polarized, while data points inside the sphere are partially polarized. Since silicon-based photodetectors cannot measure the polarization properties of light directly, multiple light measurements employing a set of polarization filters are needed. This set commonly consists of linear polarization filters and retarders which are used to modulate the light intensity of the measured light as a function of the incoming polarization state. Equation (1) shows the relationship between an incoming Stokes vector and the measured intensity after the light has gone through a retarder with retardance $\phi$ and a linear polarizer with a rotation angle of $\theta$, in that order. As shown in Equation (4.1), four independent measurements are enough to solve for the four Stokes parameters; however, an over-constrained system is usually preferred to reduce the measurement noise.
\[
I(\theta,\phi) = \frac{1}{2} \left( S_0 + S_1 \cos 2\theta + S_2 \sin 2\theta \cos \phi + S_3 \sin 2\theta \sin \phi \right)
\]  

(4.1)

Other figures of merit are also used to describe particular characteristics of a polarization state. The degree of polarization (DoP) is the distance, on the Poincaré sphere's coordinate system, from the data point to the origin, normalized with respect to \( S_0 \). Similarly, the degree of circular polarization (DoCP) is the distance from the data point to the \( S_1S_2 \) plane normalized by \( S_0 \), and the degree of linear polarization (DoLP) is the magnitude of the data point's projection to the \( S_1S_2 \) plane normalized by \( S_0 \). Lastly, the angle of polarization (AoP) is half the data point's azimuth angle [32].

### 4.2.2 Polarization Characterization

The optical setup, shown in Figure 4.2, was used to create a collection of different incoming polarization states which are measured before and after going through the endoscopes. The optical setup comprises three parts: (a) the Stokes vector generator, (b) the endoscope under evaluation, and (c) the polarization analyzer or polarimeter. The Stokes vector generator was built with (1) a DPSS Green (532 nm) laser (GL532T3-200, Shanghai Laser & Optics Century), (2) an integrating sphere (819D-SF-4, Newport), (3) an adjustable iris (SM2D25, Thorlabs), (4) an aspheric collimating lens (ACL7560, Thorlabs), (5) a 532 nm laser clean-up filter (LL01-532-12.5, Semrock), (6) a linear polarizer (20LP-VIS-B, Newport), (7) a zero-order quarter wave retarder at 532 nm (20RP34-532, Newport), and (8) two nano-rotator stages (NR360S, Newport), one for the linear polarization filter and one for the quarter wave retarder. The analyzer was built with (1) a quarter wave retarder, (2) a linear polarizer, (3) two rotation stages, and (4) a calibrated photodiode (S130C, Thorlabs) driven by (5) a power meter (PM100D, Thorlabs), with elements (1) through (3) being identical to those used to generate the Stokes vectors.
analyzer setup, the retarder and polarizer can switch places to effectively create zero retardance while keeping the same transmission ratio.

![Optical setup for polarization characterization.](image)

**Figure 4.2** Optical setup for polarization characterization.

### 4.2.3 Polarization Calibration

The Mueller matrix is a powerful mathematical tool that describes any optical component that may reflect, refract, or scatter the light and thereby change the initial polarization state of incoherent light. By the same principle, it is possible to reconstruct an unknown input Stokes vector if the optical component’s Mueller matrix and the output Stokes vector are known. This relation is shown in Equation (4.2), where the 4-by-4 matrix $M$ is known as the Mueller matrix.

$$ S_{out} = M \cdot S_{in} $$

(4.2)

Two learning algorithms are presented here that enable correct estimation of the Mueller matrix for two rigid endoscopes widely used in clinical and pre-clinical settings.

**Modeling the rigid endoscopes as double rotated retarders**

Mueller matrices for ideal polarization components, e.g. linear polarizers, retarders, rotators, or diattenuators, are well documented in the literature [32]. For more complex optical elements with multiple polarization effects, which can be difficult to interpret from a single matrix, it is possible to decompose their non-degenerate Mueller matrix into a set of simpler Mueller matrices describing single optical effects. These effects can be decomposed into depolarization, retardance, and diattenuation [122]. We can hypothesize that the birefringent crystals at the input
and output endoscope windows of a rigid endoscope can be modeled as two rotated retarders.

This hypothesis is based on the fact that the DoP calculated on the output data for both endoscopes is virtually one, meaning no depolarization occurs in the endoscope, and that the endoscopes’ optics contain minimal diattenuation effects. It is possible to replace the endoscope crystals with fused silica or other non-birefringent materials to create a customized endoscope; however, the intention of this method is to calibrate off-the-shelf unmodified commercial endoscopes that can be easily implemented in clinical trials and require no further approval by a regulatory body, e.g., the U.S. Food and Drug Administration (FDA). The Mueller matrix of a rotated retarder is presented in Equation (4.3), where $\theta$ represents the retarder’s rotation angle and $\phi$ represents the retardance or phase shift.

$$M(\phi, \theta) = \begin{pmatrix}
1 & 0 & 0 & 0 \\
0 & \cos^2 \theta + \cos \phi \sin^2 \theta & \cos \phi \sin \theta & -\sin \phi \sin \theta \\
0 & \cos \phi \sin \theta & \cos \phi \cos \theta & \sin \phi \cos \theta \\
0 & \sin \phi \sin \theta & -\sin \phi \cos \theta & \cos \phi 
\end{pmatrix}$$

(4.3)

The training algorithm consists of finding the four angle terms that minimize the error between the training Stokes vector sets $S_{out}$ and $S^*_{out}$. Equations (4.4) and (4.5) define $S^*_{out}$ and the error between two sets of Stokes vectors, respectively.

$$S^*_{out} = M\left(\phi_1^*, 2\theta_1^*\right) \cdot M\left(\phi_2^*, 2\theta_2^*\right) \cdot S_{in} = M_{endoscope} \cdot S_{in}$$

(4.4)

$$\text{error} = \frac{\sum_{i=1}^{n} \left\| S_{1,i} - S_{2,i} \right\|}{n}, \text{ for } n \text{ data points}$$

(4.5)

To find the parameters that minimize this error function, the algorithm first does a four-dimensional coarse grid search with the objective of finding a good pair of initial guesses. After acquiring the initial search points, the Broyden-Fletcher-Goldfarb-Shanno (BFGS) Quasi-Newton algorithm [123], an off-the-shelf optimization algorithm, was used. The grid searches...
revealed that there is only one local minimum for each endoscope on the non-redundant range of the angle parameters. Hence, more sophisticated optimization algorithms are not needed. Once the four angle parameters—and thus the endoscope’s Mueller matrix—are found, its pseudo-inverse can be calculated and used to compute the input Stokes vectors.

**Linear regression with cross validation**
Linear regression algorithms are typically used to extract more robust and unconstrained models constructed from a dataset. This approach has the benefit of being immune to physical assumptions about the data acquired. However, linear regression can suffer from overfitting, which happens when errors from the measurements, such as noise, are incorporated in the model instead of the linear relationship between the variables. K-fold cross validation was used to test for overfitting, with the standard k = 10. The data was randomly divided into 10 subsets of equal size. The algorithm consists of 10 iterations, where for each iteration the model (Equation (4.6)) is trained using the merged data from the other 9 subsets and tested (Equation (4.7)) on the left-out subset. For each iteration a different subset is left out such that each data point is used 9 times for training and once for testing. For each iteration, the out-of-sample error is computed between $S_{out\_test}^*$ and $S_{in\_test}$ (Equation (4.5)). The mean and the standard deviation of these 10 errors are used to evaluate the performance of the algorithm. For data outside the initial calibration, an averaged $M^*$, computed from the 10 training iterations, is used.

$$M^* = S_{out\_train} \cdot S_{in\_train}^p$$  \hspace{1cm} (4.6)

$$S^*_{in\_test} = \left(M^*\right)^p \cdot S_{out\_test}$$ \hspace{1cm} (4.7)

Since the individual values in the 4-by-4 Mueller matrix $M^*$ can be any real number, it is important to check that the transmittance is greater than zero and less than one, i.e. that the optical element does not amplify light or produce negative intensities. The Mueller matrix should
also not overpolarize the light, i.e. produce Stokes vectors with DoP > 1. These two cases may happen if the model tries to solve for data that has been acquired incorrectly or is significantly noisy.

4.2.4 Optical Characterization
A thorough optical characterization of optical elements in a customized optical setup is critical, particularly when the optical elements are intended for use outside of their conventional use, e.g. near-infrared fluorescence or polarization imaging, since commercially available optical specifications may not give enough information to assess whether a given element is adequate for the system. I performed a series of optical tests on both endoscopes to completely evaluate the endoscopes’ optical performance. The optical transmission as a function of wavelength, the DOF number, the MTF curve, and the optical resolution were measured. These metrics delineate the characteristics of the sensor behind the endoscope as well as appropriate experimental conditions.

The optical setup shown in Figure 4.3 was utilized to measure the spectral response. The setup consisted of (1) a monochromator (Acton SP2150, Princeton Instruments), (2) an integrating sphere (819D-SF-4, Newport), (3) an adjustable iris (SM2D25, Thorlabs), (4) an aspheric collimating lens (ACL7560, Thorlabs), and (5) a calibrated photodiode (S130C, Thorlabs) driven by (6) a power meter (PM100D, Thorlabs). The optical power was measured from 400 nm to 1000 nm with and without the endoscope of interest on the optical path to calculate a transmission ratio.
To measure the DOF numbers, the MTF curves, and the optical resolutions for the two endoscopes, a DOF target (DOF 5-15, Edmund Optics), an ISO 12233 resolution chart (1X-13A/ISO, Edmund Optics), and a 1951 United States Air Force (USAF) resolution chart (USAF Resolution Target, Edmund Optics) were used, respectively. For all three experiments the endoscopes were directly coupled to a digital camera (B1923, Imperx) containing a CCD sensor (KAI-02170, ON Semiconductor). The sensor has an effective resolution of 1920-by-1080 pixels, with a 7.4 μm pixel pitch. For the DOF measurements the endoscope imaging axis was placed at a 45° angle with respect to the target’s plane. The endoscope was focused on top of the target such that the focused portion represented the uppermost portion of the field depth. No additional iris elements were added to the optical path to keep the endoscopes’ DOFs unmodified. For the MTF measurements, different sections of the ISO 12233 resolution chart were used as knife-edge targets to compute individual MTF curves, and their response was averaged and reported. For the resolution measurements, high contrast images were taken of the 1951 USAF resolution chart and the standard resolution formula was used to calculate the number of line pairs per mm, \( \text{lp/mm} = 2^{\text{group+(element-1)/6}} \).
4.3 Results and Discussion

4.3.1 Polarization

Permutations of different rotation angles for the linear polarizer and retarder were used to generate a Stokes vector cloud that served as the input polarization states for the endoscopes. A representation of these 1,800 input data points on the Poincaré sphere is shown in Figure 4.4(a). Figure 4.4(b) and Figure 4.4(c) show the output Stokes vectors after passing through the 5 mm and 10 mm endoscopes, respectively. The data in Figure 4.4 is clustered into 10 groups of color-coded data points. This is done solely with the purpose of visually identifying the polarization migration of the initial input Stokes vectors (Figure 4.4(a)) caused by the rigid endoscopes to the final output Stokes vectors (Figure 4.4(b) and Figure 4.4(c)); i.e., this color coding indicates corresponding pairs of input and output Stokes vectors. From Figure 4.4 the significant change of polarization is evident, e.g. the dark blue cluster has a relatively low and constant DoCP for all its members at the input; however, the outputs for both endoscopes show highly variable DoCP that approaches one on some data points. This effect is more pronounced in the 10 mm endoscope. The cluster arrangement suggests that the data has suffered a 2 DOF rotation around the Poincaré sphere. To further understand the consequences of not using a polarization calibration scheme to counteract this data rotation, the output figures of merit have been plotted as a function of the input figures of merit. Since this data is multi-dimensional, I have set some of the input parameters to a fixed value while sweeping one of the input variables. This is analogous to plotting data points from the Poincaré sphere that lie on a particular vector. Figure 4.5 shows the output AoP as a function of the input AoP with a constant input DoLP of one for both endoscopes. The AoPs have not only suffered a significant shift but lost their linearity. Furthermore, for the 5 mm endoscope the relationship is monotonically decreasing. A misreading on the AoP can lead to misalignments of the optical components and improper tissue pairing for
different DoLPS. Figure 4.6 shows the output DoLP as a function of the input DoLP with a constant input DoP and AoP of one and zero, respectively, for both endoscopes. For the 10 mm endoscope the relationship is monotonically decreasing. Also, the high output DoLP at low input DoLPS, for both endoscopes, suggests a birefringent effect. Figure 4.7 shows the output DoLP and the output DoCP as a function of the input AoP with a constant input DoLP of one. Figure 4.7 confirms the retardance behavior of the endoscopes. This output response seems to be periodic, where the DoLP decreases below 40% and 20% and the DoCP increases from a null value to above 85% and 95% for the 5 mm and 10 mm endoscope, respectively. The birefringent effect is more pronounced on the 10 mm than the 5 mm endoscope, due to the larger optics used, causing the light to travel farther in a birefringent medium. These graphs show the alarming change of the input polarization state caused by the endoscopes. The polarimeter readings will fall outside the acceptable tolerance for most of the polarimeter’s readable polarization domain. It is imperative to characterize and apply a calibration scheme to these optics if they are intended for polarimetry in biomedical applications.

The calibration results for the double retarder model are shown in Table 4.1 and Figure 4.8 for both endoscopes. Table 4.1 shows the angle parameters estimated by the double rotated retarder model for the two rotated retarders, the obtained Mueller matrices characterizing the endoscopes obtained by applying the angle parameters in the model, and the error (Equation (4.5)) and standard deviation between the input and the reconstructed input Stokes vectors using the obtained Mueller matrix. Figure 4.8 shows the input Stokes vector data and the reconstructed input Stokes vector data on the Poincaré sphere as well as the error histograms. For both endoscopes the mean error is less than 4%, meaning that the error on the DoCP, DoLP, and DoP has been reduced to less than 4% and the standard deviation is significantly lower, indicating that
there are few outliers that fall outside of the double retarder model. The calibration results for the linear regression model are analogously shown in Table 4.2 and Figure 4.9. Table 4.2 shows the average Mueller matrices characterizing the endoscopes and average cross validation error (Equation (4.5)) between the input and the reconstructed input Stokes vectors in the test datasets obtained by the 10 iterations of the linear regression model as well as the standard deviation in the cross-validation errors. For the linear regression model, the Poincaré sphere and the error histograms show the reconstructed data and errors on test data, from a single iteration out of 10, of the cross-validation algorithm. For both endoscopes the cross-validation error is less than 3% with very low standard deviation. These errors may be due to variation of the field of view caused by the optics’ non-uniform spatial response and could be minimized by using a spatially discretized calibration scheme. These results indicate that the linear regression method will outperform the double rotated retarder model in additional test data.

It is interesting that both models output very similar Mueller matrices for both endoscopes. This similarity is preserved on each of the 16 matrices’ elements. Note that no physical assumptions were made on the linear regression model. Elements on the first row and column, with the exception of (1,1), are virtually set to zero, indicating non-significant diattenuation effects. As well, the other 9 elements have similar values that vary by a few percentage points between models. This suggests that the hypothesis of modeling the endoscopes as retarders is accurate. However, the linear regression algorithm has lower error than the double rotated retarder model. Considering that the linear regression used a cross-validation approach to minimize overfitting and the low standard deviation over the out-of-sample errors, it is reasonable to hypothesize that the linear regression algorithm is also modeling other smaller retarder effects caused by the rest of the inner endoscope optics, e.g. small lenses and coatings. The double retarder model can be
expanded to include additional DOFs, e.g. diattenuation or extra retardation effects, by adding Mueller matrices to the model; however, a reasonable assumption about the endoscope’s polarization optics needs to be made, while the linear regression model yields the advantage of treating the endoscope as a black box with unknown individual polarization elements, but this powerful scheme can suffer from overfitting. It is important to notice as well that both models agree that the last column and last row of the Mueller matrices contain non-zero elements; i.e., energy has been exchanged between the linearly and circularly polarized components. Therefore, to reconstruct the initial input Stokes vector, it is necessary to measure all four parameters of the output Stokes vector, even if the input $S_3$ parameter is not of interest or is known to be null. In other words, a full polarimeter [51, 124, 125] is needed for polarimetry with endoscopes that have retardance effects, whereas most real-time polarimeters in the literature capture only the first three Stokes parameters [25].

<table>
<thead>
<tr>
<th>Optimal angle parameters</th>
<th>Mueller matrix</th>
<th>Error and standard deviation</th>
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<tbody>
<tr>
<td></td>
<td>$5 \text{ mm endoscope}$</td>
<td></td>
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<tr>
<td>$\theta_1 = 20.44^\circ$</td>
<td>$M_{\text{endoscope}} = \begin{pmatrix} 1 &amp; 0 &amp; 0 &amp; 0 \ 0 &amp; -0.1812 &amp; 0.6416 &amp; 0.7453 \ 0 &amp; -0.4215 &amp; -0.7354 &amp; 0.5306 \ 0 &amp; 0.8885 &amp; -0.2180 &amp; 0.4037 \end{pmatrix}$</td>
<td>$error = 3.62%$</td>
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<tr>
<td>$\phi_1 = 140.48^\circ$</td>
<td></td>
<td>$std(error) = 2.04%$</td>
</tr>
<tr>
<td>$\theta_2 = 59.55^\circ$</td>
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<td></td>
</tr>
<tr>
<td>$\phi_2 = -111.51^\circ$</td>
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<td></td>
<td>$10 \text{ mm endoscope}$</td>
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<td>$\theta_1 = 28.52^\circ$</td>
<td>$M_{\text{endoscope}} = \begin{pmatrix} 1 &amp; 0 &amp; 0 &amp; 0 \ 0 &amp; -0.2563 &amp; 0.3163 &amp; -0.9134 \ 0 &amp; 0.0378 &amp; 0.9475 &amp; 0.3175 \ 0 &amp; 0.9659 &amp; -0.0468 &amp; -0.2548 \end{pmatrix}$</td>
<td>$error = 3.99%$</td>
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<tr>
<td>$\phi_1 = -38.62^\circ$</td>
<td></td>
<td>$std(error) = 1.86%$</td>
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<tr>
<td>$\theta_2 = 39.44^\circ$</td>
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</tr>
<tr>
<td>$\phi_2 = 141.75^\circ$</td>
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Table 4.1 Double rotated retarder model calibration results.
<table>
<thead>
<tr>
<th>Mueller matrix</th>
<th>Error and standard deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 mm endoscope</td>
<td></td>
</tr>
</tbody>
</table>
| $M_{\text{endoscope}} = \begin{pmatrix} 1 & 0 & 0 & 0 \\ 0.0354 & -0.1664 & 0.6651 & 0.7776 \\ 0.0009 & -0.4227 & -0.7356 & 0.5174 \\ 0.0254 & 0.9112 & -0.2051 & 0.4289 \end{pmatrix}$ | $CV_{\text{error}} = 2.13\%$  
 $\text{std}(CV_{\text{error}}) = 0.13\%$ |
| 10 mm endoscope |                             |
| $M_{\text{endoscope}} = \begin{pmatrix} 1 & 0 & 0 & 0 \\ -0.0004 & -0.2963 & 0.2876 & -0.9084 \\ 0.0008 & 0.0320 & 0.9596 & 0.3328 \\ 0.0026 & 0.9608 & 0.0204 & -0.2370 \end{pmatrix}$ | $CV_{\text{error}} = 2.72\%$  
 $\text{std}(CV_{\text{error}}) = 0.06\%$ |

Table 4.2 Linear regression model calibration results.

Figure 4.4 Data plotted on the Poincaré sphere. Input Stokes vector data (a) and output Stokes vector data for the 5 mm (b) and 10 mm (c) endoscopes. Three-dimensional representation (left) and two-dimensional profiles (right). The data is clustered into 10 groups of color-coded data points with the purpose of visually identifying the polarization migration of the initial input Stokes vectors (a) caused by the rigid endoscopes to the final output Stokes vectors (b and c); i.e., this color coding indicates corresponding pairs of input and output Stokes vectors.
Figure 4.5 Output AoP vs. input AoP, with DoLP = 1, for the 5 mm (left) and 10 mm endoscope (right).

Figure 4.6 Output DoLP vs. input DoLP, with DoLP = 0 & AoP = 0, for the 5 mm (left) and 10 mm endoscope (right).

Figure 4.7 Output DoLP, and output DoCP vs. input AoP, for the 5 mm (left) and 10 mm endoscope (right).
Figure 4.8 Input Stokes vector data (red cubes) and reconstructed input Stokes vector data (green spheres) on the Poincaré sphere (top) with error histograms (bottom) for the 5 mm (left) and the 10 mm (right) endoscopes using the double rotated retarder model.

Figure 4.9 Input Stokes vector data (red cubes) and reconstructed input Stokes vector data (green spheres) on the Poincaré sphere (top) for one cross-validation iteration with error histograms (bottom) for the 5 mm (left) and the 10 mm (right) endoscope using the linear regression model.
4.3.2 Optical Characterization
Figure 4.10 shows the spectral responses of the two endoscopes, where P and P_e indicate the optical power generated by the photon flux at the input and output windows, respectively. The 5 mm endoscope has a lower transmittance than the 10 mm endoscope due to its smaller aperture. The maximum transmittances are ~6% and ~16% for the 5 mm and 10 mm endoscopes, respectively. These low maximum transmission ratios are to be expected from optical systems with low optical apertures and long optical trains. Both endoscopes reach maximum transmittance around 660 nm which may be optimal for tissue hues; however, after 670 nm both transmittances decay rapidly— to ~2% and ~6%, respectively, at 800 nm. If the endoscopes are intended for use in a complementary imaging modality, such as near-infrared fluorescence imaging, the imaging sensor would need to have an increased fluorophore sensitivity to compensate for this low transmission ratio. The near-infrared fluorescence imaging setting is already limited by the preference of low dye concentrations, low fluorophore quantum yields, and low sensor quantum efficiencies in the near-infrared spectrum. Endoscopes with a more generous transmittance curve would be preferred for this imaging modality.

Figure 4.11 shows the images acquired from the DOF target for both endoscopes. The 5 mm endoscope resolves 5 lp/mm at ~9 mm while the 10 mm endoscope can only resolve 5 lp/mm at ~4 mm. The DOF gain is attributed again to the smaller aperture on the 5 mm endoscope. This transmittance versus DOF trade-off is a typical dilemma in optical setup designs, especially when real-time frame rates are desired. Figure 4.12(a) and Figure 4.12(b) show the sections of the ISO 12233 resolution chart that were used as knife-edge targets to compute the MTF curves for the 5 mm and 10 mm endoscope, respectively. Figure 4.12(c) shows the MTF plots for both endoscopes. The normalized spatial frequency is calculated as the spatial frequency divided by the cutoff or Nyquist frequency which is half-cycle per pixel pitch. The 5 mm endoscope has an
MTF of 44.6% at a normalized spatial frequency of 0.1 while the 10 mm endoscope has an MTF of 69.5% at the same normalized spatial frequency. This difference is again attributed to the higher aperture on the 10 mm endoscope in comparison to the 5 mm endoscope. As expected, the endoscopes’ MTF curves are lower than commercial lenses, but such inferior performance is expected from this complex and compact set of optics. Figure 4.13 shows the images acquired from the 1951 USAF resolution chart for the 5 mm (a) and 10 mm (b) endoscopes. The 5 mm endoscope can resolve the 2nd element in group 3, giving 8.97 lp/mm or 55.68 µm/λ. This is equivalent to an imaging pixel with a 22.51 µm pitch. For the 10 mm endoscope, the smallest resolved pattern is the 5th element in group 3, giving 12.69 lp/mm or 39.37 µm/λ. This is equivalent to an imaging pixel with an 18.51 µm pitch. Both of these results are far from optimal, i.e. the sensor’s pixel pitch of 7.4 µm. However, knowing this information can influence the optical design in a positive way. It is possible to use sensors with higher pixel pitch (and hence higher quantum efficiency), leading to better performance in low light conditions which translates to higher polarization and fluorophore sensitivity, without compromising the overall system’s optical resolution. The 10 mm endoscope has much higher transmittance, a better MTF curve, and higher optical resolution, but it is outperformed on the DOF measurement. These comparisons should be kept among endoscopes built similarly but with different apertures. However, if the experimental setup permits the utilization of an endoscope with a bigger aperture, the DOF can be improved by adding a variable iris on the optical path, at the expense of accuracy in the other metrics.
Figure 4.10 Spectral response as a function of wavelength for the 5 mm (left) and 10 mm (right) endoscope.

Figure 4.11 DOF target images for the 5 mm (a) and 10 mm (b) endoscopes.

Figure 4.12 Sample images used to calculate the MTF curve for the 5 mm (a) and 10 mm (b) endoscope and MTF curve plots for the 5 mm and 10 mm endoscope (c).

Figure 4.13 1951 USAF resolution chart images for the 5 mm (a) and 10 mm (b) endoscope and magnified versions (right).
4.4 Conclusions
Advances in nanotechnology have led to the proliferation of a vast range of polarization-sensitive imagers or polarimeters suitable for emerging biomedical applications. These polarimeters can be used in conjunction with rigid endoscopes or other optics to create a tailored imaging system for specific applications. A trimodal endoscopic imaging technique has been proven to increase the overall sensitivity and specificity of identifying cancerous tissue in the diagnosis of colitis-associated cancer. However, little effort has been made to evaluate and characterize the optical and polarization properties of rigid endoscopes or additional optics that couple with these polarimeters. Previous works do not delineate comprehensive calibration methods; consequently the errors have not been qualitatively documented. Here, I present a framework to analyze the endoscopes’ optical limitations and influence over polarized light. Two calibration schemes are shown to reconstruct the original polarization state of interest before light goes through the endoscope. These calibration methods can easily be expanded to flexible endoscopes given that polarization-maintaining optical fibers are used and the rotation of the distal tip coordinate system with respect to the proximal tip coordinate system is known.
Chapter 5: A Bio-Inspired Imager Improves Sensitivity for Near-Infrared Fluorescence Image-Guided Surgery

Image-guided surgery can enhance cancer treatment by decreasing, and ideally eliminating, positive tumor margins and iatrogenic damage to healthy tissue. Current state-of-the-art near-infrared fluorescence imaging systems are bulky, costly, lack sensitivity under surgical illumination, and lack co-registration accuracy between multimodal images. As a result, an overwhelming majority of physicians still rely on their unaided eyes and palpation as the primary sensing modalities to distinguish cancerous from healthy tissue. Here I introduce an innovative design comprising an artificial multi-spectral sensor inspired by the Morpho butterfly’s compound eye which can significantly improve image-guided surgery. By monolithically integrating spectral tapetal filters with photodetectors, I have realized a single-chip multi-spectral imager with 1000-fold higher sensitivity and 7-fold better spatial co-registration accuracy compared to clinical imaging systems in current use. Preclinical and clinical data demonstrate seamless integration of this technology in the surgical work flow while providing surgeons with real-time information on the location of cancerous tissue and sentinel lymph nodes, respectively. Due to its low cost, the bio-inspired sensor will provide resource-limited hospitals with much-needed technology to enable more accurate value-based health care.

5.1 Motivation for Near-Infrared Fluorescence for Image-Guided Surgery

Surgery is the primary curative option for patients with cancer, with the overall objective of complete resection of all cancerous tissue while avoiding iatrogenic damage to healthy tissue. In addition, sentinel lymph node (SLN) mapping and resection is an essential step in staging and managing the disease [1]. Even with the latest advancements in imaging technology, incomplete
tumor resection in patients with breast cancer is at an alarming rate of 20–25%, with recurrence rates of up to 27% [2]. The clinical need for imaging instruments that provide real-time feedback in the operating room is unmet, largely due to the use of imaging systems based on contemporary technological advances in the semiconductor and optical fields, which have bulky and costly designs with suboptimal sensitivity and co-registration accuracy between multimodal images [3-7].

Here I demonstrate that image-guided surgery can be dramatically improved by shifting the design paradigm away from conventional advancements in the semiconductor and optical technology fields and instead adapting the elegant 500-million-year-old design of the Morpho butterfly’s compound eye [8, 9] – a condensed biological system optimized for high-acuity detection of multi-spectral information. Nature has served as inspiration for many engineering sensory designs with performances exceeding state-of-the-art sensory technology and enabling new engineering paradigms, such as achromatic circular polarization sensors [12], artificial vision sensors [13-15], silicon cochlea [16, 17] and silicon neurons [18]. Our artificial compound eye, inspired by the Morpho butterfly’s photonic crystals, monolithically integrates pixelated spectral filters with an array of silicon-based photodetectors. Our bio-inspired image sensor has the prominent advantages of (1) capturing both color and near-infrared fluorescence (NIRF) with high co-registration accuracy and high sensitivity under surgical light illumination, which allows simultaneous identification of anatomical features and tumor-targeted molecular markers; (2) streamlined design – at 20 g including optics, the bio-inspired image sensor does not impede surgical work flow; and (3) low cost, which will provide resource-limited hospitals with much-needed technology to enable more accurate value-based health care.
5.1.1 Nature-Inspired Design
Light has imposed significant selection pressure for perfecting, optimizing, and miniaturizing animal visual systems since the Cambrian period some 500 million years ago [26]. Sophisticated visual systems emerged in a tight race with prey coloration during that time, resulting in a proliferation of photonic crystals in the animal kingdom used for both signaling and sensing [27, 28]. For example, not only are the tree-shaped photonic crystals of the Morpho butterfly the source of its wings’ magnificent iridescent colors (Figure 5.1A), which can be sensed by conspecifics from a mile away, but these crystals also can sense vapors [29] and infrared photons [30] with sensitivity that surpasses state-of-the-art man-made sensors. Similar photonic crystals are also present in the compound eye of the Morpho butterfly. These photonic crystals, known as tapetal filters, are realized by stacks of alternating layers of air and cytoplasm, which act as interference filters at the proximal end of the rhabdom within each ommatidium (Figure 5.1D). The light that enters an individual ommatidium and is not absorbed by the visual and screening pigments in the rhabdom will be selectively reflected by the tapetal filters and will have another chance of being absorbed before exiting the eye. The spectral responses of the tapetal filters together with screening and visual pigments in the rhabdom determine the eye shine of the ommatidia [8] and the inherent multi-spectral sensitivity of the butterfly’s visual system (Figure 5.1B). Individual ommatidia have different combinations of visual pigments and tapetal filter stacks, enabling selective spectral sensitivity across the ultraviolet, visible, and near-infrared (NIR) spectra.
Figure 5.1 Our bio-inspired imaging sensor uses a new design paradigm to capture color and near-infrared fluorescence information. A, *Morpho* butterfly (*Morpho peleides*) blue color is due to tree-shaped photonic crystals in its wings. Similar photonic crystals can be found in the butterfly’s ommatidia. B, Close-up of the *Morpho* ommatidia indicating ‘eye-shine’ due to tapetal filters together with screening and visual pigments in the rhabdom, which enable multi-spectral target detection. C, Our compact bio-inspired multi-spectral imaging sensor combines an array of imaging elements with pixelated tapetal spectral filters. D, Transmission electron microscope image of an individual *Morpho* ommatidium, which monolithically integrates tapetal filters with light-sensitive rhabdom. E, Cross-sectional scanning electron microscope image of the bio-inspired imaging sensor. Tapetal filters combined with silicon photodetectors allow for high co-registration accuracy in the detected spectral information. Scale bar, 2 μm.
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<td>$$$$$&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> The detection limit was performed under visible light illumination. ICG fluorescence marker was used.

<sup>b</sup> The detection limit was performed with no surgical light illumination. IRDye800CW fluorescence marker was used.

<sup>c</sup> The cost of the commercially available NIRF systems range from $20,000 to more than $200,000 depending on instrument capabilities and options. The estimate cost to manufacture our sensor is less than $20.

Table 5.1 Optical performance of various state-of-the-art near-infrared fluorescence (NIRF) imaging systems and our bio-inspired NIRF imager.
By imitating the compound eye of the Morpho butterfly using dielectric materials and silicon-based photosensitive elements, I developed a multi-spectral imaging sensor that operates radically differently from the current state-of-the-art multi-spectral imaging technology (Figure 5.1C; Table 5.1). The tapetal spectral filters are constructed using alternating nanometric layers of SiO$_2$ and TiO$_2$, which are pixelated with a 7.8-µm pitch and deposited on the surface of a custom-designed silicon-based scientific complementary metal-oxide semiconductor (sCMOS) imaging array (see Methodology). The alternating stack of dielectrics acts as an interference filter, allowing certain light spectra to be transmitted while reflecting others (Figure 5.1E). Four distinct pixelated spectral filters are replicated throughout the imaging sensor in a 2-by-2 pattern by modulating the thickness and periodicity of the dielectric layers in individual pixels. Three of the four pixels are designed to sense the red, green, and blue (RGB) spectra, respectively, and the fourth pixel captures NIR photons with wavelengths greater than 780 nm. An additional stack of interference filters is deposited across all pixels to block fluorescence excitation light between 770 nm and 800 nm. The proximity of the imaging array’s four base pixels inherently co-registers the captured multi-spectral information, similar to its biological counterpart.

5.2 Methodology
5.2.1 Animal study
Animal study protocols were reviewed and approved by the Animal Studies Committee of Washington University in St. Louis. Female PyMT mice (n=5) were obtained from Washington University Medical Center Breeding Core. Mice developed multiple mammary tumors as early as 5-6 weeks and were injected with 100 µl of 60 µM LS301, a tumor-targeted NIRF contrast agent, via the lateral tail vein. Images were taken 24 h post injection for best contrast. During the in vivo study, the bio-inspired multi-spectral imaging sensor was set up at 1 m working distance, and the illumination module was placed at a 1 m distance. The animals were imaged under
simultaneous surgical light illumination (60 kLux) and laser light excitation power of 20 mW/cm² at 785 nm. Collimating lenses and diffusers were used to create a uniform circular excitation area with a 15-cm diameter. The RGB pixels’ exposure time was set to 0.1 ms, and the NIR pixels’ exposure time was set to 40 ms to ensure imaging rates of 25 frames/sec.

A double-blind protocol was used for tissue specimen collection. Randomization was not used in this study and all animals were included in the data analysis. During the imaging experiments, animals remained anesthetized through inhalation of isoflurane (2-3% v/v in O₂). The surgeon used fluorescence information detected by the bio-inspired sensor to locate and resect all tumor tissues. After the removal of each tumor, the surgeon resected two additional samples: a tissue sample next to the tumor identified as tumor margin and a fluorescence negative muscle tissue. Total of 6 to 8 tumors per mouse were resected. Total of 102 samples were collected from all 5 mice. All harvested tissues samples were also imaged using Lycor Pearl small animal imaging system. All tissue samples were then preserved for histology evaluation. Each sample was sliced with 8 µm thickness, stained with hematoxylin and eosin (H&E) and examined by clinical pathologist. The pathologist was blind to the fluorescence results.

For the 4T1 studies, six week old Balb/C female mice were obtained from Jackson Laboratory and were injected with 5 × 10⁵ 4T1 murine cancer cells. In the first study, the 4T1 cells were implanted into either left or right inguinal mammary pad. In the second study, the 4T1 cells were implanted next to the sciatic nerve. At 5–7 mm tumor size (7–10 days post-implantation), these mice were injected with 100 µl of 60 µM LS301 agent via the lateral tail vein. The animals were imaged 24 h post-injection.

5.2.2 Human study
Human study protocols were approved by the Institutional Review Board of Washington University in St. Louis. The human procedure was carried out in accordance with approved
guidelines. The inclusion criteria for patients in this study were newly diagnosed clinically node-negative breast cancer, negative nodal basin clinical exam, and at least 18 years of age. The exclusion criteria from this study were contraindication to surgery; receiving any investigational agents; history of allergic reaction to iodine, seafood, or ICG; presence of uncontrolled intercurrent illness; or pregnant or breastfeeding. All patients gave informed consent for this HIPAA-compliant study. The study was registered on clinicaltrials.gov website (trial ID no. NCT02316795).

The mean ± SD age and body mass index of all patients were 64±14 years and 32.7±6.9 kg/m². Before the surgical procedure, 99mTc-sulfur colloid (834 μCi) and ICG (500 μmol, 1.6 mL) were injected into patient’s tumor area, followed by site massage for approximately 5 min. At 10-15 min post injection, surgeons proceeded with the surgery per standard of care. Once the surgeon identified the SLNs using the visible properties of ICG (i.e., green color) and radioactivity using the gamma probe, the surgeon used the bio-inspired imaging system to locate the SLNs. The patients were imaged under simultaneous surgical light illumination (60 kLux) and laser light excitation power of 20 mW/cm² at 785 nm. The surgeon then proceeded with the resection of the SLNs. The imaging system was set up at a 1 m working distance, and the illumination module was placed at a 1 m distance. The RGB pixels’ exposure time was set to 0.1 ms to ensure non-saturated color images were recorded, and the NIR pixels’ exposure time was set to 40 ms to ensure imaging rates of 25 frames/sec. The average imaging time with the bio-inspired sensor was 2.5±0.6 min.

5.2.3 Cell culture

4T1 breast cancer cells were used for in vivo tumor models. This cell line was obtained from American Type Culture Collection in Manassas, VA, USA. Mycoplasma Detection Kit from Thermo Fisher Scientific was used to verify negative status for mycoplasma contamination in the
cell line. Cells were cultured in Dulbecco’s modified eagle medium from Thermo Fisher Scientific, with 10% fetal bovine serum and antibiotics.

5.2.4 Fluorescence concentration detection limits under surgical light sources
Ten different ICG concentrations in plastic vials were imaged under surgical light illumination (60 kLux) and excited with laser light excitation power of 20 mW/cm² at 785 nm. Six different vials at each concentration, as well as a control vial with deionized water, were imaged with two different pixelated imaging sensors. The first sensor was the bio-inspired imager, which has two separate exposure times per every frame: the RGB pixels’ exposure time was set to 0.1 ms to ensure non-saturated color images were recorded, and the NIR pixels’ exposure time was set to 40 ms to ensure imaging rates of 25 frames/sec. The second sensor was a pixelated CMOS imaging sensor with single exposure time for both RGB and NIR pixels in the array. The exposure time for the second sensor was set to 0.1 ms because longer exposure times would saturate the color image due to the high photon flux from the surgical light source illumination. A region of interest within each vial was selected to avoid edge artefacts. An average intensity value and standard deviation of the NIR pixels were computed on the region of interest, excluding 5% of the pixel’s outliers. The detection threshold was determined as the average NIR signal plus three standard deviations of the control vial.

5.2.5 Temperature dependent co-registration accuracy measurement
The imaging instruments were individually placed in a custom built thermal chamber with a transparent viewing port, where the operating temperature was accurately controlled using a proportional-integral-derivative controller. The imaging instrument resided at 15 °C for 24 h to reach thermal equilibrium. The disparity matrix between the NIR and color image for the beam splitter image was computed at 15 °C temperature. The temperature of the thermal chamber was increased in increments of ~2.5 °C, and the instrument was held at the new temperature for 15
min before an image of a calibrated checkerboard target was captured with both NIR and color sensors. These new images were co-registered using the disparity matrix computed at 15 °C, and the co-registration error across the entire image was evaluated. The operating temperature of the instrument was increased to 35 °C and then reduced back to 15 °C. The co-registration error was computed at each temperature point.

5.2.6 **Scientific CMOS imager with pixel-level multi-exposure capabilities**

The custom scientific CMOS imager, which is used as a substrate for the NIRF sensor, is custom designed and fabricated in 180 nm CMOS image sensor technology. The imager is composed of an array of 1280 by 720 pixels, programmable scanning registers, and readout analogue circuits comprising switch capacitors, amplifiers, bandpass filters, voltage reference circuits, and analogue-to-digital converters (Figure 5.2). An individual pixel comprises a pinned photodiode and four transistors that control the access of the pixel to the readout circuitry and the exposure time of the pixel.

The digital scanning registers interface with the individual pixels and control the transistors’ gates within each pixel. The scanning registers are designed to be programmable by inserting different digital patterns and altering the clocking sequence via an external file programmable gate array. This enables pixel-level control of the exposure time for individual photodiodes and specialized readout sequence of individual pixels. Hence, individual groups of pixels (color or NIR) can be read out at different times, and the exposure time for both types of pixels is optimized to ensure acquisition of high signal-to-noise (SNR) color and NIR images, respectively.
Figure 5.2 Block diagram of the bio-inspired color-near infrared (NIR) scientific complementary metal-oxide semiconductor imaging sensor. An array of photodetectors and pixelated interference spectral filters are monolithically integrated. The two exposure control registers enable individual control of the times the NIR and red, green, blue photodiodes are collecting photons.

During a single frame readout, NIR and color pixels can have different exposure times, and the frame rate is limited by the longest integration time of the two. For example, during intraoperative procedures, the light intensity from the surgical light sources that is reflected from the tissue is much higher than the NIRF signal from the molecular dye. In this scenario, to ensure high-SNR and non-saturated images, the integration time for the color pixels is set to ~0.1 ms and the exposure time for the NIR pixels is set to 40 ms to ensure imaging rates of 25 frames/sec. The timing sequence for two neighboring pixels with different spectral filters is shown in Figure 5.3. Initially, both pixels are reset, and then they start to collect photons on the photodiodes’ intrinsic capacitance. Since the photon flux for the visible-spectrum photons in the operating room is typically much higher than that of the NIRF photons, the photodiode voltage in the color pixels drops faster than in the NIR pixels over time. At the end of the exposure period, the photodiode voltages from the color pixels are sampled first on the column parallel readout capacitors. A readout control registers scans through the column parallel capacitors and digitizes
the analog information after it has been amplified with a programmable gain amplifier. The same readout sequence is repeated with the NIR pixels at the end of the 40-msec exposure time.

![Timing diagram of two neighboring pixels with different spectral filters. Top, Photodiode voltage on the near infrared (NIR) pixel. Bottom, Photodiode voltage on the visible (red, green, blue) pixels. Because of the difference in the photon flux between the NIR fluorescence and reflected visible-spectrum light under surgical light illumination, the exposure time is adjusted accordingly to capture high-SNR and high-contrast images. The photodiode signal is sampled at the end of the exposure time, which is 0.1 ms for the visible spectrum pixel and 40 ms for the NIR pixels.](image)

**5.2.7 Fabrication of pixelated spectral filters**
The pixelated spectral filters were fabricated via a set of optimized microfabrication steps. Here are the steps necessary to fabricate the pixelated filters:

The carrier wafer was soaked for 30 minutes in isopropanol alcohol and rinsed with DI water. The wafer was coated with 20 nm of chromium, which was used to block stray light between pixels (Figure 5.4(a)).

SU8 2000 photoresist was spin coated at 500 rpm for 10 seconds and then at 3000 rpm for 50 seconds with 500 rpm per second acceleration.
The sample was baked at 65 °C for 1 min and then at 95 °C for 2 min on a hot plate (Figure 5.4(b)).

The photoresist was exposed at 375 nm wavelength for 22 seconds at 5mW/cm² intensity using a Karl Zuess mask aligner.

The sample was post-baked at 65 °C for 1 min and then at 95 °C for 3 min. The sample was cooled down to 65 °C for 1 min to gradually decrease the temperature and minimize stress and cracking on the photoresist. The photoresist was developed for 3 min in an SU-8 developer using an ultrasound bath and gently rinsed with isopropyl alcohol at the end of the procedure (Figure 5.4(c)).

The exposed chromium was etched in an Oxford reactive ion etching inductively coupled plasma instrument (Figure 5.4(d)).

Omnicoat was spin coated at 4000 rpm and baked at 150 °C for 1 min.

SU8 2000 photoresist was spin coated at 500 rpm for 10 seconds and then at 3000 rpm for 50 seconds with 500 rpm per second acceleration.

The sample was baked at 65 °C for 1 min and then at 95 °C for 2 min on a hot plate.

The photoresist was exposed at 375 nm wavelength for 22 seconds at 5mW/cm² intensity using a Karl Zuess mask aligner.

The sample was post-baked at 65 °C for 1 min and then at 95 °C for 3 min. The photoresist was developed for 3 min in an SU-8 developer using an ultrasound bath and gently rinsed with isopropyl alcohol at the end of the procedure.

The exposed Omnicoat was etched using chlorine in an Oxford reactive ion etching inductively coupled plasma instrument (Figure 5.4(e)).
Alternating layers of silicon dioxide and silicon nitrate were deposited across the entire sample using physical vapor deposition. The thickness of the dielectric layers was optimized for transmitting NIR light with high transmission ratio (Figure 5.4(f)).

The sample was immersed in PG removal and ultrasound bath for 30 minutes to lift off the unwanted structures (Figure 5.4(g)).

Steps 2 through 13 were repeated three times to fabricate red, green and blue pixels (Figure 5.4(h) and Figure 5.4(i)).

The final sample had all four different types of pixels: NIR and visible spectrum pixels (Figure 5.4(j)).

![Microfabrication procedure for fabricating pixelated spectral filters.](image)

**5.3 Results**

**5.3.1 Optoelectronic performance of the bio-inspired sensor**

The optical density and transmission spectrum of the four base pixels from the artificial compound eye is presented in Figure 5.5(A) and Figure 5.5(B), respectively. The sensor was evaluated with uniform monochromatic light impinging normal to the surface of the imaging
plane. The individual tapetal filters are optimized to achieve transmission of 60% in the visible spectrum and 80% in the NIR spectrum. The high optical density of ~12 ensures effective suppression of fluorescence excitation light between 770 nm and 800 nm. The spatial uniformity or fixed pattern noise (FPN) before calibration for the RGB and NIR filters is 6.5%, 1.9%, 4.5%, and 5%, respectively (Figure 5.5(C)). After first-order gain and offset calibration, the FPN is around 0.1% across different illumination intensities (Figure 5.5(D)). The spatial variations in the optical response of the tapetal filters are primarily due to variations in the underlaying transistors and photodiodes within individual pixels, which can be mitigated via calibration, improving spatial uniformity under various illumination conditions. The peak quantum efficiencies for the RGB and NIR pixels are 28%, 35%, 38%, and 28%, respectively (Figure 5.5(E)).

5.3.2 Acquiring NIR fluorescence and color under surgical light illumination
Simultaneous and real-time imaging of both NIR fluorescence and RGB information is essential in surgical settings as it will enable the surgeon to identify the location of the tumor on the correct anatomical feature. U.S. Food and Drug Administration (FDA) regulations require the optical power of visible-spectrum surgical illumination to be between 40 and 160 kLx. The optical power for NIR laser-based excitation sources typically does not exceed 30 mW/cm². Hence, the intensity of the NIRF molecular probe, which could be emitted from tumors several centimeters deep in the tissue, is at least 5 orders of magnitude or more weaker than the intensity of the reflected visible-spectrum light [126]. To enable simultaneous color and NIR imaging in the operating room, most FDA-approved instruments work with dimmed surgical illumination, which significantly impedes the surgical workflow: physicians stop the resection, dim the surgical lights, evaluate the surgical margins with NIRF instrumentation, and then continue the surgery under either dim illumination or normal illumination but without NIRF guidance [127].
This significant drawback short-circuits the intrinsic benefits of NIRF, preventing wide acceptance of this technology in the operating room, which can also lead to positive margins and iatrogenic damage.

Figure 5.5 A and B, Measured optical density (A) and transmittance (B) of the four tapetal spectral filters in the bio-inspired multi-spectral imaging sensor. C, The spatial uniformity or fixed pattern noise (FPN) before calibration as a function of light intensity. D, The calibrated FPN shows that the bio-inspired camera captures data with ~0.1% spatial variations for uniform intensity targets. E, Quantum efficiency of the bio-inspired image sensor. F, Fluorescence detection limits under surgical light illumination for the bio-inspired sensor, which utilizes a multi-exposure method (green) compared with single-exposure method (blue) used in state-of-the-art pixelated color-NIR sensors. The dashed vertical lines are the individual detection thresholds for both sensors estimated as the mean value plus three standard deviations of the control vial. The bio-inspired sensor’s 1000-fold improvement in detection limit is achieved due to multi-exposure (P<0.0001), high NIR optical density, and high NIR quantum efficiency. Data are presented as mean ± s.d.

To address the wide-dynamic-scene imaging demands in the operating room, the custom sCMOS imager has programmable readout circuitry that enables independent exposure control for the visible and NIR pixels within a single frame (see Methodology). For example, due to the high
visible-spectrum illumination in the operating room, the exposure time for the RGB pixels is
typically set to ~0.1 ms or lower to ensure that non-saturated and high-contrast color images are
recorded. Since the NIRF emission is weaker than the visible light reflected from tissue, the
exposure time is set to 40 ms to ensure imaging rates of 25 frames/s and acquisition of high-
contrast NIR images. This contrasts with current state-of-the-art pixelated NIRF systems that
either utilize polymer-based [128] or Fabry-Perot absorptive pixelated spectral filters with low
optical density (<1.5) coupled with CMOS sensors (see Table 5.1 and Table 5.2), allowing only a
single exposure time for all pixels, or utilize a multicamera approach, which lacks co-registration
accuracy between multimodal images [127]. The multi-exposure capabilities of the sCMOS
coupled with the high optical density and high quantum efficiency of the NIR pixels enables
detection of 100 pM fluorescence concentrations of indocyanine green (ICG) under 60 kLux
surgical light illumination (P<0.0001), which is a 1000-fold improvement over current state-of-
the-art single-exposure pixelated sensors [128] (Figure 5.5(F)).

5.3.3 Multi-exposure imaging under surgical light illumination
We demonstrate the preclinical relevance of the multi-exposure capabilities of the bio-inspired
sensor by imaging a 4T1 breast cancer model under 60 kLux surgical light illumination and laser
light excitation power of 5 mW/cm² at 785 nm. The results are compared with a single-exposure
pixelated CMOS camera (Figure 5.6). Using the tumor-specific NIRF marker LS301 (a cypate-
based contrast agent that typically accumulates in the periphery of tumors [129]), I obtained high
target-to-background contrast images due to the tissue’s low auto-fluorescence, low scattering,
and absorption in the 700- to 950-nm spectral bands. The images in Figure 5.6, top and middle
rows, were obtained with a single-exposure CMOS camera with exposure times of 0.1 ms and 40
ms, respectively. When the animal was imaged with an exposure time of 0.1 ms, the color image
is well illuminated, while the NIR image has very low contrast. The animal was then imaged
with 40-ms exposure time, resulting in a well-illuminated NIR image but a saturated color image. This is due to the large difference between the visible and NIR photon flux in the operating room. Utilizing a single exposure time in a pixelated camera enables only one of the two imaging modalities to have satisfactory contrast and high signal-to-noise ratio, rendering this technology incompatible with the demands of intraoperative imaging applications.

<table>
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<td>Reference</td>
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<td>Maximum optical density NIR pixels in visible spectrum</td>
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<td>NIR excitation light</td>
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<td>ICG fluorescence detection limit</td>
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<td>Sensor bit depth</td>
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<tr>
<td>Exposure Time</td>
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<td>Maximum FPS</td>
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<td>Background correction</td>
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Table 5.2 Optical performance comparison between pixelated NIRF imaging systems and our bio-inspired NIRF imager.
Single-exposure cameras have limited capabilities for simultaneous imaging of color and near-infrared (NIR) images with high contrast under surgical light illumination. Top row, exposure time of 0.1 ms produces good color but poor NIR contrast images. Middle row, exposure time of 40 ms produces oversaturated color image but good NIR contrast image. Bottom row, the bio-inspired camera captures color data with 0.1-ms exposure time and NIR data with 40-ms exposure time. This multi-exposure feature enables high-contrast images from both imaging modalities.

The bottom row in Figure 5.6 presents data collected with the bio-inspired imaging sensor. The exposure times for the color and NIR pixels were set to provide optimal contrast in both color and NIR channels: 0.1 ms for the color pixels and 40 ms for the NIR pixels. The combined images contain high signal-to-noise ratios and non-saturated information from both imaging modalities. Hence, the operator can clearly identify the anatomical features of the patient while accurately determining the location of the tumor as tagged by the molecular probe.

5.3.4 Multi-spectral co-registration accuracy
Co-registration accuracy between color and NIF images is one of the most important attributes for an instrument to be clinically relevant. Yet, state-of-the-art NIF instrumentation comprising a beam splitter and dichroic mirrors suffers from temperature-dependent co-registration inaccuracy due to thermal expansion and thermal shifts of individual optical components. These FDA-approved instruments are rated to function between 10 °C and 35 °C, though they fail to maintain co-registration accuracy in this range. In contrast, the bio-inspired sensor
monolithically integrates filtering and imaging elements on the same substrate and is inherently immune to temperature-dependent co-registration errors.

We evaluated co-registration accuracy as a function of temperature for both the bio-inspired sensor and a state-of-the-art NIRF imaging system composed of a single lens, beam splitter, and two imaging sensors (Figure 5.7). The sensors were placed 60 cm from a calibrated checkerboard target to emulate the distance at which the sensor will be placed during preclinical and clinical trials. At the starting operating point, the beam-splitter NIRF system achieves subpixel co-registration accuracy using standard calibration methods. However, the disparity between the two images increases as the instrument’s operating temperature increases, leading to large co-registration errors. And, as the instrument is cooled, the trajectory of the co-registration error differs from that when the instrument is heated up. Hence, placing a temperature sensor on the instrument will not sufficiently correct for thermal expansion of the individual optical elements.

In contrast, in the bio-inspired sensor the worst-case co-registration error at the sensor’s plane is $\sqrt{2}$ pixels due to the pixelated filter arrangement. Compared to the beam-splitter NIRF system, the bio-inspired sensor exhibits 7-fold improved spatial co-registration accuracy at the imaging plane when the sensors operate at 35 °C.
5.3.5 Implications on co-registration accuracy in murine cancer model

The implication of the temperature-dependent co-registration error between the NIR and RGB images in state-of-the-art NIF systems is demonstrated in a murine model where 4T1 cancer cells are implanted next to a sciatic nerve. At ~2 weeks post-implantation, the tumor size is ~1 cm and is imaged with the tumor-targeted agent LS301. The animal is imaged with a beam-splitter NIF imager placed inside a thermal chamber, which has a viewing port that allows imaging of the animal without perturbing the temperature of the instrument. The animal is kept on a heated thermal pad to maintain constant body temperature of ~37 °C.

Figure 5.8(A) is a composite image taken with the NIF system at 15 °C operating temperature. The green false color indicates the NIF signal from the tumor targeted agent LS301. The fluorescence signal from the tumor tissue underneath the sciatic nerve is much weaker than the fluorescence signal from the surrounding tumor tissue. After thresholding the fluorescence signal, the location of the sciatic nerve is observed due to the absence of fluorescence signal (Figure 5.8(A), arrow). Since the image sensor is calibrated at 15 °C operating temperature, the
NIR image (i.e., location of the tumor) is correctly co-registered on the color image (i.e., anatomical features).

Figure 5.8. Color–near infrared (NIR) composite images recorded with a beam-splitter NIRF system while the instrument is at an operating temperature of 15 °C (A) and 32 °C (B). The tumor-targeted probe LS301 is used to highlight the location of the tumor, which is under the sciatic nerve. The instrument is calibrated at 15 °C, so the NIR and color images in (A) are correctly co-registered, and the location of the sciatic nerve is visible due to absence of fluorescence signal (arrow). In contrast, at 32 °C (B) the NIR fluorescence image is spatially shifted and superimposed on the incorrect anatomic features (arrow) due to the thermal shift of individual optical elements comprising the instrument. Thus, the location of the sciatic nerve is incorrectly highlighted as cancerous tissue.

Figure 5.8(B) is another set of images recorded with the NIRF sensor at 32 °C. Because of the thermally induced shift in the optical elements of the NIRF instrument, the fluorescence image is shifted with respect to the color image. The NIRF image incorrectly marks the sciatic nerve as cancerous tissue, while the cancerous tissue immediately next to the sciatic nerve has low fluorescence signal. This incorrect labelling of cancerous and nerve tissue can lead to iatrogenic damage to healthy tissue, which might not be visible to surgeons, while leaving behind cancerous tissue in the patient. In contrast, the bio-inspired sensor suffers no thermally induced co-
registration error and accurately depicts the location of the tumor and sciatic nerve at both temperatures due to the monolithic integration of pixelated spectral filters and imaging elements.

5.3.6 Imaging spontaneous tumors under surgical light illumination

We used the bio-inspired sensor to identify spontaneous tumor development in a transgenic PyMT murine model for breast cancer (n=5). All animals developed multifocal tumors throughout the mammary tissues by 5-6 weeks, and some of the small tumors blended in well with surrounding healthy tissue due to their color and were difficult to differentiate visually with the unaided eye. However, because the bio-inspired sensor has high co-registration accuracy and NIRF sensitivity, we could easily locate the tumors, resect them, and ensure that the tumor margins were negative (Figure 5.9(A)-(C)). When we compared results obtained with the bio-inspired imaging sensor against histology results, we found that the sensor together with the tumor-targeted probe LS301 had a sensitivity of 80%, a specificity of 75%, and an area under the receiver operator curve of 73.4% using parametric analysis. In addition, while visible-spectrum imaging picks up only surface information, fluorescence imaging in the NIR spectrum enables deep-tissue imaging, which helps identify the location of tumors before surgery (Figure 5.9(D)). Compared to the state-of-the-art, non-real-time, bulky Pearl imaging system, with a receiver operator curve of 77.9% and relevant standard error of 6.3% [130], the bio-inspired sensor provides similar real-time accuracy under surgical light illumination.
Figure 5.9 A-C, Snapshot images obtained with the bio-inspired imaging sensor during surgery with a spontaneous breast cancer murine model. A, Near-infrared (NIR) image highlights the locations of the tumors, as well as the liver, where the tumor-targeted contrast agent LS301 is cleared. B, Color image of the animal obtained by the bio-inspired sensor during tumor removal. C, A combined image of both NIR and color information as it is presented to the surgeon to assist with tumor resection. D, A combined image of both NIR and color information indicating the location of the tumors under the skin. (See Supplementary Videos S1 and S2.)

5.3.7 Clinical translation of the bio-inspired technology
The current standard of care for tracking SLNs is to inject into the patient both a visible dye, such as ICG or methylene blue, and a radioactive 99mTc Sulphur-colloid tracer. The SLNs are generally first identified by the unaided eye, due to the coloration of the accumulated dye at the tissue’s surface, followed by gamma probe to check for radioactivity. In a pilot clinical trial, I investigated the utility of the bio-inspired imaging sensor to locate SLNs in human patients (n=11) with breast cancer using the ICG lymphatic tracer. ICG naturally exhibits a green color due to its absorption spectra, as well as NIRF at 800 nm. ICG passively accumulates in the SLNs and is cleared through the liver and bile ducts within 24-36 h post-injection.

Our bio-inspired imaging system provided the surgeon with real-time intraoperative imaging of the tissue in color that is enhanced with NIRF information from the ICG marker under surgical light illumination (Figure 5.10). The surgeon could identify 100% of the SLNs when using
information registered with the bio-inspired imaging system alone. In contrast, the surgeon identified 90% of SLNs when using the green color from ICG with the unaided eye, and 87% when using information from the radioactive tracer.

Noteworthy, during one of the procedures, the surgeon identified and resected two uninvolved SLNs using information from the green color of the ICG probe, which the gamma probe did not detect. With the assistance of the gamma probe, another two involved SLNs were identified and resected, which did not exhibit visible green color accumulation of ICG. However, the bio-inspired imaging system correctly identified all four SLNs during this operation. We anticipate that this is because the green color of the ICG dye can be visually identified only at the tissue’s surface when viewed with the naked eye; in contrast, the bio-inspired sensor identifies NIRF signals several centimeters deep in the tissue [7]. The gamma ray detector failed to detect the radioactive tracer in two of the four SLNs because the limited space in the surgical cavity limited inserting the relatively large radioactivity detection probe. In contrast, since both surgical and excitation light could clearly illuminate the surgical cavity, the bio-inspired sensor provided accurate visualization of both anatomic features and the location of the ICG dye in the SLNs.
Figure 5.10 Clinical use of the bio-inspired imaging sensor for mapping sentinel lymph nodes (SLNs) using indocyanine green (ICG) contrast agent. A, Fluorescence image obtained with the bio-inspired camera highlights the location of the SLNs. B, Color image obtained with the sensor captures anatomical features during the surgical procedure. C, Synthetic image obtained by combining color and near-infrared images presented to the surgeon in the operating room to assist in determining the location of the SLNs (see Supplementary Video S3).

5.4 Discussion
We have designed, fabricated, tested, and translated into a clinical setting a bio-inspired multispectral imaging system that provides critical information to health care providers in a space-, time-, and illumination-constrained operating room. Because of its compact size and excellent multi-spectral sensitivity (see Table 5.3), this paradigm-shifting sensor can assist physicians without impacting normal surgical work flow. The high co-registration accuracy between NIR and visible-spectrum images and the high NIR imaging sensitivity under surgical light illumination are key advantages over current FDA-approved instruments used in the operating room. The initial clinical results, which corroborate results from previous studies [131] describing the benefits of using fluorescence properties of ICG for mapping SLNs in cancer
patients, suggest that the improved accuracy and sensitivity of the bio-inspired sensor can greatly improve surgical management of cancer and may even be able to spearhead new directions in cancer diagnosis that could have a profound impact on health care.

<table>
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</tr>
</thead>
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</tr>
<tr>
<td>Frames per second</td>
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<tr>
<td>Full well capacity</td>
<td>72,000 e⁻</td>
</tr>
<tr>
<td>Sensitivity</td>
<td>9 μV/e⁻</td>
</tr>
<tr>
<td>Dynamic range</td>
<td>62 dB</td>
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<tr>
<td>Signal-to-noise ratio</td>
<td>48 dB</td>
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<td>Fixed-pattern noise</td>
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<tr>
<td>Number of spectral bands</td>
<td>4</td>
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<tr>
<td>Detection limit</td>
<td>100 pM indocyanine green with 20 mW/cm² laser excitation, 60 kLux surgical illumination at 25 frames/sec</td>
</tr>
<tr>
<td>Power consumption</td>
<td>250 mW</td>
</tr>
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</table>

Table 5.3 Summary of the optoelectronics performance of our bio-inspired imaging sensor
Chapter 6: Near-Infrared Angiography for Critical Limb Ischemia in a Diabetic Murine Model [132]

Peripheral arterial disease (PAD) is a highly prevalent disease process that afflicts more than 20% of individuals with diabetes. Progression of PAD in the setting of diabetes can lead to critical limb ischemia (CLI), which is associated with increased risk of wounds, gangrene, and limb loss. Prompt noninvasive evaluation of limbs affected by PAD progression and CLI is currently limited. Here, I evaluate the utility of a novel custom-designed multi-spectral imaging system for fluorescence-based near-infrared angiography and compare it to the existing gold standard of laser-scanning Doppler perfusion assessments. Due to its higher resolution and fluorescence sensitivity, near-infrared angiography demonstrates a greater capacity to characterize altered dynamic arterial perfusion in a clinically-relevant diabetic murine model for CLI. Furthermore, I demonstrate that the imaging system can accurately track arterial perfusion recovery over time following induced ischemia, and reveal unique phenotypic differences in the setting of diabetes.

6.1 Motivation for Near-Infrared Angiography
Nearly 10 million Americans suffer from peripheral arterial disease (PAD) [133] – a disorder that affects nearly 20% of individuals above the age of 60 [134], and is the third leading cause of atherosclerotic cardiovascular morbidity [135]. PAD is caused by the buildup of atherosclerotic plaque in the extremity arteries [136]. This results in the chronic inadequate supply of oxygen and nutrients to the extremities, leading to advanced critical limb ischemia (CLI), non-healing wounds, infection, and gangrene [137]. After smoking, diabetes is the second most important risk factor for the development of PAD [135]. PAD also begins earlier and progresses more rapidly in
patients with diabetes[138]. Furthermore, diabetic patients are more likely to suffer from complications related to progressive PAD and CLI, which can lead to increased risk of limb loss, and higher morbidity and mortality[139].

Despite the high prevalence of CLI in the setting of diabetes, our understanding of the pathophysiology of this recalcitrant disease process is limited. Furthermore, it is currently unknown whether CLI manifestations in the setting of diabetes present with unique molecular, anatomic, or dynamic flow principles. Accordingly, recent investigations have attempted to characterize arterial perfusion in the setting of CLI. However, few tools are currently available to accomplish this goal in the clinical setting. Therefore, there is a profound need to develop an imaging system that can facilitate noninvasive evaluation of arterial perfusion in the lower extremities of individuals afflicted with CLI and diabetes.

Here, I present a novel, custom multi-spectral camera for fluorescence-based near-infrared angiography (FBA). This high-resolution imaging system can detect in real-time and with high sensitivity the fluorophore signals emitted by the fluorescent dye indocyanine green (ICG), which is widely used in perfusion diagnostics. Our findings demonstrate that the low-cost, mobile, FBA imaging system can provide accurate, highly sensitive, dynamic assessments of arterial tissue perfusion in a murine diabetic model of CLI. These findings will provide the foundation for future translational bench-to-bedside human clinical trials that may show benefit from the use of this novel imaging platform.

Our FBA imaging system is significantly more sensitive in evaluating peak tissue perfusion than conventional tissue-perfusion assessments using laser-scanning Doppler technology. Specifically, the FBA imaging system’s higher image resolution measures, with high accuracy, low levels of perfusion in the ischemic hind-limbs of C57BL/6 mice of both phenotypes, diabetic
or nondiabetic. Similarly, the FBA imaging system helps to detect subtle but potentially relevant differences in tissue perfusion at specific hind-limb regions in diabetic mice, a known clinical risk factor for CLI. In addition, unlike the gold-standard laser-scanning Doppler imager, the FBA imaging system can differentiate the distinctive phenotypes based on real-time dynamic perfusion inflow. Prior to the induction of ischemia, the study thus reveals dynamic perfusion variables (rate of inflow) that are useful for both laboratory-based studies and future translational bench-to-bedside human clinical trials.

6.2 Instrumentation and Methods

6.2.1 Multi-spectral camera for FBA
To acquire real-time near-infrared fluorescence (NIRF) data for optical blood perfusion angiography, a custom multi-spectral camera (RGB-NIR camera) was used. The camera, outlined in Figure 6.1, consists of an array of CCD photodetectors with a total resolution of 1600 by 1200 pixels and a maximum frame rate of 40 fps. The CCD sensor has a dynamic range of 68 dB and a readout noise of 16 e− with a maximum well capacity of 20 ke−. An array of pixel pitch-matched spectral filters is overlaid on top of the imager sensor for spectral (NIR and visible spectrum) sensitivity. The pixelated filter topology consists of a 2-by-2 super pixel pattern repeated across the imaging array, where the individual pixelated filters are effectively interference filters optimized for sensing RGB and NIR (>800 nm) spectra. The spectral interference filters are achieved through a nanofabrication process, where multiple interleaved layers of materials with low and high dielectric constants are deposited on top of each other by physical vapor deposition. Because of an optimized nanofabrication process for realizing pixelated spectral filters, the filters have high transmission ratios (>80%) and high optical densities (>6 OD), which are necessary for high fluorophore sensitivity. In addition to the pixelated layer, a notch filter (Semrock, NF03-785E-25), with an optical density of ~6 at 780
nm, is added to the system to further suppress the fluorophore excitation light source from reaching the sensor. The compounded result of this architecture achieves a maximum ICG dye sensitivity of 10 nM under surgical light illumination (40 klux) with minimal spatial co-registration error among the spectral channels, i.e. color and NIR information. This leads to high blood flow detectability and co-registration of this information with the corresponding anatomic features. The camera is connected to a laptop via an Ethernet cable to transmit 12-bits per pixel data at 40 fps. The laptop screen displays a color image, a fluorophore signal image in false color, and an overlaid version of both data frames through custom-developed software.

6.2.2 Imaging setup
The imaging setup shown in Figure 6.1 consists of the RGB-NIR camera, a laptop, a 780 nm laser (B&W TEK Inc., BWF2-780-0.8) and its optics, a visible-light LED panel (Genaray, LED-7100T), and a heating pad (Kaz, 7788-R). The laser is coupled to the custom laser optics through an optical fiber. The laser optics consist of a shaping line filter at 780 nm (Semrock, LL01-780-12.5), an aspheric condenser lens (Thorlabs, ACL25416U-B), and a diffuser (Edmund Optics, 47-994) to create a 10 cm uniform illumination pattern with ~6 mW/cm² of excitation power. The LED panel produces 5 klux of visible light at the surface of the heating pad with a light temperature of 5,000 K. Visible short-pass filters (3M, Cool Mirror Film 330) are placed on top of the LED panel to suppress any leakage of NIR light that could deteriorate the fluorescence signal acquired by the RGB-NIR camera. The RGB-NIR camera sits orthogonally above the heating pad at a distance of ~60 cm, with the laser optics and the LED panel placed on each side of the camera (Figure 6.1). The anesthetized mouse is placed at the center of the heating pad in the supine position, as shown in Figure 6.1, while keeping the heating pad at a temperature of ~37 ºC to ensure normal cardiovascular system behavior in the animal.
6.2.3 In vivo small animal angiography
All animal studies were performed according to protocols approved by at Washington University School of Medicine Animal Studies Committee for humane care and use of laboratory animals.

Optical blood perfusion angiography was performed on diabetic (n = 10) and nondiabetic (n = 7) mice. All mice underwent unilateral femoral artery ligation, and their arterial perfusion was assessed before and after ligation and through their recovery process (day 7 and day 14 post ligation). The assessment was performed both with the FBA imaging system and with a laser-scanning Doppler imager for comparative reasons. The experimental approach comprised the three following steps:

Murine models for CLI and diabetes
One month prior to experiments, a cohort of adult (8 to 10 week-old) wildtype mice on a C57BL/6 genetic background received a single intraperitoneal injection of streptozotocin (STZ)
to induce a diabetes-like phenotype with a blood glucose of >300 mg/dL. These diabetic mice, as well as nondiabetic mice (control wildtype mice that did not receive STZ), underwent unilateral femoral artery ligation at a level distal to the epigastric vessel origin to induce a moderate form of hind-limb ischemia (an established model for human CLI).

**Laser-scanning Doppler blood perfusion imager of murine CLI models**
Diabetic and nondiabetic mice underwent serial arterial perfusion assessments of ischemic and non-ischemic hind-limbs at day 0 (pre-unilateral femoral artery ligation), day 1 (post ligation), day 7, and day 14. Mice were anesthetized (using a ketamine/xylazine cocktail), and kept on an underbody warming pad set at ~37 ºC, and hair was shaved from the distal abdominal, and medial hind-limb regions. Each mouse underwent serial measurements using a high-resolution laser-scanning Doppler blood perfusion imager (Perimed, PeriScan PIM 3). Adductor, gastrocnemius, and hind-paw segments of ischemic and non-ischemic hind-limbs (Figure 6.2), were analyzed by an independent investigator (who was blinded to murine cohort) using the manufacturer’s software (LDPIwin). Both mean and peak perfusion units for each anatomic region of interest were collected.

**Fluorescence-based NIR angiography of murine CLI models**
Immediately following each laser-scanning Doppler imager evaluation, all anesthetized mice also underwent dynamic perfusion assessment using the custom FBA imaging system for optical blood perfusion angiography. Through a unilateral retro-orbital infusion, mice received ICG 0.25 μg/μL adjusted to a volume-to-mass ratio of 200 μL/30 g. Immediately upon ICG infusion, the fluorescence response of ICG was captured by the FBA imaging system with a laser excitation at 780 nm and fluorescence emission at 800 nm and above. Similarly to previous assessments, an investigator blind to the murine diabetic status evaluated the perfusion variables using a custom-developed algorithm in MATLAB and C++. Variables collected included the rate of fluorescent
signal inflow and peak fluorescent signal in ischemic and non-ischemic hind-limbs. To reduce
anatomic variance and enhance consistency of the data perfusion variables, the hind-limb
adductor, gastrocnemius, and hind-paw segments of ischemic and non-ischemic hind-limbs were
compared. This division gives a total of six anatomic regions of interest per mouse as shown in
Figure 6.2.

![Figure 6.2 Sample color image with outlines of the anatomic regions of interest.](image)
The image was taken on day 1, just after ligation. The left hind-limb and right
hind-limb are labeled as ischemic (blue outline) and non-ischemic (black
outline), respectively. The hind-limbs have been conceptually divided into three
anatomic regions: gastrocnemius, adductor, and hind-paw.

6.3 Data Analysis
Variables collected from laser-scanning Doppler imager and the custom multi-spectral FBA
imaging system were then used to compare sensitivity between the two imaging modalities, and
to determine whether diabetes can influence perfusion dynamics in ischemic hind-limbs. To
evaluate this, a hind-limb perfusion ratio was derived by dividing the perfusion variables of the
ischemic versus non-ischemic hind-limb for each anatomic region of interest[140, 141].
Each technology is capable of outputting spatially co-registered intensity and signal images, with the signal image proper to each architecture (Figure 6.3). For the laser-scanning Doppler imager, the intensity image is monochromatic, while the signal image is the wavelength change (Doppler shift) of the imager’s laser and is caused by the movement of blood cells, which is directly correlated to the animal’s perfusion. For the RGB-NIR camera, the intensity image is a true color image of the area of interest, and the signal image is the NIRF light emitted by the fluorescent dye. This fluorescence magnitude is directly correlated to, among other factors, the dye concentration, dye volume, and plasma-bonded volume. Since in all mice the dye concentration was kept constant and the volume was proportional to the animal’s weight, the blood volume bonded to ICG was the only main independent variable. This variable changes dynamically as the dye circulates through the vascular system.

In Figure 6.3 the signal image for each technology is depicted in false color, where red represents a strong signal, either high fluorescence or high perfusion, and blue represents a weak signal, either low fluorescence or low perfusion. Note the striking difference of the data refresh rates between these two technologies: the Doppler imager takes several seconds to scan an area of interest for a single snapshot, while the RGB-NIR camera acquires 40 full-resolution data frames every second. This technology limitation of the Doppler imager prevents its use to assess dynamic arterial perfusion, necessary to study microvascular complications in patients with diabetes mellitus.

To extract the input hind-limb perfusion metrics for the Doppler imager, the manufacturer’s software was operated by a trained technician. Each anesthetized and shaved animal (n = 17) was placed on the Doppler imager’s imaging area in the supine position. The Doppler imager performed a scan for each mouse. Using built-in software functions, the technician marked the
six anatomic regions of interest per each mouse and then extracted the mean perfusion value per area for a total of six perfusion metrics per mouse per experimental day, three for the ischemic hind-limb and three for the non-ischemic hind-limb.

For optical blood perfusion angiography utilizing the RGB-NIR camera, the setup depicted in Figure 6.1 was utilized. The mice were imaged for several minutes while successfully capturing the injection point and the input and output perfusion variables. We focused on the input perfusion variable since it is expected to be the most consistent between mouse groups and would capture whether arterial inflow is altered in the setting of diabetes. The inflow variable commonly lasts only a few seconds. For the RGB-NIR camera, a trained technician used custom-developed software to draw the six areas of interest on each of the animals. The software then extracted the raw pixel-data that constitute each of the areas on each frame. These pixel-data
were averaged, taking out 20% of the outliers, and passed through a low-pass filter to reduce high-frequency noise. Figure 6.4 shows the six normalized angiography vectors over time acquired with the RGB-NIR camera from a nondiabetic and a diabetic mouse on day 0 (before ligation), day 1 (after ligation), and day 7 (during recovery). Figure 6.4 also shows three fluorescence snapshots on each of the three experimental days at the injection point, halfway between the injection point, and the first local maximum point. Of note, each data point on this vector is directly correlated to the accumulation of ICG in the blood but not necessarily to its flow. To extract a blood perfusion metric, the fluorescence gradient on each of the six vectors was computed. As shown in Figure 6.1, points A and B on the inset denote the injection and first local maximum points, respectively, on a sample fluorescence plot. Using these two points, a fluorescence gradient was computed for each of the six time vectors, giving a total of six perfusion metrics per mouse per experimental day that are homologous to those extracted by the Doppler imager. Since the rate of ICG accumulation in the blood is calculated, rather than its momentary signal emission, this metric is more closely related to blood flow than the single-frame data points. This numerical approach has the additional advantage of suppressing any signal baselines caused by imperfections of the optics or nonuniformities on the excitation light source distribution.

Once the six perfusion metrics were extracted for each technology, the hind-limb perfusion ratios were computed by dividing the anatomic regions of the non-ischemic hind-limb by the corresponding regions of the ischemic hind-limb. These perfusion ratios are the basis of the comparative analysis for these experimental trials.
6.4 Results and Discussion

6.4.1 Distal arterial perfusion during the ischemic recovery process

Here, I summarize the differences between the gold-standard scanning laser Doppler technology and the novel FBA imaging system in evaluating hind-limb perfusion recovery in the setting of ischemia and diabetes. We present each technology’s ability to track the recovery process of each phenotype group, as well as data supporting the hypothesis that the FBA system reveals
distinct differences in distal arterial perfusion during the recovery process of diabetic mice. The hind-limb perfusion ratios should be minimal immediately following unilateral femoral artery ligation since the ischemic vascular system has been seriously compromised. As time progresses the ischemic hind-limb should progressively recover arterial inflow and distal perfusion. This process is assumed to be impaired in the setting of diabetes.

To assess the recovery process, I have clustered the mice by diabetic status and experimental day. Note: Data are geometric centroids of adductor, gastrocnemius, and hind-paw perfusion ratios and geometric distances between the centroids from day 0 (D0; before ligation) and those on days 1, 7, and 14. Table 6.1 shows the geometric centroids, equivalent to the cluster-member means, of each anatomic hind-limb perfusion ratio (adductor, gastrocnemius, and hind-paw) at day 0 before ligation, day 1 right after ligation, and days 7 and 14 after recovery for the RGB-NIR camera and the Doppler imager. Both technologies report maximum and minimum hind-limb perfusion ratios on day 0 and day 1, respectively, for all three anatomic regions. To more systematically evaluate the recovery process, I have computed the geometric distances between the centroids for days 1, 7, and 14 and the initial vascular-unaltered centroid for day 0. As expected, the geometric distance for both phenotype groups and for both technologies is greatest on day 1, when ischemic hind-limb perfusion is most compromised. Both technologies show that this geometric distance decreases with time in both phenotypes, confirming that both diabetic and nondiabetic mice are recovering from the induced ischemia. Interestingly, both technologies very similarly demonstrate that hind-limb perfusion on day 1 is more compromised in diabetic mice. These findings combined with the results from the RGB-NIR camera on day 7 (described below), provide strong evidence that diabetic mice recover more slowly than their nondiabetic counterparts. Finally, the RGB-NIR camera detects a slower recovery, based on the geometric centroid distance, in both diabetic and nondiabetic mice on days 7 and 14 compared to the
Doppler imager, possibly indicating that the FBA imaging system is additionally providing microvascular perfusion data that the Doppler imager is less sensitive to.

<table>
<thead>
<tr>
<th></th>
<th>Diabetic (Adductor, Gastroc, Hind-paw)</th>
<th>Dist. to D0</th>
<th>Nondiabetic (Adductor, Gastro, Hind-paw)</th>
<th>Dist. to D0</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>RGB-NIR camera</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Day 0</strong></td>
<td>(0.910, 1.024, 1.145)</td>
<td></td>
<td>(0.777, 0.754, 1.131)</td>
<td></td>
</tr>
<tr>
<td><strong>Day 1</strong></td>
<td>(0.618, 0.136, 0.063)</td>
<td>1.430</td>
<td>(0.421, 0.063, 0.084)</td>
<td>1.304</td>
</tr>
<tr>
<td><strong>Day 7</strong></td>
<td>(0.750, 0.434, 0.190)</td>
<td>1.134</td>
<td>(0.575, 0.286, 0.125)</td>
<td>1.127</td>
</tr>
<tr>
<td><strong>Day 14</strong></td>
<td>(0.779, 0.479, 0.271)</td>
<td>1.038</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Doppler imager</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Day 0</strong></td>
<td>(1.129, 0.936, 0.998)</td>
<td></td>
<td>(1.000, 0.882, 1.075)</td>
<td></td>
</tr>
<tr>
<td><strong>Day 1</strong></td>
<td>(0.458, 0.102, 0.048)</td>
<td>1.431</td>
<td>(0.635, 0.133, 0.074)</td>
<td>1.302</td>
</tr>
<tr>
<td><strong>Day 7</strong></td>
<td>(0.936, 0.621, 0.343)</td>
<td>0.752</td>
<td>(1.044, 0.654, 0.353)</td>
<td>0.759</td>
</tr>
<tr>
<td><strong>Day 14</strong></td>
<td>(0.956, 0.810, 0.587)</td>
<td>0.464</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Note: Data are geometric centroids of adductor, gastrocnemius, and hind-paw perfusion ratios and geometric distances between the centroids from day 0 (D0; before ligation) and those on days 1, 7, and 14. Table 6.1 RGB-NIR camera and Doppler imager centroid coordinates and recovery distances. A more comprehensive view of these distributions is shown in Figure 6.5, which offers a three-dimensional visualization of the perfusion ratio data for each phenotype for the RGB-NIR camera and Doppler imager. Figure 6.5 also shows the centroid locations for each experimental day cluster and their projections to the coordinate planes. These projections visually show how each of the three perfusion ratios agree with the findings in Note: Data are geometric centroids of adductor, gastrocnemius, and hind-paw perfusion ratios and geometric distances between the centroids from day 0 (D0; before ligation) and those on days 1, 7, and 14. Table 6.1. Figure 6.5 also shows the two-dimensional profiles of gastrocnemius versus adductor and hind-paw versus adductor. On both profiles, the cluster distribution trend can be appreciated for both phenotypes and for both technologies: day 0 data points cluster at the top right of the profile or coordinate plane, indicating high perfusion ratios; day 1 data points cluster at the bottom left, indicating low perfusion ratios; and day 7 and 14 data points gravitate toward day 0 with those on day 14 being the closest.

**6.4.2 Dynamic arterial perfusion variables distinguish diabetic from nondiabetic mice prior to ligation**

We hypothesized that the subtle differences caused by the diabetic status on hind-limb perfusion should be sufficient to successfully distinguish diabetic mice from nondiabetic mice before induction of the CLI mode, and indeed, based on the dynamic arterial perfusion variables, we
could identify the distinctive phenotypes. Based on the fact that diabetes causes microvascular complications, I hypothesized specifically that ICG propagation through the cardiovascular system would have different dynamics in different phenotypes as it approaches the hind-limb extremities. We support and frame this hypothesis with reference to the chemical properties of ICG and plasma: (a) ICG is known to bind to plasma and this binding is strongly dependent on the albumin protein content[142], and (b) the albumin content in plasma is altered greatly in diabetic patients[143].

To evaluate the hypothesis, I assessed each mouse hind-limb (n = 34) separately, using the initial perfusion metrics on day 0 (three measures per hind-limb), before ischemia was introduced to the model. To frame these metrics in the hypothesis, the analysis scheme divides the hind-limb perfusion metrics by those of the hind-paw, the most distal anatomic region. Figure 6.6 shows the clear cluster separation between the two phenotypes for the RGB-NIR camera, in contrast to the Doppler imager results, and the ability to draw a decision boundary between the two phenotype clusters computed with a support vector machine. The nondiabetic data points tend toward the top right section of the plot, while the diabetic ones tend in the opposite direction. This data separation does not appear with the data acquired from the Doppler imager.
Figure 6.5 Data plotted for RGB-NIR camera (a) and Doppler imager (b) for diabetic vs. nondiabetic mice: 3D visualization of the hind-limb perfusion ratios for each mouse with their respective cluster centroids and projections to the coordinate planes (left column) and 2D profiles for gastrocnemius vs. adductor (middle column) and hind-paw vs. adductor (right column).
Figure 6.6 Phenotype clustering for the RGB-NIR camera and the Doppler imager. Unlike data from the Doppler imager, the RGB-NIR camera data allows a clear decision boundary to be drawn between the two phenotype clusters. Day 0 mice hind-limbs, n = 20 diabetic, 14 nondiabetic.

We attribute this difference, first, to the superior resolution and sensitivity of the FBA imaging system and its ability to capture dynamic perfusion variables in real time, and second, to the inherent architectural disparity coming from the different physical phenomena captured. The Doppler imager relies on measuring, for one point in space at a time, the back-scattering of light caused by red blood cells. This measurement can suffer from heterogeneities, where the blood perfusion measurement can be affected by spatial variations and optical properties of the tissue[144-146]. This effect indicates that the perfusion signal can be compromised if the measurement site is changed, jeopardizing analysis of blood perfusion changes over time. The RGB-NIR camera, in contrast, measures the dye’s emitted fluorescence in an anatomic area of interest at a single point in time. This fluorescence is correlated not only to the blood volume and hence its perfusion but also to its chemistry, providing valuable knowledge about arterial perfusion angiography.

6.5 Conclusion

PAD is a serious health problem for Americans, with life-threatening consequences. PAD can evolve into CLI in the setting of diabetes, causing the patient to undergo arterial surgery or amputation. Current technology is greatly limited in noninvasively evaluating peripheral arterial
perfusion in the setting of CLI and diabetes. An imaging system that can provide real-time assessments of arterial hind-limb perfusion would greatly improve the ability to evaluate the pathophysiology of the disease process, which may translate into better management and treatment strategies. Here, I present the novel, custom multi-spectral FBA imaging system for angiography. Our RGB-NIR camera system can track the ischemic recovery of diabetic and nondiabetic mice in a CLI model, involving arterial ligation more accurately than the gold-standard laser-scanning Doppler imager. Our system can also identify differences between diabetic and nondiabetic murine cohorts prior to arterial ligation (i.e. day 0 mice of the experiment), based on dynamic perfusion parameters that are not detectable with laser-scanning Doppler imaging. The greater accuracy and expanded capability this system offers will be useful for both laboratory-based studies and future translational bench-to-bedside human clinical trials. No conflicts of interest, financial or otherwise, are declared by the authors.
Chapter 7: A Bio-Inspired Hexachromatic Imager for Near-Infrared Fluorescence Image-Guided Surgery

I have designed, fabricated, and tested a hexachromatic imager for near-infrared fluorescence image-guided surgery capable of simultaneously imaging three visible and near-infrared spectral channels. The sensor is comprised of 1280 by 720 pixels yielding a 48 dB signal-to-noise-ratio and achieving a minimum indocyanine green concentration detectability of 5 nM. The imaging sensor combines the vertically stacked photodiode technology and an array of pixelated spectral interference filters in a single-chip single-snapshot architecture yielding high sensitivity and clinical translation.

7.1 Motivation for a multi-spectral imager

Image-guided surgery (IGS) has proved to be an exceptional operational strategy for surgeons – aiding in performing far less invasive and much safer procedures [147]. Among the optical imaging techniques available for IGS, near-infrared fluorescence (NIRF) imaging has been one of the main protagonists in research performed by both industrial and academic laboratories. The development of less toxic and far more specific molecular targeted probes constitutes a potential major improvement in the patient’s surgery outcome by giving the surgeon the ability to make real-time clinically relevant decisions based on molecular labels. NIRF imaging has multiple advantages over visible fluorescence imaging, such as low tissue auto-fluorescence and low tissue scattering and absorption which enables capturing fluorescence signals several millimeters deep in the tissue. In addition, the excitation and emission spectra are invisible to the unaided human eye and so do not hamper the clinical workflow [127].
Depending on the nature of the procedure, the surgeon could choose to use one or more of the many commercially available molecular probes to highlight different biological systems including tumor tissues, blood vessels, lymph nodes or vessels, nerve networks, or bone structures [25, 132, 148-153]. To complement and exploit the benefits provided by these molecular probes, an imaging system for NIRF IGS should provide the surgeon with reliable real-time information about the magnitude and location of the signals generated by the NIRF probes as well as time and space co-registered high-resolution color frames to identify the patient’s anatomical features. At the same time, the system should occupy a small and ergonomic form factor that can blend seamlessly into the current surgical workflow and be brought effectively into the operating theater. Furthermore, it is desirable that multiple near-infrared (NIR) fluorophores can be detected and identified simultaneously in real time to increase the overall sensitivity and selectivity labeling, e.g. diminishing false negatives in the identification of cancerous tumors, or to decrease iatrogenic damage to healthy tissue during surgery, e.g. avoiding function loss due to the improper laceration of a nerve during a tumor resection procedure [154, 155].

In response to the proliferation of NIRF molecular probes, a variety of optical systems for NIRF imaging have been developed over the past decade. However, FDA-approved NIRF imaging systems that are commonly used lack the fundamental capabilities to be incorporated in standardized procedures. For example, Hamamatsu PDE and Novadaq SPY [127] cannot co-register the color and NIRF video feeds, forcing the surgeon into a cumbersome mode of operation. Other FDA-approved technologies, e.g. VisionSense Iridium [127], use beam splitters and other complex optics in conjunction with multiple imaging sensors to overcome, although not entirely, the co-registration issue between data frames. Apart from incurring a temperature-
dependent co-registration error, these technologies sacrifice compactness and portability, preventing their implementation in a time- and space-constrained operating room.

Due to advances in nano-technology, these technological limitations have been solved by the monolithic integration of spectral interference filters with a panchromatic imaging array, abandoning the use of burdensome and mobile optics and yielding high optical densities that translate to compact and highly sensitive architectures capable of operating under real surgical light conditions [3, 47, 128, 156, 157]. Nevertheless, the topology implemented in this approach, a tetrachromatic imaging system, is still incapable of multiple fluorophore detection; a single NIR-broadband spectrum is captured by one fourth of the pixels, and no spectral discrimination can be effectuated on the NIR window.

Here, I describe a novel, high-resolution hexachromatic imager for NIRF imaging suitable for IGS. Our imaging sensor combines an array of vertically stacked CMOS photodetectors with pixelated spectral interference filters. The three-dimensional photodetector array exploits the silicon wavelength-dependent depth absorption coefficient to resolve trichromatic information at each pixel location. The pixelated interference filters following a chessboard layout act as shortpass and longpass filters on half of the pixels, respectively. High optical density (OD) is achieved due to a specialized nano-fabrication process where material layers with high and low dielectric constants are stacked. The overall result is a monolithically integrated hexachromatic imager capable of color reconstruction in half of the pixels and NIR-shade sensitivity in the other half of the pixels for multiple NIR fluorophore imaging for IGS.
7.2 Imaging sensor architecture

I have developed, fabricated and tested a hexachromatic imager for NIRF IGS by integrating pixelated spectral interference filters with an array of vertically stacked photodetectors. A block diagram of the sensor with the spectral responses is presented in Figure 7.1.

The imaging sensor comprises an array of 1280 by 720 pixels, and each pixel contains three vertically stacked photodiodes. The stacked photodiode structure is achieved by epitaxial growth on three separate layers on a positively doped silicon wafer and alternating the doping of six n+ and p+ layers. The depths of the PN junctions are modulated to achieve maximum quantum efficiency (QE) at targeted wavelengths while maintaining a broadband absorption spectrum, necessary for good sensitivity in the NIR window. Three transistors are utilized per photodiode and a standard difference double-sampling readout scheme is implemented (Figure 7.2). The imager’s pixel pitch is 7.8 μm with a well capacity of 72 ke− and a conversion gain of 9 μV/e− with a root-mean-square readout noise of 70 e− yielding a dynamic range of 62 dB and a
maximum signal-to-noise ratio of 48 dB while consuming 250 mW. A 14-bit off-chip analog-to-digital converter (ADC) is utilized per photodiode layer.

Figure 7.1 shows the 2 by 1 pixelated filter pattern, which is laid out in a chessboard fashion and contains two different types of spectral filters, acting as shortpass and longpass filters for the purpose of color reconstruction and NIR-shade sensitivity and discrimination, respectively. Each of the two types of pixelated filters covers half of the pixels, increasing the overall resolution and reducing the edge artifacts when compared to multi-spectral imagers that use a 2 by 2 pattern where each spectral channel covers one fourth of the array. The spectral interference filters are achieved by using an advanced nano-fabrication process where physical vapor deposition (PVD) is employed to stack interleaved layers of SiO₂ and TiO₂ with low and high dielectric constants, respectively. The dielectric material profiles are modulated to create the appropriate interference pattern and achieve the desired spectral response. To achieve a high ratio between the transmission and reflection bands, i.e. high OD, many layers are stacked. Photolithography is used to pattern the spin-coated photoresist on the surface of the filter array. A combination of reactive ion-etching and lift-off processes is later used to remove unwanted sections of the targeted spectral filter.

The procedure is repeated one more time to create the second type of spectral filter. Since the procedure steps are iterated only twice, the defects on the nano-fabrication process are reduced. Chromium lanes are added at the beginning of the procedure to reduce the optical crosstalk in between adjacent filters. Finally, the filters are monolithically integrated with the three-dimensional CMOS imaging array to create a 6-channel spectral sensor; the pixels that sit under the shortpass filters yield visible-trichromatic vision while the pixels that sit under the longpass filters yield NIR-trichromatic vision, both decoupled from each other due to the high ODs.
achieved. High ODs are important both to avoid false negatives during fluorophore imaging caused by the bright surgical light and to achieve high fluorophore sensitivity.

A field-programmable gate array (FPGA) is used to transfer the 42-bit-per-pixel raw data via USB3.0 at a maximum full-frame rate of 24 fps to a computing unit where the data is processed and displayed in real time to the user. The raw data is de-mosaicked and interpolated to recover the full resolution. A gain-and-offset calibration algorithm is applied to the NIR data to compensate for optical and electronic mismatches across the imaging array. As well, the color response is adjusted using a 4 by 3 color-mixing matrix to closely replicate the human eye’s color perception.

Figure 7.2 Cross sectional diagram of the pixel’s circuitry. Three vertically stacked photodiodes are shown with a three-transistor readout scheme. The first positively doped epitaxial layer is grown on top of the silicon wafer with ~1.5 μm thickness, followed by selective negative doping to create the bottom photodiode. The second epitaxial layer is grown on top of the first epitaxial layer with a thickness of ~2 μm to create the middle photodiode. The third epitaxial layer for the top photodiode, with ~0.8 μm thickness, is grown last.
7.3 Optoelectronic Characterization

The imaging sensor was tested to characterize its optoelectronic performance, which ultimately translates to fluorophore sensitivity and clinical implementation feasibility. To measure the spectral responses of the filters and the photodiodes (Figure 7.1) a monochromator (Acton SP2150, Princeton Instruments) was connected to the input of an integrating sphere (819D-SF-4, Newport). The output of the integrating sphere was aligned to an adjustable iris (SM2D25, Thorlabs) and an aspheric collimating lens (ACL7560, Thorlabs) to create uniform collimated light orthogonal to the imager’s plane. A calibrated photodiode (S130C, Thorlabs) driven by a power meter (PM100D, Thorlabs) was placed at the end of the optical train to measure the total optical power. The monochromator produced light from 400 nm to 1000 nm in steps of 10 nm. The photodiode then was swapped with our imager sensor to estimate the photodiode QE curves, and a bare sensor, i.e. without spectral filters, to estimate the filter transmission curves. This characterization experiment found that the shortpass (visible) and longpass (NIR) filters have constant and high transmission ratios of ~95% over the desired targeted spectra: 400 – 670 nm and 680 – 1000 nm for the visible and NIR filters, respectively. As well, high ODs were achieved over the desired blocking bands: ~2.5 and ~4 for the shortpass and longpass filters, respectively. Maximum quantum efficiency ratios were found to be ~30% at 430 nm, ~24% at 550 nm, and ~26% at 670 nm for the top, middle, and bottom layers of photodiodes under the visible spectral filters, respectively, and ~7%, ~14%, and ~27% all at 690 nm for the top, middle, and bottom layers of photodiodes, respectively, under the NIR spectral filters. Additional spectral notch filters are added to the system in between the camera lens and the imager to bring the total OD to ~12 at the excitation wavelengths of the fluorophores chosen, thereby blocking undesired laser reflections.
A variation of the previously described optical setup was utilized to calculate the uniformity and fixed pattern noise (FPN) of our imager. The input of the integrating sphere was replaced with a halogen light source (OSL1, Thorlabs) which was current controlled and driven by a direct current (DC) power supply (N5746A, Agilent Technologies). The raw-data histograms, i.e. uncalibrated data, of the six spectral channels over a region of interest (101 by 101 pixels, n = 10201) are shown in Figure 7.3. The low FPN of ~1.4% on all six channels is due to the advanced nano-fabrication process employed on the filter fabrication, necessary for accurate fluorophore identification.

The high QE and low FPN of the imager translate to a minimum indocyanine green (ICG) detection limit of 5 nM when using the International Union of Pure and Applied Chemistry (IUPAC) detection limit model and a numerical factor of 3 for a confidence level of 99.86%. This low detection limit is critical to identify small cell clusters and ensure that no positive margins, in the context of cancer resection, are left behind when the surgery is over.
7.4 Shades of NIR
Sample images showing the color- and NIR-trichromatic vision capabilities as well as the fluorophore classification capability of the hexachromatic imager is given in Figure 7.4. The scene includes two vials with quantum dots (QDs) with emissions at 800 nm and 720 nm, respectively, and a small printed color chart. It is observed that the substance of the QDs inside of the glass vials has a much stronger near-infrared signal than the rest of the scene, due to the intrinsic fluorescence properties of the QDs. In addition, the QDs show an orange-red hue, due to the dominant QE of the bottom photodiode layer on the NIR spectrum. In this figure, a great signal-to-background ratio, which is fundamental to good fluorophore detectability, is shown.

Due to the trichromatic vision capabilities of our imaging system, we can accurately differentiate different shades of NIR which translates to differentiating several molecular probes simultaneously that have different spectral profiles, e.g. differentiating methylene blue (MB) with a peak emission of ~680 nm versus ICG with a peak emission of ~800 nm. Fluorophores with different spectral curves will have a characteristic fingerprint on the response of the three spectral channels. This problem is on a similar complexity level as identifying different colors, i.e. visible hues, such as the precision of a color camera at accurately differentiating yellow from orange, regardless of the scene color temperature. It is important that this differentiation can be accurately performed independently from the fluorophore volume or concentration which determines the NIR signal’s intensity. For this reason, the NIR-trichromatic data is transformed to a hue-saturation-value (HSV) color-space. In this color-space the volume- or concentration-dependent brightness of the sample is mapped to the value component of the color space, and hence can be ignored.

Instead, fluorophore classification can be made by clustering the pixel data points on the plane formed by the hue and saturation components, or $S\cos(2\pi H)$ and $S\sin(2\pi H)$ for their Cartesian
counterparts where $S$ and $H$ represent the saturation and hue components, respectively. The clustering of two fluorophores, ICG and MB, on the $S\cos(2\pi H)-S\sin(2\pi H)$ Cartesian plane with different spectral curves is shown in Figure 7.5. Each data point on this cluster represents a pixel exposed to the fluorescence signal. The cluster includes a total of $126E+3$ and $142E+3$ pixels for ICG and MB, respectively. It is shown that a very clean clustering per pixel is easily achievable by our imaging system. The two fluorophore clusters, ICG in green circles and MB in blue squared, do not have any overlapping among the hundreds of thousands data points, showing the high spectral resolution of our system. This data was collected simultaneously, which is critical for IGS [7]. Furthermore, experimental data shows that our imaging system is capable of differentiating the same fluorophore dissolved in two different media giving emission peaks 5 nm apart, e.g. ICG dissolved in 100% water vs. ICG dissolved in 50% water 50% fetal bovine serum (FBS).

Figure 7.4 Sample images showing the color- and near-infrared-trichromatic vision capabilities and fluorophore classification capability of our hexachromatic imager for near-infrared fluorescence. An RGB color space was utilized for all three images. For a) and b) the bottom, middle, and top photodiodes data are mapped to the red, green, and blue channels and c) is the hue-saturation-value transformation of b) with low and high valid thresholds on the value component. The scene includes, from left to right: quantum dots with peak emission at 800 nm, a small printed color chart, and quantum dots with peak emission at 720 nm. a) Visible spectrum trichromatic image, i.e. color image, after color calibration. b) Near-infrared-trichromatic image; the substance of the quantum dots inside of the glass vials is observed to have a much stronger near-infrared signal than the rest of the scene, due to the intrinsic fluorescence properties of the quantum dots. c) Different hue values are observed for the two types of quantum dots, i.e. fluorophore classification is possible.
Figure 7.5 Pixel clustering of two fluorophores with different spectral profiles. Indocyanine green with peak emission at ~800 nm and methylene blue with peak emission at ~680 nm are represented by green circles and blue squares, respectively. The data plane is constructed by the hue and saturation components. The x- and y-axes are equivalent to $S\cos(2\pi H)$ and $S\sin(2\pi H)$, where $S$ and $H$ represent the saturation and hue components, respectively.

7.5 Conclusion
Here I have shown that by integrating the vertically stacked technology with pixelated spectral interference filters a radically new spectral imager was conceived. The imager is capable of hexachromatic vision for the purpose of color reconstruction and NIR-shade discrimination. This capability enables the differentiation of multiple fluorophores in real time, yielding meaningful clinical information that will help surgeons make real-time decisions about patient outcomes.

The imager inherently co-registers in time and space all the spectral channels, giving the user an accurate matching between anatomical features and fluorophore labeling. Due to the low weight and compact design of our imaging system, it can be brought into the time- and space-constrained operating room, seamlessly blending into the clinical workflow.
Chapter 8: Conclusions
During my doctorate studies I investigated the implementation of biological concepts found in the compound eyes of the mantis shrimp and Morpho butterfly to create radically different and compact imaging sensors for image-guided surgery. Firstly, I show that by mimicking the vertically stacked photosensor design found in the compound eye of the mantis shrimp, I have developed a highly sensitive color-polarization camera. This polarimeter is the first single-chip imaging system reported in the literature that can reliably capture the orthogonal information provided by both color and polarization. Its compactness allows it to be deployed in situations where other state-of-the-art polarimeters, based on conventional advances in optics, cannot operate. A clear example of its optimized usefulness is its integration with an underwater system, capable of recording high-framerate underwater polarization phenomena in situ.

In this thesis, I have shown the paramount advantages as well of using near-infrared fluorescence for image guided surgery. By combining the vertically stacked photosensor technology with interference tapetal filters, biologically inspired in the plasmonic structures found in the eyes of the Morpho butterfly, I have created a multi-spectral imaging sensor capable of detecting and identifying multiple fluorophores under real surgical settings. This bio-inspired sensor can capture simultaneously spatially co-registered color and trichromatic near-infrared videos. Its compact and robust design allows it to be brought into the operating room, effectively blending with the surgical workflow. Characterization data shows its high fluorophore sensitivity and specificity, product of the high optical density numbers achieved by the pixelated spectral filters, necessary to identify small cell clusters and diminish positive margins and iatrogenic damage. This research solves long standing polarization and multi-spectral imaging research problems – investigating an architectural imaging design that can capture two orthogonal optical data planes.
in a spatially- and timely-co-registered fashion without any scene assumptions. Finally, by mimicking and adopting the elegant designs found in nature, I have shown that radical improvements can be achieved on the imaging fields, translating to the use of these technologies by specialists and non-specialists alike.
References


