DECONVOLUTION OF LIGHT SCATTERING AND DIFFUSE REFLECTANCE SIGNATURES FOR DELINEATION OF MUCOSAL CANCER CELLS USING WAVELET ANALYSIS

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Esta tesis está dedicada a mi familia. Mis padres, Manuel y Lupita, que siempre me han dado su apoyo incondicional y me han enseñado a sobrellevar todos los obstáculos, y mis hermanas, Rosa y Mari, que siempre me apoyan en todas mis decisiones locas.

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ABSTRACT

Deconvolution of Light Scattering and Diffuse Reflectance Signatures for Delineation of Mucosal Cancer Cells Using Wavelet Analysis

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This thesis presents a technique to measure cells using a Fabry-Perot optical model along with wavelet decomposition applied to the reflected or transmitted optical spectrum. Cell size measurement is used as an indicator in early diagnosis of cancer. This research is based on developing a non-invasive, optical system to detect dysplasia in mucosal tissues; this is achieved by analysis of the reflectance or transmittance spectrum to determine the mutated cells' sizes. The collected spectrum contains information about the cell's internal structure. To decode this information, an optical cell model was formulated to create a custom mother wavelet to be used to deconvolve the absorption and light scattering components of the collected spectra. Finally, the previous results are fitted to different models for prediction of cell sizes and the results with the least error are presented as a Gaussian distribution.
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CHAPTER I

1. INTRODUCTION

The motivation for this thesis is to present a new technique to measure size and biological structure of cells using a Fabry-Perot model along with wavelet decomposition applied to the transmitted spectrum. Cell size and biological structure measurements are important because they are indicators in the early diagnosis of cancer; thus allowing physicians to take preventive measures that keep cancer from growing or spreading to more advanced stages where treatment is more aggressive and has a lower success rate.

This chapter provides the motivation and goals of the research presented in this thesis. It also provides a review of the current state of technology in the field of optical cancer diagnosis.

1.1. CANCER STATISTICS

Two very common types of mucosal cancers are stomach and cervical cancer. According to the American Cancer Society [1], about 21,800 Americans will be diagnosed with stomach cancer during 2005, and there will be an estimated 11,550 deaths from this type of cancer. Worldwide this is the second cause of cancer deaths, reaching about 700,000 deaths in 2002 [1]. In the United States the average 5-year relative survival rate is about 23 percent; this is due to late diagnosis of the stomach cancer. If it is diagnosed early (stage I – Localized cancer), the 5-year relative survival rate elevates to nearly 60 percent, as shown in 1.
Fig. 2. Approaches for diagnosis in the case of stomach cancer are gastroscopy, x-rays and biopsy.

Cervical cancer ranks the 14th most common type of cancer in the USA [2], with about 10,370 cases projected for 2005. Worldwide, it ranks the 2nd most common type of cancer with 490,000 cases projected. Detecting the cancer in its early stages leads to a successful eradication and, as with any type of cancer, the later it is detected, the lower the chances of successful treatment, [2] see Fig. 1.

Dr. Papanicolaus created the Pap-smear test in the early 1940's. This test has become a routine exam for woman because it was shown to reduce by 70 percent the amount of deaths caused by cervical cancer due to diagnosis [3]. The pap-smear test consists of taking sample cells from different parts of the cervix, placing them on a microscope slide, using alcohol to preserve the sampled cells, and having a cytologist analyze the cells for symptoms of dysplasia or other morphological changes.

![SEER Relative Survival Rates by Race Cervix Uteri Cancer, All Ages SEER 9 Registries for 1988-2001](image)

Fig. 1. Relative survival rate for cervix cancer [2].
One of the challenges of the pap-smear test is that the samples are taken randomly and the cytologist has to examine thousands of cells for a few dozen cells that present symptoms of dysplasia. The problem with the current detection techniques for this type of cancer is that they require histopathological examination by clinicians, which introduces the potential for human error. Further, the tissue regions sampled depends solely on the physician collecting the sample, in most cases guided by limited visual observation of the suspected area. Given that macroscopic mutations of cells are generally not visible to the human eye until late stages of cancer development, a system that can guide physicians to sample regions of tissue in early stage dysplasia is of great importance.

1.2. **GOALS FOR THIS RESEARCH**

The motivation to design this system is to aid in the detection stage, by enhancing areas where there are cells with a wide spread of nuclei diameters. The complete system is a three part system using fluorescence, reflectance spectroscopy and light scattering spectroscopy (LSS). These methods are used because each method does not resolve at 100 percent accuracy and, by weighing the outcome from all the tests, it increases the final accuracy. This research covers the reflectance part of the system along with a wavelet analysis to delineate the resonance structure produced by the cell.

Both cervical and stomach cancer can be probed using the technique presented here. These are slow growing types of cancer that usually have no symptoms until they reach late stages, where the treatment is confined to major surgery, chemotherapy and/or radiation. It
is hard to detect stomach cancer because the early symptoms are often not present or are confused with other illnesses until the cancer has reached a stage of growth where it can not respond to treatment. In these cases, surgical removal of the organs is the most frequently recommended treatment. However, because of the slow growth rate, detection using techniques such as the pap-smear, endoscopy and colposcopy guided by the measurement technique presented here could have a significant impact on the average five-year relative survival rate. Moreover by guiding physicians to sample tissues most likely to be dysplastic, the efficiency and efficacy of the examination process can be improved.

![SEER Relative Survival Rates by Stage at Diagnosis Stomach Cancer, Both Sexes SEER 9 Registries for 1988-2001](image)

**Fig. 2.** Relative survival rate for stomach cancer [4].

Needless to say, early detection is imperative for either type of cancer, as shown in Fig. 2 and Fig. 1. These figures show how important the early detection of the cancer is in the survival rate.

Reflectance spectroscopy can aid in the early detection of cervical and stomach cancers because it can differentiate the cancer cells from normal cells. This is because there
are clear morphological differences between normal cells and cancer cells at this early stage that can be detected by the optical response of the nucleus. One of these differences is the ratio between the cell size and the size of its nucleus. Normal cells have a nucleus that constitutes 20-25 percent of the cell structure, while cancer cells tend to have nuclei above 50 percent the cell size. Moreover, normal cells are homogeneous in shape and in size and cancer cells are not homogeneous in either. Cancer in early stages is known as dysplasia and is mostly present on the surface of the tissues [5] allowing for a tissue reflection technique to be applied for detection purposes. A system that employs the techniques presented here would aid gynecologists and gastroenterologists in the early detection of cervical and stomach cancers, respectively, because such a system can detect the early indicators and guide physicians to the best regions to biopsy for cancer.

1.3. BACKGROUND

Other different approaches have been taken in order to detect the changes produced by dysplasia. In recent years, many efforts have emerged for improving sensitivity and specificity of early diagnosis techniques for gastric and cervical cancer. These include autofluorescence imaging [6], light-scattering spectroscopy [7], optical coherence tomography [8], photodynamic fluorescence [9], double-barium radiography [10], laparoscopic and endoscopic ultrasound [11, 12], and genetic screening [13]. Of these, the minimally invasive optical techniques have attracted the most attention for their potential to perform "optical biopsy" over sizable regions of the epithelium. These techniques could allow for more thorough screening and possibly have a substantial impact on mortality rates if implemented as a routine component of endoscopy and gynecological examination.
1.3.1. **Fluorescence Spectroscopy**

Fluorescence spectroscopy is a real-time optical technique for probing a material by which the material absorbs high-energy light and emits light at a lower energy. The absorption and emission of light are related to the chemical structure of the material, and therefore, can be used to identify the material. Fluorescence techniques are useful in cancer detection because of the differences in fluorophores present in dysplastic and malignant tissues. Examples of such fluorophores include porphyrins, reduced nicotinamide adenine dinucleotide (NADH), flavin adenine dinucleotide, and collagen.

Vo-Dinh et al. [14] have demonstrated promising results for the detection of high-grade dysplasia using autofluorescence excited at 410 nm due to porphyrins. The intrinsic auto-fluorescence properties of biological tissues can change depending on alterations induced by pathological processes. Evidence has been reported concerning the application of autofluorescence as a parameter for *in situ* cancer detection in several organs. For example, Bottiroli et al. [15] have reported autofluorescence properties of normal and tumor tissue in the brain and demonstrated autofluorescence as a suitable technique for a real-time diagnostic application. In their research, data were obtained both on *ex vivo* samples, by microspectrofluorometric techniques, and *in vivo*, during surgical operation, by means of fiber-optic probe. Significant differences were found in auto-fluorescence emission properties between normal and tumor tissues, in terms of both spectral shape and signal amplitude, which confirm the potential of autofluorescence as a parameter to distinguish neoplastic from normal cells. The noninvasiveness of the technique opens up interesting prospects for improving the efficacy of neurosurgical operations, by allowing an intraoperative delineation of tumor resection margins.
Richards-Kortum et al. [16] have developed a method for defining diagnostic algorithms for pathologic conditions based on fluorescence spectroscopy. They have applied this method to human colon tissue and showed that fluorescence can be used to diagnose the presence or absence of colonic adenoma. Fluorescence excitation-emission matrices (EEM) have been used to identify optimal excitation regions for obtaining fluorescence emission spectra. These data can be used to differentiate normal and pathologic tissues. In the case of normal and adenomatous colon tissue, optimal excitation regions were found to be: 330nm, 370nm, and 430nm ± 10nm. At these excitation wavelengths, emission wavelengths for use in diagnostic algorithms are identified from average difference and ratio of the spectra from normal and pathologic tissues.

While autofluorescence has demonstrated much promise as an in vivo technique for distinguishing between malignant and normal tissue for various types of cancer [6, 9, 17], diagnoses are based on subtle differences in the emission spectra, such as a reduction in the fluorescence yield or slight deformities in the fluorescence line shape. In practice, these effects can easily be caused by naturally occurring dynamics within the patient's epithelia, as well as factors external to the measurement. Further, the specificity of this technique for identifying low-grade dysplasia is poor. To exploit the benefits of autofluorescence measurement techniques and improve specificity, precise referencing from additional probing methods can be used.
1.3.2. **Reflectance Spectroscopy**

![Reflectance Spectrum](image)

Fig. 3. Reflectance spectrum from nondysplastic Barrett's esophagus site [18]. This figure shows substantial structure that can be probed.

Reflectance spectroscopy has been used in many of the initial optical surveys of dysplastic and cancerous tissues [8, 17, 19, 20]. Reflectance spectroscopy can probe changes in epithelial nuclei that are important in pre-cancer detection, such as mean nuclear diameter, nuclear size distribution and nuclear refractive index. This technique has two beneficial aspects: (1) it can be used to derive morphological data by analysis of cell crowding by light-scattering spectroscopy (LSS) and (2) it can be used to derive tissue composition by analysis of diffuse reflection and absorption. Additionally, nearly all broadband reflectance studies show regions of the spectral signature that differ between healthy and malignant tissues [20], indicating a potential for this technique in diagnosis of dysplasia and cancer.

An example of a reflectance spectrum obtained from normal tissue within Barrett's esophagus is given in Fig. 3. The major features seen in this spectrum at 420nm, 540nm, and 580nm result from oxyhemoglobin absorption. As tissue changes from nondysplastic to dysplastic to cancerous, there are discernable changes in the reflectance spectral structure.
and a reduction in the overall scattering coefficient [18]. However, the resolution of these cancer-revealing features is low to moderate at best, leaving much of the determination to be based on, subtle differences scrutinized by multivariate analysis.

It is established in [7, 18, 21, 22] that cell crowding is an indicator of dysplasia. The nuclear spacing is manifested in the reflected spectrum by small oscillations as a function of the scattered wavelength. The higher the frequency of these oscillations, the larger the cell's nucleus, which can be used as a measure of the nuclear crowding associated with dysplasia. Employing LSS analysis on the reflected spectra can give a measure of this morphology and an indication of dysplasia; however, this signature can be a small portion of the return spectrum. While the LSS method has shown good results [20-22]; one point that is very important to make in regard to its application is that there are certain limitations, such as the distance between scatters and its size in relation to the wavelength applied when using Mie Theory to model the scattering [23]. This is the motivation for using the Fabry-Perot resonator approach in conjunction with wavelet analysis, a technique that is able to separate small signatures embedded in a large background signal.

1.3.3. **Multimodal Spectroscopic Techniques**

Fluorescence and reflectance spectroscopy together provide the ability to assess tissue structure and metabolism in vivo, providing improved diagnosis of pre-cancerous lesions. Reflectance spectroscopy can probe changes in epithelial nuclei that are important in pre-cancer detection, such as mean nuclear diameter, nuclear size distribution and nuclear
refractive index. Fluorescence spectroscopy can probe changes in epithelial cell metabolism, by assessing mitochondrial fluorophores, and epithelial-stromal interactions, by assessing the decrease in collagen cross link fluorescence that occurs with pre-cancer. Thus, fluorescence and reflectance spectroscopy provide complementary information useful for pre-cancer diagnosis [24].

Georgakoudi et al. [18] have studied these optical biopsy techniques, i.e., autofluorescence, diffuse reflectance, and light-scattering spectroscopies, for their efficacy as independent measurements, as well as combined/correlated measurements. They found that each of these techniques provides complimentary information on the biochemical and morphologic changes that occur during the development of dysplasia. Further, they show that the combination of these measurements can provide a sensitive and specific means for detection of dysplasia of the Barrett’s esophagus.

In their multimodal setup, they performed Laser-induced fluorescence (LIF) with Nitrogen pumped dye-laser cells at various wavelengths and reflectance spectroscopy, both diffuse reflectance (DF) and light scattering (LS), with a xenon flash lamp. They analyzed the reflectance for absorbing feature, which were then used to obtain the intrinsic fluorescence spectrum, i.e., that of the fluorophore alone. An example of this referencing technique is shown in Fig. 4, where the intrinsic fluorescence (dashed line in (a)) is calculated based on the measured oxyhemoglobin absorption derived from the reflectance spectrum (b).
Fig. 4. Data published by Georgakoudi et al. [18] (a) Fluorescence from a nondysplastic BE site, 337-nm excitation. Measured spectrum, solid line; extracted intrinsic fluorescence, dashed line. (b) Corresponding reflectance spectrum.

Georgakoudi et al. [18] show that this correlation technique improves the sensitivity and specificity of classification methods for dysplastic tissues of the Barrett's esophagus. Table 1 shows the results of their findings.

Table 1. HGD, High-grade dysplastic; LGD, low-grade dysplastic; NDB, nondysplastic Barrett's esophagus. Accuracy of Spectroscopic Classification of Nondysplastic, Low-grade, and High-grade Dysplastic Tissue in BE. Data published by Georgakoudi et al. [18].

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1.4. THESIS STRUCTURE

The remaining chapters of this thesis focus on the theoretical development of the system described above, and its applications in the early detection of cervical and stomach cancers. Chapter 2 presents the theoretical background on the technique applied, starting with the explanation of the cell structure, the optical properties of the cell, the different samples used and their sizes, the design of the optical cell model, and a description of the wavelet transform. Chapter 3 reviews the experiments realized to measure different optical parameters such as the Brewster angle, the description of the set up, the confocal microscope data acquisition, the wavelet transform for the model, the algorithms for data analysis, and the results obtained using the described technique. Chapter 4 provides a discussion of the results as well as the limitations and enhancements that can be done to the system and software. Chapter 5 concludes the thesis with a discussion of the importance and potential impact of the research presented here. Programs, tables, and general derivations are provided in the appendices.
CHAPTER II

2. THEORETICAL DEVELOPMENT

This chapter provides a brief overview of cells, their structure, and their basic optical properties. It also contains a brief introduction to the early stage of cancer, called dysplasia, where healthy cells begin to mutate into cancer by increasing the protein storage leading to a greater size of the cell’s nucleus. These basic assumptions are presented with the idea of explaining the model used, which is based on the Fabry-Perot optical resonator. In this case, the cells are modeled as an optical resonator using the optical characteristics of each type of major cell component. A model is created for normal cells and dysplastic cells. Finally, this chapter presents a brief introduction to the use of wavelets as a cell size indicator. By producing a wavelet from the normal cell, it is possible to look for specific changes within cells that indicate cancerous mutations.

2.1. ANIMAL CELLS

All living organisms are composed of cells, which are the smallest, self-replicating organic unit. Some organisms are composed of a vast collection of specialized cells with a unique goal, such as in the case of a tissue which surrounds or becomes an organ in an animal, while others organisms are composed entirely of a single cell, such as an amoeba.
2.1.1. **CELL STRUCTURE**

Each cell grows and reproduces with a determined genetic makeup. This allows a group of cells to perform a very specialized task and create a structure called a tissue. This differentiation allows a group of cells to have unique physical and physiological characteristics. A better understanding of this can be found in a text on physiology by Cotran and Robbins [5].

With few exceptions, all cells have a common structure. This structure is composed by a cell body called cytoplasm, which is almost clear and is surrounded by a wall of fat and proteins that control traffic of nutrients in and out of the cell. Within the cytoplasm is a globular body, the nucleus, which is composed of a protein, named chromatin. These two larger structures have different optical properties, specifically, different indices of refraction. A diagram illustrating the cell components is shown in Fig. 5.

![Cell diagram](image.png)

**Fig. 5.** Cell and its basic structural components.
Alterations to the cell structures can create problems, such as cancer. The early mutations a cell goes through while developing cancer is called dysplasia. This mutation of the cell is manifested as morphological changes, such as a reduction or enlargement of the nucleus in relationship to the size of the cell. When dysplasia is present, the nucleus changes its size by means of protein storage, while the overall cell size remains the same. Another change that occurs is the loss of homogeneity among the cells of the same type within a tissue, giving rise to cell clustering. These morphological changes can be detected by optical processes such as fluorescence and light scattering spectroscopy (LSS).

2.2. **OPTICAL CELL MODEL**

The first step in creating this model was to understand the underlying structure of the cell and to decide which factors are most important given the optical properties of the structures within the cell. As is possible to see from Fig. 5, the largest parts of the cell are the cytoplasm and the nucleus [5, 25]. This constitutes the primary components of the model presented here.

2.2.1. **REFLECTION COEFFICIENT**

Assuming that the cells are large in comparison to the applied optical wavelength (greater than ten times the wavelength used (\(\lambda\))), their surfaces can be assumed to be parallel to each other along any optical axis within the optical domain. With this simplification in mind, a cell model can be created based on an analysis of the optical interference produced
by multiple reflections. Reflection is created by the difference in the indices of refraction between the boundaries of the large surfaces (the nucleus and the cytoplasm) of the cell. It is also assumed for simplicity that the cells are all part of the same type of tissue, and therefore, are homogeneous along the surface of the observed tissue.

A detail analysis of the reflection coefficients resulting from an index of refraction boundary is given in Appendix I. From this analysis, the reflection coefficient for the cell boundary at normal incidence for the nucleus/cytoplasm surfaces is given in Eq. (1).

\[ R = \left( \frac{nn(\lambda) - nc(\lambda)}{nn(\lambda) + nc(\lambda)} \right)^2 \]

The detailed model has a reflection coefficient \( R \) at the boundary between the cytoplasm and the nucleus caused by the difference of the indices of refraction, where \( nn(\lambda) \) and \( nc(\lambda) \) are refractive indices for the nucleus and the cytoplasm respectively.

### 2.2.2. MULTIPLE BEAM INTERFERENCE WITHIN THE CELL

An optical cell model was created based on multiple beam interference, which occurs via internal reflection off of the parallel surfaces. The structure of the cell and the resonator model are shown in Fig. 6. In the model, there is an absorption coefficient characteristic of each chemical within the nucleus. This coefficient, \( \alpha \), will be considered a loss and will be dependent on the chemical properties, and density of the nuclei.

As light is applied to the cell, all these variables interact creating a unique spectral pattern within the transmitted and reflected light. This is further explained in Fig. 7. One
very important factor in determining the size of a particle using this approach is that the free spectral range (FRS) changes with the wavelength creating a specific pattern for different values of d, the diameter of the nucleus.

Assuming that a non-polarized optical beam, $E_0$, is incident on a boundary with a different index of refraction as the one it is propagating. On the first incidence, there will be a reflection; this produces a reflected beam, $E_{or}$, and a transmission beam, $E_{ot}$. Following this ray, it is found that its intensity is attenuated due to absorption on a spectral range related to the chemistry and the length of the traveling medium. At this point, the beam will be described by,
Where $l = d \cos(\theta)$,

$$E_0 t \cdot e^{-\alpha l / \cos \theta},$$

Fig. 7. Light waves being reflected and transmitted between mediums surrounded by a media with a different refractive index.

Once this beam reaches the next boundary, it will be transmitted and reflected once again. The transmitted signal reaching the next layer is described by Eq. (3). This behavior continuous indefinitely, as shown in Fig. 7, and is described by equations (4)-(11). However, for the purposes of this research, only the transmitted light factors are used for the model. These factors will create a spectral signature with information related to the boundaries and physical properties. If the experiment was to be based on the reflection from the cells, the reflected beams would be used for this analysis [26, 27].
The total transmitted waves are the sum of the Electric fields:

\[ E_{\text{TRANS}} = E_2 + E_6 + E_{10} + \ldots \]

The 8 factor in \( E_{\text{TRANS}} \) is the net phase shift and is equal to:
\[
\delta = \frac{2 \pi}{\lambda_0} nd \cos \theta + \delta_r
\]

in a round trip, where \( n \) is the index of refraction inside the resonator, \( \theta \) is the incidence angle, for this purpose 90 degrees, \( \lambda_0 \) is the vacuum wavelength of the source light, and \( \delta_r \) is the phase change due to reflection at the boundaries. Substituting the electric field expressions for their respective variables gives:

\[
E_{\text{TRANS}} = E_0 t^2 e^{-\alpha l} +
\]

\[
E_0 t^2 r^2 e^{-\frac{3 \alpha l}{\cos \theta}} e^{i\delta} + E_0 t^2 r^4 e^{-\frac{5 \alpha l}{\cos \theta}} e^{2i\delta} + ......
\]

These elements can be grouped to create this geometric series:

\[
E_{\text{TRANS}} = E_0 t^2 e^{-\frac{\alpha l}{\cos \theta}} \left( 1 + \frac{r^2 e^{-2\alpha l/\cos \theta}}{e^{i\delta}} + \frac{r^2 e^{-2\alpha l/\cos \theta}}{e^{i\delta}} + ......ight)^2
\]

\[
+ \left( \frac{r^2 e^{-2\alpha l/\cos \theta}}{e^{i\delta}} + ......ight)^3 + ......
\]

And it can be represented by the geometric series with coefficients

\[
r^2 e^{i\delta} e^{-\frac{2 \alpha l}{\cos \theta}}
\]
as shown in the next equation:

\[
E_{\text{TRANS}} = \frac{E_0 t^2 e^{-\frac{\alpha t}{\cos \theta}}}{1 - r^2 e^{-\frac{2\alpha t}{\cos \theta}} e^{i \delta}}
\]  

(14)

Transforming the wave electric field into intensity, the following equation is used.

\[
I_{\text{TRANS}} = \frac{|E_{\text{TRANS}}|^2}{2 \eta}
\]

(15)

Where,

\[
\eta = \sqrt{\frac{\mu}{\varepsilon}} \quad \text{and} \quad I_0 = \frac{E_0^2}{2 \eta}
\]

Expanding (15) from (14) gives:

\[
I_{\text{TRANS}} = \frac{I_0 t^4 e^{\left(-\frac{2\alpha t}{\cos \theta}\right)}}{1 - 2r^2 e^{\left(-\frac{2\alpha t}{\cos \theta}\right)} \cos \delta + r^4 e^{\left(-\frac{4\alpha t}{\cos \theta}\right)}}
\]

(16)

Making \( \beta = 2\alpha \) in (16)

\[
I_{\text{TRANS}} = \frac{I_0 \cdot t^4 \cdot e^{\left(-\frac{\beta t}{\cos \theta}\right)}}{1 - 2r^2 \cdot e^{\left(-\frac{\beta t}{\cos \theta}\right)} \cos \delta + r^4 \cdot e^{\left(-\frac{2\beta t}{\cos \theta}\right)}}
\]

(17)

Defining \( R = |\mathbf{r}|^2 \) and \( T = |\mathbf{t}|^2 \), where \( |\mathbf{t}|^2 = \mathbf{t} \cdot \mathbf{t}^* \) and \( |\mathbf{r}|^2 = \mathbf{r} \cdot \mathbf{r}^* \) gives,
\[ I_{\text{TRANS}} = \frac{I_0 \cdot T^2 \cdot e^{-\frac{\beta V}{\cos \theta}}}{\left(1 - R \cdot e^{-\frac{\beta V}{\cos \theta}}\right)^2 + 4 \cdot R \cdot e^{-\frac{\beta V}{\cos \theta}} \sin^2 \left(\frac{\delta}{2}\right)} \]  

(18)

Where,

\[ \delta = \frac{2\pi \mathbf{n} d \cos \theta}{\lambda_0} + \delta_r, \]

\( \delta_r \) ranges from zero to \( \pi \) depending on the polarization of the source and the incident angle. In this case, a value of \( \pi \) is assumed due to normal incidence.

Finally, the intensity of the optical model of a cell based on multiple beam interference is:

\[ I_{\text{TRANS}} = \frac{I_0 \cdot T^2 \cdot e^{-\frac{\beta V}{\cos \theta}}}{\left(1 - R \cdot e^{-\frac{\beta V}{\cos \theta}}\right)^2} \quad \rightarrow \quad 1 = \frac{1}{1 + \left(1 - R \cdot e^{-\frac{\beta V}{\cos \theta}}\right)^2 \sin^2 \left(\frac{\delta}{2}\right)} \]  

(19)

Backman et al. [22] used an optical cell model based on Mie theory, and the scattering cross section for this model is given in Eq (20) below.

\[ \sigma_s (\lambda, I) = \frac{1}{2} \pi I^2 \left[ 1 - \frac{\sin(2 \delta_\lambda)}{\delta \lambda} + \left(\frac{\sin(\delta_\lambda)}{\delta \lambda}\right)^2 \right] \]  

(20)
With, \( \delta = \pi n_c (n-1) \), where, \( n_c \) is the refractive index of cytoplasm, \( n \) is the relative difference between cytoplasm and nucleus index of refraction, \( \lambda \) is the vacuum wavelength and \( l \) the diameter of the particle to be modeled. Based on Val Del Hust's analysis in reference [9], the application of scattering theory for detection of cancer cells is questionable due to the close proximity of cells. Van Del Hust [9] claims that this model is not recommended for particles relative close to each other.

Fig. 8. Comparison of results between Backman and the optical cell model resonator.

Backman et al. [22] show good agreement between this model and their experimental data. In comparing our optical resonator model with that of Backman's [22] it is found that the end result is essentially the same as seen in Fig. 8, where both models are plotted for a
particle size of 3 μm. The primary difference is that the resonator model does not include a damping coefficient as function of wavelength where their model does. However, this is taken care of in our approach by the wavelet analysis (to be shown in section 2.3). Thus, the model described in Eq. (19) is what was used as the basis of the mother wavelet for decomposing the LSS signal from the diffuse reflection signal.

The key components of Eq. (19) are the coefficients of reflection, transmission, and absorption, as well as the size of the resonator, in this case, the cell nucleus. The reflection and transmittance coefficients are found through the relative refractive indices (see Appendix I). The difference in the index of refraction determines the magnitude of \( R \), and consequently changes the shape of the resonant spectrum through a parameter known as the coefficient of finesse, \( F \). This is described by Eq. (21), where absorption and incident angle are accounted for.

\[
F = \frac{4 \cdot R \cdot e^{-\frac{\beta}{\cos \theta}}}{\left(1 - R \cdot e^{-\frac{\beta}{\cos \theta}}\right)^2}
\]  

(21)

The free spectral range, which is defined as the spectral separation between adjacent transmission peaks, is related to the size of the resonator. An expression for the FSR is given by Eq. (22). Notice the inverse relationship between the Free Spectral Range (FSR) and the resonator (19).

\[
FSR = \frac{\lambda_0^2}{2nd}
\]  

(22)
Here, \( n \) is the index of refraction, \( d \) is the thickness of the layer or diameter of the nucleus, and \( \lambda_0 \) is the wavelength in vacuum. An important characteristic is that the FSR, and thus, the spectral structure depend on the size of the particle and not on the difference of the index of refraction. This is shown by the plots given in Fig. 9, where Eq. (19) is plotted for different coefficients of finesse Eq. (21) and an FSR based on a 3 \( \mu \text{m} \) particle/resonator. This plot shows that as the reflectance changes, only the magnitude of the signal changes, but not the FSR. Thus the analysis requires finding the FSR on the final transmission signal, where the spectral magnitude of the signal will be dependent only in the difference in the refraction indices of the particle and its surrounding medium.

![Optical cell model for different indexes of refraction](image)

**Fig. 9.** Resonance of a 3 \( \mu \text{m} \) particle with different indices of refraction

In this specific application, the importance of the index of refraction is the difference between the resonator and its surrounding medium, and not so much the index behavior.
along the spectral range. This is because both, cytoplasm and nucleus indices have similar but subtle dependencies on wavelength. This is shown in the results section.

A plot of resonators with two different sizes (cells of 2 µm and 3 µm) is shown in Fig. 10, where no absorption coefficient is used. This plot shows that for the same reflection coefficient the signal amplitudes are the same, but clearly the FSR has changed. In a simplistic approach the FSR can be used to derive the resonator’s size.

![Intensity for Different Cells](image)

Fig. 10. Free Spectral Range due to two different cell sizes. No absorption coefficient used. Simulated cells of 2 µm (blue color) and 3 µm (red color) sizes.

To determine the size of a cell producing the spectrum, equation (23) is used; the result is based on the expansion of the oscillation throughout the wavelength range Δλ:
\[
\hat{d} = \frac{\lambda^2}{2 \, n \, \Delta \lambda}
\]  

Equation (23) measures the size of a particle, \(d\), by obtaining the distance between peaks, \(\Delta \lambda\), the index of refraction of the medium, \(n\), and the wavelength where the peaks are located. As mentioned before, it is assumed that a tissue is a thin homogeneous structure of certain types of cells. We insure this by analyzing the reflectance or transmittance spectrum from only the first few layers of cells in a tissue. To account for the large number of cells in the model, all of the light intensities from each individual model of the cell are taken into account, creating a weighted sum of multiple patterns as shown in Fig. 11.

This pattern will be different depending on the modeled spread of cell sizes. In Fig. 11, where only two cells exist, with sizes of 2\(\mu\)m and 3\(\mu\)m, using equation (23) with data obtained from this figure produces a series of high and low peaks shown in Table 2. Taking the mean values in the single peaks column gives a result of 3.1 \(\mu\)m with a standard deviation of 0.779 \(\mu\)m. As more particles of different sizes are added, the signal becomes more complex, making it more difficult to derive the resonator size from a physical measurement of the FSR.
Fig. 11. Intensity from two cells with sizes of 2 and 3 μm shown in Fig. 10

To illustrate this point further, Fig. 12 through Fig. 14, show the transmission spectrum produced by 100 cells, with a mean resonator size of 15 μm and a spread of 0.5 μm, 5 μm and 10 μm, respectively. In addition to this, the Fourier transform of each spectrum is also given to show the spread in FSR.
Table 2. Peaks from Fig. 11, distance between peaks and calculated sizes using Eq. (23).

<table>
<thead>
<tr>
<th>wave peaks, (nm)</th>
<th>positive peaks n-(n-2)</th>
<th>difference 2 peaks</th>
<th>Sizes based on single peak</th>
<th>double peak</th>
</tr>
</thead>
<tbody>
<tr>
<td>353.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>358.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>366.35</td>
<td>12.85</td>
<td>4.79637E-06</td>
<td></td>
<td></td>
</tr>
<tr>
<td>375.15</td>
<td>16.65</td>
<td>3.7017E-06</td>
<td>2.08927E-06</td>
<td></td>
</tr>
<tr>
<td>384.5</td>
<td>18.15</td>
<td>3.39578E-06</td>
<td>1.77107E-06</td>
<td></td>
</tr>
<tr>
<td>393.75</td>
<td>18.6</td>
<td>3.31362E-06</td>
<td>1.6771E-06</td>
<td></td>
</tr>
<tr>
<td>400.7</td>
<td>16.2</td>
<td>3.80453E-06</td>
<td>1.77107E-06</td>
<td></td>
</tr>
<tr>
<td>406.9</td>
<td>13.15</td>
<td>4.68695E-06</td>
<td>2.09994E-06</td>
<td></td>
</tr>
<tr>
<td>417.45</td>
<td>16.75</td>
<td>3.6796E-06</td>
<td>2.06132E-06</td>
<td></td>
</tr>
<tr>
<td>429.1</td>
<td>22.2</td>
<td>2.77628E-06</td>
<td>1.58237E-06</td>
<td></td>
</tr>
<tr>
<td>441.2</td>
<td>23.75</td>
<td>2.59509E-06</td>
<td>1.34131E-06</td>
<td></td>
</tr>
<tr>
<td>452.85</td>
<td>23.75</td>
<td>2.59509E-06</td>
<td>1.29754E-06</td>
<td></td>
</tr>
<tr>
<td>461.65</td>
<td>20.45</td>
<td>3.01385E-06</td>
<td>1.39442E-06</td>
<td></td>
</tr>
<tr>
<td>471.3</td>
<td>18.45</td>
<td>3.34056E-06</td>
<td>1.5844E-06</td>
<td></td>
</tr>
<tr>
<td>485.355</td>
<td>23.705</td>
<td>2.60001E-06</td>
<td>1.46206E-06</td>
<td></td>
</tr>
<tr>
<td>500</td>
<td>28.7</td>
<td>2.1475E-06</td>
<td>1.1761E-06</td>
<td></td>
</tr>
</tbody>
</table>
Fig. 12. Top) 15 μm particle size with a 0.5 μm spread. Bottom) Fourier transform of the signal.

Fig. 13. Top) 15 μm particle size with a 5 μm spread. Bottom) Fourier transform of the signal.
As these figures show, the broader the cell size distribution the wider the FSR (frequency) spread, making it more difficult to pinpoint a specific cell size (mean resonator size). This creates an interesting challenge while trying to recognize the LSS pattern created by the reflectance spectrum.

The FSR is inversely proportional to the cell size, and as the number of cells increases, the FSR becomes more difficult to define due to the spread in cell sizes. This means that Fourier analysis cannot provide information about the signal, because the FSR of the pattern changes as the wavelength and cell size changes. After trying different approaches, the analysis tool that showed the best results was wavelet analysis.
2.3. **WAVELET ANALYSIS**

Wavelet analysis is a new and flexible mathematical tool for de-noising and signal processing; it provides information on the time and frequency components of a signal. Unlike Fourier transform, which only provides frequency information, the use of wavelet transformation provides frequency and time information. This kind of transform can be used for voice analysis, biological signals, earthquake signals, etc. It provides a more flexible analysis than Short Time Fourier transforms (STFT) due to the fact that it changes the “time window” size as part of the analysis.

While time-frequency information is provided, it is important to be aware of a physical attribute described by the Heisenberg Uncertainty Principle. This is significant because it means that the more information there is about the time when the event happened, the less is known about frequency, and vice versa. An example of this is the Fourier transform, where frequency information is very accurate but there is not time information of when a specific frequency is happening (i.e., there must be an infinite wave train to describe the Fourier coefficients). The same effect occurs when only a few points in time are considered, which results in inaccurate frequency information. For more information on wavelets and Fourier transforms, the readers refer to the *Illustrated Wavelet Transform Handbook* by Addison [28].

A wavelet is a function that satisfies certain mathematical criteria. There are several different wavelets one could choose from; the best one for a given application depends on the characteristics of the signal one wants to analyze and the features one wants to enhance.
A very common example of a wavelet is the Mexican hat wavelet, shown in Fig. 15. This wavelet is given by Eq. (24):

\[ \psi(t) = \left(1 - t^2\right) \cdot e^{-t^2/2} \]  

(24)

The wavelet described in equation (24) is known as the mother wavelet. It is the basic form of a wavelet that is dilated and translated as the wavelet transform is applied.

2.3.1. WAVELET REQUIREMENTS

As mentioned earlier, for a function to become a wavelet it has to meet certain mathematical criteria. These criteria are:
(1) A wavelet needs to have finite energy:

\[
\text{Energy} = \int_{-\infty}^{\infty} |\psi(t)|^2 \, dt < \infty
\]  

(2) Given the Fourier Transform of the wavelet \( F(\psi(t)) \) the following must hold true:

\[
C_g = \int_{0}^{\infty} \frac{|F(\psi(t))|^2}{\psi(t)} \, d\psi(t) < \infty
\]  

The wavelet has no zero frequency components, \( F(\psi(t)) = 0 \). This is called the admissibility constant condition; for the Mexican hat wavelet, its value is equal to \( \pi \).

(3) For complex wavelets the Fourier transform must be real and vanish for negative frequencies.

Wavelets that satisfy equation (26) are in fact bandpass filters, which is the desired characteristic. Therefore, scaling is equal to selecting different bandpass frequencies, and this selected frequency is the one tested against the signal. This results in a signal filtered by different pass-band filters.

2.3.2. WAVELET TRANSFORM

After having a wavelet, the next step is to make it more flexible by adding two different basic manipulations: Translation and dilation. To do so, it is necessary to change the original equation (24) by adding a translating factor \( b \) and a dilation factor \( a \), as shown in equation(27).
Translation, as its name indicates, is the process of translating the wavelet along the time scale by changing the value of b. This can be seen in Fig. 16. By translating the wavelet thorough the time scale, it allows one to compare each wavelet with the function being tested at a given time with a specific wavelet.

On the other hand, by dilating the wavelet, its frequency is changed with respect to the function under testing, as shown in Fig. 17. Moreover, by adding the process of translation and dilation, there is a frequency analysis that changes as the time changes. This allows one to work with a function such as voice or chirps or, as is presented in this research, with optical signals that change with respect to the wavelength.

\[
\psi(t) = \left(1 - \left(\frac{t-b}{a}\right)^2\right) \cdot e^{-\left(\frac{t-b}{a}\right)^2 / 2}
\]

(27)

Fig. 16. Translating a function by changing the b parameters.
In order to apply the wavelet transform it is necessary to normalize it, particularly in the case of the Mexican hat. After integrating the wavelet in equation (24), the value under the curve is $\left(3 \sqrt{\pi/4}\right)^{-1/2}$. This value has to be added into the mother wavelet, and all the resulting wavelets will be normalized to a constant value under the curve.

Finally, the wavelet in Equation (28) can be used to obtain the continuous wavelet transform (CWT) of a function $x(t)$ defined by:

$$T(a,b) = w(a) \int_{-\infty}^{\infty} x(t) \psi^* \left( \frac{t-b}{a} \right) dt$$

(28)

Here $w(a)$ is a weighting function, $\psi((t-b)/a)$ the dilation $a$ and translation $b$ of the wavelet, and $x(t)$ the signal to be transformed. In this case, $x(t)$ is the reflectance of the cells.
contained in the tissue. Normally, the wavelet transform uses the complex wavelet function. However, in the case of a real function, there is no complex part, and thus, a real function is used. In equation (28), the product of the wavelet and the signal are multiplied and integrated along the signal range in a process called convolution. As the wavelet is translated and dilated over the whole signal range while convoluting signal and wavelet, a new signal is created with new information about the $x(t)$ signal.

Figure 18 shows the signal obtained from the model of the two cells with sizes of 2 $\mu$m and 3 $\mu$m. This signal will be analyzed using the Mexican hat wavelet transform.

![Analyzed Signal](image)

**Fig. 18.** Signal to be transformed using the wavelet toolbox and the fpr wavelet.

Figure 19 shows the signal transformed by the wavelet. In this figure, along the y axis are the different scales or dilations performed on the signal. It is possible to see how the signal changes as the wavelet is dilated and translated along the x axis. It is also possible to spot a cross used to select a point in this figure. This selection is used to plot a scale in a different window.
Fig. 19. Wavelet transform of data in Fig. 5, 2 and 3 μm cell model.

In Fig. 20, a scale is selected from the transformed signal and is plotted in the window. This scale is an a value of dilation convoluted with the x(t) signal, as was shown in Fig. 18.

Fig. 20. Selected wavelet scale from Fig. 19.

On the right side of the Fig. 21 are all the parameters used to obtain these results; the wavelet used, a custom type, \texttt{fpr} the wavelet created based on Eq. (19); the scales or dilations
1 thru 30; and the sampling period. All of these values can be changed depending on the desired representation and scales.

![Wavelet Toolbox Parameters](image)

Fig. 21. Parameters used on this wavelet example. The program used is the wavelet toolbox from Matlab™.

To achieve the goals of this research, a unique wavelet for detection of specific patterns produced by the resonance of the light in cell structures was created. The analysis applied to the optical cell model will be presented in more detail in the next chapter. The procedures to follow are exactly the same as the ones shown in this example.
CHAPTER III

3. EXPERIMENTAL RESULTS

This chapter presents the experiments, procedures, and results obtained by following the ideas presented in theoretical development. The final part of this chapter addresses the algorithm for data fitting used on the samples to measure the oscillations in the reflected spectrum.

3.1. TRANSMISSION EXPERIMENT

The purpose of the transmission experiment is to apply a known light field to a sample and to record the transmitted spectrum. This is repeated using different samples that are measured beforehand for the purpose of corroborating the results obtained in the analysis section. The recorded reflectance spectrum contains two different components: a large DC signal, and an oscillation with respect to wavelength due to multi-pass interference.

The goal of this setup is to obtain a transmission field across the sample. One of the required specifications for testing is that the incident light field makes a normal with the sample plane. The experimental setup is illustrated in Fig. 22 and as shown, the set up is very simple. The spectrometer is positioned behind the sample as close as possible and forming a parallel line with the sample. In this case, the obtained spectrum will be the transmittance spectrum. To obtain the reflectance spectrum from the sample, it is necessary to use optical fibers to send and collect the reflected light. The incident light also needs to be polarized, so
that only the surface cells are probed. The light reflected from the surface cells maintains the same polarization as the source, because it only experiences a single scattering event. Conversely, the backscattered light from within the tissue contains a random polarization because of multiple scattering events and can be filtered out with another polarizer.

![Diagram](image)

**Fig. 22. Experimental Setup for the light reflectance test.**

### 3.1.1. EXPERIMENTAL SETUP

The test setup is designed to be simple and to avoid introducing any variables that cannot be accounted for. It consists of a light source, a sample holder, and a spectrometer. The components of the experiment are shown in Table 3.
Table 3. Parts used in the test setup for the reflectance spectroscopy experiment.

<table>
<thead>
<tr>
<th>Part description</th>
<th>Part Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tungsten lamp</td>
<td>BPS 100 BW TEK</td>
</tr>
<tr>
<td>Spectrometer</td>
<td>BRC100E-VIS BW TEK serial interface</td>
</tr>
</tbody>
</table>

Figure 23 shows the results obtained from a data capture on a dry blood sample. It is possible to see the absorbance in the lower 500 nm-580 nm range region (blue through orange spectrum). In addition, a low magnitude reflectance signal produced by the layer of cells is embedded throughout this spectrum.

![Transmittance Spectrum for Fresh Blood](image)

Fig. 23. Spectrum obtained from the transmittance spectrum from a blood sample.
3.1.2. Procedure

Previous to any measurement, it is necessary that the spectrometer has its original calibration data loaded; this is to avoid any wrong wavelength placement in the data obtained.

In order to obtain repeatable data, the following procedure was created:

1. The light source and the spectrometer are placed facing each other at about half a meter distance (1.5 ft) apart.
2. A measurement of the spectrum is taken to make sure the spectrometer is not saturated. If the spectrometer has more than 3000 relative units of intensity (∼73 percent of full scale), a sample of the light source spectrum is saved. This data is used as calibration and future reference.
3. With the room and source lights turned off, a measurement of the dark noise is taken and saved for reference.
4. A sample is placed on a microscopy slide of 1 mm of thickness. The clear side of the microscope slide is then placed in flush contact to the surface of the spectrometer. A spectrum measurement is taken. If the spectrum of the signal produced by the sample is above 1000 relative units of intensity (∼25 percent of full scale), then three samples are taken and saved along with the reference data for dark noise and light source.
5. Finally, because the spectral data capture range of the spectrometer is not equal to the actual visible spectral range of the source, it is necessary to truncate all of the data below 475 nm.
One thing worth mentioning is that, because of the resolution of the spectrometer, it is possible to use this 1 mm microscope slide without it producing any interference. This is because to see a resonance pattern from this slide would require a resolution of less than 0.1 nm. The spectrometer averages the signal over 1 nm samples.

3.2. MEASUREMENT OF THE INDEX OF REFRACTION

Measuring the index of refraction is a key part of the modeling and testing of the cell sizing. This is because the reflection is directly connected to the index of refraction and it changes the shape of the resonance spectrum, as the quality of the cavity is dependent on this very value. Moreover, the wavelet must be directly connected to the shape of the reflectance spectrum. Thus, by measuring the index of reflection it is possible to change the wavelet shape used on the data analysis.

3.2.1. INDEX OF REFRACTION EXPERIMENTAL SETUP

The index of refraction $n$ in our model is important because the magnitude and shape of the resonating signal will be dependant on its value. The reflectance depends on the difference in indices of refraction between the cytoplasm and the nucleus of the cell. Thus, it is necessary to measure these values on the samples used for testing to create a customize wavelet for the given value of index of the refraction coefficient.

The goal of this experiment was to measure the index of refraction of the background, and of the blood samples. This was achieved by measuring the Brewster angle. The Brewster angle is a specific angle where the polarization of the light reflected by the
boundary between two different media has only vertical polarization relative to the plane of incidence. This angle is related directly to the value of the refractive index of the boundaries. Blood is one of those rare cells that do not have nucleus, thus the measured index of refraction is only produced by one main component in the blood. Also, the reflectance happens with the outer walls of the cell and the boundaries carrying the light. In this case, air.

3.2.2. Procedure

The procedure is to measure the angle where the weakest reflection happens (Brewster angle), a diagram of the testing setup is shown in Fig. 24. This angle is found by maintaining the laser on a fix position and rotating the sample until the lowest intensity is achieved. A detector is used to determine the minimum intensity of the reflected beam. The Brewster angle is recorded when the weakest point on intensity is found. This procedure is detailed in the Brewster angle section 3.2.

Fig. 24. Setup to measure the Brewster angle from the blood sample.
The equipment used in testing is listed on Table 4. The results are as follows: For the Nd:YAG and He-Ne lasers the lowest point of intensity is located along 51~53 degrees. It was not possible to measure with more accuracy this angle because the instruments used could not provide more resolution than the recorded. Thus taking 52 degrees as the middle point and using equation $n_2 = n_1 \tan(\theta_B)$, an index of refraction of 1.3 is obtained for the dry blood sample with a variation of ±0.05. Schweitzer et al. gives a value of average over the wavelength range of interest of 1.4, which is close with the obtained results. This also shows agreement with Schweitzer [29] data, which shows very little variation along the visible range for the index of refraction of the blood.

**Table 4.** Equipment used for index of refraction measurement.

<table>
<thead>
<tr>
<th>Equipment used</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>HeNe Laser</td>
<td>635 nm 3mW</td>
</tr>
<tr>
<td>Frequency doubled Neodimium YAG laser</td>
<td>532 nm 10 mW</td>
</tr>
<tr>
<td>Rotating base</td>
<td>Controls/measures rotation</td>
</tr>
<tr>
<td>Crystal slide 1 mm thick</td>
<td>Sample</td>
</tr>
<tr>
<td>Dried blood</td>
<td>Sample</td>
</tr>
</tbody>
</table>

### 3.3. SIGNAL ANALYSIS

For the signal processing stage, there were several programs coded for analysis, simulation, and presentation of results. This section explains how the signal is analyzed
based on a comparison with the optical cell model, using the wavelet signal analysis to decompose the transmission spectrum. The process is basically a multilevel comparison of the transformed data using the wavelet function and the model over a wide variety of particle sizes. The programs used for modeling and analysis are MathCAD and Matlab, with its signal analysis, wavelet and curve fitting toolboxes.

The main programs are functions for data conditioning, cell size modeling, wavelet transform, and data fitting and plotting. The programs are shown in Appendices III, IV and V and they can be changed to expedite the calculations or improve the results. The main goal is the proof of concept, and in order to achieve this, the functions are left as simple as possible to clarify the process.

3.3.1. CELL MODELING

In the case of the modeling of cells, the program is simple. First, the user defines the desired distribution of the cell population and selects a saved absorption spectrum over the wavelength range of interest. These data are used to create a spectrum where the modeled cell data are embedded. As an example, the results from the model for 2 cells with a mean size of 7.5 μm and a spread of 3 μm, using a spectrum obtained from butter is shown in Fig. 25.
3.3.2. **WAVELET TRANSFORM BASED ON THE OPTICAL MODEL OF A CELL**

To create a wavelet that allows separating the different fréquences produced in the transmission spectrum, the optical cell model is used. To obtain a shape, only one period of this oscillation is used and added as an **fpr** (custom wavelet) wavelet, as mentioned in Chapter 2. Finally, after adding the wavelet to the matlab toolbox, it is possible to display it by using the `waveren` toolbox, where selecting “display wavelet” and “**fpr**” presents the wavelet shown in Fig. 26.
This signal is used as the wavelet to analyze the transmission spectrum obtained from the samples. As shown in chapter 2, this signal is translated and dilated along the sample range, in order to give a collection of convolutions with the sampled signal. This produces a two dimensional array that is analyzed with the data fitting algorithm, presented in the next section. The idea is to utilize a unique wavelet based on the difference in the indices of refractions for the samples. For this research a custom wavelet is used, this wavelet is based on the optical cell model presented in Chapter 2 with the appropriate index of refraction of blood.

### 3.3.3. Model Fitting Algorithm and Results

The data fitting algorithm works by decomposing the signal using a customized wavelet transform and comparing that signal with the optical model of the cell. As
mentioned earlier, the spectrum has a free spectral range (FSR) for a given particle size; its magnitude can be small for a given index of refraction difference, but the FSR is constant (see Fig. 9.) The algorithm works on the premise of matching these peaks with the peaks from the model.

The block diagram in Fig. 27 shows the simplified version of the program used for data fitting. For the code, please refer to appendix III.

Fig. 27. Block diagram for the data fitting algorithm.
The steps are: 1) The spectrum is loaded along with the chosen wavelet scales in order to convolve them using the fpr wavelet transform. At this point, a figure is generated showing the reference light source spectrum, the transmission spectrum, the absorbance and the Fast Fourier transform applied to the transmitted spectrum. Fig. 28 shows an example of these data sets.

![Data from the blood test sample.](Image)

2) A wavelet transform is obtained by using the absorbance data. This generates a wavelet window as shown in Fig. 29. The displayed values are the absolute values of the transform. This is done to generate better contrast for the results shown in the transform.
A limitation of the system is the resolution and range of the spectrometer; this creates a limit for the measurable particle sizes. Based on 1312 data points that are useful from the optical spectrum and the resolution of the spectrometer, it is only possible to see particles sized within the range of 0.1 μm to 120 μm; see Appendix II for the calculation of these values.

Fig. 29. Wavelet transform of the transmission spectrum.

After the wavelet transform is calculated, a bi-dimensional array is produced with a-scales (see Eq. (27)) by 1312 data points. Each one of these scales is compared against different models created with different particle sizes. This is presented in Fig. 29 and it is known as a wavelet transform display.
In the size matching algorithm, a range of particle sizes is generated for comparison with the wavelet scales, these values can vary from 2 \( \mu \text{m} \) to 100 \( \mu \text{m} \) with a minimum step 0.1 \( \mu \text{m} \) between them. These ranges are user selected. Each of these comparisons (wavelet scale vs. range of sizes) results in a minimum error, all are saved, and then the minimum is chosen for that specific wavelet a-scale. This generates as many points as a-scales chosen for the transform. The results are then displayed as shown in Fig. 30. Finally, one way to display the collected results more clearly is to fit the data to a distribution. Matlab offers one way to do this by using a Gaussian error fitting function as shown in Fig. 31, by using the data from Fig. 30. This code is in the Appendix III.
Fig. 31. Gaussian fitting of the data produced by the data fitting algorithm.

The normal approach for this system is to give a broad testing range for the particles and narrow it to where the error is smaller. This final selection of the minimum error can be changed and made to work in a more efficient way by following other decision parameters. For this case, the proof of concept is the main goal, and the results are very positive, as will be shown in the next chapter.
CHAPTER IV

4. RESULTS

This chapter presents the results obtained from five different measurements using the model and algorithm mentioned earlier in Chapters 2 and 3. It also presents results obtained to corroborate the results from this optical method. The samples included a microscope slide cover, three different samples of lysed dried blood, and red blood cells. In the case of the microscope slide, a caliper was used as our reference data. In all the other samples a confocal microscope was used to take images at different depths of view, these images were stacked together to later build a 3D image that would render the depth which is used to corroborate the Fabry-Perot optical model results.

4.1. MICROSCOPE SLIDE COVER

First, the simplest test was to measure a microscope slide cover. The direct measurement is done using a caliper. The result is shown in Table 5.

<table>
<thead>
<tr>
<th>Sample Volume</th>
<th>Thickness</th>
<th>Method used</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microscope slide cover</td>
<td>120 µm</td>
<td>caliper</td>
</tr>
</tbody>
</table>

This microscope slide was used to obtain a transmittance spectrum, and after applying the algorithm to the spectrum, the results in Fig. 32 and Fig. 33 are obtained. Taking the peak value from the curve in Fig. 33, the analysis technique presented here resulted in a measured thickness of 115 µm, which is 4 percent different from the caliper measurement.
Fig. 32. Error vs size plot for the microscope slide cover.

Fig. 33. Gaussian fitting for the size results of Fig. 32.
4.2. **Dry Lyzed Blood**

Another test samples used were blood with different thicknesses. The idea was to spread a chosen blood volume over a known area (2.5 cm x 1.5 cm) on a slide, measure its thickness and obtain a transmission spectrum from the blood samples. However, it was found that the blood was lysed (cells are destroyed), because of this the samples were left to dry to perform measurements later. The cell measurements were done by obtaining images using a laser Biorad confocal microscope MRC 600 and a Zeiss Axiovert 10 Inverted Microscope.

This microscope can produce a collection of images at different levels in the z-direction (vertical to sample slide), which allows the creation of a 3D model by relating the images to a size in the z-direction. The error in this system is about ±2.5 μm, for a 20X amplifying lens. This presents a problem in measurement accuracy when cell sizes are less than 10 μm. In this case, a 100X lenses is used, which reduces this error by a factor of 5.

The measurements performed with the confocal microscope showed a large variation in the thickness of the sample, as shown in Fig. 34. These samples still had the index of refraction of the blood; this presented an opportunity to measure a case similar to dysplasia, where the variation of the cell nucleus radii in the diameter of the cells. The resulting sizes are shown in Table 6.
Fig. 34. 0.005 mL lysed blood picture using the confocal microscope.

**Table 6. Thickness obtained by using a known area and volume.**

<table>
<thead>
<tr>
<th>Sample Volume</th>
<th>Thickness</th>
<th>Method used</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.002 ml of lysed blood</td>
<td>1.5-4.4 μm</td>
<td>Confocal (±0.5μm)</td>
</tr>
<tr>
<td>0.003 ml of lysed blood</td>
<td>2-3 μm</td>
<td>Confocal (±0.5μm)</td>
</tr>
<tr>
<td>0.005 ml of lysed blood</td>
<td>2-7 μm</td>
<td>Confocal (±0.5μm)</td>
</tr>
</tbody>
</table>

The thickness of these samples is not constant. Fig. 35 shows a view of the 0.002 mL lysed blood slide. It is possible to see that there are different levels of thickness. This is very interesting because it is a wide spread of thicknesses in a homogeneous medium.
Fig. 35. \textit{xz} cross-section lysed blood with a figure thickness of 7.5 \textmu{}m from top to bottom. Picture obtained with the confocal microscope.

The results for the sample of 0.002 mL of blood using the algorithm are shown in figures Fig. 36 and Fig. 37. It is possible to see from these figures that the most repetitive value is about 2.8 \textmu{}m but there exist a variation in the results of almost 1 \textmu{}m. The calculated error is of about 8\% from the mean value of the confocal microscope reading. The confocal microscope has an inherent error of \pm{} 0.5 \textmu{}m which is within the range of the measured results. This makes the calculated difference vary largely.

![Minimum Error and Size Results for the Wavelet Matching Algorithm](image)

Fig. 36. Error vs size plot for the 0.002 mL of lysed blood.
Fig. 37. Gaussian fitting for the results of Fig. 36.

Fig. 38 shows a view of the 0.003 mL lysed blood 3D confocal reconstruction. It is possible to see that there are different levels of thickness, but it is more homogeneous in thickness than the previous sample.

Fig. 38. **xz** cross-section lysed blood with a thickness of 6 μm. Picture obtained with the confocal microscope.

The results for sample of 0.003 mL of blood using the algorithm are shown in figures Fig. 39 and Fig. 40. It is possible to see from these figures that the most repetitive
value is about 2.8 μm but there exist a variation in the results of almost 0.5 μm. This is in agreement with Fig. 38. The calculated difference is 20%.

![Minimum Error and Size Results for the Wavelet Matching Algorithm](image1)

**Fig. 39.** Error vs size plot for the 0.003 mL of lysed blood.

![Resulting Size Distribution](image2)

**Fig. 40.** Gaussian fitting for the size results of Fig. 39.
Figure 41 shows a view of the 0.005 mL lysed blood 3D confocal reconstruction. It is possible to see that thickness is more homogenous, but there is still a variation in thickness in the surfaces.

Fig. 41. xy cross-section lysed blood with a thickness of 15 μm. Picture obtained with the confocal microscope.

Fig. 42. Error vs size plot for the 0.005 mL of lysed blood.
The results for sample of 0.005 mL of blood using the algorithm are shown in figures Fig. 42 and Fig. 43. It is possible to see from these figures that the most repetitive value is about 3.5 μm but there exist a variation in the results of about 1 μm. This is in agreement with Fig. 41. The calculated difference is 33%.

![Resulting Size Distribution](image)

**Fig. 43.** Gaussian fitting for the size results of Fig. 42.
4.3. **Red Blood Cells**

Finally, for the measurements using red blood cells, the same procedure as before was used. A confocal picture of the test sample is shown in Fig 44. The volume reconstructions are shown from Fig. 45 thru Fig. 47.

![Fig. 44. Blood smear from the confocal microscope, this is one picture of the 22 pictures on the stack.](image)

![Fig. 45. xy image view of the volume reconstruction.](image)
The measurement of the thickness of the blood cell pictures obtained with ImageJ program is done by calculating the thickness of the image in pixels and making it equal to the value of the thickness of the stacks. The total picture size is equal to 336 pixels and this image consists of 22 stacks at 0.5 μm of distance between them, giving a result of 10.5 μm.
total. The yellow color on the top of the pictures is the blood cell. Its thickness is of 78 to 98 pixels, which is equal approximately to 2~3 μm thickness.

Table 7. Calculated results from different volumes of blood smeared on a microscope slide.

<table>
<thead>
<tr>
<th>Sample Volume</th>
<th>Thickness</th>
<th>Method used</th>
</tr>
</thead>
<tbody>
<tr>
<td>Red Blood cells</td>
<td>2-3 μm thickness / 7-10 μm height</td>
<td>Confocal (±0.5μm)</td>
</tr>
</tbody>
</table>

The results from the algorithm are shown next. The error is about 11 percent; these results are from different cells that are illuminated, in the case of the confocal it is one specific cell.

Fig. 48. Results from the fitting algorithm.
Fig. 49. Gaussian fitting of the data produced by the data fitting algorithm.

These results are very encouraging and prove that these conceptual ideas can be applied successfully to measure homogeneous cell structures as long as there is a difference on the index of refraction enough to create a pattern that can be recognized by the wavelet transform.
CHAPTER V

5. CONCLUSIONS

Initially, the goal was to be able to measure cells based in the optical properties of their larger structures, and thus to provide an aid to the physician while performing a pap smear or an endoscopy by optically analyzing the diameter distributions of cell's nuclei. This idea leads us to the development of the optical model of a cell and the wavelet decomposition which produced positive results with a short calculation time; this is shown in Chapter III. And, from the results of Chapter IV, it can be concluded that this method is promising and can be implemented easily with a very inexpensive setup system as originally planned.

Chapter III, also makes a comparison between this model and Backman et. al. [22], in doing so, it can be determined that both models are very similar. However, Backman's model [22], is based on Van Del Hust [9] scattering model, for which the author estimates the minimum distance should be three times the diameter of the particles to maintain the condition for independence. In Backman's work, this is not the case given that nuclei with dysplasia grow close to the cell size. However, results from his work are good and after a comparison, both ours and his models show good agreement although arrived to from different assumptions. In the optical system created, it is assumed that this is one layer of homogeneous (cells from the same tissue with similar optical properties) resonating cells. To apply this approach in a in-vivo testing it is necessary to polarize the source light, and to use
a polarizer with the same orientation that the source light before the spectrometer. This permits the recovery of only light with polarization similar to the source. This light contains only the reflectance spectrum of the upper layer of cells. The lower layers will scatter the light, and thus, changing its polarization. By filtering this light, all this randomly polarized light will not reach the detector.

As mentioned before, one of the strengths of this system is the creation of a wavelet database for a given set of reflections and indices of refraction. This permits the measurement of other type of cells with different optical properties as long as a difference between the indices of refraction exists. It is also worth clarifying that this research is done under the transmittance spectrum, and that working with the reflected spectrum is possible but the set-up will require a fiber optic system that guides the source and reflected light beams for delivery and signal processing. This approach will most likely be used as an attachment of an endoscope/colposcope system, which will allow the doctor to test while performing an optical inspection or gathering samples (pap-smear or biopsy).

One point that is very important to mention, is the simplicity of the setup; a white light source and a spectrometer are the only components used; compared to other results from researchers mentioned in Chapter II, where bulky lasers, high power light sources and scattering algorithms are used, this approach allows for the fastest measurement with the least expense. This gives us a clear advantage in cost and implementation and with the results obtained, an opportunity to take this work to a commercial for biopsies and early cancer detection. Moreover, if miniaturization is possible, this could be implemented as a pill size device that can be swallowed, gathering data of the gastrointestinal system for later analysis. This system would aid in reducing costs of endoscopy and create an instrument to detect
early stomach cancer. One point that has to be clear that in order to increase the chances to determine positively if cancer is present or not at any stage, is a tri-modal of approach. By doing this, not only dysplasia can be found but also polyps and cancer in advanced stages.

One of the challenges in this work was the need of better equipment to corroborate the results while analyzing cancerous and live tissue. However, the results presented here prove that it is possible to measure cells and micro-structures, and it is a future goal to test cancerous tissues and perform in-vivo testing. Finally, the algorithm for size matching can be greatly enhanced by changing the fitting method, the error reduction, and the selection of the results from each wavelet scale case. In this case the goal was a positive conceptual proof and this software structure provides the results and desired simplicity.
6. REFERENCES


APPENDIX

I REFLECTANCE COEFFICIENTS AND INDEX OF REFRACTION

The index of refraction n in our model is very important because the magnitude and shape of the resonating signal will be highly dependant on its value. This difference is the difference in indices of refraction between the cytoplasm and the nucleus of the cell. Thus, it is necessary to measure these values to create a customize wavelet.

For the model it is necessary to measure the index of refraction of the background and of the sampled cells. This is achieved by measuring the Brewster angle. The Brewster angle is a specific angle where the light reflected from the boundary between two different media having different refraction indices has only vertical polarization to the incident plane. This angle is related directly to the value of the relative refractive index of the boundary. By detecting this polarization angle it is possible to measure the index of refraction of the material. To further explain this, assume that there is a beam of light traveling in air striking a surface with a different indices of refraction. This results in a refracted and a reflected beam of light as shown in Fig. 50.

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Fig. 50. Light incident on a plane with a different index of refraction generates two beams, one reflected and one refracted.

These three beams create a plane called plane of incidence. The incident and reflected angle are equal, but opposite, from the normal; the refracted angle is given by Snell's law in equation (29).

\[
n_1 \sin \theta = n_2 \sin \theta' \tag{29}
\]

Note that the only factor changing the refracted angle is the ratio of the index of refraction.

To continue with the analysis of polarization, it is necessary to review the Fresnel equations for the reflected transverse electric (TE) and transverse magnetic (TM) fields.

The reflected TE is \( r_\perp \):

\[
r_\perp = \frac{E_r}{E_i} = \frac{\frac{n_1 \cos \theta}{\mu_1} - \frac{n_2 \cos \theta'}{\mu_2}}{\frac{n_1 \cos \theta}{\mu_1} + \frac{n_2 \cos \theta'}{\mu_2}} \tag{30}
\]

If \( \mu_1 = \mu_2 = 1 \), which is the case for dielectrics the equation becomes:
\[ r_\perp = -\frac{\sin(\theta - \theta'_1)}{\sin(\theta + \theta'_1)} \]  

and for the reflected TM \( r_\parallel \):

\[
\frac{E_\parallel}{E_j} = \frac{\frac{n_2}{\mu_2} \cos \theta - \frac{n_1}{\mu_1} \cos \theta'}{\frac{n_1}{\mu_1} \cos \theta' + \frac{n_2}{\mu_2} \cos \theta}
\]  

Again, if \( \mu_1 = \mu_2 = 1 \), which is the case for dielectrics, the equation becomes:

\[
r_\parallel = \frac{\tan(\theta - \theta')}{\tan(\theta + \theta')}
\]  

Plotting equations (31) and (33), for a boundary of 1:1.5 in Fig. 51, shows that only the reflected TE field reaches a zero at about a 60 degree angle, while the reflected TM never reduces its magnitude. By plotting the magnitude of the electric field (see Fig. 52), it is possible to see that there is a point where the intensity of the reflected TE is equal to zero. In this case it is about 60.9 degrees.

The value of \( n_2 \) is related to the angle by finding the zero intensity point \( \theta_B \) and solving equation (34) and shown in Fig. 52 and Fig. 51:

\[
n_2 = n_1 \tan(\theta_B)
\]
Fig. 51. Reflected transverse electric (TE) and transverse magnetic (TM) fields.

Fig. 52. Intensity of Reflectance for reflected TE and TM fields.
### FSR Range for a Given Particle Size

<table>
<thead>
<tr>
<th>Index of Refraction</th>
<th>Data Points</th>
<th>Beginning Wavelength [m]</th>
<th>Delta [m]</th>
<th>Range [m]</th>
<th>Particle Size [m]</th>
<th>FSR of Particle [m]</th>
<th>Data Points Needed</th>
<th>FRS in 1312 Points</th>
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</thead>
<tbody>
<tr>
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III MAIN PROGRAM TO ANALYZE THE REFLECTANCE DATA

% Program to display the data acquired from the spectrometer in the 
% resonance measurements
% The arrays are 11x2048 data points
% from excel exported from file worksheets (origin is a txt file)
clear;
excel=0;

% #1 is the index
% #2 is the data - reference
% #3 is the wavelength
% #4 is the dark noise
% #5 is the reference
% #6 is the transmission
% #7 is the Absorbance
% #8 is the and ahead do not matter

% from csv files
% #1 is the pixel or index
% #2 is the wavelength
% #3 is the dark noise
% #4 is the reference
% #5 is the raw data #1
% #6 is the dark subtracted from raw #1
% #7 is the % TR #1
% #8 is the absorbance #1
% #9 is the raw data #2
% #10 is the dark subtracted from #2
% #11 is the % TR #2
% #12 is the absorbance #2
% #13 is the raw data #3
% #14 is the dark subtracted from #3
% #15 is the % TR #3
% #16 is the absorbance #3
First import the archive you want to work with.

```matlab
load('E:\MATLAB7\work\dryblood\horizontal\thin\arealsample 1.csv')
```

M = dlmread('filename', delimiter, R, C) reads numeric data from the ASCII-delimited file filename, using the specified delimiter. The values R and C specify the row and column where the upper left corner of the data lies in the file. R and C are zero based, so that R=0, C=0 specifies the first value in the file, which is the upper left corner. M = dlmread('filename', delimiter, range) reads the range specified by range = [R1 C1 R2 C2] where (R1,C1) is the upper left corner of the data to be read and (R2,C2) is the lower right corner.

You can also specify the range using spreadsheet notation, as in range = 'A1..B?'.

```
[file path] = uigetfile('.csv','Choose the csv file u want to open');
data = dlmread(fullfile(path,file),',',1,0);
figure('name',file);
```

if (excel)
raw = data(:,2);
index = data(:,1);
wave = data(:,3);
trans = data(:,6);
ref = data(:,5);
else
% for wavelet de-noising level 5 we need 1312 values
raw = data(736:2047,5);
index = data(736:2047,1);
wave = data(736:2047,2);
trans = data(736:2047,7);
ref = data(736:2047,4);
```
dark = data(736:2047,3);
raw = data(736:2047,6);
end

%%% Calculate the absorbance
%%% absorbance raw/reference
absorbance = (raw-dark)/(ref-dark);
subplot(4,1,1), plot(wave,ref-dark);
title('reference vs wave (dark substracted)');
subplot(4,1,2), plot(wave,raw-dark);
title('raw data vs wave (dark substracted)');
subplot(4,1,3), plot(wave,absorbance);
title('absorbance (dark substracted)');
subplot(4,1,4), plot(wave,dark); title('dark noise');
valfft = fft(trans,1024);
valfft(1)=0; % first component
Pyy = (valfft.* conj(valfft))/512;
f = 500/512*(0:254);
subplot(4,1,4), plot(f,Pyy(1:255)); title('FFT');
figure;
kk = cwt(trans,1:23,'fp','plot');
[vvv xxx]= errorfitwithwavelet(wave,kk,2e-6,0.1e-6,300);
IV DATA FITTING FUNCTION

function [siz errxJ = errorfitwithwavelet(wave, arraY, sp, step, iterations)

%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%
%%%%%%%%Program to find the best fit for the fabry perot model of a cell%
%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%
%% Receives a wavelength and an array of data that will be compared and the
%% error reduced
%% Parameters used: this will work on the 20-90% data range
%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%
% Changes 8-3-05
%% changes to the error reducing by making the comparisons smaller in size
[length, col] = size(wave);
array = array';
datpoints = 100;
compsteps = ceil(length/9);
[b, row] = size(array);
fullwrange = wave(length) - wave(1);
%compsteps = (fullwrange/9)'10'-9;
wresolution = (fullwrange/length)'10'-9;

for i=1:row, %wavelet controlled
for test = 1:iterations,
   dla = (wave(compsteps)*10^-9)^2/(2*pi*(sp+step*test));
datpoints = floor(dla/wresolution);
[wav, I] = new_errorfit(datpoints, wresolution, wave(compsteps)*10^-9, sp+step*test);
onda = makeone2(array(compsteps:datpoints+compsteps-1,i));
I = makeone2(I);
erroressize(test) = (std(I-onda));
tamanios(test) = sp+step*test;

end;
% Plot only the min error and the particle size comparison that
% produced it
[xx,i] = min(erroressize);
siz(i,1) = tamanios(ii);
errx(i,1) = xx;

%% Plot error for all size comparison for each wavelet
figure
plot(tamanios,erroresssize);title('tamanios vs error')
end;
figure
eje = 1:row;
plotyy(eje,siz,eje,errx,'stem','plot');title('error vs tamano');
[mu,s,muci,sci] = normfit(siz);
devia = std(siz);
normspec([mu-devia mu+devia],mu,s);
V Program To Generate Optical Cell Models

%%% Program to model a cell as a fabry perot resonator %%%
%%% Ver 4.0 April 05 Luis Ortiz. %%%

clear;

% If the data is already smoothed and saved
% If the data is already smoothed and saved
%10 = load('mat', 'smootheddata1.mat')
%ref = [0. smootheddata1.y];

%% Load file with wavelengths %%

[file path] = uigetfile('.csv','File to use as a Iref, Abs');
data = dlmread(fullfile(path, file), ',', 1, 0);
figure('name', file);

% data = dlmread('E:\MATLAB7\workdir\blood\unknown sample from Damcc d horizontal thin area\sample 1.csv', ',', 1, 0);

raw = data(736:2047, 5); % for wavelet de-noising level 5 we need 1312 values
raw2 = data(736:2047, 9);
raw3 = data(736:2047, 13);
rawT = (raw + raw2 + raw3)/3;
index = data(736:2047, 1);
wave = data(736:2047, 2);
trans = data(736:2047, 7);
Iref = data(736:2047, 4);
dark = data(736:2047, 3);
rm = data(736:2047, 6);
abs = data(736:2047, 8);

% Smooth data for elimination of residuals %%

r = smooth(wave, raw, 25, 'moving', 0);
Irefs = smooth(wave, Iref, 25, 'moving', 0);
darks = smooth(wave, dark, 25, 'moving', 0);
abss = smooth(wave, abs, 25, 'moving', 0);
xwave = wave(1):(wave(2) - wave(1))/5:wave(1311);
xrefs = interp1(wave, Irefs, xwave);
xtrans = interp1(wave, trans, xwave);
xraws = interp1(wave, raws, xwave);
xdarks = interp1(wave, darks, xwave);
[row xcol] = size(xwave);

%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%
%% Variables definition %%
%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%

% Index of refraction of the nucleus  \( nn \)
nn = 1.5 % in this case blood
% Index of refraction of the cytoplasm \( nc \)
nc = 1.0 % in this case air.

% Reflection
R = ((nn - nc)/(nn + nc))^2; %Question, shall it be \( 1 - T(w) \)
disp ('The reflection coefficient is: '); R
T = 1 - R;

% number of cells
ncells = 1
orz = 7*10^-6;
orz2 = 3*10^-6;
mu = 7.5*10^-6;
sigma = 2*10^-6;
cells = (random(Normal', orz, orz2, ncells, 1));
%cells = normmd(mu, sigma, 1, ncells);

%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%
%% Now we have an array of ncells size, we need to calculate delta %%
%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%

%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%
%% Transmission %%
%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%

% This is the raw signal divided by the reference signal.  
% Optical absorvance or Optical density
% Where \( A = -\log_{10}(T) \)
% This are useful when using Beer Lambert's law, particularly at low
% concentration. Where the absorbance is proportional to the concentration
Another quantity that can be measured is the absorption coefficient we start from Bouger's law which relates $I$ to $I_0$ via the equation:


\[ I = I_0 \exp(-\text{absorption} \times \text{thickness}), \text{t is in cms and alpha in cm}^{-1} \]

Where absorption ($\alpha$) = ($2.3 \times 10^3 A$)lt

$\text{xtransmitance} = (\text{xtransmits} - \text{xdarks}).(\text{xls} - \text{xdarks})$;

$\text{xtransmitance} = \text{xtransmitance}'$;

$\alpha = \frac{\text{absorption}}{\text{thickness}}$, $t$ is in cms and $\alpha$ in cm$^{-1}$

Where absorption ($\alpha$) = ($2.3 \times 10^3 \text{xAbs})/t$; $t$ should be the volume in ml divided by the area size, calculate also from the confocal pictures.

$\beta = 2 \alpha$;

theta = $\cos(\theta)$; % make it less confusing (just make it a constant and make a reference to its value.

$\text{l} = \text{zeros(ncells,xcol)}$;

for $x=1:ncells$,

for $y = 1:xcol$,

\[ \text{delta} = (4 \times \pi \times n \times \text{cells}(x) \times \theta) / (\text{xwave}(y) \times 10^{-9}); \]

\[ A = \exp(-1 \times \text{beta}(y) \times \text{cells}(x))/\text{theta}; \]

\[ l(x,y) = \left(\frac{T^2 \times A}{(1 - R \times A)^2}\right) \times \left(1 + \frac{4 \times R \times A}{(1 - R \times A)^2} \times (\sin(\delta I))^2\right); \]

% \[ \text{A} = (-1 \times \text{beta}(y) \times 8 \times 10^{-6}); \]

% \[ l(y,x) = \left(\frac{\text{Iref}(y) \times \text{transmitance}(y) \times 2 \times \exp((-1 \times \text{beta}(y) \times 8 \times 10^{-6}))}{(1 - \text{R} \times \exp((-1 \times \text{beta}(y) \times 8 \times 10^{-6})) \times 2} \times \right. \]

% \[ \left. \frac{1}{(1 + \frac{4 \times R \times \exp((-1 \times \text{beta}(y) \times 8 \times 10^{-6}))}{(1 - R \times \exp((-1 \times \text{beta}(y) \times 8 \times 10^{-6})) \times (\sin(4 \times \pi \times n \times 8 \times 10^{-6} \times \theta / \text{xwave}(y) \times 10^{-9}))^2}}; \right) \]

end;

end;

hold;

plot(xwave,l(1,:));

Transmitance = zeros(1,xcol);

for $i=1:ncells$,
Transmitance(1,:) = l(:,1) + Transmitance(1,:);
end;
Transmitance2 = Transmitance ./ncells;
figure
plot(xwave,Transmitance2); Title('Adding all cells');
modelabs = zeros(1,xc);
modelabs = Transmitance2 ./ xrefs;
figure
Absx = (modelabs);
plot(xwave,Absx); Title('absorption spectrum');
hold off;
VI CONFOCAL MICROSCOPE USAGE PROCEDURE

The confocal microscope produces stacks of images in the Biorad format. To visualize and analyze them, it is necessary to use a program that can work with this format. ImageJ [30] and its plug-ins presented the solution for this case. ImageJ is a tool for image analysis based on Java and allows the creation of scripts and tools.

**Warning**: The confocal microscope uses an Argon-Ion laser, which is a class IV laser. It has a high power output at a very low wavelength; if one plans to use this equipment, it is imperative that the safety warning and operating manual are read prior to use. This is not a manual that describes the use of the microscope, but rather presents the procedure used to obtain the images needed for this thesis.

The procedure to obtain images and analyze them using ImageJ is as follows.

1. Be sure that the mechanical switch from the Biorad system is turned to the left to stop any output from the laser to reach the microscope.
2. Turn on the equipment in this order: computer and monitors, laser cooling-fan, microscopy lights, Biorad system, and the Ar+ Laser.
3. Run the program, Comos. This program will allow the user to use the scanning function at different rates and to create a Z series scan.
4. At this point, it is possible to use the system as a microscope. Again, be sure that the switch is to the left. Place the sample on the slide holder of the microscope, select the chosen lens, adjust the intensity of the microscope light, and find the focal point of the sample to visualize.
5. Once the focal point is found, use the computer step motors to control the lens’s focal point and be sure that the settings on the computer screen match the correct microscope lens. This will set the default steps for the step motors.

6. Find the lowest point at which the sample is in focus and select this point as the minimum point.

7. Find the highest point at which the sample is in focus and select this point as the maximum point.

8. Use the motors to place the microscope on the focal point and move the mechanical switch from the Biorad system to allow the laser light to pass through the microscope. Then, turn off the microscope lights.

9. In the Biorad system, make sure that all automatic controls are switched off.

10. Set the Black level to about 2 and the gain to about 6. If the screen is too obscure, increase the black level. Once it is possible to see a clear image, use the gain to make it clearer or to increase the shadows. Repeat step 9 until you can see a clear image.

11. Move the step motors from the lower and upper positions found on step 6 and 7. Make sure the image is clear through the range. If it is not, stop and return to step 10 until the image is clear within the selected range.

12. In the program’s menu, go to -obtain Z-series,- make sure that the minimum and maximum positions match the values found earlier, and select the minimum Z-increment between pictures. You can select special filters, such as Kalman, to increase the sharpness of the images obtained. Save the file.

14. Increase the contrast/sharpness, change or expand the histogram, and change the colors as desired. It is possible to use a different Look Up Table (LUT) to increase the contrast by changing colors of the Region of Interest (ROI), in this case the LUT editor plug-in [31]. Fig. 44 is an example of the results at this point.

15. Now the image stacks are ready to be analyzed. To do so, the volume viewer [32] Plug-in is used. This plug-in has different visualizations: the slice and volume dots are used to measure the thickness of the red blood cells.

Use any LUT and obtain pictures of the xy, zy, zx views.