

SPECTROPHOTOMETRIC QUANTIFICATION OF BILIRUBIN  
IN SCLERA OF THE EYE USING VISIBLE DLP®  
HYPERSPECTRAL IMAGING

by

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## ABSTRACT

### SPECTROPHOTOMETRIC QUANTIFICATION OF BILIRUBIN

#### IN SCLERA OF THE EYE USING VISIBLE DLP®

#### HYPERSPECTRAL IMAGING

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In clinical settings doctors strive to give best care to the patients by looking for methods to diagnose the illness early and intervene with the necessary treatment. In the case of patients with liver problems (e.g. cirrhosis, hepatitis) an early indicator of impaired liver function is an increased concentration of bilirubin in the blood circulation. Bilirubin concentration is currently quantified by blood sampling, which is invasive, and it would be desirable to have a technology that quantifies bilirubin non-invasively. The goal of this work was to help address this clinical need and develop a methodology for determining non-invasively a measure of bilirubin concentration. This technology consisted of a Digital Light Processing hyperspectral imaging (DLP HSI) system coupled to a fundus microscope. In this system light from a polychromatic visible light source was dispersed onto a micro mirror array of a DLP chip so that each column of micromirrors could reflect only a narrow band of monochromatic light. Columns of mirrors were programmed to turn

towards or away from light illumination to thus enable control of the spectral output of the light source. The DLP chip was programmed so that light in 5nm wavelength bins was reflected sequentially across a 440-555 nm range. The back-reflected light from the scleral area was collected by the fundus microscope optics and projected onto a CCD camera, which was time-synced with the DLP chip so as to acquire images separately for each wavelength bin. The detected hyperspectral reflectance data cubes from the scleral area were subsequently analyzed to quantify the relative absorbance of Bilirubin in that vicinity. Quantification of these chromophores was based on their known absorbance spectra for each image pixel. A limitation of this work that precluded absolute quantification of chromophores was that the scleral tissue optical properties were not taken into account in the analysis. Nevertheless, the estimated relative bilirubin absorbance values showed reasonably good correlation with independent quantitative measurements of bilirubin concentration from blood samples obtained from each patient being imaged. Therefore, the proposed technique shows good potential as a non-invasive tool for the detection of Hyperbilirubinemia and warrants more detailed future studies in a larger patient population to assess its sensitivity and specificity for the early diagnosis of this condition.

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## CHAPTER 1

### INTRODUCTION

Hyperspectral imaging helps us visualize the gross chemical composition of tissue by probing the absorbance of tissue-embedded biomolecules with different wavelengths of light. In contrast to invasive methods that can only sample tissue chemistry in a limited number of selected locations, non-invasive methods such as hyperspectral imaging offer the opportunity to survey over extended areas of tissue. The capacity to non-invasively measure multiple tissue regions in one shot can help identify small regions of abnormal composition that can otherwise be missed. Measuring multiple tissue regions can also allow a more statistically robust picture of the overall tissue health to emerge, which can help doctors make more informed decisions about diagnostic or treatment options. Furthermore if Hyperspectral imaging can be validated in its diagnostic potential against a standard invasive technique, then it could eventually replace it. The objective of this work was just along these lines. Specifically, it was attempted to assess the feasibility of using Hyperspectral imaging to identify the chemical (absorbance) signature of bilirubin in the human eye as a surrogate to the standard method of measuring bilirubin levels by spectrophotometry in blood samples withdrawn from the patient. Bilirubin is a yellowish pigment found in bile, a fluid made by the liver. Large amounts of bilirubin in the blood are an early indicator of jaundice and liver cirrhosis. A daily monitoring of total bilirubin from laboratory blood assays is necessary for Doctors to assess the state of a patient's liver.

A hyperspectral imaging system integrated with a fundus microscope was used as a new method to monitor total bilirubin levels in the blood non-invasively from image samples taken

from sclera of the eye. The data presented in this thesis was collected from human patients from Parkland Hospital.

### 1.1 Hyperspectral imaging

Hyperspectral imaging is best understood from the process of interpretation of color. Every object that one remembers is associated with a color, green grass, white moon and red rose. It's is the processing of visual images of the object with the human brain's unique interpretation of different wavelengths of light in the visual cortex of the human brain that helps us define and associate an object.

Hyperspectral images obtained from the desired individual wavelength range have information about the function of the tissue of interest. Each chromophore's response depends on the absorption and reflective properties of tissue at different wavelengths. A Hyperspectral imaging system can be programmed to sequentially scan specifically in the wavelength region to which the chromophores are a known to have a characteristic spectral response

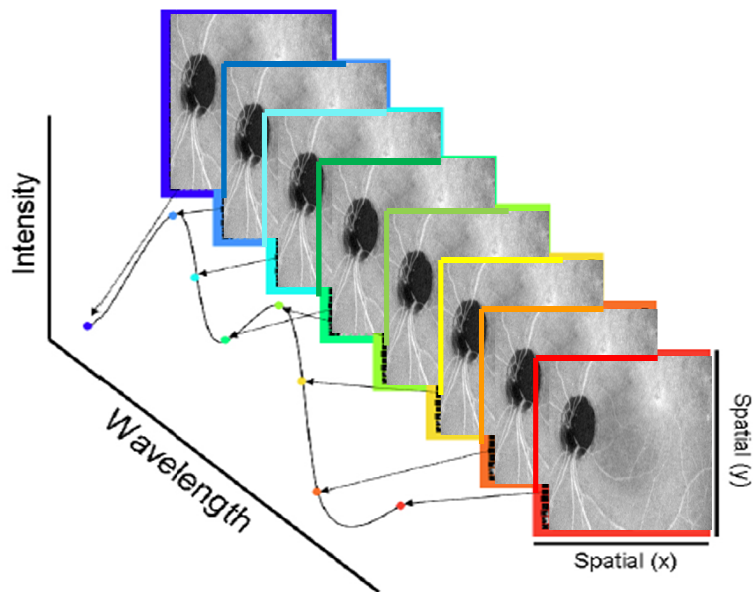


Figure 1.1 A three dimensional Hyperspectralcube (right) and the absorbance spectra (left) derived from this image cube (image of a Retina)

The hyperspectral imager presented in this thesis is a combination of an Agile light source (OL 490) and detector (DVC Camera system). Since the hyperspectral imager collects individual wavelength images, we can generate a 3-Dimensional (use three-dimensional or 3D, search and replace throughout) data cube over a period of time and we can generate the overall spectral information of the object being imaged.

The data presented in this thesis is mainly focused on the relative bilirubin absorbance changes in scleral blood vessels in hyperbilirubinemia. Other current applications of hyperspectral imaging technology are the imaging of bile duct and major nearby blood vessels during a cholecystectomy, determining the degree of tissue biochemical changes in burn victims, and determining a better perfused skin flap to be used during plastic surgery [6],

## 1.2 Research Hypothesis

The hyperspectral imager used for the collection of Scleral Images is a visible spectrum system and the range of data collected using the system is 440 nm to 555 nm. This particular range of wavelengths is chosen based on the distinctive absorbance peak in the spectrum of bilirubin at 470 nm. The system is based on the Digital Light Processor (DLP®) technology by Texas Instruments and can be used to detect changes in relative bilirubin, oxy and deoxy Hemoglobin concentration non-invasively. The images are captured in a 3-dimensional spectral cube. This non-invasive system can be used to provide clinically useful information regarding the level of relative bilirubin concentration. The research hypothesis is that by using the visible DLP® hyperspectral imaging system one can deduce quantitative metrics from the reflectance spectrum of the sclera that correlate with the level of bilirubin measured in the blood samples collected from the same patients.

## CHAPTER 2

### HYPERSPECTRAL IMAGING SYSTEM

#### 2.1. Spectral Illumination Source

The illumination source is a DLP® unit developed by Gooch & Housego (previously known as Optronics Laboratories, Orlando, FL). It consists of a 500 Watt xenon lamp, power housing, and the OL490, which is a Texas Instruments (T.I.) DLP® chip. A desired slit can be used to focus the broadband light from the lamp housing onto the diffraction grating in OL490. The diffraction grating then separates the monochromatic light into its polychromatic components; these individual wavelengths are then projected onto individual columns of DMD micromirrors in the DLP® chip. The DMD electronics can be programmed by the user in order to control the spatial and/or temporal profiles of the illumination spectrum thus allowing the illumination source to precisely control spatial and temporal wavelength profile. Figure 2.1 explains the light path inside an OL490 unit.

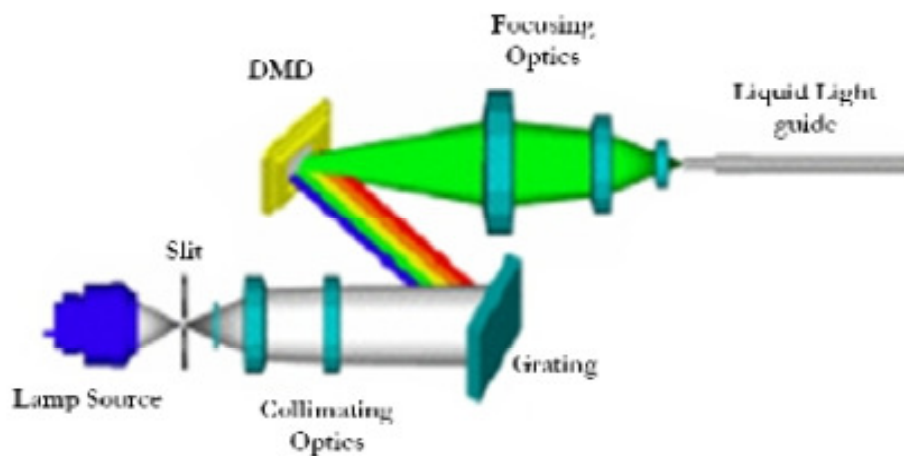


Figure 2.1 Light path from the lamp housing through the OL490 to the liquid light guide (LLG) [6]

### *2.1.1. Texas Instrument DLP® Chip*

The DLP® chip developed by Texas Instruments is based on the Digital Micro-mirror Device (DMD) technology. The DMD was integrated into a DLP® chip (figure 2.2) by Larry Hornbeck of Texas Instruments, Inc. (Plano, TX) in 1987. The DMD is a 768 x 1024 array of micro-mechanical mirrors, which can each be rotated by +/- 12 deg on its own at 3.68 µm pitch. When a mirror is 'on', the light incident on that mirror is reflected for illumination, and when a mirror is 'off', the light incident on that mirror is reflected to an internal heat sink [6].

A DMD device is an  $m \times n$  array of approximately two million micro mirrors that can be switched on or off electronically. This electronic control of the DMD mirrors can be programmed by the user thereby enabling spatial and temporal modulation of light.

Figure 2.2 is a schematic representation of a DMD. The micro-mirrors seen in the figure are made of aluminum and are hinge-mounted on a CMOS substrate. Controlling the voltage bias to each individual electrode creates an electrostatic attraction between the mirror and an address electrode and an opposite force between the yoke and an address electrode thus switching the mirrors 'on' or 'off' very quickly [6].



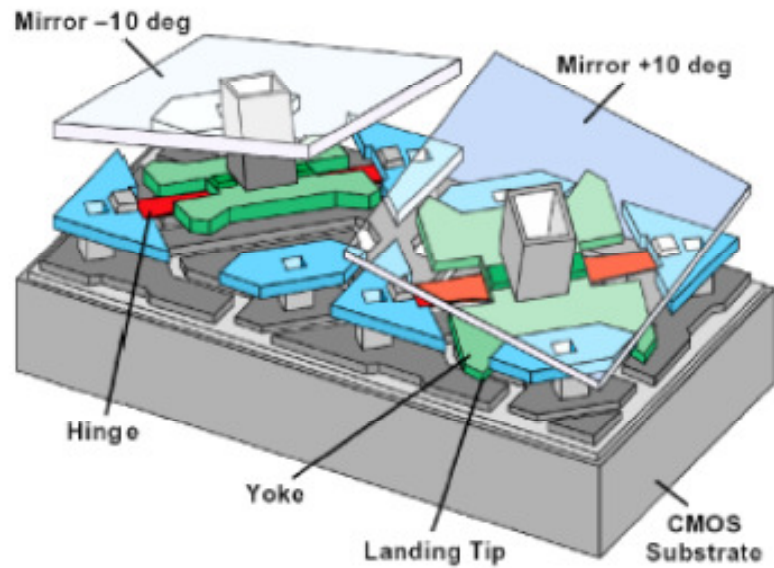


Figure 2.2 Two DMD pixels (mirrors are shown as transparent)[6]. The mirrors are in different states shown at +/-10 deg, as in the original DMD design. The mirrors in the DMD of the OL 490 hinge between +/-12 deg [6]

Positive and negative deflections of the mirror are based on the electrostatic force which is developed between the mirror and its address electrode and also between the yoke and its address electrode. This produces a torque which acts against the restoring torque of the hinges hence allowing the mirror and the yoke to make positive and negative deflections [6].

The light from the DMD is then projected on to the target via a projection lens. The lens and the DMD are aligned in such a way that desired sections of target are illuminated, by turning 'on' the mirrors corresponding to that section. For modulation and fine tuning of the spectral output pulse width modulation is done thereby increasing the grayscale levels for illumination. Figure 2.3 illustrates how a DMD is used to project various grey scale levels.

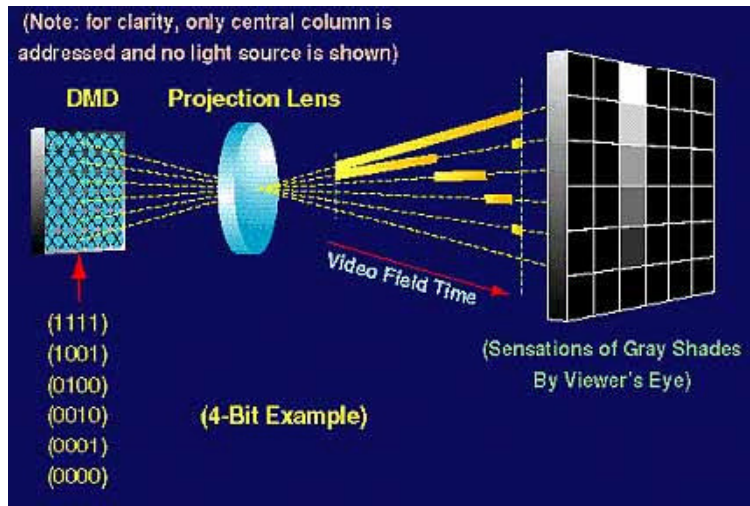


Figure 2.3 Example of DMD array projecting variable gray levels [6]

### 2.1.2 Spectral filtering with the DMD

The basis of spectroscopy is determining the differential effects of light on an object along the frequency or wavelength axis. To measure these effects, broadband light passed through a thin diffraction grating or prism is angularly dispersed into different wavelength components.[6] Spectral spatial analysis is the physical basis of a common point spectrometer, in which a point sample of light is dispersed through a grating and detected by a line array of photodetectors, With each detector in the array corresponding to a narrow band component of the original broadband sample. Similarly, in the OL 490, broadband light from the Xe lamp is angularly dispersed onto the DMD array, and each column of the array corresponds to a unique narrow band component of the broadband light. To illuminate with one of the narrow bands of light at maximum intensity, all 768 of the mirrors in the corresponding DMD column are switched 'on' and all of the mirrors in every other column are 'off'. To reduce the intensity to half of maximum, only 384 mirrors are switched 'on'. Therefore, by turning 'on' a variable number of mirrors in each column, the user can precisely determine the spectrum of light illuminated by the OL 490.

### *2.1.3 Software control of the OL 490*

When the OL 490 Agile light source is shipped to its end user, it comes with OL 490 Application Software (AS) and a software development kit (SDK). The SDK contains sample applications written in C# programming language and the dynamic link libraries (DLLs) necessary to write programs to control the OL 490 hardware. The OL 490 AS features a graphical user interface (GUI). The user selects which slit is between the Xe lamp and the entrance port to the optics head so that the appropriate calibration profile may be loaded in software. The calibration profile indicates which columns of mirrors correspond to which wavelengths.

To program the OL 490 to sweep through illuminations of contiguous bandpasses with constant intensity and bandwidth, the user can select the "Create Sweeps" button on the far right above the sequence area. The user then defines a sweep by selecting the start and end center wavelengths, the wavelength increment, a single intensity value applied to each bandpass, and a single bandwidth applied to each bandpass. The "Create Sweeps" option is very useful for quickly generating a sweep of contiguous bandpasses as required by traditional hyperspectral imaging

## 2.2. Detector

The visible hyperspectral system uses a wide array of scientific grade detectors based on the application. This thesis presents the use of a DVC camera for imaging the human sclera. The DVC Camera consists of Sony ICX285 CCD Chip with a very small form factor and requires minimal to no cooling.

### *2.2.1. DVC Camera*

The DVC is a monochrome camera that consists of a Sony ICX285 CCD chip with a 12 bit @ 40MHZ digitizer. Speed of the system is very critical for clinical setup. In order to acquire a single 3D hyperspectral image cube, multiple slices at each individual wavelength have to be

obtained over time. Hence the speed of the detector is determined by the time required to capture individual images. The technical specifications of the camera are listed in Table 2.1.

Table 2.1. Imaging specifications of the detector which is integrated into the HSI system

<b>Model</b>	DVC 1500M
<b>CCD Sensor</b>	Sony ICX285
<b>Pixel Array</b>	1392 x 1040
<b>Pixel Size</b>	6.45 x 6.45 $\mu\text{m}$
<b>Imaging Area</b>	8.98 x 6.71 mm
<b>A/D Transfer</b>	12 bits @ 40 MHz

The characterization of DVC 1500M is presented in detail in the thesis “Post-Operative Renal Function Analysis Using Visible DLP® hyperspectral imaging System” by Robert Francis [6]. An experiment was performed to see how the frame rate changed with variable exposure time, It was determined that DVC frame rate was independent of the detector exposure time. In another experiment where the exposure time was kept constant, the relationship between the readout time and the number of frames was observed to be linear.

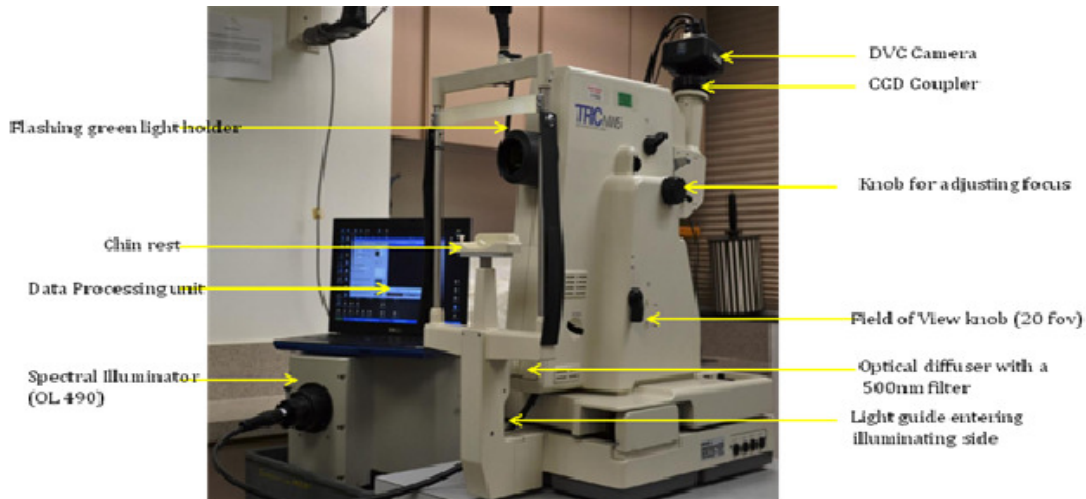


Figure 2.4 Showing the DLP hyperspectral imaging fundus camera

The DVC camera is attached to the C-mount of Topcon TRC NW5s Fundus Camera (Figure. 2.4). To couple light into the camera in this setup, the proximal end of a 5 mm diameter LLG (Liquid Light Guide) must be coupled to the output of the OL490 optics head and a set screw screwed firmly in place. The terminal end of the LLG is inserted halfway into the custom coupler in the illumination housing of the Topcon NW5s, into a chamber containing the optical diffuser. The fundus camera has a knob to adjust the field of view between 20 degree field of view and 45 degree field of view and also a knob to adjust focus.

The Patient placed their chin on the chin rest of the machine which was identical to the one used in the eye exam. Subsequently, reflectance data was acquired by scanning across the 440 nm to 555 nm wavelength range as described above. The light intensity was not more than that used in a regular eye exam. The light that was reflected back from the sclera was detected by the DCV camera and saved in the computer. There was no contact between the instrument and the eye and the procedure was painless and there were no dyes that were injected into the eye. Short

acting anesthetic drops and numbing drops (same drops used in all ophthalmology clinics) were used in order to be able to image the sclera. For some patients that caused irritation of the eyes.

## CHAPTER 3

### SYSTEM SETUP

#### 3.1 Data acquisition and processing

##### *3.1.1 Setup instructions*

The following setup instructions illustrate the set of standard procedures and precautions that must be adhered to while setting up the system for imaging purposes.

##### 3.1.1.1 Computer

A copy of the 'HSI-FC-GUI' folder should be located on the computer's 'C:\' drive, and a shortcut to 'HSI-FC-GUI.exe' placed on the desktop. This is the executable containing the main user interface. Also a copy of 'hsiFC.dll' must be located in 'C:\Windows\System32\'. This is the compiled MATLAB library which contains the processing algorithms for analyzing data cubes.

The Matlab Compiler Runtime Ver. 7.8 must be deployed, so that there is access to 'mclmcr78.dll' and 'vcredist\_x86.exe' should be run to ensure that Microsoft Visual Studio 2008 run-time libraries are on the computer. The DVC camera software, gigabit Ethernet, OL490 ALS software, Ocean Optics SpectraSuite software and USB driver for the Ocean optics spectrometer must be previously installed.

The system must have latest version of Microsoft Visual C# Express and all associated runtime libraries. A copy of 'image\_cube\_copier.exe', 'sweep\_creator.exe', and 'ELCANalyzerFC.exe' should be located on the desktop for saving image cubes into appropriately named folders, creating new illumination sweeps, and easy viewing of captured image cubes and spectra. A copy

of illumination configuration files and GXT files in individually named folders should be located in the parent directory 'C:\DLPDATA\Illumination\'. Most importantly, Wireless networking must be disabled in order for DVC camera connection to work.

All power cords should be plugged into an isolation transformer in order to isolate unsafe leakage current and prevent the potential for electrical shock. OL490 Agile Light Source (VIS - 380 nm to 780 nm) a OL490 Optics Head and OL490 Xe lamp must be coupled on-axis and also make sure that the slit between the lamp and the optics head is 750  $\mu\text{m}$ . Note the last four digits of the serial no. for the OL490 Optics Head as "Filter: OLXXXX" and the last four digits of the serial no. for the OL490 Xe Lamp as "Illuminator: OLXXXX" An AC power cord must be plugged into the optics head and connected to an AC power supply. A USB 2.0 cord must be plugged into the optics head and connected to a control laptop.

An AC power cord must be plugged into the power supply and connected to an AC power supply. The massive power cord must be properly connected to the OL490 Power Supply, with its other end connected to the OL490 Xe lamp. If all is connected correctly, three green LEDs will be lit up.

The proximal end of a 5 mm diameter LLG (Liquid Light Guide) must be coupled to the output of the OL490 optics head and the set screw be screwed firmly in place. The terminal end of the LLG must be inserted halfway into the custom coupler in the illumination housing of the Topcon NW5s. Use the angled hex-driver to tighten set screw which holds the LLG in place in the coupler.

If the coupler becomes disconnected from the fundus camera illumination tube, unscrew the long set screw all the way. Slide the coupler up the fundus camera illumination tube as far as it will slide (you may need to jostle it from side-to-side). Then, retighten the long set screw

To couple the LLG, the cover to the illumination housing must first be removed. After coupling the LLG, replace the cover to the illumination housing. To couple the camera, the bayonet to C-



mount adapter must first be securely tightened to the digital adapter of the fundus camera. All FOV switches on the side of the fundus camera should be set to '45o' and the diopter selector should be pressed all the way in and read '0'.

Plug an AC power cable into the fundus camera and turn the unit on. If the external fixation LED is not blinking, toggle the *Intern. Fix* switch to the 'off' position. Remove the lens cap from the front objective, and an image should appear on the TRC NW5s monitor. The brightness and contrast of this image can be adjusted with the labeled knobs on the left of the fundus camera.

Mount the camera to the C-mount on the digital adapter of the fundus camera. Connect the camera to the computer with a gigabit Ethernet cord and plug the power cable into the camera. After the computer is turned on and booted up, turn on the camera power and wait for the Windows network icon to indicate the status of the Local Area Connection is Limited or no connectivity. Use DVC's GIGE camera finder software to ensure that the computer is communicating with the camera.

Connect the 6-pin terminal from the synchronization electronics box to the I/O port on the back of the DVC camera. Make sure the pins are properly aligned. Connect the BNC terminal from the synchronization electronics box to the "Advance" trigger input on the rear of the OL490-OH. Plug the DC power adapter into the isolation transformer.

#### 3.1.1.2 Capturing hyperspectral cubes with a calibration target

If the slit between the OL490 Xe lamp and the OL490 Optics Head is even slightly off-center, a wavelength shift will be introduced. To correct for this shift, the three set screws must be tightened precisely in the right order and to the correct torque. Even still, a wavelength shift may exist. To measure this wavelength shift, a spectrometer should be placed in front of the TRC-NW5s front objective at a distance such that the illumination beam is incident on the input aperture of the spectrometer. Open OL-490 ALS software and tune the light source to 520 nm

center wavelength with a minimum bandwidth and maximum intensity. Open the spectrometer software (i.e. SpectraSuite) and make sure that the actual center wavelength of the bandpass is at 520 nm. Actual center wavelength should be calculated as the average of the two half-maxima wavelengths.

For Spatial uniformity calibration, place a 20% white Spectralon target in the FOV of the fundus camera. The beam spot reflected from the Spectralon target should appear to be a uniform donut. Now move the fundus camera axially toward and away from the Spectralon target and ensure that the donut grows and shrinks uniformly. If it appears that one side of the beam spot is weighted more than another side, then you can adjust the LLG as in Setup 2C.

#### 3.1.1.3 Folder setup

Create a folder named 'Ufret Clinic – YYYYMMDD' Within that folder create a subfolder named 'Run 1'. Copy illumination wavelength folders into the Run 1 folder depending on what patient population you will be imaging. For bilirubin – \_adjraw\_2b\_440nm\_5nm\_555nm\_31cols\_0b\_. This is a sweep from 440 nm by 5nm to 555 nm, forward and then in reverse, with two blank frames at the beginning. The resulting data cube should contain 24 images. Name one of the copies 'Background'. For Capturing background cubes, Turn off all room lights. Run the executable 'HSI-FC-GUI.exe' by double clicking the desktop shortcut. It may take up to 2 minutes for the executable to load the first time, If it does not load after a longer period, make sure you followed all of the Setup steps correctly. Set "Light Source Calibration Profile" to "750  $\mu$ m," which should be the 3rd option in the pull down menu. When the hyperspectral imaging GUI opens up, load a configuration file located in the folder containing the illumination wavelengths you wish to use for imaging. Parent directory should be named 'Background' and contain the following folders, depending on patient population. Make sure that the processing algorithm is "Show Middle Image." For bilirubin – \_adjraw\_2b\_440nm\_5nm\_555nm\_31cols\_0b\_

'Acquire' five data cubes with the imaging parameters for each set as described in Table 3.1

*NOTE: Do not click "Capture Background Cube".*

Table 3.1 Illumination parameters for acquiring background cube

<b>Column1</b>	<b>Tempcube0</b>	<b>Tempcube1</b>	<b>Tempcube2</b>	<b>Tempcube3</b>	<b>Tempcube4</b>
Binning	1x1	1x1	1x1	1x1	1x1
Exposure Time(ms)	5	10	15	20	20
Gain (dB)	0.2	0.2	0.2	0.2	0.2

### *3.1.2 Image Procedure for Acquiring Patient Data*

Prepare the patient surfaces prior to seating the patient. Wipe the chin rest and forehead rest thoroughly with a previously unopened cleaning swab. Make sure that the LLG and other cords do not present a tripping hazard for the patient when sitting down or getting up from the chair. The fundus camera body should be near the front edge of the ophthalmic instrument table, but ensure that all four rubber feet are completely supported by the table.

For the height adjustment we can adjust the height of the ophthalmic instrument table by stepping on the foot peddle and manually raising or lowering the table also we can adjust the height of the chin rest using the rotary knob on the base of the fundus camera nearest to the examiner. The height of the chin rest should allow the patient to comfortable rest his/her chin and forehead simultaneously without straining his/her neck. Adjust the height of the front objective of the fundus camera by rotating the joystick about its vertical axis.

For focus adjustment we use the fundus camera joystick to move the front objective back from the patient's eye, and position the front objective such that the cornea and pupil are fully visible. Still using the joystick, move the front objective toward the patient's eye, while maintaining the

pupil in the center of the image. When the front objective is near enough to the eye, the pupil will no longer be visible and the internal structures of the eye will fill the camera's FOV. Use the fine focus knobs on the side of the fundus camera body to adjust the focus of the image without moving the front objective. If the focus knob is rotated backwards as far as it will go, but the image is still not in focus, pull the diopter tab out 1 (one) click so that it reads '-'. If the focus knob is rotated forward as far as it will go, but the image is still not in focus, pull the diopter tab out 2 (two) clicks so that it reads '+'. Continue adjusting the focus knob until the scleral vasculature is in focus. If an edge of the image appears saturated, try to re-center the front objective with the pupil. If all edges of the image appear saturated, move the front objective nearer or further from the patient until you are at an optimal distance where the beam of light and the camera's FOV pass entirely through the patient's pupil.

The external fixation LED fixation should be positioned nasally, so that if the patient's right eye (OD) is being imaged he or she will look left and if the patient's left eye (OS) is being imaged he or she will look right. The LED can be moved superiorly or inferiorly in order to look above or below the optic disc. For accurate positioning of the sclera, refer to the Topcon monitor instead of the software GUI.

Table 3.2 Illumination parameters for acquiring data

Column1	Tempcube0	Tempcube1	Tempcube2	Tempcube3	Tempcube4
Binning	1x1	1x1	1x1	1x1	1x1
Exposure Time(ms)	5	5	10	15	20
Gain (dB)	0.2	0.2	0.2	0.2	0.2

## 3.2 Data Processing

### *3.2.1 Calculating Absorbance*

According to the Beer-Lambert law the quantitative relationship between the absorbance of radiant energy, the concentration of the sample solution and the length of the cuvette "

$$A = \log\left(\frac{I_0}{I}\right) = \epsilon lc \quad \text{eq 3.1}$$

A = Absorbance

C= Concentration

$\epsilon$  = extinction coefficient

l=length of the cuvette

With the DLP hyperspectral imaging system an image of the 20% white Spectralon target is acquired and this is considered to be the 'I<sub>0</sub>' value for the calculation in Eq. 3.1. The reference path of light and the sample cube of the area of interest from the measurement on the patient's eye is considered to be 'I' value. The background cube is saved after initial acquisition and accessed for processing after every sample cube acquisition. To understand our calculation we replace I with R to represent reflectance in Eq. 3.2, where the subscripts xy represent each spatial pixel and the lambda variable refers to the wavelengths slice of the hyperspectral cube. Hence this means the data is the logarithm of the ratio between background and sample is calculated for each pixel at each illumination wavelength. A 348×260×3, (3 wavelengths) data requires 271440 operations of equation [3.2] and a 1392×1040×23 (23 wavelengths) data cube requires a 1.17 million operations just to calculate absorbance.

$$A_{xy}(n) = \log(R_{xy}(\lambda_n) / R_{xy}(\lambda_0)) \quad \text{eq 3.2}$$

Spatial heterogeneity of the illumination profile is eliminated by sampling a small area in the center of the background. Then, the area under the curve value from each frame of acquired data in the 1040 \* 1392 \* 23 matrix is divided by this calibration target value.

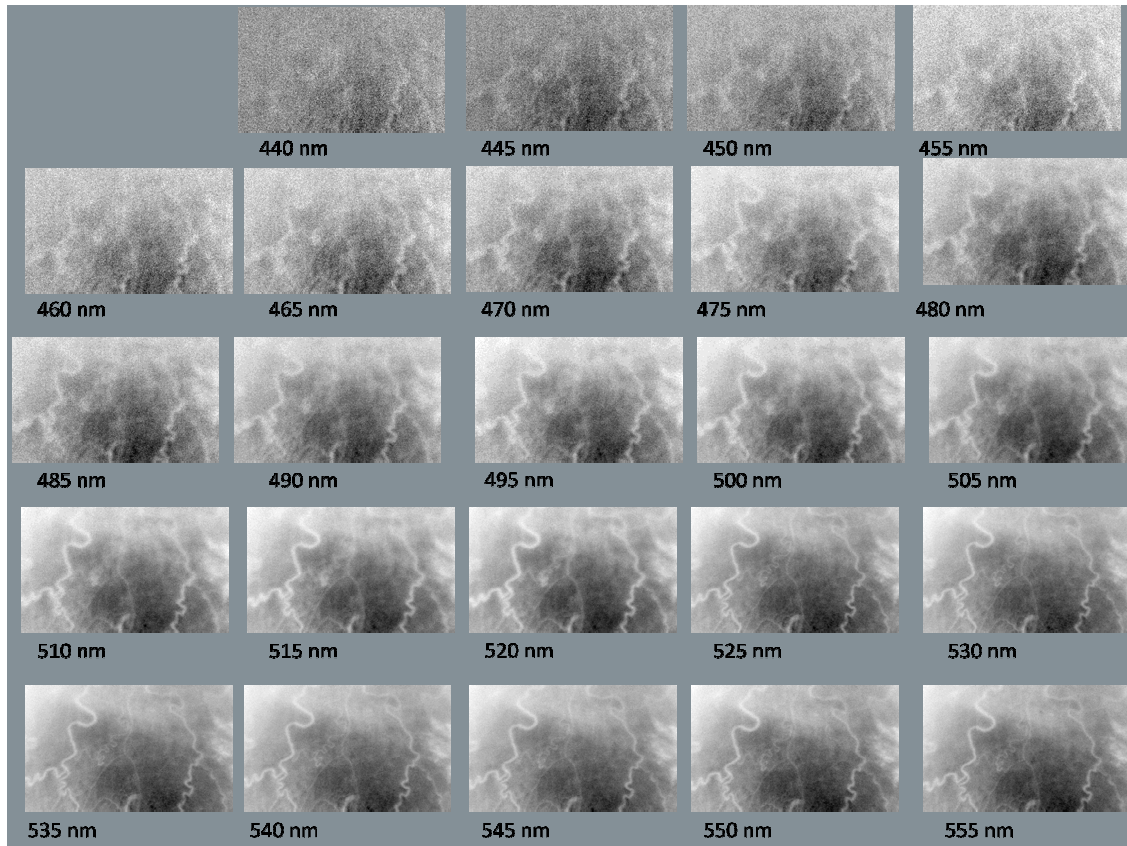


Figure 3.1 Hyperspectral absorbance Images of normal right eye from a hyperbilirubinemia patient with a total bilirubin value of 10.5 mg/dl

Images obtained by hyperspectral imaging are spatially shifted due to movement of the eye. We use a i2k align retina program to register all spectral images for a series of measurements automatically to a base image. The base image is a target image that we can choose before aligning. The computer transformations combine all the images, mapping them into a common 'aligned' coordinate system. The software employs a 'unbiased' estimation technique that produces accurately-aligned images with minimal geometric distortion despite very low inter image overlaps

### 3.2.2 Alignment software

#### i2k Align Software, Step 1

We select the Images we need to be aligned and then we check on the align options and set the parameters to Aggressive registration and click on Align Images

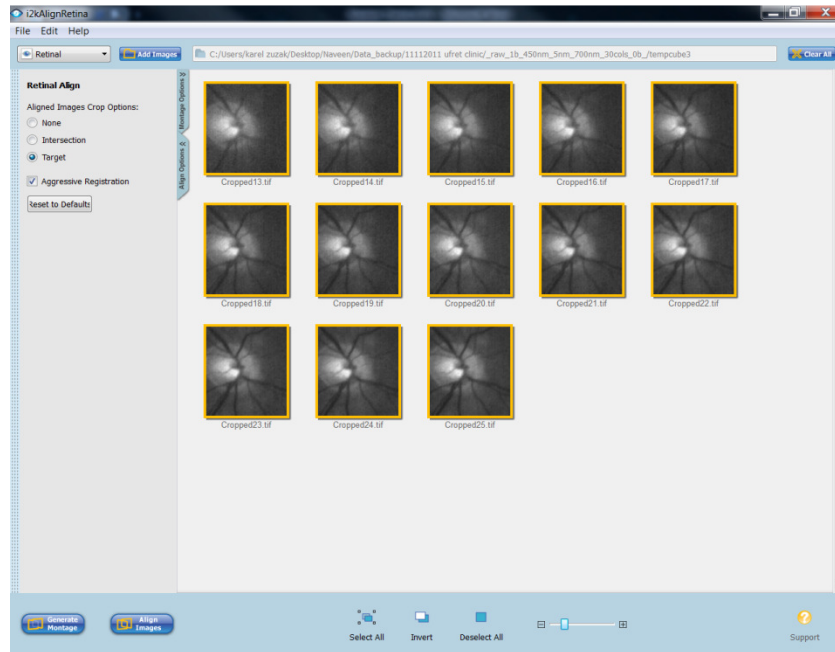


Figure 3.2 Showing alignment process using i2k align retina software step 1

Step 2, The i2k align retina software with Preference window displayed

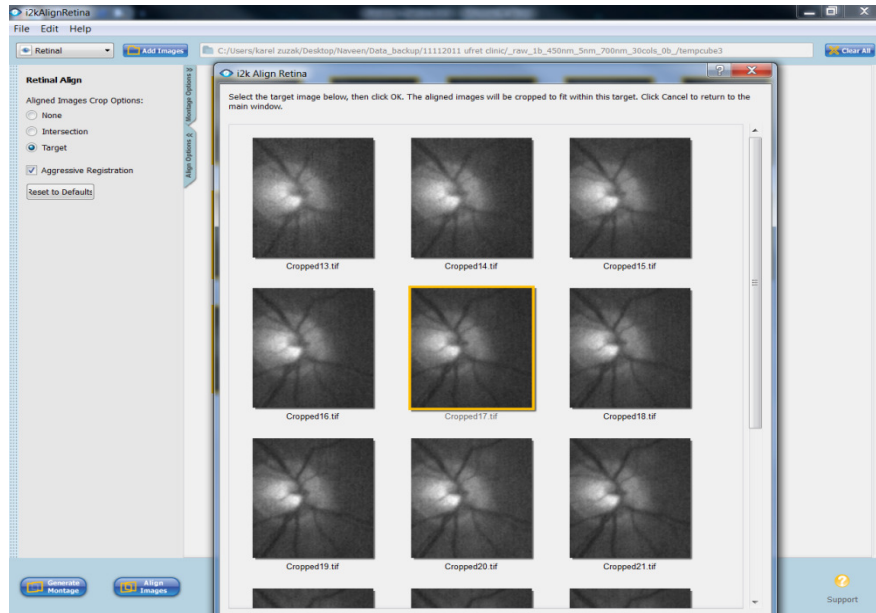


Figure 3.3 Showing alignment process using i2k align retina software step 2

STEP 3

The i2k align retina software with progress bar displayed

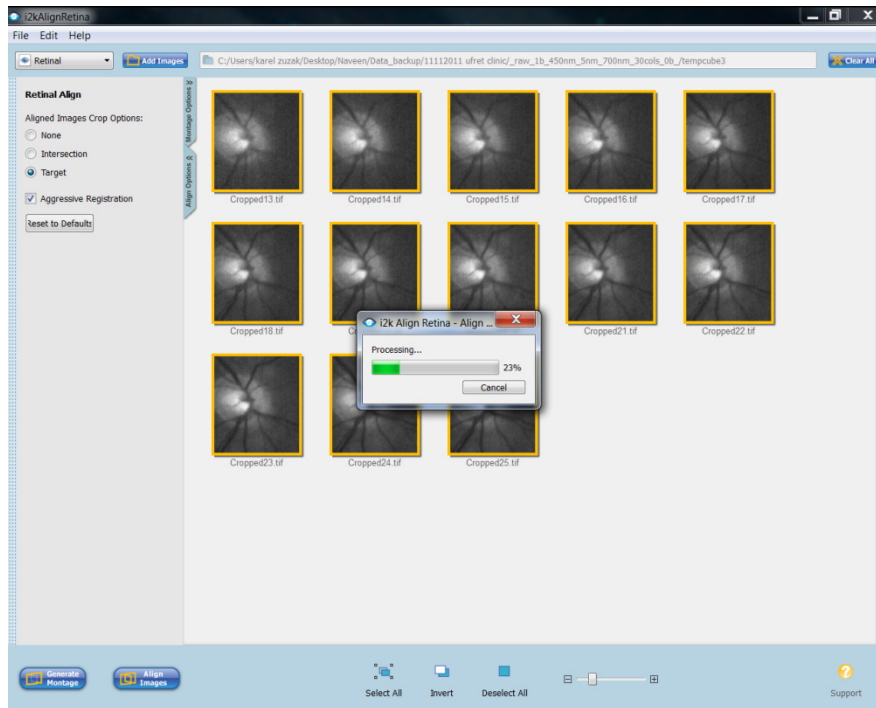


Figure 3.4 Showing alignment process using i2k align retina software step 3



## STEP 4 i2k align retina software displaying Preview window

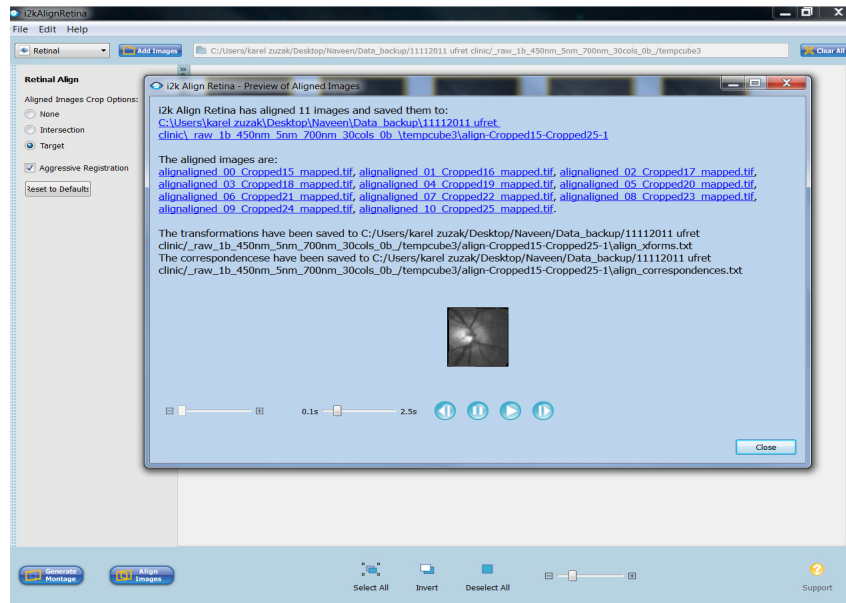


Figure 3.5 Showing alignment process using i2k align retina software step 4

## STEP 5

i2k align Retina saves the aligned images in the root folder and also it saves the transformation function it uses in 'align\_correspondences.txt'.

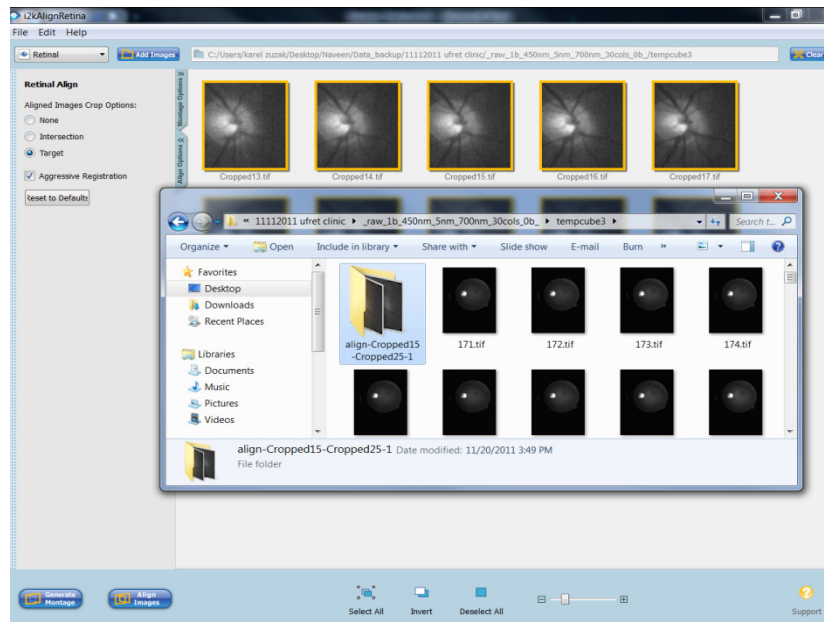


Figure 3.6 Showing alignment process using i2k align retina software step 5

CHAPTER 4  
APPLICATION OF HYPESPECTRAL IMAGING TO THE ASSESSMENT OF  
HYPERBILIRUBINEMIA

A total of 18 patients were imaged for this study but only 14 cases have been included in the final study due to the reduce image quality in the other 4 patients due to motion artifacts.

4.1 Assessment of Relative Bilirubin Absorbance from Hyperspectral Imaging with a Fundus  
Microscope

*4.1.1 Methods*

Patient recruitment occurred at Parkland Hospital. Patients seen at the Outpatient Liver Clinics or by the Inpatient Liver Consult service were selected as candidates. Consent was only obtained if the subject completely understood the Informed Consent form and the withdrawal provisions, and agreed to take part in the study. The consent form was signed by the patient and by the principal, sub-investigator, or clinical research coordinator. An executed copy was given to the patient; another was placed in the patient medical chart. The original Informed Consent was placed in the regulatory binder along with other important study documents. The regulatory binder was kept in a secure area in the research coordinator office.

4.1.1.1 Criteria for Inclusion of Subjects

1. Patients of any sex and race, ages 18 and up. Consent forms in Spanish were be available.
2. Patient willing to review, understand, and sign written Informed Consent.

3. Written authorization for use or release of health and research study information
4. Patients had blood samples collected and tested for bilirubin levels as part of their routine medical care.

#### 4.1.1.2 Criteria for Exclusion of Subjects

1. Uncontrolled systemic disease or the presence of any significant illness or condition that could, in the judgment of the investigator, interfere with interpretation of the study results.

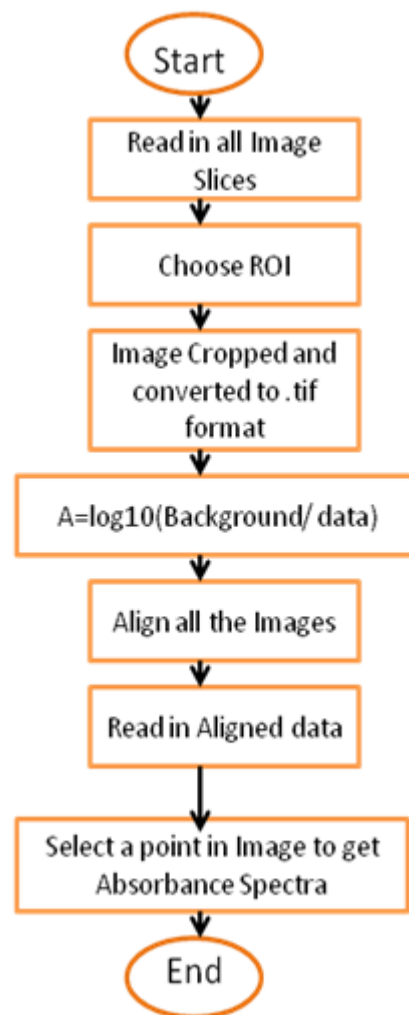


Figure 4.1 Process describing Image Processing and analysis

#### 4.1.1.3 Data Processing of the Data Acquired in the Clinic

The flowchart of how the acquired hyperspectral data from the patient's eye were processed is shown in Figure. 4.1. The matlab program reads in all the images from the destination folder specified and we choose our region of interest away from the lower eyelid and the cornea. Absorbance calculation as specified in equation [3.2] is done to each and every pixel of the image. Movement of eye during data collection can be checked by comparing images manually of each frame and if there is a lot of movements alignment software i2k align retina is used to help align the images as described in section 3.3.1. We read in the aligned absorbance images and we select a sampling area in between the blood vessels to acquire the averaged absorbance value at each frame for the entire wavelength range and plot them respectively against wavelength from (440-555)nm in 5 nm increments. The sampling is repeated three more times using the same sampling size as that of the first one.

This optical absorption measurement of bilirubin in blood was made by J. Li, using a Cary 3 commercial spectrophotometer [16]. The absorption values were collected using a spectral bandwidth of 1.0 nm with a signal averaging time of 0.133 sec, a data interval of 0.25 nm, and a scan rate of 112.5 nm/min. These measurements were scaled to make the molar extinction coefficient match the value of  $55,000 \text{ cm}^{-1}/\text{M}$  at 450.8 nm [15].

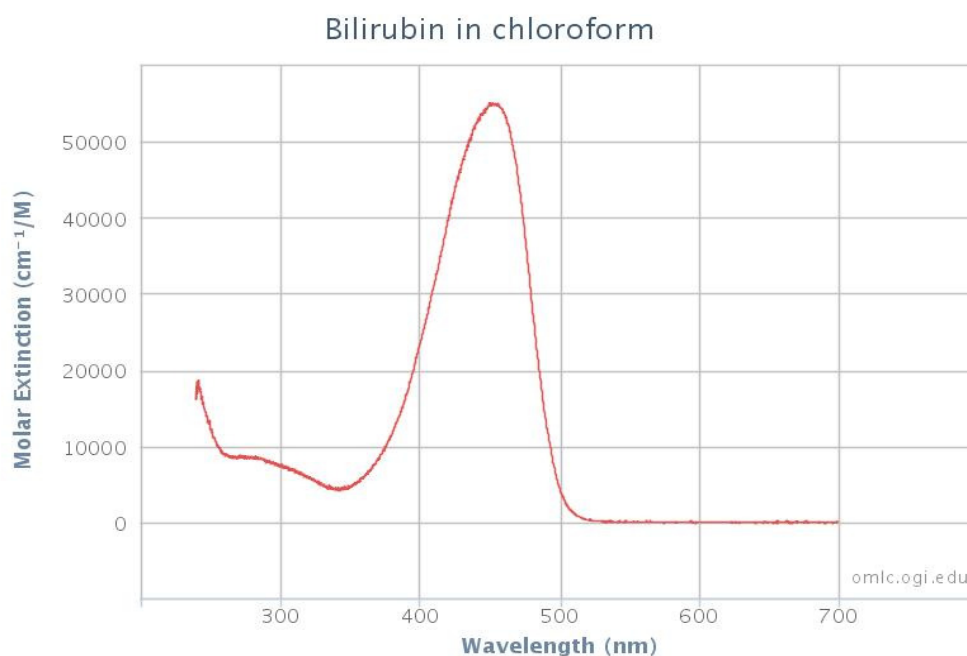


Figure 4.2 Absorbance spectra of bilirubin in chloroform [15]

For hyperbilirubinemia patients a 16-ml blood sample will be acquired from the patient's arm. The health professional taking a sample the blood from the patient upper arm after wrapped with an elastic band to stop the flow of blood. This makes the veins below the band larger so it is easier to put a needle into the vein and the site of needle sampling is cleaned with alcohol. The needle was inserted into the vein. A tube (BD Vacutainer® Lithium Heparin<sup>N</sup>) was attached to the needle to fill it with blood. The band was removed from the arm when enough blood was collected. No other follow-up care or treatment was needed for this study. Subject participation was only one visit. After collection, each sample was centrifuged and serum separated immediately and stored at -80 degree Centigrade in the laboratory for further analysis. The serum level of total bilirubin was assayed with a commercially available spectrophotometer and analysis software

#### 4.1.2 Results

Scleral vasculature was successfully visualized by the microscopic DLP hyperspectral imaging system in both View Finder and Spectral Sweep modes. In View Finder mode, white light illumination was created by turning all of the DMD mirrors on in the OL 490 as detected by the DVC camera through the optics of the Fundus microscope at 1x magnification. This mode effectively visualized the physical anatomy of the eye as seen in Figure 4.3(a). The Absorbance spectra obtained from imaging the sclera of the patients with hyperbilirubinemia were compared to those from normal volunteers to determine if there was any readily identifiable Hyperspectral signature for this condition.

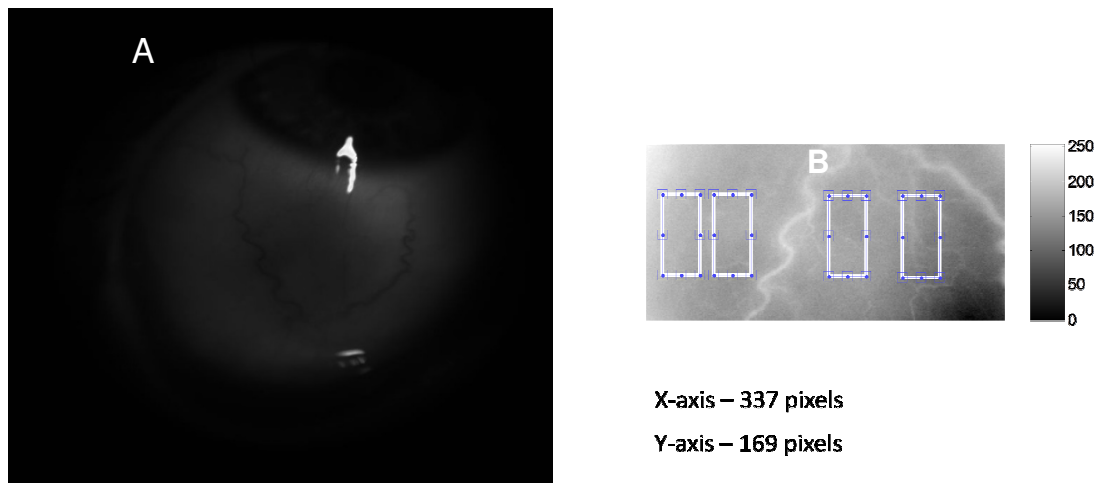
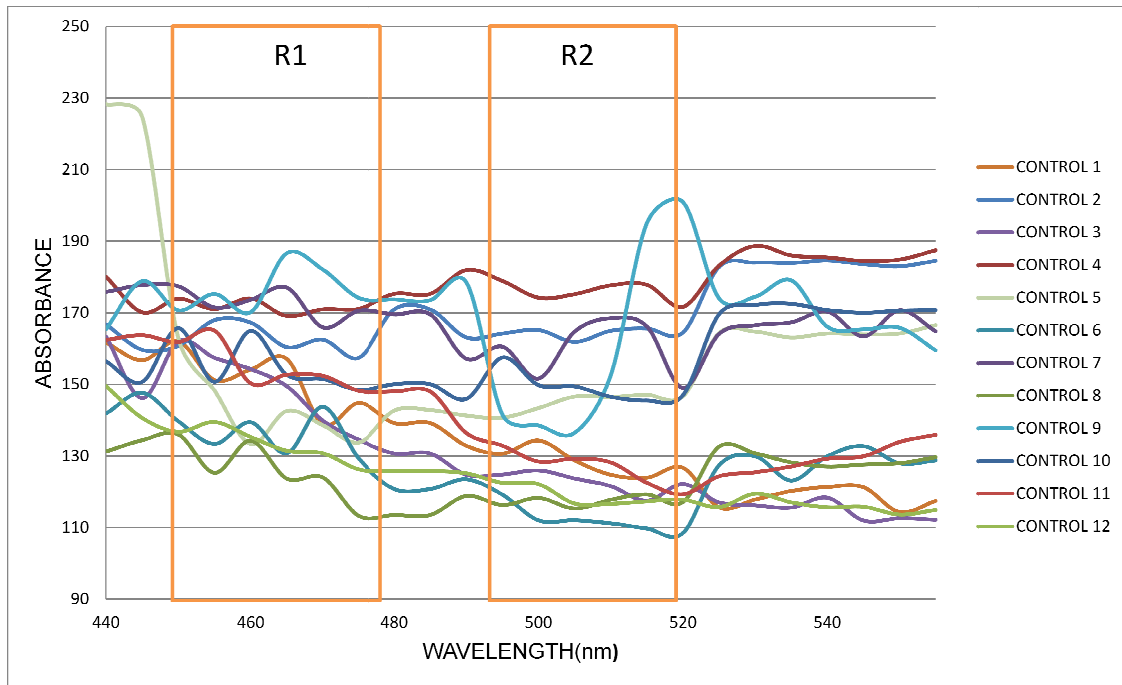


Figure 4.3 Hyperspectral Image of the sclera of the human eye in (a) shows the anatomical features of the sclera while the processed image in (b) shows the absorbance image acquired from processing the image cube from 440 to 555 nm



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Figure 4.4 Graph showing sclera Absorbance spectra from normal (control) volunteers

The above figure [4.4] shows the absorbance spectra for all the control subjects measured successfully in this study. As all these measurements were relative, a method had to be found to remove the variable baseline of the results. The relative absorbance was calculated by the subtracting minimum in Region 2. This region was chosen because in this wavelength window oxy- and deoxy-hemoglobin absorbance should be high, whereas bilirubin absorbance should be negligible. The baseline of the data was thus normalized for both controls and hyperbilirubinemia subjects using the following formula:

$$\text{Relative normalized absorbance} = A(\text{each data point}) - B \quad \text{eq 4.1}$$

A is the data point

B = min (R2)

We can see from figure [4.4] that there is a lot of variability in the absorbance spectra obtained in between the control group. This might be due to variations in the control group age, race and gender. Other factors that might play a role in interfering with the data measurement are there was no record to check control group history of intake of medication that might interfere with the measurement and also red eye to allergic conjunctivitis which is possible since these measurements were performed during the spring season. It is preferable to collect the data from subjects who can diet up to 4 hours prior to imaging; this procedure is generally followed in clinical testing to find bilirubin level from sampling blood. Variations in subject eye angle during data acquisition will also affect the data. Accumulation of fluid in the abdomen for some patients with liver disorder will lead to bloated stomach and because of that positioning the chin in the chin rest and holding in the position till the imaging procedure finishes becomes difficult for the patient even with necessary height adjustments. Patients with eye melanoma have spots in the sclera region that might contaminate the readings, careful precautions should be taken by the consenting personnel to check for this condition before recruiting the patient so that data collected will prove incompatible for processing.



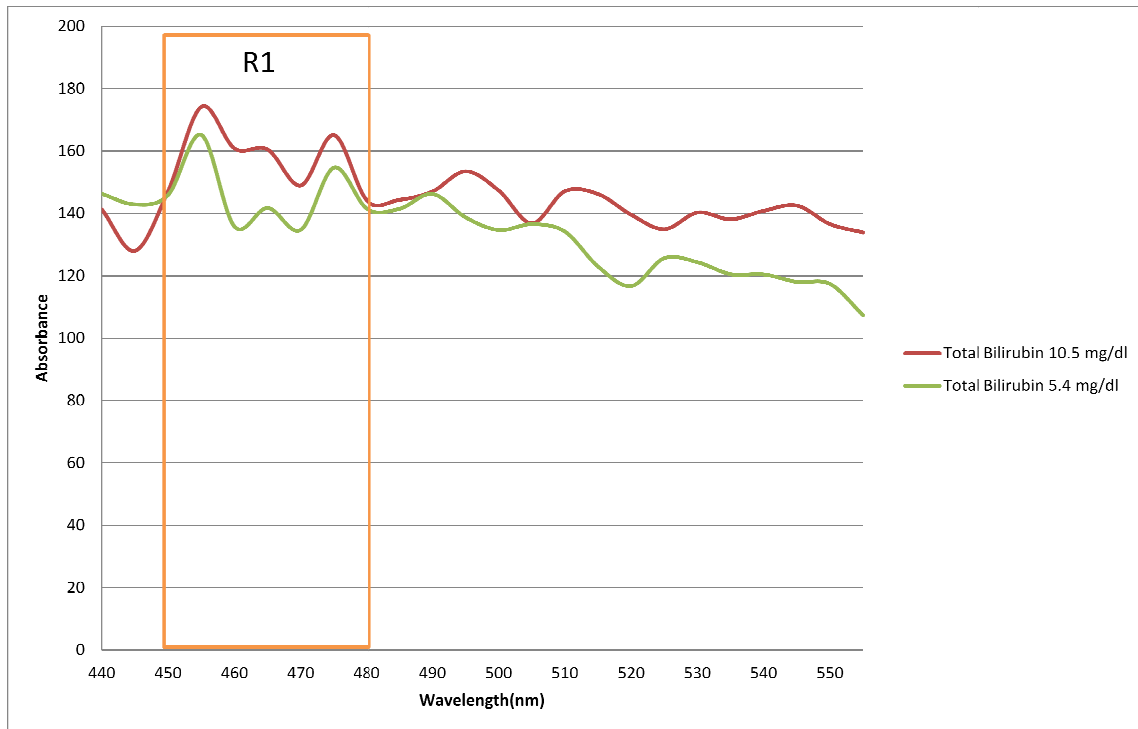


Figure 4.5 Graph showing Absorbance spectra of Hyperbilirubinemia Patient data

After subtracting the minimum from each reflectance curve so that all patient data had similar baseline amplitude values, the next hurdle was to identify any prominent spectral features that could be associated with bilirubin. Bilirubin has a peak at in the 440 – 485 nm range [15]. However, the reflectance measurements from the human eye depended on its scattering optical properties in combination with absorption from other chromophores, mainly oxy- and deoxy-hemoglobin. As a result, the reflectance spectra seen in figure [4.5] from the two subjects measured with high bilirubin blood content showed a trend of having a double peak and a double trough in Region 1 ( R1). Based on this observation we decided to heuristically normalize relative absorbance by dividing the sum of maximum of the first peak (P1) and second peak (P2) by the sum of the minimum values of first (T1) and second trough (T2):

$$\text{Relative normalized absorbance value} = [P1 + P2] / [T1 + T2] \quad \text{eq 4.2}$$

P1=maximum of peak 1

P2=maximum of peak 2

T1=minimum of trough 1

T2=minimum of trough 2

The relative bilirubin metric value for the region R1 was calculated for all the subjects and plotted in figure [4.6] against blood bilirubin. It should be clarified that the total bilirubin blood content was only measured in the blood samples of the patients, as that was a standard part of their care, but was not sampled in the normal controls. Therefore the x-axis value shown in figure [4.6] is the mean value of bilirubin content for normal controls found in the literature [16].

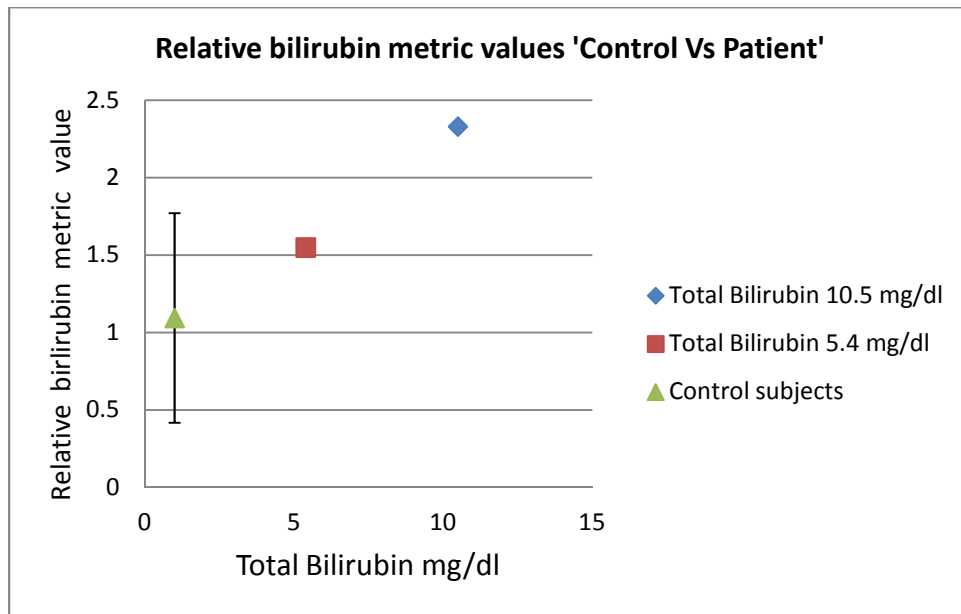


Figure 4.6 shows the Relative bilirubin metric values plotted against the total bilirubin value in (mg/dl) for control subjects and hyperbilirubinemia patients.

It should also be noted there was clearly a high level of variability in the normal control data. In fact the double-peak, double-trough pattern was only identifiable in 8 of the 12 normal controls, presumably due to the low levels of bilirubin in these subjects. The data figure [4.6] for normal controls represent the mean and standard deviation for only these 8 subjects. Nevertheless, the hyperbilirubinemia patient relative absorbance values, in comparison to controls, showed good correlation to the total bilirubin values measured in their blood. This ability to distinguish between patients with normal and high bilirubin levels based on their differing relative absorbance supports the usefulness of hyperspectral imaging for this particular medical application. Normal clinical practice to find serum level of total bilirubin requires collection of blood from a vein which is invasive. Hyperspectral imaging allows us to determine total bilirubin levels noninvasively.

When considering other possible confounding factors, the chronicity of the disease should also to be taken into account and the duration of the disease or the liver condition must also be a factor in these calculations. Chronicity might lead to accumulation of bilirubin pigments in other forms such as biliverdin, Biliverdin has been found in excess in the blood of humans suffering from hepatic diseases. Jaundice is caused by the accumulation of biliverdin or bilirubin (or both) in the circulatory system and tissues. Sclera (whites of the eyes) is a fibrous tissue and overlaying it is a mucous membrane called the conjunctiva which is richly supplied with blood vessels and there is a possible presence of other indigenous chromophores that might be present in the sclera that is not taken into account in this analysis. Every patient's sclera has a different pattern of blood vessels running through the background and for patients with widely branched small blood vessels it is harder to choose a sampling area without inclusion of blood vessels that interferes with the measurement. While sampling blood due to dilution effect, no liquid heparin products are used. Dry heparin products are available that virtually eliminate the effect of interference by heparin.

#### *4.1.3 Conclusion*

Hyperspectral imaging of the sclera is at an investigational stage. The objective of this study was to determine if clinically-useful data from the scleral epithelium can give us information on the relative bilirubin metric values in correlation with the total bilirubin level using this technique. The limited number of recruited patients so far precludes a definitive answer, but our preliminary data indicates that a correlation could exist between the image-derived relative bilirubin metric and the total bilirubin measured in blood. We are currently recruiting more patients to further explore the validity of this correlation.

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## BIOGRAPHICAL INFORMATION

Naveen Balaji, Balasubramanian has an inquisitive and explorative attitude which he believes will lead to a constant learning process. He did his under graduation in the field of electronics and instrumentation in Sathyabama engineering college and later he joined Biomedical Engineering Program at the University of Texas at Arlington and the University of Texas Southwestern Medical Center in the fall of 2009 to pursue his interests. He has gained a lot of skills and knowledge by working with doctors at UT Southwestern and also dealing with patients to visualize how a technological advancement will help the doctors serve patients better by early diagnosis or by monitoring the patient condition with the technology available noninvasively. He plans to graduate and has a realistic idea of his short term career goal upon completing my Masters program. He wish to take up a job in a professionally reputed technology and advanced-research company to gain first-hand experience in solving real world problems applying his technical expertise gained from his academic endeavors.