3D Image Processing of Two-Photon Microscopy Images
Depicting Nanoprobes in Skin

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ABSTRACT

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Choosing a deconvolution algorithm can be beneficial when imaging nanoprobes in skin by means of two-photon microscopy. By design, deconvolution algorithms can increase the signal to noise ratio of the raw images and thus make it easier to identify discrete, subresolution nanoprobes from blurry two-photon image data. This poses the favorable benefit of knowing more precise locations of nanoprobes inside skin.

This thesis demonstrates how the Expectation-Maximization deconvolution algorithm (EM algorithm) can be applied to three-dimensional, two-photon images depicting quantum dot nanoprobes inside human skin. This was accomplished in part by devising a way to deliver nanoprobes inside skin by means of low frequency ultrasound. Many nanoprobes become sparsely scattered inside skin when using this nanoprobe delivery methodology.

The scattered nanoprobes resulting from the nanoprobe delivery pose a unique benefit in acquiring an experimental point spread function of the imaging system. This in turn gives an accurate representation of the point spread function that can be used as an input to the EM algorithm. The methodology of utilizing the EM algorithm in this manner is presented.
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# TABLE OF CONTENTS

ACKNOWLEDGEMENTS ........................................................................................................... 3
LIST OF FIGURES .................................................................................................................. 5
LIST OF ACRONYMS .............................................................................................................. 7
I INTRODUCTION ................................................................................................................... 8
II FUNDAMENTALS .................................................................................................................. 9
  2.1 Two-Photon Fluorescence Microscopy ........................................................................... 9
  2.2 Quantum Dot Nanoprobes ............................................................................................ 13
  2.3 Skin ................................................................................................................................ 18
  2.4 Transdermal Nanoprobe Delivery .................................................................................. 23
  2.5 Keck 3D Fusion Microscope .......................................................................................... 31
III EXPERIMENTATION AND ANALYSIS ............................................................................ 34
  3.1 Transdermal Nanoprobe Delivery Experimentation ...................................................... 34
    3.1.1 Skin Preparation and Imaging Procedures .............................................................. 34
    3.1.2 Imaging Results and Analysis .............................................................................. 36
  3.2 Background Noise Experimentation ............................................................................. 46
IV EXPERIMENTAL POINT SPREAD FUNCTION ................................................................. 48
  4.1 Procedure to Detect and Analyze the Experimental Point Spread Function (PSF) ...... 48
  4.2 Experimental PSF Analysis ........................................................................................... 58
V THREE-DIMENSIONAL IMAGE DECONVOLUTION ......................................................... 60
  5.1 Microscope Signal Model ............................................................................................. 60
  5.2 The EM Algorithm ....................................................................................................... 62
  5.3 Application of the EM Algorithm .................................................................................. 65
VI RESULTS AND FURTHER ANALYSIS ............................................................................. 78
  6.1 Deconvolution Without Nanoprobes Inside Skin ......................................................... 78
  6.2 Qualitative Assessment ................................................................................................. 85
  6.3 Concluding Remarks .................................................................................................... 87
APPENDIX A — SPECIFICATIONS ....................................................................................... 89
APPENDIX B — EM ALGORITHM MATLAB SCRIPT ............................................................. 93
**LIST OF FIGURES**

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>Two-photon excitation volume within Gaussian focus beam [54]</td>
<td>12</td>
</tr>
<tr>
<td>2.2</td>
<td>Photoluminescence of a semiconductor [50]</td>
<td>14</td>
</tr>
<tr>
<td>2.3</td>
<td>DTE scaler tip GD2 with arrows depicting the locations of transient cavitation streams</td>
<td>26</td>
</tr>
<tr>
<td>2.4</td>
<td>Ultrasound water pressure measurements</td>
<td>27</td>
</tr>
<tr>
<td>2.5</td>
<td>Sonophoresis assembly</td>
<td>28</td>
</tr>
<tr>
<td>2.6</td>
<td>Sonophoresis assembly mounted with ultrasound transducer attached</td>
<td>29</td>
</tr>
<tr>
<td>3.7</td>
<td>x-y data slice at position z=71 in a 20-frame-averaged z-stack</td>
<td>39</td>
</tr>
<tr>
<td>3.8</td>
<td>z-y data slice at position x=75 in a 20-frame-averaged z-stack</td>
<td>40</td>
</tr>
<tr>
<td>3.9</td>
<td>y-z plane Maximum Intensity Projection (MIP) depicting quantum dots inside skin</td>
<td>42</td>
</tr>
<tr>
<td>3.10</td>
<td>Varying number of frames averaged when each frame is the same subset of the x-y slice at z=71 (containing pixels x=1-200 and y=100-300). All images are using the same intensity scale between 0 and 255. Entropy is computed for all frames averaged and all x &amp; y values at z=71</td>
<td>44</td>
</tr>
<tr>
<td>3.11</td>
<td>Histograms for the varying number of averaged frames mentioned in Figure 3.10. This figure was enlarged and rotated so localized histogram peaks can be easily distinguished</td>
<td>45</td>
</tr>
<tr>
<td>3.12</td>
<td>Background PMT noise measurements</td>
<td>46</td>
</tr>
<tr>
<td>3.13</td>
<td>Background PMT noise histogram</td>
<td>47</td>
</tr>
<tr>
<td>4.14</td>
<td>Maximum voxel value vs. depth into skin</td>
<td>50</td>
</tr>
<tr>
<td>4.15</td>
<td>Number of voxels vs. nearby signal average</td>
<td>51</td>
</tr>
<tr>
<td>4.16</td>
<td>Histogram: Pixel volume of candidate nanoprobes inside skin</td>
<td>52</td>
</tr>
<tr>
<td>4.17</td>
<td>Weighted standard deviation of candidate nanoprobes in x, y &amp; z axes</td>
<td>54</td>
</tr>
<tr>
<td>4.18</td>
<td>Weighted standard deviation of candidate nanoprobes in major, medium &amp; minor axes</td>
<td>56</td>
</tr>
<tr>
<td>4.19</td>
<td>Averaged PSF at different z-axis increments. Each z-axis increment is 1 µm</td>
<td>57</td>
</tr>
<tr>
<td>4.20</td>
<td>FWHM of major axis</td>
<td>58</td>
</tr>
<tr>
<td>4.21</td>
<td>FWHM of median axis</td>
<td>59</td>
</tr>
<tr>
<td>4.22</td>
<td>FWHM of minor axis</td>
<td>59</td>
</tr>
<tr>
<td>5.23</td>
<td>Comparison of automatic and manual segmentation. Both images were normalized so that their sum is equal to one</td>
<td>66</td>
</tr>
</tbody>
</table>
5.23 Artifacts detected as a function of deconvolution iterations. The PSF yielding the least number of artifacts is the best performing PSF (the averaged PSF). The worst performing PSF is the one yielding the most number of artifacts (PSF with Id 12 in Table 1). Performance of other PSF is not shown due to space constraints.

5.24 Comparison of deconvolution artifacts.

5.25 Varying the number of deconvolution iterations when using the averaged PSF.

5.28 Number of intensity changes for each deconvolution iteration. Deconvolution was performed using the averaged PSF.

6.29 Surface of skin sample that has not undergone sonophoresis.

6.32 Histograms of images shown in Figures 6.30 and 6.31. The histogram figures show histograms before and after deconvolution was applied.

6.33 Nanoprobes on surface of skin.

6.34 Nanoprobes at 76 µm inside skin.

6.35 High signal strength at 50 µm inside skin.

6.36 Cell at 46 µm inside skin.
LIST OF ACRONYMS

BFM  Bright Field Microscopy
CRM  Confocal Reflectance Microscopy
DMSO Dimethyl Sulfoxide
FOV  Field of View
FWHM Full Width at Half Maximum
LFS  Low Frequency Sonophoresis
LTR  Localized Transport Regions
MIP  Maximum Intensity Projection
MSE  Mean Squared Error
OCT  Optical Coherence Tomography
OTF  Optical Transfer Function
PMF  Probability Mass Function
PMT  Photomultiplier Tube
PSF  Point Spread Function
PSNR Peak Signal-to-Noise Ratio
PVP  Polyvinylpyrrolidone
SC   Stratum Corneum
SNR  Signal-to-Noise Ratio
SVD  Singular Value Decomposition
TBE  Tris/Borate/EDTA
TPFM Two-Photon Fluorescence Microscopy
CHAPTER I

INTRODUCTION

This thesis investigates using the Expectation-Maximization algorithm (EM algorithm) as a deconvolution algorithm that will improve the Signal-to-Noise Ratio (SNR) of 3D images depicting nanoprobes inside skin. The EM algorithm is an iterative deconvolution algorithm that can help restore the image using the microscope’s Point Spread Function (PSF) and image noise characteristics. Therefore, the SNR will increase when the EM algorithm is correctly applied. This in turn will make it easier to discern discrete nanoprobes from blurry two-photon image data.

Quantum dots were used as nanoprobes in the experimentation conducted for this thesis. These nanoprobes are far smaller than the imaging resolution of the two-photon microscope. Therefore, they serve as an accurate object for characterizing the experimental PSF. The experimental PSFs obtained from these nanoprobes were used as an input to the EM algorithm. A procedure that delivers nanoprobes inside skin was devised by means of using low frequency ultrasound. This procedure causes enough nanoprobes to be scattered so that the experimental PSF can be obtained.

Chapter 2 discusses the fundamentals relating to steps prior to using the EM algorithm. These fundamentals include an overview of two-photon microscopy, quantum dot nanoprobes, optical properties of skin and transdermal nanoprobe delivery. Chapter 3 includes imaging procedures for all the two-photon experimentation conducted for this thesis. An automated procedure was developed to detect nanoprobes that would serve as good candidates for an experimental PSF. This procedure is discussed in Chapter 4.

A derivation of the EM algorithm is presented in Section 5.2. This section is then followed by an analysis of the optimal experimental PSF and how many iterations of the EM algorithm to compute. Supporting evidence that the EM algorithm is working is presented in Chapter 6. Section 6.2 shows images resulting from the EM algorithm that make discrete nanoprobes much easier to identify.

Nanoprobes could become be a valuable tool when they are observed inside skin. The extent of the value could be further enhanced by using deconvolution techniques such as the one presented in this thesis.
CHAPTER II

FUNDAMENTALS

The topics in this chapter include fundamental concepts that are helpful to understand when reading subsequent chapters. Much of the equipment and materials used for experimentation in this thesis are also discussed.

2.1 Two-Photon Fluorescence Microscopy

Maria Göppert-Mayer was the first to predict that two photons can be simultaneously absorbed by an atom or molecule in the same quantum event [25]. This absorption allows fluorophores to be imaged in an entirely different manner when compared to traditional single photon fluorescence. Göppert-Mayer’s prediction was made in 1931; however, many applications of the theory were not realized until decades later when high power lasers were available. It wasn’t until 1990 that Göppert-Mayer’s theory was successfully applied to create a two-photon microscope [14]. The following paragraphs will explain how two-photon fluorescence is utilized for imaging as applied to this thesis.

Two photons need to be absorbed by an atom or molecule within about $10^{-16}$ seconds for the absorption to be considered a candidate for a simultaneous quantum event [15, p. 41]. Once this event occurs the atom or molecule is in an excited state. The atom or molecule almost immediately drops back to a metastable state and then to its normal state, all within a few nanoseconds. As the atom or molecule decays from the metastable state to the normal state an emission photon is released that has a different wavelength than the exciting photons. This emission photon has the same wavelength as the emission photon that is produced by a normal single-photon fluorescence event from the same metastable state. One underlying benefit of two-photon microscopy is the freedom to choose different wavelengths for the two exciting photons. The fluorescent light emission relationship between single photon and two photon fluorescence can be seen in the equation

$$\lambda_{1-photon} \approx \left( \frac{1}{\lambda_A} + \frac{1}{\lambda_B} \right)^{-1}$$  \hspace{1cm} (2.1)

where $\lambda_{1-photon}$ is the excitation wavelength for the atom or molecule when excited by a single photon and $\lambda_A$ & $\lambda_B$ are the wavelengths for each of the corresponding photons that are absorbed simultaneously during two-photon fluorescence [15, p. 43].

Equation (2.1) suggests that two-photon fluorescence is a useful alternative for exciting fluorophores instead of using the traditional one-photon fluorescence technique. The experiments in this thesis utilize a single pulsed laser to excite fluorophores by means of two photons. Therefore, $\lambda_A = \lambda_B$ and Equation (2.1) becomes

$$\lambda_{1-photon} \approx \frac{\lambda_{2-photon}}{2}$$  \hspace{1cm} (2.2)

where $\lambda_{2-photon} = \lambda_A = \lambda_B$. Single photon fluorescence requires a higher energy excitation wavelength to achieve a lower energy fluorescence emission wavelength from the fluorophore.
The energy of a photon is inversely proportional to the wavelength such that

\[ E = \frac{hc}{\lambda} \]  

where \( h = 6.6 \times 10^{-34} \text{ J} \cdot \text{s} \) is Planck’s constant, \( c = 3 \times 10^8 \text{ m} \cdot \text{s}^{-1} \) is the speed of light in a vacuum and \( \lambda \) is the wavelength. It therefore can be seen that two-photon fluorescence works uniquely in that a fluorophore can be excited by a light source that has a longer wavelength than the emission wavelength, which is typically not true for single-photon fluorescence. The ability to image a fluorophore using a longer excitation wavelength is a significant benefit for skin imaging. This will be discussed in a subsequent section within this chapter.

The probability of an atom or molecule absorbing \( n \) photons is proportional to the probability of finding \( n \) photons within the volume it occupies in a given time period. Therefore, it can be generally deduced that single photon absorption is much more probable than two photon absorption. This has been proven mathematically when laser light is modeled as a Poisson distribution of photons [4]. The result of this mathematical proof can be observed as the Poisson distribution equation

\[ P_n = \left( \frac{m^n}{n!} \right) e^{-m} \]  

where \( P \) is the probability of \( n \) photons being in a volume when \( m \) is the mean number of photons. The decreased probability of two-photon absorption implies that two-photon fluorescence requires much more power than single-photon fluorescence. The power of laser light is proportional to the irradiance \( (I) \) which has units \( \text{E}_m/(l^2 \cdot \text{s}) \), where \( \text{E}_m \) is a unit of energy, \( l^2 \) is a unit of area and \( s \) is a unit of time.

The high power requirement drawback of two-photon fluorescence is somewhat mitigated by its non-linear excitation relationship. The probability of two photons being absorbed is proportional to the square of the irradiance. This can be seen in the relationship

\[ P_2 \propto \Upsilon I^2 \]  

where \( \Upsilon \) is a proportionality factor [15, p. 45]. A major contribution to \( \Upsilon \) is the quantum efficiency of the fluorophore. The quantum efficiency is simply the probability of a photon being emitted by a fluorophore after the fluorophore absorbs one or more photons in the same quantum event.

A common implementation of a two-photon fluorescence microscope involves an excitation laser beam traveling through a magnifying objective lens which is then focused inside a sample that sits on a coverslip. The emission beam then travels back in the same path as the excitation beam and later is decoupled by a dichroic mirror. The decoupled emission beam subsequently is sent to a photon detection device made for low light levels. The two-photon microscope used in this thesis utilizes a photomultiplier tube as a photon detector [60].

The two-photon microscope used in this thesis produces a focused excitation laser beam that behaves as a Gaussian beam such that the irradiance distribution can be described by Gaussian functions. The contour lines of equal irradiance are shaped like an egg near the focal point. This three-dimensional distribution is represented by the equation

\[ I(u, v) = \left| 2 \int_0^1 J_0(v \rho) e^{-i u \rho^2 / 2} \, d\rho \right|^2 \]  

where \( J_0 \) is the Bessel function of the first kind and \( v \) is the spatial frequency. The contour lines of equal irradiance are shaped like an egg near the focal point.
where \( J_0 \) is the zeroth-order Bessel function, \( \rho \) is a radial coordinate in the pupil plane, \( u \) is the axial coordinate and \( v \) is the radial coordinate. \( u \) and \( v \) are given by

\[
\begin{align*}
    u &= \frac{8\pi z \sin^2 \frac{1}{2} \alpha}{\lambda}, \\
    v &= \frac{2\pi r \sin \alpha}{\lambda}
\end{align*}
\]

where \( \lambda \) is the wavelength of light ([49]; [15, p. 49]).

It is significant to note that Equation (2.6) suggests two-photon fluorescence has a decrease in resolution due to the focus volume when compared to single-photon fluorescence. The two-photon excitation beam has about twice the wavelength of the single-photon excitation beam. Therefore, the \( u \) and \( v \) coordinates become reduced by a factor of two in the case of two-photon and the focus volume increases accordingly.

As with the excitation beam, the emission beam has an irradiance distribution at the focal point which is characterized by Equation (2.6). This distribution represents the excitation volume and is called the Point Spread Function (PSF). When the PSF is calculated the term calculated PSF is sometimes used. Comparatively, the term experimental PSF is sometimes used when the PSF is obtained by imaging a particle that is significantly smaller than the calculated PSF. Physical aberrations in the optics can cause these two point spread functions to differ because the calculations in the calculated PSF do not compensate for these aberrations, when not taken into account. An index of refraction mismatch located within the optical path can also cause a difference in the PSF. The experimental PSF is a more accurate representation of the imaging system because the three-dimensionally scanned image is ultimately constructed from it. Therefore this PSF was obtained to be used in this thesis for image processing tasks (see Chapter 3).

Perhaps the biggest benefit of two-photon microscopy’s non-linear property is that it helps create very good optical sectioning. The excitation beam irradiance falls as the square of the distance from the focal point, as implied in Equation (2.6). In addition, Equation (2.5) suggests that the fluorescence emission radiance given by the fluorophore decreases quadratically depending on the excitation beam radiance. Therefore, the emission radiance decreases by a power of four from the focal point and the excitation volume looks similar to the shape of an egg (see Figure 2.1). This abrupt reduction allows for better resolution since the fluorophore is excited only very close to the focal point. This also allows for wide-field detection since all detected light has to come from the focal region.

Confocal fluorescence microscopy is worth mentioning because of its similarity to a two-photon fluorescence microscopy. The details of confocal fluorescence microscopy will not be discussed, but it is important to note that this microscopy is not preferable to two-photon microscopy in many skin imaging applications. This is primarily because it cannot produce adequate fluorescence images of skin at a depth that a two-photon microscope is capable of. This imaging depth deficiency is due to a combination of reasons. The shorter wavelength of the confocal fluorescence microscope’s excitation beam does not allow for as much depth penetration in a highly scattering medium such as skin. In addition, confocal fluorescence microscopy lacks the beneficial non-linear property of two-photon fluorescence microscopy that produces good optical sectioning. The lack of good optical sectioning exhibits poorer resolution at increasing depths in a scattering medium because an abundance of out of focus light scatters back to the detector. Confocal fluorescence microscopes require a pin hole detector in attempt to reject some of the scattering effects of out of focus light.

The lower power required for the excitation laser beam is the primary benefit of confocal fluorescence microscopy when compared to two-photon fluorescence microscopy. Another benefit is the ability to build a confocal fluorescence microscope with cheaper hardware.
Despite these advantages, there are many obvious skin imaging applications that are best fit for a two-photon fluorescence microscope.

The experiments in this thesis sometimes utilize a confocal reflectance microscope to capture skin structure data at a shallow imaging depth. These images are then registered with fluorescence images obtained by two-photon fluorescence microscopy. Confocal reflectance microscopy is the same as confocal fluorescence microscopy except that the detected light is at the same wavelength as the excitation.

**Figure 2.1:** Two-photon excitation volume within Gaussian focus beam [54]
2.2 Quantum Dot Nanoprobes

Experimentation conducted for this thesis utilize quantum dots as fluorescent nanoprobes inside skin. Quantum dots have gained increasing interest in the last couple of decades. They have unique electrical properties that are appealing to many research applications. In particular, they behave as superior fluorophores in biological substances when excited by an excitation laser beam in a two-photon fluorescence microscope [34]. Skin imaging diagnostic applications will likely benefit from quantum dots because quantum dots can be conjugated so that they may bind to various biological materials [40]. One major application of conjugation is the ability to target tissue pathologies such as cancer [22].

A quantum dot is made from a semiconducting material that has such small volume that it becomes physically constrained [18]. The photoluminescence workings of traditional, unconstrained semiconductors will first be discussed before mentioning how quantum dots behave as fluorophores in the experiments conducted for this thesis.

An unconstrained semiconductor notoriously has a small band gap between the valence band and conduction band [50]. The valence band is a low energy band that contains all of the potentially excitable electrons when the temperature is at 0°C. The conduction band is a high energy band that contains the excited electrons which have moved from the valence band. This movement can be achieved by interaction with an external energy that has a magnitude greater than or approximately equal to the band gap energy characterized by the semiconductor. The band gap energy is measured in electron volts, eV, and is roughly 1-2 eV for most semiconductors [6, p. 373]. Quantum dots typically have a band gap energy near the upper boundary of this range, as will be shown later in this section.

In the case of photoluminescence, an electron in a semiconducting material may move from the valence band to the conduction band when a photon having energy greater than or approximately equal to the band gap energy interacts with the material [50]. The energy of a photon is represented by Equation (2.3). Therefore, the energy of the photon increases as the wavelength decreases. It is important to note that thermal energy can also cause an electron to jump from the valence band to the conduction band. Such thermally induced electron movement always occurs in semiconductors under normal conditions (temperatures above 0°C) [50]. However, the number of electrons so excited is negligibly small when compared to electron movement induced by a high powered excitation laser beam, such as the case in a two-photon fluorescence microscope.

An electron-hole pair is created once an electron moves from the valence band to the conduction band of a semiconductor. This excited state occurs for a very short time until the electron moves back to the valence band with no external influence. Energy can be released in the form of a photon as the electron moves back to the valence band. The energy of the emitted photon is usually a little less than the energy of the semiconductor’s band gap due to small energy losses during the photon emission process. This will later be discussed for the case of quantum dots. See Figure 2.2 for a pictorial description of the excitation and emission events of a semiconductor undergoing photoluminescence.

The white circles in Figure 2.2 represent electron holes when the electrons have moved to the conduction band. These holes exhibit a positive charge. The dark circles in Figure 2.2 represent electrons that exhibit a negative charge. Insulating materials have such a high band gap energy that few to no electrons are able to move to the conduction band. In contrast, conducting materials have a small enough band gap energy that electrons are able to move to the conduction band with ease. This ease of movement poses little resistance to current flowing when a voltage potential occurs across the material [50].
Quantum dots behave similarly to traditional, bulk semiconductors except they are so small that the energy of their band gap is dependent on their size as well as the type of semiconducting material of which they are composed [2]. The unique properties arising from such physically constrained materials are described by the term quantum confinement. The first person to describe quantum confinement for semiconductors representing quantum dots was Alexei Ekimov in 1982 ([18]; [19]). For this reason, Alexei Ekimov is commonly credited as the inventor of quantum dots.

The quantum dots used in this thesis have a physically constrained semiconducting material composed of cadmium-selenium (CdSe). These quantum dots were ordered from Ocean NanoTech (catalog number QSH550). A shell made out of zinc-sulfide (ZnS) exists around the perimeter of quantum dot QSH550 in order to enhance photoluminescence [12]. See Appendix A.1 for specifications pertaining to quantum dot QSH550. Carboxylic acid is bound to the surface of the shell of quantum dot QSH550, as mentioned in the specifications. The carboxylic acid coating assists the quantum dots to be biocompatible and water soluble [11]. Additional coatings can optionally be bound to these quantum dots so that quantum dots can attach to various biological substances [5]. Such methods using quantum dots to label biological substances have been avoided in this thesis so that the analysis of the image processing algorithms is generalized and simplified.

The CdSe-ZnS inorganic core diameter has not been reported in the specifications. This diameter can be estimated to be about 3.8 nm according to Dabbousi et al. [12, Figure 2]. The carboxylic acid surface attachment contributes to a hydrodynamic size that can substantially increase the diameter of fully assembled quantum dots. The specifications sheet for QSH550 reports a hydrodynamic size that is about 8 – 10 nm larger than the inorganic core size. Therefore, the quantum dots in this thesis can be estimated to be about 11.8 – 13.8 nm in diameter while in skin.

The emission peak for quantum dot QSH550 is 550 nm +/- 10 nm, as reported in Appendix A.1. Quantum dots are generally known to have a narrow emission peak, such as...
The case of QSH550. The Full Width at Half Maximum (FWHM) of the QSH550 emission peak is less than 35 nm. The narrow emission peak occurs because it is directly related to the band gap energy of the applicable quantum dot [50]. In other words, excitation wavelengths higher than the band gap will induce higher states that will relax to the state with energy closest to the band gap before emission occurs [50]. The experimental results from He et al. have proven that the quantum dot emission wavelength of one-photon excitation is nearly identical to that of two-photon excitation [29].

The peak emission wavelength of QSH550 can be used to approximate the band gap energy by using Equation (2.3). This can be described as

\[ E_g \approx \frac{hc}{\lambda_{QSH550}} = 3.6 \times 10^{-19} \text{ J} = 2.247 \text{ eV} \]  

where \( E_g \) is the approximate band gap energy of quantum dot QSH550 and \( \lambda_{QSH550} \) is the approximate peak emission wavelength. There is some red-shift involved with the emission wavelength when compared to the lowest energy excitation wavelength. The main cause of this red-shift is a common red-shift phenomenon in photoluminescence known as the Stokes shift [50]. The ZnS coating further enhances the red-shift of quantum dots with a CdSe core (see results in Dabbousi et al. [12, Figure 2]).

Absorption cross section is a term commonly used to describe the amount of light being absorbed by a particular fluorophore when exposed by a given wavelength. The two-photon absorption cross-section is commonly measured in Göppert-Mayer (GM) units, where 1 GM = \( 10^{-50} \text{ cm}^4 \cdot \text{s} \cdot \text{photon}^{-1} \). The unit term \( \text{cm}^4 \) results from the product of two areas that correspond to each of the excitation photons. The unit term \( \text{s} \) relates to the time needed for both photons to be absorbed. Finally, the unit term \( \text{photon}^{-1} \) represents the emission photon.

Emission does not necessarily take place even if absorption occurs. Therefore the term two-photon action cross section is used to characterize the amount of light being absorbed and used for emission by a particular fluorophore at a given excitation wavelength. The two-photon action cross section is also commonly measured in GM units. The equation for the two-photon action cross section is simply expressed as the product of the two-photon absorption cross section and the quantum yield of the fluorophore. Quantum yield is described by the equation

\[ \Phi = \frac{n_e}{n_a} \]  

where \( \Phi \) is the quantum yield, \( n_e \) is the number of photons emitted and \( n_a \) is the number of paris of photons absorbed. In terms of photoluminescence, the quantum yield is sometimes referred to as the emission efficiency. The specs for QSH550 report an emission efficiency of > 50% (see Appendix A.1). These quantum dots can be considered to have relatively good quantum yield properties. In comparison, Dabbousi et al. measured various sized CdSe-ZnS quantum dots to not exceed a quantum efficiency of > 50% [12].

Quantum dots generally have a very broad one-photon and two-photon absorption spectra. Appendix A.1 shows the one-photon absorption spectrum of quantum dot QSH550, in arbitrary units. The one-photon absorption wavelengths must be doubled when compared to two-photon excitation (as implied in Section 2.1). The QSH550 specifications show that this type of quantum dot has a well defined absorption peak at 540 nm (approximately equivalent to 1080 nm using two-photon excitation). This peak is known as the quantum-confinement peak.

The experimental results from Larson et al. show a relatively consistent two-photon action cross section of water soluble CdSe-ZnS quantum dots similar to QSH550 [34]. These
results show two-photon action cross section measurements for excitation wavelengths between 700 – 1000 nm. The two-photon action cross section is a little less than 10000 GM at most of these wavelengths, with the exception of wavelengths between 960 nm and 1000 nm. A wavelength of 920 nm was chosen for experimentation conducted for this thesis primarily to avoid the laser excitation beam being overly absorbed by gas and liquid forms of H$_2$O molecules. This is of high concern, of course, because H$_2$O molecules are abundant in skin tissue as well as in humid air. Optical characteristics pertaining to H$_2$O molecules and skin composition will be discussed in Section 2.3.

The characteristic broad excitation spectrum of quantum dots is made possible by the many excitation states that quantum dots typically have [11]. This behavior poses an additional benefit when concurrently taking advantage of the narrow emission wavelength of quantum dots. The broad absorption and narrow emission characteristics together allow different types of quantum dots to simultaneously fluoresce at different emission wavelengths while being excited at only one excitation wavelength. This multiplexing functionality can be very beneficial in biological applications [11].

Quantum dots have been known to have an aggregate photoluminescence that is extremely high when compared to other two-photon fluorophores used in biomedical imaging [34]. This feature arises mostly from the high absorption of quantum dots, rather than their quantum yield. Quantum dots have been shown to have a two-photon action cross-section as high as 47,000 GM [34]. There is currently no other two-photon induced fluorophore brighter than quantum dots [34]. The experiments in this thesis benefit from the high photoluminescence of quantum dots by being able to image deeper in skin tissue. This high photoluminescence also prevents having to increase the excitation laser power to levels that increase the probability of damaging skin cells.

The fluorescence intensity decay curve of quantum dot QSH550 can be approximately characterized by the double exponential equation

$$I = 0.5e^{-t/4 \times 10^{-9}} + 0.5e^{-t/32 \times 10^{-9}}$$

where $I$ is the emission intensity and $t$ is the time in seconds. This equation has been deduced from experimental results of a similar CdSe quantum dot by He et al. [29, Figure 2]. Therefore, the total emission intensity decay time of CdSe is expected to be unobservable after no more than about 40 ns. This time is negligible when compared to the pixel acquisition rate of 325 ns (see Section 2.5). The insignificantly small emission intensity decay time means there is expected to be no blurring effects while imaging adjacent pixels during experiments conducted for this thesis.

One of the most acclaimed benefits of quantum dots is their outstanding resistance to photobleaching. The term photobleaching is commonly used to represent the susceptibility of a fluorophore to lose its ability to fluoresce due to previous exposure by an excitation light source. Photobleaching resistance of quantum dots is especially beneficial to biological applications because traditional fluorophores used in biology have been known to show signs of photobleaching very rapidly. For example, rhodamine 6G photobleaches after only 10 minutes [23]. By comparison, CdSe-ZnS quantum dots have shown to show no signs of photobleaching well over an hour of continuous excitation [23]. The experimentation and analysis for this thesis greatly benefits from a quantum dot’s resistance to photobleaching. This is ultimately because captured images do not vary much depending on how long the sample was exposed to the excitation beam.

Quantum dots have been known to oscillate their emission intensity at a widely varying rate [33]. This blinking characteristic of quantum dots many times poses the most nuisance
when working with these fluorophores. The cause of blinking is not entirely understood at the time of this writing; however, this characteristic can be suppressed [30]. Fortunately, many experimental setups do not cause quantum dots to blink, as the case in this thesis. There was no evidence of a quantum dot blinking in any of the experiments conducted for this thesis. Similar conclusions were found by Larson et al. when investigating photophysical properties of water soluble CdSe-ZnS quantum dots that were nearly identical to quantum dot QSH550 [34].
2.3 Skin

Optical and structural properties of a human skin sample vary based on the skin sample's body location as well as the skin donor's age, sex, race and medical condition. All these factors have contributed to make cutaneous research challenging. Fortunately, basic properties of skin are reasonably well understood at the time of this writing. Properties relevant to this thesis will be discussed below.

The human skin samples used in experimentation for this thesis were obtained from the Greater MetroWest DermSurgeons, LLC. Many thanks are given to this organization for their outstanding commitment to skin research. Samples attained at the time of experimentation likely have come from people with fair skin (skin types one through four). This was determined by visual inspection of the skin's pigmentation. Only skin samples without lesions were utilized. The skin was stored in the refrigerator between the time it was excised and the time it was imaged. This time period was less than 24 hours. Accurate information about skin donors' age, skin donors' sex and skin surface locations cannot be easily deduced and were not disclosed due to privacy concerns. Therefore, this information has not been reported.

The composition of human skin is complex. The outermost layer of skin is usually composed of dead skin cells known as the stratum corneum. These cells are sometimes not present because they can be easily rubbed off. Multiple layers of cells exist underneath the stratum corneum. The layers directly underneath the stratum corneum are the stratum granulosum (granular cells), stratum spinosum (spinous cells) and stratum basale (basal cells), as mentioned in descending order. All these layers of cells (Including the stratum corneum) form the area of skin known as the epidermis. Cells in the stratum corneum, stratum granulosum and stratum spinosum originate from basal cells. Basal cells become flatter and longer as they age and move to different cell layers closer to the skin surface. These cells take about 52-72 days to navigate across the epidermis [10].

Hair follicles and sweat glands commonly exist in the epidermal area of human skin. Glabrous skin found on one's palms and soles does not contain hair follicles and is significantly different than skin in most other areas of the body [10]. This type of skin was not used in any of the experimentation conducted for this thesis. All skin samples were visually inspected to contained vellus hair or otherwise long hair such as hair commonly found on a one's scalp. Vellus hair is fine "peach-fuzz" hair that covers most of the human body.

The area of skin known as the dermis exists underneath the epidermis. The epidermal-dermal boundary has an uneven characteristic that acts as a partial barrier against large molecules while concurrently contributing to mechanical support [10]. Main components in the dermis are collagen, elastin and blood vessels. Underneath the dermis is a layer of subcutaneous fat that is beyond the imaging depth of the two-photon microscope used in experimentation conducted for this thesis.

Results by Rajadhyaksha et al. report the approximate depth and size of many skin cell types of non-glabrous samples of healthy human skin [43]. This publication reports the Stratum Corneum (SC) extending 10 – 30 µm deep, granular cells to be 25 – 35 µm in size at a depth of 15 – 20 µm beneath the SC, spinous cells to be 15 – 25 µm in size at a depth of 20 – 100 µm beneath the SC and a single layer of basal cells that are 7 – 12 µm in size at an average depth of 100 µm beneath the SC. These skin layer characteristics are useful when observing three-dimensional images of human skin, such as those shown in this thesis.

The total hydrodynamic size of quantum dot QSH550 was estimated to be about 11.8 – 13.8 nm while in skin (see Section 2.2). While conducting experimentation for
this thesis, these nanoprobes were observed to be unable to penetrate through the surface of normal, healthy skin without any additional assistance. It was surprising to see such difficulty of penetration despite the hydrodynamic size of the quantum dots being orders of three magnitude smaller than the smallest skin cell. Ultrasound was needed to aid the diffusion process (see Section 2.4 for details). Perhaps the difficulty of skin penetration and diffusion is due to intercellular cutaneous substances. These substances serve as mechanical, biochemical and signaling interactions between cells and partially consist of desmosomes, adherens junctions, gap junctions and tight junctions [10].

Laser light traveling through skin can be analyzed when modeling skin using parameters $g$, $\mu_a$, $\mu_s$ and $\mu_s'$. The anisotropy factor, $g$, represents the average cosine angle of photon scattering. Therefore, $g = 1$ represents only forward scattering, $g = -1$ represents only backwards scattering and $g = 0$ represents isotropic scattering. Skin is well known to behave as a strong forward scatterer. The anisotropy factor for skin can be estimated by the equation

$$g \approx 0.62 + \lambda 0.29 \times 10^{-3} \quad (\lambda \text{ in nm}) \quad (2.10)$$

where $\lambda$ is the wavelength of light in nanometers [61]. Therefore, $g_{920} = 0.8868$ can be used to represent the anisotropy factor of skin when exposed to the excitation beam used in experimentation for this thesis. Similarly, $g_{550} = 0.7795$ can be used to represent the anisotropy factor of the same skin sample when exposed to the emission beam. There is a small variation of anisotropy as the wavelength changes.

The absorption coefficient, $\mu_a$, is the measure of absorption effectiveness of a material. This coefficient has units of cm$^{-1}$. A dimensionless value will result when $\mu_a$ is multiplied by a length that represents the travelling distance of a laser beam in skin. According to Bashkatov et al., the absorption coefficient of human skin can be approximated to be $\mu_a,550 \approx 1.2$ cm$^{-1}$ when exposed to a laser beam having a wavelength of 550 nm, and $\mu_a,920 \approx 0.37$ cm$^{-1}$ when exposed to a laser beam having a wavelength of 920 nm [7]. The substantially higher absorption coefficient at wavelength 550 nm is primarily due to absorption caused by hemoglobin [20, Figure 1]. This is somewhat of an unfortunate occurrence since this absorption peak could have been avoided by using quantum dots that have an emission wavelength of 620 nm (Ocean NanoTech catalog number QSH620). Nonetheless, this potential increase in absorption did not pose a great threat to the experimentation conducted for this thesis because quantum dots were able to be observed deep inside the epidermis. Hemoglobin does not exist in the epidermis. The absence of problems caused by the 550 nm absorption peak might also be contributed by scattering dominating over absorption when skin is exposed to a laser beam having a wavelength approximately between 300 nm and 1000 nm for non-pigmented skin [61]. Therefore, absorption appears not to be the limiting factor for skin samples used in experimentation for this thesis.

As implied above, scattering dominates absorption for skin samples exposed to the two-photon excitation and emission laser beams. Scattering in skin can be parameterized by the scattering coefficient, $\mu_s$, which is the measure of a material’s susceptibility to scatter a photon. Similarly to the absorption coefficient, the scattering coefficient has units of cm$^{-1}$. The reduced scattering coefficient, $\mu'_s$ is frequently used in order to compensate for some of the light that is scattered back. The reduced scattering coefficient is defined by

$$\mu'_s = \mu_s (1 - g) \quad (2.11)$$

where $g$ is the anisotropy factor. According to experiments by Bashkatov et al., the reduced scattering coefficient of skin is approximately 24 cm$^{-1}$ when exposed to laser beam having
a wavelength of 550 nm and approximately 15 cm\(^{-1}\) when exposed to a laser beam having a wavelength of 920 nm [7, Figure 3]. We can therefore define \(\mu_{s,550}^\prime \approx 24 \text{ cm}^{-1}\) and \(\mu_{s,920}^\prime \approx 15 \text{ cm}^{-1}\). Given the reduced scattering coefficient, \(\mu_s\) can be calculated by

\[
\mu_{s,550} = \frac{\mu_{s,550}^\prime}{1 - g_{550}} = 108.84 \text{ cm}^{-1}
\]  
(2.12)

when a skin sample interacts with a laser beam having a wavelength of 550 nm. Likewise, \(\mu_s\) can be calculated by

\[
\mu_{s,920} = \frac{\mu_{s,920}^\prime}{1 - g_{920}} = 132.51 \text{ cm}^{-1}
\]  
(2.13)

when a skin sample interacts with a laser beam having a wavelength of 920 nm.

As a laser beam propagates through skin there is a component of this propagation solely due to absorption. This component of propagation is commonly expressed as Beer’s law. Beer’s law can be shown by the equation

\[
I_c(z, r) = I_L(r) (1 - r_{sp}) e^{-\mu_a z}
\]  
(2.14)

where \(z \text{ [cm}^{-1}\) is the light penetration depth, \(r \text{ [cm}^{-1}\) is the radial distance of the collimated laser beam, \(I_c(z, r) \text{ [watt m}^2\) is a measure of fluence at the given distances, \(I_L(r) \text{ [watt m}^2\) is the profile of the incident laser beam and \(r_{sp}\) is the specular reflectance coefficient. The form of this equation was derived by the equation given by Gemert et al. [61]. Beer’s law poses a good propagation model to follow when \(\mu_a \gg \mu_s\).

All experimentation conducted for this thesis utilizes an excitation laser beam that is positioned perpendicular to the surface of skin. Therefore, the specular reflectance coefficient may be neglected. Equation (2.14) can then be reduced to

\[
I_c(z, r) = I_L(r) e^{-\mu_a z}
\]  
(2.15)

The scattering and absorption coefficients discussed earlier imply that \(\mu_s \gg \mu_a\) for a skin sample when the skin sample is exposed to a laser beam having a wavelength of 550 nm or 920 nm. Therefore, Beer’s law alone cannot adequately model laser beam propagation inside a skin sample. An equation similar to Equation (2.15) can be used to approximate the intensity of laser light that is focused inside skin. This equation is represented by

\[
I_c(z, r) = I_L(r) e^{-\mu_t z}
\]  
(2.16)

where \(\mu_t\) is considered to be the total attenuation coefficient and is defined as \(\mu_t = \mu_a + \mu_s\). All other parameters are the same as in Equation (2.15). Equation (2.16) does not compensate for light that is scattered back. However, light that is scattered back into the focus volume is minimal since the focal volume is so small.
The refractive index of human skin is a very important parameter to consider because artifacts can occur when imaging with an objective lens that is affected by boundaries having a mismatch of the index of refraction. Any high numerical aperture lens will cause spherical aberrations if the lens’ immersion medium has an index of refraction that is significantly different from skin (spherical aberrations will be further discussed in Section 2.5.) Experimentation conducted by Ding et al. estimate the epidermis’ index of refraction to be 1.46 when the incident laser beam has a wavelength of 550 nm, and 1.43 when that wavelength is 920 nm. Similarly, these experiments estimated the dermis’ index of refraction to be 1.39 when the incident laser beam has a wavelength of 550 nm, and 1.38 when that wavelength is 920 nm [16].

Both the excitation and emission laser beams penetrate through a significant amount of aggregated $H_2O$ molecules that can be in the form of gas or liquid. These aggregations of molecules can interact with the laser beams as the beams pass through skin and humid air. $H_2O$ molecules in the form of liquid are abundantly present inside skin. The pure water immersion medium is another area where the laser beams can interact with an aggregation of $H_2O$ molecules in liquid form. Humid air can be present in the microscope’s operating environment. Humid air is an aggregation of $H_2O$ molecules taking the form of gas.

It is for the above reasons that the optical characteristics of an aggregation of $H_2O$ molecules should be reasonably understood for when this aggregation takes the form of a gas (water vapor) or liquid (water). Experimentation from Hale et al. reported the absorption of water to steadily increase from about 475 nm to 3 $\mu$m [27]. The data from this publication reports an extinction coefficient of $1.96 \times 10^{-9}$ at a wavelength of 550 nm and $1.06 \times 10^{-6}$ at a wavelength of 925 nm. The extinction coefficient rapidly rises soon after 925 nm before reaching a peak of 0.135 at 3.15 $\mu$m. Similarly to the absorption coefficient, the extinction coefficient is a measure of a medium’s susceptibility to absorb light. Both these coefficients increase as the magnitude of absorption increases.

Analysis of the data obtained by Hale et al. suggests that a laser beam having a wavelength of 550 nm is not affected by water absorption so much, but a laser beam having a wavelength of 920 nm might be significantly impacted. It is obvious that water does not significantly absorb light with a wavelength of 550 nm because all wavelengths in the visual spectrum of about 380 nm to 750 nm can penetrate through water easily. Problems due to light scattering in water are of little concern. This is because pure water has very little scattering [68].

The optical characteristics of water vapor are significantly different than water in liquid form. There is a narrow, but strong absorption peak at 940 nm [26]. This peak must be avoided for fear of the excitation laser beam being substantially attenuated inside the laser cavity. The magnitude of this peak can vary drastically depending what time of year [31, Figure 1]. The remaining optical properties of water vapor do not appear to be of great concern. Atmospheric scattering properties of air containing water vapor can be seen in the results of Tomasi et al. [59, Table 4]. The scattering peaks near the wavelengths of interest are much less in magnitude when compared to the absorption peaks of water vapor.
A beam of laser light usually penetrates deeper in skin as the wavelength of the incident beam increases [3]. Therefore, choosing an excitation beam wavelength of 920 nm makes sense because it is high enough to penetrate skin significantly, yet low enough to avoid a steep rise in absorption that occurs at slightly higher wavelengths. The absence of significant excitation or emission beam attenuation during the experimentation further supports this reasoning.

Two-photon fluorescence emitted from human skin was not seen to cause any imaging problems. As discussed in Section 2.2, the nanoprobes used have one of the highest two-photon action cross sections of any fluorophores. This creates a large contrast when compared to the relatively weak fluorescence caused by the skin sample.

Experimentation by Masters et al. reported a skin emission peak at 520 nm when excited by a laser beam having a wavelength of 960 nm [39]. These wavelengths are similar to the excitation and emission beam wavelengths used in experimentation for this thesis. The emission peak at 520 nm can be concluded to be relatively small for the skin samples used in experimentation because no substantial background fluorescence was detected while collecting two-photon images.
2.4 Transdermal Nanoprobe Delivery

Delivering nanoprobes into skin can be challenging; however, transdermal nanoprobe delivery had been achieved while conducting research for this thesis. Quantum dots QSH550 were utilized as nanoprobes (see Section 2.2 for details about quantum dot QSH550). This section discusses some of the research involved when trying to find an adequate method to assist quantum dots QSH550 into skin. The preferred method to deliver these nanoprobes into skin will be discussed in detail later in this section.

Adequate penetration of quantum dots can be considered to correspond with penetration depths greater than 20 µm from the surface of a skin sample. As implied in Section 2.3, 20 µm is the average depth of the layer of dead skin cells known as the stratum corneum. Therefore, imaging at depths less than 20 µm can be less useful to researchers because it is well known that most skin diseases originate in areas of live skin cells.

Chemical enhancers were used in some of the first attempts to assist quantum dots QSH550 into skin. Methods involving chemical enhancers were not chosen for the experimentation discussed in Chapter 3, but an overview of these methods will be mentioned below for the purpose of communicating the research that was performed.

The use of chemical enhancers was appealing because of their low cost and proven use to assist some medications into skin [9]. Oleic acid, Lauric acid, Dimethyl Sulfoxide (DMSO) and Polyvinylpyrrolidone (PVP) were used. Oleic acid and lauric acid are fatty acids that can be mixed with water while in liquid form. DMSO and PVP are water-soluble compounds. Chemical mechanisms involved with aiding penetration of molecules into skin will not be discussed. See Chapter 2 of Percutaneous penetration enhancers for additional information on this subject [51].

Adequate transdermal delivery of quantum dots QSH550 was not observed when using the chemical enhancers previously mentioned. This surprising conclusion might be due to the many environmental and skin differences that make it difficult to determine optimal conditions. Perhaps adequate transdermal delivery of quantum dots QSH550 can be achieved if optimal conditions are found. The absence of quantum dots deeper that 20 µm in skin was verified by using two-photon microscopy. Occasionally, some nanoprobes were seen to penetrate into a hair follicle shaft; however, this data could not be used because those nanoprobes were not seen to show evidence of migrating into the skin.

Additional procedures involved with increasing temperature, increasing penetration time, changing quantum dot concentration and tape stripping were combined with and without the use of the chemical enhancers mentioned above. Temperature was controlled by using a humidity controlled incubator set at 39°C. Variations in penetration time typically ranged from 30 minutes to 36 hours. Quantum dot concentration were varied from approximately 0.2 mol·µL\(^{-1}\) to 8 mol·µL\(^{-1}\). Cellophane tape was sometimes lifted off the skin surface about 20 times to reduce the thickness of the stratum corneum. Alas, despite all these efforts, inadequate transdermal delivery was concluded.

Little or no quantum dot penetration was observed by another research group as well. Zhang et al. reported most of the quantum dots primarily remained in the stratum corneum layer of porcine skin after many hours in a flow-through diffusion cell [67]. The experimentation conducted by Zhang et al. did not involve chemical enhancers.

However, a couple of research groups have reported quantum dots being delivered into skin by means of chemical enhancers or by little or no assistance. Results by Lo et al. suggest quantum dots can penetrate into skin slightly past 20 µm with the assistance of oleic acid [37]. Ryman-Rasmussen et al. concluded that different types of quantum dots in
concentrations between 2 mol $\cdot \mu$L$^{-1}$ and 8.7 mol $\cdot \mu$L$^{-1}$ can penetrate through the entire epidermis of porcine skin after many hours [45].

Similar outcomes did not occur when trying to replicate Lo et al.’s and Ryman-Rasmussen et al.’s experiments after several attempts. The experimentation conducted by Lo et al. involved using mouse skin. The difference in skin might explain why the outcome of this experiment could not be replicated. The experiments conducted by Ryman-Rasmussen et al. could not be exactly repeated because a flow-through diffusion cell was required and could not be easily obtained. No penetration was observed after conducting similar experiments to Ryman-Rasmussen et al.’s when using porcine and human skin. An incubator was used for some experiments that had execution times up to 36 hours. The means by which quantum dots penetrate skin with chemical enhancers or by themselves appear to be inconclusive at the time of this writing.

Procedures involving sonophoresis were attempted to assist penetration of quantum dots inside skin after procedures involving chemical enhancers were abandoned. Much deeper penetration of quantum dots QSH550 was accomplished when applying sonophoresis. Sonophoresis is a method that helps move molecules into skin by means of ultrasound. This method was utilized in the experimentation explained in Chapter 3 because it has been observed to assist quantum dots QSH550 into skin at far greater depths than 20 µm.

Two characteristics of ultrasound that may contribute to transdermal delivery are heat and transient cavitation. Heat has been long known to enhance percutaneous penetration of molecules [8]; however, as previously mentioned, the use of heat when utilizing an incubator set at 39°C did not appear to significantly contribute to penetration of quantum dots QSH550. In comparison, the heat from ultrasound during sonophoresis experiments was measured to be at most 40.9°C. This measurement is discussed later in this section when the ultrasound was applied for only three minutes. Therefore, it would seem that heat is not likely to be the dominant cause for transdermal delivery of quantum dots QSH550. Transient cavitation represents the occurrence of bubbles in fluids that rapidly expand and contract. These bubbles occur as negative pressure builds up during rarefaction of an ultrasound wave. Negative pressure causes bubbles to form by allowing dissolved gasses to undissolve. The book titled Diagnostic Ultrasound: Principles and Instruments by Kremkau is helpful to understand the basics of ultrasound and ultrasound applications to biological substances [32].

Low Frequency Sonophoresis (LFS) utilizes ultrasound in the frequency range of 20 kHz to 100 kHz [36]. Cavitation is known to be more prevalent in lower ultrasound frequencies such as those used for LFS. This is because the dissolved gasses in liquid do not have enough time to become undissolved as the rarefaction time decreases at higher frequencies. The conductivity of skin has been observed to increase significantly when exposed to these lower ultrasound frequencies [57]. Hence, an increase in skin conductivity is directly correlated to the ability for molecules to penetrate skin. Results by Tezel et al. have suggested cavitation to be the primary mechanism involved with decreasing skin barrier function [56].

Cavitation bubbles collapsing near the skin surface have been theorized to create shock waves that disturb the lipid bilayers existing around some skin cells [56]. It seems reasonable to assume that all intercellular substances can be affected by these shockwaves, such as the ones mentioned in Section 2.3. A powerful microjet extending with a length approximately the length of the bubble can extend away from a collapsing bubble [35]. The microjet usually extends toward a nearby solid surface and creates shock waves of its own. Therefore, microjets extending from cavitation bubbles can also contribute to transdermal nanoprobe delivery.
Whether or not cavitation occurs inside skin appears to be inconclusive at the time of this writing. An article by Tezel et al. has suggested that cavitation is unlikely to occur inside skin because the cavitation bubble size is relatively too large [56]. An estimation of a cavitation bubble radius is approximately between 10 $\mu$m and 100 $\mu$m at frequencies used during LFS. Cavitation can possibly act destructively if it were to occur inside the body. In any case, many drugs have been known to be able to penetrate skin by means of sonophoresis [52].

The areas in which molecules penetrate into skin during sonophoresis are commonly called Localized Transport Regions (LTR). The size of these regions can vary a bit, but can roughly be 1 mm in diameter on the skin surface [58]. The LTR observed in experimentation using the procedure mentioned in Chapter 3 have been observed to have a diameter a little less than 1 mm.

A paper by Paliwal et al. reported experimental results that suggest quantum dots can be assisted into skin by means of LFS [42]. Experimentation conducted for that paper utilized a quantum dot with a diameter of 20 nm. This size can be assumed to correspond with the hydrodynamic size, which is slightly smaller than that of quantum dots QSH550 (see Section 2.2). Additional information about the type of quantum dot used in experimentation by Paliwal et al. cannot be determined. This research group cited using a quantum dot with a catalog number from Quantum Dot Corporation; however, this company no longer exists.

Paliwal et al. used a transducer that produces an ultrasound frequency of 20 kHz. This instrument was placed about 4 mm from the surface of a porcine skin sample in a Franz diffusion cell. The ultrasound transducer was set to a 50% duty cycle with a period of 10 seconds. The total experimentation time was three minutes. Paliwal et al. have concluded that the quantum dots penetrated as deep as 60 $\mu$m and primarily resided in intercellular lacunar regions.

An industrial grade low frequency ultrasonic transducer such as the one used by Paliwal et al. could not be easily acquired when conducting experimentation for this thesis. Therefore, a low cost solution was needed. Ultrasonic piezoelectric tooth scalers commonly used by dentists utilize low frequency ultrasound transducers that produce a continuous ultrasound wave having a frequency of about 28 kHz. This frequency is within the range of frequencies that can be used for LFS and is relatively close to the 20 kHz frequency used by Paliwal et al. These transducers have been obviously designed so that their tip can be submerged into liquid and can come in contact with biological substances. Dentists use these scalers to clean the surface of the tooth that is internal to the patient’s body. This is accomplished by touching the outer side of the transducer tip to the tooth.

A new scaler was acquired from eBay at a reasonable cost. The brand of this scaler is DTE. The model number is D1. Specifications for this ultrasound transducer report that this device produces a continuous ultrasound wave having a frequency of 28 kHz $\pm$ 3 kHz. Additional technical specifications for DTE scaler D1 can be found in Appendix A.2.

Different scaler tips can be attached to the DTE ultrasonic scaler. The scaler tip having model number G2 was used. This tip was chosen because it appears to produce more cavitation bubbles than any of the other scaler tips packaged with the instrument. Individual cavitation bubbles are too small to be seen with the naked eye; however, aggregations of these bubbles can be seen. These aggregations look like a white haze when the scaler tip is submerged in water. A picture of what these cavitation bubbles look like can be found in a paper by Felver et al. [21].

Figure 2.3 shows a photograph of scaler tip GD2. The transient cavitation bubbles that
interact with the skin sample stream from two locations perpendicular to the outer side of the tip. These streams are labeled as streams A and B, as indicated in Figure 2.3. Unlabeled cavitation streams are represented by white arrows pointing away from the inner side of the transducer tip. Nearly all the cavitation produced by this transducer is transient. Sometimes a few stable cavitation bubbles meander in the water as the transducer is operating. Unlike transient bubbles, stable bubbles do not rapidly expand and collapse. The effects of stable bubbles is considered to be negligible since very few are produced and they do not appear to disturb anything.

Experimentation was performed to investigate the operation of the ultrasound transducer. A tank was filled with tap water so that underwater acoustical pressure measurements can be made. The volume of water had dimensions $25.4 \times 20.3 \times 19.1$ cm. A hydrophone (brand: Brüel & Kjær, model: 8103) was submerged 3.2 cm underneath the surface of the water and positioned so that its tip was pointing towards the short side of the tank. The side of the hydrophone was 7.6 cm away from the long side of the tank and the tip was 24.1 cm away from the short side of the tank. The temperature of the water inside the tank was 19.5°C. The hydrophone was sitting in the water for at least 10 minutes prior to experimentation so that accurate measurements could be acquired.

The hydrophone was connected to a conditioning amplifier (brand: Brüel & Kjær, model: 2692). This amplifier was configured with the following settings: low frequency cutoff (amplifier setup menu): 10 Hz, high frequency cutoff (amplifier setup menu): 100 kHz, output sensitivity (amplifier setup menu): $31.6 \mu V \cdot Pa^{-1}$, transducer sensitivity (transducer setup menu): $0.096 pC \cdot Pa^{-1}$, input float (floating / correction menu): No, out float (floating / correction menu): No, application correction (floating / correction menu): 0.8414. Some of these settings can be derived from the hydrophone’s specification and calibration sheets. The application correction value of 0.8414 was chosen to compensate for the approximate signal loss of 1.5 dB, according to the calibration graph at 28 kHz.

The output of the conditioning amplifier was connected to an oscilloscope. Acoustical measurements were taken at varying distances from two locations on the scaler tip.
These locations are represented as stream A and stream B because they correspond to the two streams of cavitation bubbles shown in Figure 2.3. Streams A & B were positioned to intersect with the side of the hydrophone because the hydrophone has less frequency dependence in this direction. The power setting on the ultrasound transducer was set to maximum. During sonophoresis these streams were directed towards the surface of the skin sample (see Chapter 3).

Figure 2.4 shows the resulting sound pressure measurements taken at varying distances from the start of streams A and B. Voltage amplitude measurements observed from the oscilloscope were converted to sound pressure measurements (Pascals) by dividing the voltage by the conditioning amplifier’s output sensitivity setting of $31.6 \, \mu V \cdot Pa^{-1}$. Acoustical reflections inside the tank were causing some difficulty when reading the oscilloscope, but average amplitude values were able to be approximated.

![Graph showing sound pressure measurements](image-url)

**Figure 2.4:** Ultrasound water pressure measurements

The results in Figure 2.4 suggest that the ultrasound intensity could be largest in the area of stream B at distances closest to the surface of the scaler tip. The maximum pressure amplitude reading of approximately 31 kPa is relatively small when compared to the pressure of diagnostic ultrasound. Diagnostic ultrasound has been known to exceed pressures of 1 MPa [66, p. 6]. The ultrasound water pressure measurements observed closest to the scaler tip are towards the bottom end of the range specified in the machine’s specifications (see Appendix A.2). The results in the table further suggest that the sound pressure was
approximately the same for both streams at a distance of 10 mm.

Additional information about the ultrasound was observed while measuring the ultrasound pressure. An oscilloscope was used to validate the ultrasound frequency of 28 kHz. The ultrasonic waveform was also validated to be continuous as the specifications reported. Although the ultrasound experimentation in the tank of water is noteworthy, the information gathered from these tests cannot accurately represent sonophoresis conditions mentioned in Chapter 3. The volume of water used during sonophoresis is so small that measuring the acoustical pressure would be nontrivial and unrelated to the topic of this thesis.

Quantum dot nanoprobes QSH550 are manufactured so that they can be dissolved in water (see Appendix A.1 for quantum dot specifications). The sonophoresis procedure discussed in Chapter 3 mentions that deionized water is added to the factory shipped quantum dot solution to make a total volume of approximately 700 µL. A custom made sonophoresis assembly was developed to accommodate this very small volume of quantum dot solution. Figure 2.5 shows this assembly is made of a base and a chamber. The base and lower part of the chamber are made from a plastic vial (brand: Fischer Scientific, cat. No.: 0566957). The bottom of this tube was cut off so that the top portion of a test tube (manufacturer: Fisher Scientific, model: 05-408-23C) can be inserted over the plastic vial. Test tube 05-408-23C was cut at approximately 20 mm from the top. The test tube and plastic vial were then adhered together by using Duco Cement. Together, the test tube and plastic vial form the chamber. The total length of the chamber was measured to be 40 mm. The diameter of the plastic vial is 12 mm.

![Figure 2.5: Sonophoresis assembly.](image)

The base of the sonophoresis assembly was previously the screw-on top of the plastic vial. The hollow portion of the base was filled with LOCTITE marine epoxy. Two small pins were positioned parallel to each other and then inserted into the epoxy so that the pointed sides extrude 7 mm from the surface of the epoxy. Once the epoxy dried these pins were bent to form an angle of approximately 45°, as shown in Figure 2.5. These pins were designed to hold the skin sample in place during sonophoresis. During experimentation the base remains screwed into the bottom of the chamber as the ultrasonic scaler tip is inserted into the top end of chamber. The cap attached to the top portion of the chamber is beneficial to keep the nanoprobe solution inside the chamber while unscrewing the base to remove or insert a skin sample.
The temperature of the 700 μL of nanoprobe solution is expected to increase as ultrasound is applied. This is a significant concern because skin cells can be damaged if the temperature increases to temperatures greater or equal to 44°C [41]. The procedure discussed in Chapter 3 involves applying ultrasound to the nanoprobe solution for three minutes at a 50% duty cycle with a period of 10 seconds. Therefore, a heat experiment was conducted to measure how much this ultrasound exposure can change the temperature of the 700 μL of nanoprobe solution. This temperature measurement gives a worst case skin surface temperature approximation. It is considered to be worst case because the experiment was performed without the skin in the sonophoresis assembly. The temperature measurement of the ultrasound conduction medium would likely decrease if the heat experiment was performed with the skin present because the limited amount of thermal energy produced by ultrasound will be diffused over a larger area. A skin sample was omitted from the heat experiment primarily because variations of skin sample sizes can induce significant uncertainty when approximating skin surface temperature of different sonophoresis experiments.

Deionized water having a volume of 700 μL was inserted into the chamber of the sonophoresis assembly prior to conducting the ultrasound heat experiment. The sonophoresis assembly was then clamped onto a mounting assembly as shown in Figure 2.6. The ultrasound transducer was placed into the chamber in a fashion similar to the sonophoresis experimentation described in Chapter 3. The ultrasound transducer was set to the maximum setting and turned on for three minutes at a 50% duty cycle with a period of 10 seconds, similarly to the procedure discussed in Chapter 3.

![Figure 2.6: Sonophoresis assembly mounted with ultrasound transducer attached.](image)

The water inside the chamber of the sonophoresis assembly has a tendency to sometimes spill as the ultrasound transducer is operating. Nearly all the heated water remained contained for the duration of the experimentation; however, one or two drips managed to leak out the top of the chamber. Therefore, there is some margin of error.

The chamber of the sonophoresis assembly is too small to allow an available thermometer to be inserted. Therefore, the heated water was added to a vial containing a thermometer and 4.3 mL of deionized water. This water was measured to have an initial temperature of 19.5°C. The measured rise in temperature was observed to be 3°C after the heated water was added. In comparison, the measured temperature change was 2.5°C after the heat experiment was repeated. The rise in temperature of 3°C was taken for subsequent calculations in order to measure a worse case scenario temperature increase.
The change in temperature of the 700 $\mu$L of water in the sonophoresis assembly can be determined by using the equation

$$(\Delta T_1)(m_1) = (\Delta T_2)(m_2) \quad (2.17)$$

where $\Delta T_1$ is the change in temperature of the combined heated and non-heated water, $m_1$ is the mass of that combined water, $\Delta T_2$ is the change in temperature of the 700 $\mu$L of ultrasound heated water, and $m_2$ is the mass of that heated water. The specific heat of water is approximately $0.9982$ g · mL$^{-1}$ for the temperatures observed in this experimentation. Therefore, $m_1$ can be calculated to be $m_1 = (0.9982$ g · mL$^{-1}$) (5 mL) = 4.991 g. Similarly, $m_2$ can be calculated to be $m_2 = (0.9982$ g · mL$^{-1}$) (0.7 mL) = 0.69874 g. The term $\Delta T_1$ is already known to be 3°C. Therefore, $\Delta T_2$ can be calculated to be 21.43°C by substituting all applicable values into Equation (2.17).

The 700 $\mu$L of water in the sonophoresis assembly was at 19.5°C prior to being heated by ultrasound. Therefore, this water can be estimated to have been at 40.93°C after applying ultrasound. This value was calculated by adding the initial temperature of 19.5°C to $\Delta T_2$. Fortunately, this temperature is significantly lower than a potentially destructive threshold temperature of 44°C. Experimentation conducted by Moritz et al. implies skin is likely not to be burned when the surface skin temperature is below 44°C for an extended period of time [41].

Unfortunately, it is unknown how low frequency ultrasound affects quantum dots. It can be speculated that the abrupt cavitation effects of low frequency ultrasound might alter the structure of quantum dots. Experimentation conducted for this thesis implies that low frequency ultrasound likely does not significantly decrease the likelihood of quantum dot QSH550 to perform emission. This assumption can be made because solutions of quantum dots have been successfully reused in some sonophoresis experiments with no noticeable absence of fluorescence. However, it is unknown how the carboxylic acid surface coating was affected. The effect of low frequency ultrasound on quantum dot QSH550 needs more investigation.
2.5 Keck 3D Fusion Microscope

The Keck 3D Fusion Microscope was utilized in experimentation conducted for this thesis. The Optical Science Laboratory at Northeastern University designed and built this microscope so it can be custom configured. This microscope is a useful tool because it has many different microscope modalities that can be used without repositioning the sample. Two-Photon Fluorescence Microscopy (TPFM) was the primary modality used for experimentation. Bright Field Microscopy (BFM) and Confocal Reflectance Microscopy (CRM) are two modalities that were used to extract accessory imaging data. The TPFM modality will be primarily mentioned in this section because image processing techniques discussed in a subsequent chapter are designed to be applied to images acquired by a two-photon fluorescence microscope. Supplementary information about the Keck microscope can be found in Dan Townsend’s thesis [60] and an article written by Warger et al. [65].

The two-photon excitation beam path in the Keck microscope originates from a titanium-sapphire laser (brand: Spectra Physics, type: Tsunami Titanium-Sapphire). This produces a pulsed laser beam having a pulse width of 100 fs and a pulse rate of 80 MHz. The laser beam coming out of the laser passes through two-polarizers that are positioned back-to-back to control the laser power. A periscope is the next major assembly that the beam passes through. An optical power meter (brand: Newport, model: 1830-C) is temporarily placed directly after the periscope when setting up experiments. The power measured at this location can be identified as \( P_{\text{setup}} \). The excitation beam travels to a rotating polygon mirror after it leaves the periscope. The 36 facets on the mirror rotate at a speed of approximately 7.5 ms per revolution. The purpose of this mirror is to scan the beam in the X-axis. Next, the beam travels through a 3X telescope before reflecting off a galvometric mirror. The galvometric mirror incrementally tilts the excitation beam in the Y-axis each time the beam progresses through a complete X-axis scan. The time it takes to complete a scan in the X-Y plane is approximately 100 ms [60].

The excitation beam travels through another 3X telescope after exiting the galvometric mirror. Next, the excitation beam reflects from a hot mirror that is located inside a Nikon TE 2000U microscope assembly. The beam then travels up through an objective that focuses the beam into a sample that is faced down. Thus, the Keck is an inverted microscope. See Chapter 3 for details about the objective used during experimentation.

The 550 nm emission light produced by the nanoprobes inside the skin sample scatters back down to the objective in the opposite direction of the excitation laser beam. The newly formed emission laser beam then passes through the hot mirror because it is green light. After the hot mirror the beam travels through a green filter that passes wavelengths between 485 nm and 565 nm. Next, the beam passes through a tube lens and reflects from a mirror that passes the laser beam into the Photomultiplier Tube (PMT) (brand: Hamamatsu, model: HC124-02). Details about the laser beams inside the Keck Microscope can be seen in Figure 3.3 of Dan Townsend’s thesis [60]. The broadband quarter wave plate and confocal beam splitter were removed when using the two-photon modality because they are not needed. These unneeded components cause significant laser power loss.

The objective used in experimentation conducted for this thesis has a high numerical aperture. All objectives with a high numerical aperture can create significant spherical aberrations. It is important to assess the effects of spherical aberrations that can affect the outcome of image processing approach discussed in Chapter 5. Before discussing the cause of spherical aberrations it is important to review how light refracts.

A boundary between two different materials can cause light to bend. Bending occurs
when one side of the boundary has a different index of refraction value than the other side of the boundary. Furthermore, the incident angle of the light ray must not be zero for refraction to occur. This bending of light is described by Snell’s law:

$$n_1 \sin \theta_1 = n_2 \sin \theta_2$$  \hspace{1cm} (2.18)

where $n_1$ is the index of refraction of the first medium, $\theta_1$ is the angle of incidence of a ray of light in the first medium, $n_2$ is the index of refraction of the second medium and $\theta_2$ is the angle of incidence in the second medium. In the case of a microscope objective having a high numerical aperture, the rays in the peripheral area of the laser beam refract more than those in the area of the laser beam that is perpendicular to the sample. This is caused by the physical contour of the objective’s lens. This contour allows the laser beam to become focused by directing the peripheral area of the beam at a slightly different incident angle than the light at the center of the beam. The light at the center of the beam usually has an angle of incidence that is normal to the sample, as is the case of the Keck microscope. Optical aberrations caused by this bending of light are called spherical aberrations. These aberrations cause the focal point to stretch in both of the axial directions.

The glass coverslip (brand: Corning, cat. No. 2940-225) used for experimentation has an index of refraction of 1.523. All commonly used immersion media have indices of refraction less than the refractive index of the glass coverslip. For example, air typically has an index of refraction of 1.0 and pure water has an index of refraction of approximately 1.33. The first boundary that can cause spherical aberrations is between the immersion medium and coverslip glass. Using Equation (2.18), it can be concluded that the excitation laser beam bends towards normal incidence at this boundary since $n_2 > n_1$. This can cause the focal point to stretch towards the objective since the peripheral area of the laser beam will cause the light to be focused more in that direction. The angle of incidence of the peripheral rays of the excitation laser beam can be found by using the numerical aperture equation:

$$NA = n \sin \theta$$  \hspace{1cm} (2.19)

where $n$ is the index of refraction of the medium and $\theta$ is the angle of incidence of the laser beam.

Another boundary that can cause spherical aberrations is the boundary between the coverslip and the skin sample. As mentioned in Section 2.3, the index of refraction of the epidermis is approximately 1.46. Fortunately, this boundary helps to correct the spherical aberrations caused by the first boundary since $n_2 < n_1$. Many objectives have special optics to correct for issues that can be caused by coverslips, but this is not always true. See Chapter 3 for details on what objective and immersion medium were used during experimentation.

The epidermis-dermis junction poses a boundary to consider that is inside skin. Section 2.3 mentions that the index of refraction of the dermis is approximately 1.38. The epidermis-dermis junction has a significant difference in values for the index of refraction. This difference will cause the light beam to bend away from normal incidence and therefore stretch the focal point away from the objective. Therefore, imaging inside the dermis can be compromised; however, most of the experimentation imaging took place in the epidermis (see Section 3). In reality, differences in skin components inside the epidermis and dermis cause additional variations to the index of refraction as the excitation beam penetrates. The numerous occurrences of these differences are mainly responsible for the high scattering coefficient of skin.
Effects of spherical aberrations can be reduced by using an image processing algorithm discussed in Chapter 5. There is no need to assess spherical aberrations relating to the emission light because these aberrations are not seen. This is due to the optical sectioning and wide field detection benefits present in two-photon microscopy. See Section 2.1 for a review of two-photon microscopy.

The software used to capture images from the Keck microscope was version 7 of Joe Kerimo’s Keck software. This software was written in C# and is based on the original code mentioned in Dan Townsend’s thesis [60]. A National Instruments framegrabber card (model: IMAQ PCI-1408) is used to detect the output voltage coming from the PMT and make the reading available to the Keck’s software. The framegrabber was configured so that a black pixel is recorded at 0.06 V and a white pixel is recorded at 1.26 V. Each pixel value acquired by the framegrabber is 8-bits. A black pixel relates to an 8-bit numerical value of zero and a white pixel relates to an 8-bit numerical value of 255. Furthermore, the framegrabber was set up so that there is a linear mapping between the voltage input and pixel value. The sample time period for each pixel is 325 ns (see Equation (4.4.13) in Dan Townsend’s thesis [60]). Each two-dimensional frame captured by the framegrabber is saved as an 8-bit TIFF image file having pixel dimensions 640 by 480. The Keck software saves one TIFF image file for each imaging depth inside skin. The imaging depth is configurable by the Keck software and is automatically controlled by a piezo-electric-crystal driven z-stage that is attached to the Nikon TE 2000U microscope assembly.
CHAPTER III

EXPERIMENTATION AND ANALYSIS

Details about imaging experimentation are presented in this chapter. Analysis of the resulting imaging data is also presented.

3.1 Transdermal Nanoprobe Delivery Experimentation

Experimentation was performed to achieve transdermal quantum dot nanoprobe delivery by means of sonophoresis. Subsequently, the two-photon microscopy modality of the Keck microscope was utilized to produce three-dimensional z-stacks depicting quantum dot nanoprobes inside human skin. Application of the EM algorithm to the resulting imaging data is discussed in Section 5.3.

3.1.1 Skin Preparation and Imaging Procedures

The skin preparation and imaging procedures are discussed below. The success rate of achieving transdermal nanoprobe delivery deeper than 20 \( \mu \text{m} \) is estimated to be greater than 50\% after following the procedural steps. This estimate is based on experiences. There is reasonable uncertainty to this estimate due to the small amount of times these procedures have been performed.

Skin Preparation Procedure:

1. Cut skin sample to a size of approximately 5 mm \( \times \) 5 mm.

2. Rinse skin sample in distilled water to remove any contaminants.

3. Mount skin sample on the pins that are located on the base of the sonophoresis assembly. Make sure the pins are inserted through the subcutaneous fat instead of through the dermis and epidermis. See Figure 2.5 for a picture of the base and chamber components of the sonophoresis assembly.

4. Screw the base of the sonophoresis assembly into the chamber.

5. Mount the sonophoresis assembly so that it is sturdy while applying pressure on the top. Make sure the opening of the assembly is facing upwards. See Figure 2.6 for a picture of the assembly in a mounted position.

6. Insert 680 \( \mu \text{L} \) of deionized water into the sonophoresis assembly. Mix 20 \( \mu \text{L} \) of quantum dot solution QSH550 with the deionized water.

7. Attach tip GD2 to the ultrasonic piezoelectric tooth scaler (see Section 2.4 for additional details regarding to the tooth scaler). Insert scaler tip into sonophoresis assembly such that the outer side is facing towards the skin. Note: It is recommended to place rubber around the plastic part of the scaler that touches the top of the
sonophoresis assembly. The rubber prevents liquid from escaping during sonophoresis. Any amount of escaping liquid will reduce the likeliness of successful penetration of quantum dots. Experimentation conducted for this thesis utilized latex rubber from ultrasound sheath covers.

8. Set the scaler to maximum intensity. Sonicate for three minutes at 50% duty cycle (5 seconds on / 5 seconds off). Avoid allowing the scaler tip to touch the skin while the scaler is on.

9. Remove the skin from the sonophoresis assembly after sonication is complete. Gently rinse in distilled water for about 30 seconds. This rinsing will help remove extraneous quantum dots that are attached to surface of the skin.

10. Place skin on the coverslip face-down (see Section 2.5 for details about the coverslip). Next, place the coverslip on the metal coverslip holder that is used with the Keck microscope.

11. Skin preparation procedure is now complete.

Skin Imaging Procedure:

Note: Additional information about the Keck microscope’s hardware and laser beam paths can be found in Section 2.5.

1. Power on all the relevant Keck microscope equipment except the PMT.

2. Set the Tsunami laser to produce a laser beam having a wavelength of 920 nm. The laser should be mode-locked so that the laser light is pulsed.

3. Adjust the polarizer next to the Tsunami laser until the laser power is detected to be 190 mW after the periscope. Therefore, $P_{\text{setup}} = 190$ mW ($P_{\text{setup}}$ is defined in Section 2.5).

4. Remove the confocal beam splitter and broadband quarter wave plate from the path of the excitation laser beam.

5. Affix the 20x Nikon objective to the Keck microscope (objective parameters: Immersion Medium: Air, Numerical Aperture: 0.75, Working Distance: 1.0 mm, Coverslip Correction: 170 µm).

6. Set the green filter (filter position 2) to be the filter used for the emission light to pass through.

7. Set the light path selection dial on the front of the Nikon TE 2000U microscope assembly to position 2. This position directs the emission light towards the PMT.

8. Place the coverslip assembly with the skin sample on the piezoelectric microscope stage.

9. Turn off overhead lights in room.

10. Apply power to PMT.
11. Boot up the Keck microscope software.

12. Configure the Keck microscope software for two-photon imaging. Use the software to open the Tsunami laser shutter.

13. Use the Keck microscope software to set the PMT gain to 65 (a real-time two-photon image should now be seen in the program’s window).

14. Adjust the focus depth and the horizontal and vertical positions until quantum dots can be seen under the skin’s surface. See Figure 3.7 below for how quantum dot nanoprobes should look like while inside skin.

15. **Note:** It can take as long as 20 minutes to find an area of skin where quantum dots can be seen inside skin. It is important to keep the skin hydrated by applying a couple drops of distilled water every 10 minutes while the skin sample is on the microscope.

16. Instruct Keck microscope software to capture a z-stack comprised of 160 imaging depths. Set the number of frames (z-stacks) saved to the hard disk as 20 for each z-axis location. Set the incremental z-axis imaging depth to be 1 µm.

17. The skin imaging procedure is now complete. CRM imaging can be optionally performed to obtain accessory imaging data.

### 3.1.2 Imaging Results and Analysis

The 20 images at each vertical location (on the z-axis) can be averaged to obtain a resulting image with a better SNR than a single image frame. The Poisson-distributed, random nature of laser light (see Section 2.1) is partially responsible for the increased SNR because the additional photons obtained from multiple images are used to converge the signal light to a more accurate level. The low number of photons arriving at the PMT result in producing signal shot noise after the PMT converts the photons to current. The noise from the PMT includes shot noise resulting from signal light, shot noise resulting from background light and shot noise resulting from dark current [28], which are Poisson processes. The SNR of the PMT can be represented by the equation [28]

\[
SNR_{PMT} = \frac{S_p P_i}{\sqrt{2e(S_p P_i + 2I_{da})\mu F B}}
\]  

(3.1)

where:

- \(S_p\) = anode radiant sensitivity [A/watt]
- \(P_i\) = incident light power [watts] (signal plus background)
- \(e\) = electron charge [C]
- \(I_{da}\) = anode dark current [A]
- \(\mu = S_p \cdot P_i\) [A/watt^2]
- \(B\) = circuit bandwidth [Hz]
- \(F = (\delta/(\delta - 1))\), where \(\delta\) is the secondary emission ratio.

Equation (3.1) suggests the incident light power increases the SNR, as one might expect. Therefore, the SNR would change depending on the intensity of the emission light produced by the quantum dot nanoprobes, which in turn depends on how deep the nanoprobes are. The emission light from the quantum dot nanoprobes also depends on the intensity of the
excitation laser beam, as implied in Section 2.1. All other parameters in Equation (3.1) are held constant during experimentation. Shot noise created by background light can be an additional factor that negatively impacts the SNR. This was minimized during experimentation by trying to keep the ambient light as low as possible.

The detection limit is a useful value that identifies the incident light power value that makes the SNR value unity. This value is expressed by the equation

\[ P_{\text{det}} = \frac{e\mu FB}{S_p} + \sqrt{\left(\frac{e\mu FB}{S_p}\right)^2 + 4eI_0\mu FB} \]  

(3.2)

where the parameters are the same as in Equation (3.1) [28]. Due to the Poisson nature of the signal and noise, the detection limit is most relevant when a sufficiently high number of imaging frames are averaged.

A three-dimensional data stack that has pixel dimensions of 480x620x160 can be made once the 20 images at each vertical location are averaged. However, each voxel in this z-stack does not represent equal spatial distances in the x, y and z axes. Conversion between pixel increments and spatial increments have been determined by conducting Field of View (FOV) measurements. Dan Townsend has conducted these FOV measurements for his thesis [60]. The x-axis pixel size was reported to be 0.467 \( \mu m \) when the 20x objective was affixed to the Keck microscope. Similarly, the y-axis pixel size was reported to be 0.444 \( \mu m \). Therefore, the FOV of a single frame is

\[ \text{FOV}_{\text{frame}} : (0.467 \mu m \cdot 480) \times (0.444 \mu m \cdot 620) \approx 224 \mu m \times 275 \mu m \]  

(3.3)

As mentioned in Section 3.1.1, the z-axis step size was set as 1 \( \mu m \). Therefore, the total imaging volume can be computed to be

\[ V_{\text{image}} = (0.467 \cdot 480) \times (0.444 \cdot 620) \times (1 \cdot 160) = 9.87 \times 10^6 \mu m^3 = 9.87 \text{ mm}^3 \]  

(3.4)

Figure 3.7 shows an x-y slice of the z-stack at location \( z = 71 \). This depth is approximately 45 \( \mu m \) from the surface of the skin sample. Nearly all of the signal seen in Figure 3.7 is caused by quantum dot nanoprobe (see Section 6.1). The quantum dots are primarily seen as the "white specks" in this figure. A white-ish haze can be observed when the specks are in close proximity to each other. The relatively strong image signal seen in this figure is convincing evidence that quantum dots have penetrated the skin sample. In comparison, little to no signal was observed at skin depths greater than 20 \( \mu m \) after many attempts of utilizing chemical enhancers for transdermal nanoprobe delivery. See Section 6.1 for the results of experimentation conducted with human skin not containing nanoprobe.

The circular, dark structures shown in Figure 3.7 resemble spinous skin cells. As mentioned in Section 2.3, spinous skin cells have been known to be approximately 15 − 25 \( \mu m \) in diameter at skin depths between 20 and 100 \( \mu m \). The size of the cells seen in Figure 3.7 appear to be large spinous cells. Due to the high variability of skin thicknesses, spinous cells can be at different depths inside skin. The nanoprobe shown in Figure 3.7 appear to be mostly around the circumference of the spinous cells. Therefore, it can be hypothesized that the diffusion pathways of the quantum dot nanoprobe are intercellular.

Figure 3.8 shows a z-y slice of the z-stack at location \( x = 75 \). This slice was taken from the same z-stack as the data slice shown in Figure 3.7. Contrary to the x-y z-stack slice shown in Figure 3.7, the z-y data slice shown in Figure 3.8 does not clearly exhibit cellular structures. However, quantum dot nanoprobe penetration pathways can be more
easily seen. The penetration movement can be hypothesized to have a significant lateral
direction since the bands of high signal strength extend parallel to the skin’s surface.

The z-y slice shown in Figure 3.8 is significantly different than similar data slices taken
from Optical Coherence Tomography (OCT) and CRM imaging when no nanoparticles are
present (see data presented by Jason Kellicker et al. [1]). The OCT and CRM images of
skin without nanoprobes show a more smooth texture inside skin. This is to be expected
because the rough texture in Figure 3.8 resembles quantum dots.
Figure 3.7: x-y data slice at position z=71 in a 20-frame-averaged z-stack.
Figure 3.8: z-y data slice at position x=75 in a 20-frame-averaged z-stack.
The Maximum Intensity Projection (MIP) was used to assess the approximate penetration depth of the quantum dot nanoprobes. The MIP can be represented as a two-dimensional image having pixel values that are the maximum pixel values along the third (projection) axis in a three-dimensional data stack. The MIP of the experimentation z-stack on the y-z plane is shown in Figure 3.9. The z-stack is 20-frame-averaged.

The location of the microscope’s objective is located nearest to $z = 0$ in Figure 3.9. The surface of the skin is approximately at $z = 23$. Therefore, the maximum penetration depth of the quantum dot nanoprobes can be approximated to be at least $100 \, \mu m$. The absence of significant signal seen past $100 \, \mu m$ in the skin suggests penetration might not have come from behind the skin sample. A subsequent experiment would be needed to justify this claim. Such an experiment would involve slicing the sample perpendicular to the skin’s surface and then to place the inner-side of the skin on the coverslip so that it is facing the objective. Imaging in this orientation would deduce if the nanoprobes penetrated beyond $100 \, \mu m$ into skin.
Figure 3.9: y-z plane MIP depicting quantum dots inside skin.
As seen in Figure 3.7, averaging 20 frames produces an image with reasonable visual quality. However, some imaging applications may benefit from fewer frames to decrease imaging time and laser light exposure.

Figure 3.10 shows a subset of the data in the x-y plane when \( z = 71 \). The number of frames averaged varies between 1 and 20. It can be seen that the image quality most drastically improves when the number of frames averaged is increased to 5. Marginal improvements in image quality can be observed when the number of frames averaged is between 5 and 20. The entropy of the entire 480x620 image at \( z = 71 \) was calculate for a resulting image when the number of frames averaged is 1, 5, 10, 15 and 20. Entropy is a common measure for randomness in an image. Entropy in an image can be approximated by the equation

\[
H = - \sum_{j=1}^{256} P(a_j) \log P(a_j) \tag{3.5}
\]

where \( P(a_j) \) is the probability of event \( a_j \) occurring [24]. Each event \( a_j \) can represent a pixel having a value of \( j \). Therefore, the entropy of an image is a representation of the average information in an image because a unit of information is represented as

\[
I(E) = - \log P(E) \tag{3.6}
\]

where \( E \) is an event occurring and \( P(E) \) is the probability of that event occurring [24]. This common unit of information comes from information theory.

The bottom-right image in Figure 3.10 shows an entropy plot when different numbers of frames are averaged. The x-axis is the number of frames averaged; the y-axis is the entropy values. The entropy curve shown in this plot closely corresponds to the perceived image qualities of the applicable images in the same figure.

Further analysis of varying the number of frames averaged was performed by creating a histogram when the averaged number of frames is 1, 5, 10, 15 and 20. All of these histograms have been overlaid in Figure 3.11. It can be seen that the histograms for an average of 5, 10, 15 and 20 frames are substantially different than a histogram for a single frame. This is because a single frame capture acts similarly to a photon detector in which the two most common pixel values are 0 and 255. The tiny "spikes" seen in the histograms of the averaged data result from the same photon detector principle. For example, a spike in the 5-frame average histogram occurs when the pixel value is 51. This is because \( \frac{255}{5} = 51 \).

Before ending this section, it is important to report the estimated power of the excitation laser beam at the surface of the skin sample. Section 3.1 mentions the setup power is \( P_{\text{setup}} = 190 \text{ mW} \). A transmission measurement of the Keck microscope was observed to be 0.996% between the position at \( P_{\text{setup}} \) and the location of the sample. This measurement was taken while the Keck microscope was configured for the experimentation mentioned in Section 3.1.1, except the objective was not attached. Figure 4.9 in Dan Townsend’s thesis has reported the throughput transmittance of the 20x objective to be approximately 69%. Therefore, the total transmittance of the Keck microscope can be approximated to be \( T = 0.00996 \times 0.69 = 0.687\% \) when conducting the imaging mentioned in Section 3.1.1. Hence, the laser power at the surface of the skin sample can be approximated to be \( P_{\text{skin}} = P_{\text{setup}} \times T \approx 1.3 \text{ milliwatts} \).
Figure 3.10: Varying number of frames averaged when each frame is the same subset of the \( x \)-\( y \) slice at \( z=71 \) (containing pixels \( x=1-200 \) and \( y=100-300 \)). All images are using the same intensity scale between 0 and 255. Entropy is computed for all frames averaged and all \( x \) & \( y \) values at \( z=71 \).
Figure 3.11: Histograms for the varying number of averaged frames mentioned in Figure 3.10. This figure was enlarged and rotated so localized histogram peaks can be easily distinguished.
3.2 Background Noise Experimentation

The algorithm in Section 5.2 needs a background noise measurement to enhance the performance of the computations. Figure 3.12 below shows the average pixel value of a single 2-dimensional image frame when the PMT gain is varied while the PMT was measuring background noise in the Keck microscope’s room. The Keck microscope software was configured to average 10 frames for each resulting frame that was saved to the hard drive.

The transdermal nanoprobe delivery experimentation mentioned in Section 3.1 utilizes a PMT gain of 65. Therefore, it is reasonable to use a background PMT noise value of 0.67 in the algorithm mentioned in Section 5.2.

![Graph showing background PMT noise measurements.](image)

**Figure 3.12:** Background PMT noise measurements.

Figure 3.13 shows the histogram when the PMT gain is set to 60 and 70. Pixels having a value greater than 30 were not included in the figure because none exist. The histograms are nearly identical for both PMT gains. This is not surprising since the average pixel value for both gains are approximately the same, as shown in Figure 3.12. Similarly to Figure 3.10, Figure 3.13 has a peak at a value of $255/N$, where $N$ is the number of frames averaged. Therefore, the peak value in Figure 3.13 occurs at 25 since $N = 10$. The reason for this peak was explained in Section 3.1.2.
Figure 3.13: Background PMT noise histogram.
CHAPTER IV

EXPERIMENTAL POINT SPREAD FUNCTION

As seen in Figure 3.7, the quantum dot nanoprobes can be observed as many sub-resolution point sources inside skin. Therefore, one or more of these point sources can be extracted from the skin imaging data and used as an experimental PSF. Obtaining the experimental PSF from within skin gives an accurate representation of the PSF because all deviations in the imaging environment are accounted for, including the spherical aberrations caused by heterogeneous parts of the skin sample.

Image processing techniques were used to automatically detect and analyze nanoprobes that serve as good candidates for an experimental PSF. Such image processing techniques were needed because it is too cumbersome to achieve similar results manually. Section 4.1 below discusses the procedure used to automatically detect and analyze the experimental PSF from the imaging data obtained in Section 3.1.

4.1 Procedure to Detect and Analyze the Experimental PSF

1. A two-dimensional, 3-by-3 median spatial filter was applied to each z-slice of the raw image data. This filtering step removes speckle noise so that nanoprobes will be less likely to be falsely detected.

2. A threshold of 128 was used to create a three-dimensional black and white (binary) image from the resulting three-dimensional image in Step 1.

3. Using the MATLAB image processing toolbox, six-face connected components were created for the black and white image obtained in Step 2. The volume of each of these connected components is proportional to the volumes of the PSFs exhibited from the nanoprobes.

4. The centroids were then found for all the connected components obtained in Step 3.

5. All computed data in steps 1 through 3 were then cleared from memory. The purpose of these steps is to obtain the centroids to be used in Step 8.

6. Next, a voxel threshold of 77 was used to create a black and white image from the raw data. A relatively low threshold value was used to capture as much of the PSF as possible.

7. Using the MATLAB image processing toolbox, six-face connected components were created for the black and white image created in Step 6.

8. Connected components calculated in Step 7 which do not enclose one of the centroids computed in Step 4 were filtered out.

9. Connected components calculated in Step 8 that correspond to voxels having a saturated value of 255 in the raw image were filtered out.
10. Next, the connected components in Step 9 that touch the edge of the three-dimensional image were filtered out.

11. Connected components in Step 10 that are not contiguous in at least one of the x-y planes were filtered out. The main benefit of this step is to filter out connected components that resemble multiple nanoprobes that are close enough to exhibit two PSF "touching" in the image.

12. Centroids for each of the remaining connected components after Step 11 were then computed.

13. The skin depth for each of the centroids obtained in Step 12 was then computed. The surface of the skin sample is uneven with respect to the surface of the coverslip so the depth into skin does not directly relate to the z-axis value. By visual inspection, it can be seen that the skin sample is tilted in only one of the axes. The following equation was used to obtain the z-axis value of the skin’s surface:

\[
z_{\text{skin}} = -0.0564 \times x + 51.0564
\]

(4.1)

where \(z_{\text{skin}}\) is the location of the skin’s surface in the z-axis and \(x\) is the x-axis location. Therefore, the depth into skin can be computed by using the equation

\[
d_{\text{skin}} = z - z_{\text{skin}}
\]

(4.2)

where \(d_{\text{skin}}\) is the depth into skin and \(z\) is the z-axis location. This implies that the nanoprobes detected between the coverslip and the skin’s surface have a negative value for depth into skin.

14. The maximum intensities for all the candidate nanoprobes were computed, as represented by the connected components obtained in Step 11. Figure 4.14 shows a scatter plot of the maximum voxel value of each of the candidate nanoprobes at each of the skin depths. The nanoprobes at negative skin depths are nanoprobes floating in water between the coverslip and the skin surface. These nanoprobes are filtered out after this step. All other nanoprobes mostly exist at skin depths greater or equal to 20 \(\mu\)m into skin. The dearth of candidate nanoprobes between 0 – 20 \(\mu\)m is because it can be seen by visual inspection that not many nanoprobes exist there. Figure 4.14 further shows that quantum dots are bright enough to exhibit strong signal strength deep into skin.
15. The candidate nanoprobes were then normalized so the sum of the voxel values is equal to one. This is so the nanoprobes can be easily compared with each other. The voxel location comprising each prospective nanoprobe is represented by the applicable connected component.

16. The candidate nanoprobes that were not symmetrical in the axial direction were then filtered out. Symmetry holds if the distance between the maximum value and one of the half max values is within 1 voxel of the distance between the maximum value and the other half max value. Candidate nanoprobes that are not symmetrical are likely to be nanoprobes that are moving inside skin. As mentioned in Section 2.5, the Keck microscope takes about one second for each z-slice.

17. The number of voxels for each of the candidate nanoprobes resulting in Step 16 was then computed.

18. Next, the average signal intensity adjacent to each nanoprobe was calculated. This was accomplished by computing the average voxel value of all the voxels within two voxels beyond the perimeter of each candidate nanoprobe. The candidate nanoprobes that have an average adjacent signal intensity greater than 37 were then filtered out. This step in the procedure discards non-preferred PSFs that are more likely to be

---

**Figure 4.14:** Maximum voxel value vs. depth into skin.
within or nearby a cluster comprising adjacent nanoprobes that boost up the adjacent signal strength. The threshold of 37 yields a sufficient amount of final prospective nanoprobes discussed in Step 21. Using nanoprobes with high adjacent signal strengths as an experimental PSF are not preferred because the nearby signal strength will disrupt the observation of the peripheral edge of the experimental PSF. The value of 37 can be seen in one of the axes shown in Figure 4.15. As expected, this figure shows a relationship between adjacent signal strength and the volume of prospective nanoprobes. The volume is likely to increase because the adjacent signal adds to the signal from the nanoprobe itself. Figure 3.7 is a good example of an image showing some nanoprobes that are in an area of relatively high adjacent signal strength as well as some nanoprobes that are in areas of relatively low adjacent signal strength.

![Figure 4.15: Number of voxels vs. nearby signal average.](image)

19. Step 18 reduces the prospective nanoprobe count to 18. Figure 4.16 shows the volumes of each of these nanoprobes. By visual inspection of the raw data, it was observed that the outlier having a voxel volume of approximately 200 is in fact two nanoprobes adjacent to each other. The peripheral, low signal of each of these nanoprobes were touching. Therefore, this outlier was discarded.
Figure 4.16: Histogram: Pixel volume of candidate nanoprobes inside skin.

20. The weighted covariance matrix was computed for each of the remaining 17 prospective nanoprobes from Step 19. The weighted covariance matrix was created by using the equation:

\[
C = \begin{vmatrix}
\sigma_{xx} & \sigma_{xy} & \sigma_{xz} \\
\sigma_{xy} & \sigma_{yy} & \sigma_{yz} \\
\sigma_{xz} & \sigma_{yz} & \sigma_{zz}
\end{vmatrix}
\]  

(4.3)
Each of the covariances were computed by the equations:

\[
\sigma_{xx} = \sum_{i=1}^{N} x_i^2 \left( \frac{I_i}{I_{sum}} \right) - \left[ \sum_{i=1}^{N} x_i \left( \frac{I_i}{I_{sum}} \right) \right]^2
\]

\[
\sigma_{yy} = \sum_{i=1}^{N} y_i^2 \left( \frac{I_i}{I_{sum}} \right) - \left[ \sum_{i=1}^{N} y_i \left( \frac{I_i}{I_{sum}} \right) \right]^2
\]

\[
\sigma_{zz} = \sum_{i=1}^{N} z_i^2 \left( \frac{I_i}{I_{sum}} \right) - \left[ \sum_{i=1}^{N} z_i \left( \frac{I_i}{I_{sum}} \right) \right]^2
\]

\[
\sigma_{xy} = \sum_{i=1}^{N} x_i y_i \left( \frac{I_i}{I_{sum}} \right) - \left[ \sum_{i=1}^{N} x_i \left( \frac{I_i}{I_{sum}} \right) \right] \left[ \sum_{i=1}^{N} y_i \left( \frac{I_i}{I_{sum}} \right) \right]
\]

\[
\sigma_{xz} = \sum_{i=1}^{N} x_i z_i \left( \frac{I_i}{I_{sum}} \right) - \left[ \sum_{i=1}^{N} x_i \left( \frac{I_i}{I_{sum}} \right) \right] \left[ \sum_{i=1}^{N} z_i \left( \frac{I_i}{I_{sum}} \right) \right]
\]

\[
\sigma_{yz} = \sum_{i=1}^{N} y_i z_i \left( \frac{I_i}{I_{sum}} \right) - \left[ \sum_{i=1}^{N} y_i \left( \frac{I_i}{I_{sum}} \right) \right] \left[ \sum_{i=1}^{N} z_i \left( \frac{I_i}{I_{sum}} \right) \right]
\]

(4.4)

where \(x, y\) and \(z\) are the corresponding voxel coordinates, \(N\) is the total number of voxels, \(I_i\) is the voxel intensity and \(I_{sum}\) is the sum of all the voxel intensity values that make up the nanoprobe. The covariance matrix represented in Equation (4.3) is helpful in part because the square route of the diagonal components are the standard deviations in the \(x\), \(y\) and \(z\) axis directions, respectively. Table 1 shows the standard deviation in the \(x\)-axis, \(y\)-axis and \(z\)-axis for each of the nanoprobes.

<table>
<thead>
<tr>
<th>Id</th>
<th>Skin Depth ((\mu m))</th>
<th>x-Std. ((\mu m))</th>
<th>y-Std. ((\mu m))</th>
<th>z-Std. ((\mu m))</th>
<th>(I_{sum})</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>16</td>
<td>0.454</td>
<td>0.494</td>
<td>1.842</td>
<td>08435</td>
</tr>
<tr>
<td>2</td>
<td>18</td>
<td>0.531</td>
<td>0.617</td>
<td>1.845</td>
<td>06235</td>
</tr>
<tr>
<td>3</td>
<td>18</td>
<td>0.489</td>
<td>0.525</td>
<td>2.339</td>
<td>13138</td>
</tr>
<tr>
<td>4</td>
<td>19</td>
<td>0.441</td>
<td>0.467</td>
<td>1.894</td>
<td>07884</td>
</tr>
<tr>
<td>5</td>
<td>19</td>
<td>0.548</td>
<td>0.791</td>
<td>2.025</td>
<td>12961</td>
</tr>
<tr>
<td>6</td>
<td>24</td>
<td>0.468</td>
<td>0.646</td>
<td>2.122</td>
<td>15182</td>
</tr>
<tr>
<td>7</td>
<td>31</td>
<td>0.339</td>
<td>0.578</td>
<td>1.492</td>
<td>05287</td>
</tr>
<tr>
<td>8</td>
<td>34</td>
<td>0.600</td>
<td>0.434</td>
<td>2.130</td>
<td>07303</td>
</tr>
<tr>
<td>9</td>
<td>45</td>
<td>0.544</td>
<td>0.407</td>
<td>2.235</td>
<td>07379</td>
</tr>
<tr>
<td>10</td>
<td>48</td>
<td>0.443</td>
<td>0.399</td>
<td>2.155</td>
<td>07277</td>
</tr>
<tr>
<td>11</td>
<td>52</td>
<td>0.439</td>
<td>0.583</td>
<td>1.724</td>
<td>09823</td>
</tr>
<tr>
<td>12</td>
<td>53</td>
<td>0.370</td>
<td>0.362</td>
<td>1.624</td>
<td>05662</td>
</tr>
<tr>
<td>13</td>
<td>55</td>
<td>0.509</td>
<td>0.422</td>
<td>2.178</td>
<td>06397</td>
</tr>
<tr>
<td>14</td>
<td>59</td>
<td>0.405</td>
<td>0.481</td>
<td>1.796</td>
<td>10995</td>
</tr>
<tr>
<td>15</td>
<td>61</td>
<td>0.390</td>
<td>0.404</td>
<td>1.769</td>
<td>06253</td>
</tr>
<tr>
<td>16</td>
<td>72</td>
<td>0.250</td>
<td>0.545</td>
<td>1.191</td>
<td>04081</td>
</tr>
<tr>
<td>17</td>
<td>72</td>
<td>0.556</td>
<td>0.599</td>
<td>2.005</td>
<td>10544</td>
</tr>
</tbody>
</table>

As expected, the \(z\)-axis standard deviations shown in Table 1 are the largest. This
supports the profile of the PSF shown in the schematic illustrated in Figure 2.1, where the PSF is expected to be longest in the z-axis direction. Table 1 further shows that the nanoprobes that were automatically detected are spread out across many different depths into the skin sample. Figure 4.17 shows a scatter plot of the standard deviations as a function of depth into skin.

![Figure 4.17: Weighted standard deviation of candidate nanoprobes in x, y & z axes.](image)

21. An additional step was performed to decompose $C$ in Equation (4.3) by means of Singular Value Decomposition (SVD). This was accomplished by decomposing $C$ into the three matrices:

$$C = U\Sigma V^*$$

(4.5)

where $U$ and $V^*$ are 3x3 unitary matrices and $\Sigma$ is a 3x3 diagonal matrix holding positive singular values. Since $C$ is symmetric, Equation (4.5) becomes

$$C = U\Sigma U^*$$

(4.6)

so that the columns of $U$ hold the eigenvectors and matrix $\Sigma$ holds the eigenvalues. The square root of these eigenvalues are the standard deviations of the major, median and minor axes of a "best fit" ellipsoid that approximates each of the nanoprobes. The eigenvectors in matrix $U$ specify the direction of the major, median and minor axes, respectively. Table 2 shows data for the best fit ellipsoids of the nanoprobes shown in Table 1. The columns titled "Maj-Std.", "Med-Std." and "Min-Std." correspond
to the major, median and minor axis standard deviations, respectively. The columns
titled "Maj-x", Maj-y" and "Maj-z" correspond to the major axis direction vector.
The data in Table 2 infer there is a slight tilt with respect to the z-axis direction. In
comparison, the major axis standard deviations shown in Table 2 aren’t exactly the
same values as the z-axis standard deviations shown in Table 1, as one might expect.
The major axis direction shown shown in columns Maj-x, Maj-y and Maj-z in Table 2
confirms the tilting phenomenon. We speculate that the most likely cause of the slight
tilt of the nanoprobes is likely to be from the piezo z-stage equipment on the Keck
microscope not moving exactly in line with the laser beam.

<table>
<thead>
<tr>
<th>Id</th>
<th>Maj-Std. (µm)</th>
<th>Med-Std. (µm)</th>
<th>Min-Std. (µm)</th>
<th>Maj-x</th>
<th>Maj-y</th>
<th>Maj-z</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.868</td>
<td>0.484</td>
<td>0.341</td>
<td>-0.160</td>
<td>-0.065</td>
<td>-0.985</td>
</tr>
<tr>
<td>2</td>
<td>1.937</td>
<td>0.467</td>
<td>0.308</td>
<td>-0.224</td>
<td>0.219</td>
<td>-0.950</td>
</tr>
<tr>
<td>3</td>
<td>2.358</td>
<td>0.516</td>
<td>0.398</td>
<td>-0.116</td>
<td>0.058</td>
<td>-0.992</td>
</tr>
<tr>
<td>4</td>
<td>1.924</td>
<td>0.445</td>
<td>0.315</td>
<td>-0.154</td>
<td>0.092</td>
<td>-0.984</td>
</tr>
<tr>
<td>5</td>
<td>2.148</td>
<td>0.561</td>
<td>0.309</td>
<td>-0.212</td>
<td>0.270</td>
<td>-0.939</td>
</tr>
<tr>
<td>6</td>
<td>2.165</td>
<td>0.531</td>
<td>0.411</td>
<td>-0.103</td>
<td>0.177</td>
<td>-0.979</td>
</tr>
<tr>
<td>7</td>
<td>1.555</td>
<td>0.428</td>
<td>0.275</td>
<td>-0.119</td>
<td>0.267</td>
<td>-0.956</td>
</tr>
<tr>
<td>8</td>
<td>2.198</td>
<td>0.410</td>
<td>0.299</td>
<td>-0.230</td>
<td>0.093</td>
<td>-0.969</td>
</tr>
<tr>
<td>9</td>
<td>2.272</td>
<td>0.444</td>
<td>0.315</td>
<td>-0.180</td>
<td>0.022</td>
<td>-0.983</td>
</tr>
<tr>
<td>10</td>
<td>2.168</td>
<td>0.400</td>
<td>0.369</td>
<td>-0.106</td>
<td>0.041</td>
<td>0.993</td>
</tr>
<tr>
<td>11</td>
<td>1.762</td>
<td>0.494</td>
<td>0.396</td>
<td>-0.110</td>
<td>0.184</td>
<td>-0.977</td>
</tr>
<tr>
<td>12</td>
<td>1.634</td>
<td>0.361</td>
<td>0.324</td>
<td>-0.109</td>
<td>0.029</td>
<td>0.994</td>
</tr>
<tr>
<td>13</td>
<td>2.211</td>
<td>0.424</td>
<td>0.335</td>
<td>-0.174</td>
<td>-0.004</td>
<td>-0.985</td>
</tr>
<tr>
<td>14</td>
<td>1.803</td>
<td>0.494</td>
<td>0.359</td>
<td>-0.086</td>
<td>-0.002</td>
<td>-0.996</td>
</tr>
<tr>
<td>15</td>
<td>1.786</td>
<td>0.385</td>
<td>0.328</td>
<td>-0.119</td>
<td>-0.072</td>
<td>-0.990</td>
</tr>
<tr>
<td>16</td>
<td>1.228</td>
<td>0.471</td>
<td>0.219</td>
<td>-0.060</td>
<td>-0.256</td>
<td>0.965</td>
</tr>
<tr>
<td>17</td>
<td>2.068</td>
<td>0.493</td>
<td>0.407</td>
<td>-0.186</td>
<td>0.169</td>
<td>-0.968</td>
</tr>
</tbody>
</table>

Figure 4.18 shows the major, median and minor axes standard deviations as a function
of depth into skin. Similarly to Figure 4.17, the axis having the larger values have
values that are significantly larger than the two axes having smaller standard devia-
tions. As expected, the two axes representing smaller standard deviations have values
that are nearly the same. It is also interesting to note that the standard deviations
in the major axis vary significantly more than the standard deviations respecting to
the other axes, and that none of the data points show a trend with respect to depth
into skin. This conclusion is similar to the conclusion made by Starosta et al. when
they computed the FWHM of a focused beam within multiple biological cells [55].
The last step in the procedure was to average the normalized nanoprobes that have been automatically detected. Due to variations in the composition of the skin sample it is reasonable to conclude that each of the nanoprobes that have been detected will not be exactly the same. Therefore, a good estimation of a PSF inside skin would be to average all of the normalized nanoprobes that have been detected. The normalization step is important to compensate for variations in signal intensity. As shown in Table 1, the cumulative intensity of all the voxels making a nanoprobe varies. This variation could be caused by additive signal from nearby nanoprobes.

Figure 4.19 shows the z-slices of the averaged experimental point spread function that will be used in subsequent chapters in this thesis. The distance in the horizontal direction spans 4.88 µm while the distance in the vertical direction spans 4.20 µm.
Figure 4.19: Averaged PSF at different z-axis increments. Each z-axis increment is 1 µm.
4.2 *Experimental PSF Analysis*

A reasonable experimental approximation of the PSF in skin was obtained in Step 22 of Section 4.1. The FWHM is a common heuristic for analyzing PSF size, and thus, imaging resolution. Equations (4.3) and (4.6) were first utilized to obtain the eigenvectors that represent the major, median and minor axes of a best fit ellipsoid to the averaged PSF obtained in Step 22 of Section 4.1. These eigenvectors were then scaled to obtain different \(x, y\) and \(z\) axis data points of the averaged PSF after it has been normalized. The PSF was normalized such that the intensities of all the voxels comprising the PSF sum to one.

Figure 4.20 shows the intensities of the voxel values along the major axis. It can be seen that the FWHM along this axis is approximately 4.52 \(\mu m\). In comparison, Dwyer *et al.* obtained an experimental PSF through full-thickness epidermis. The epidermis in Peter Dwyer’s experimentation was approximately 75 \(\mu m\) [17] thick. This PSF was obtained with a CRM microscope having a 10x objective (NA: 0.8, water immersion). Dwyer *et al.* reported a FWHM of 7.6 \(\mu m\) in the axial direction. This value is of the same order of magnitude as the value of 4.52 \(\mu m\) that was estimated for the FWHM shown in Figure 4.20.

![Normalized Voxel Value vs Distance](image)

**Figure 4.20:** FWHM of major axis.

Figures 4.21 and 4.22 show the graph of the voxel values along the median and minor axes, respectively. It can be seen that the FWHM in the median axis is approximately 1.31 \(\mu m\) while the FWHM in the minor axis is approximately 1.13 \(\mu m\). In comparison, Dwyer *et al.* reported a FWHM of 1.7 \(\mu m\) in the axial direction. This averaged PSF is a very close approximation to the averaged PSF reported in Section 5.3.
Figure 4.21: FWHM of median axis.

Figure 4.22: FWHM of minor axis.
CHAPTER V

THREE-DIMENSIONAL IMAGE DECONVOLUTION

5.1 Microscope Signal Model

The three-dimensional imaging output of a two-photon microscope can be modeled by the equation

\[ g(k) = h(k) * f(k) + \eta(k) \]  (5.1)

where \( g(k) \) is the observed image, \( h(k) \) is the PSF, \( f(k) \) is the actual image, \( \eta(k) \) is additive noise, \( k \) are voxels such that \( k \in \mathbb{K} \in \mathbb{R}^3 \). Since the PSF encompasses many voxels, it can be seen how the observed image results from the convolution of the PSF with the actual image. In other words, the intensity of each pixel in the observed image could be approximated to be the weighted sum of intensities of (usually nearby) emission sources that are weighted by the PSF. The resulting effect of this convolution is a reduction of resolution in the observed image due to blurring. Considering that the quantum dot nanoprobes discussed in Section 2.2 are far smaller than the PSF of the microscope, the convolution shown in Equation (5.1) will make it hard to distinguish nanoprobes that are in close proximity to each other. The noise term \( \eta(k) \) in Equation (5.1) is predominantly Poisson distributed noise for the low levels of emission light detected in the experiments conducted for this thesis. This is due to the probabalistic Poisson nature of photon emission that was discussed in Section 2.1 as seen in Figure 3.11.

Therefore, each 3-dimensional pixel from the observed image \( g(k) \) acts like a photon detector and can be modeled to be a Poisson random variable having a mean value of \( \lambda^* (k) \). This mean value represents the detected signal intensity and is proportional to the radiance of the emission beam of light. Hence, each pixel from the observed image converges to \( \lambda^* (k) \) as \( N_f \to \infty \), where \( N_f \) is the number of frames averaged.

Directly solving Equation (5.1) for \( f(k) \) yields inadequate results for low levels of light because the Poisson noise is very high and there is no way to directly know \( \eta(k) \) for each pixel. An indirect method such as the EM algorithm (see Section 5.2) produces much better results because it simultaneously accounts for the effects of Poisson noise and the PSF so the unobserved image can be estimated. The remaining discussions in this chapter will set up an adequate microscope image signal model that will then be used by the EM algorithm discussed in Section 5.2.

Consider the scenario in which there exists an input sample space consisting of the unobserved image we wish to estimate. The pixel indices in this space are denoted as \( j \in \mathbb{J} \in \mathbb{R}^3 \). The value at each of these pixels represents independent Poisson random variables having a mean \( \lambda(j) \). Therefore, these Poisson random variables directly represent emission photons that have been detected. The value of the pixels in the sampling space \( \mathbb{J} \) can be considered to be the unobserved data or complete data. Also consider an output sample space consisting of the observed image that is captured by the Keck microscope. The pixel indices in this space are denoted as \( k \in \mathbb{K} \in \mathbb{R}^3 \). As mentioned above, these Poisson random variables have a mean value of \( \lambda^* (k) \). The value of the pixels in the sampling space \( \mathbb{K} \) can be considered to the observed data.
A mapping between the sample spaces $\mathbb{J}$ and $\mathbb{K}$ can be established. When a single emission photon occurs at pixel $j$ then the probability of that photon being detected in pixel $k$ can be denoted as $p(j, k)$. Therefore, the equation below must hold if the emission photon occurring in pixel $j$ is always detected by any pixel in space $\mathbb{K}$:

$$\sum_k p(j, k) = 1 \quad (5.2)$$

The following equation will then hold if the only pixel in $\mathbb{J}$ that doesn’t have an intensity of 0 is $j_1$:

$$\lambda^*(k) = \sum_k p(j_1, k) \cdot \lambda(j) \quad \forall k \in \mathbb{K} \quad (5.3)$$

It can now be seen that the PSF relates to the term $p(j, k)$ since both the PSF and $p(j, k)$ in some sense act as an impulse response to the imaging system. This concept will be further utilized in Section 5.2.

The process of collecting individual photons can be further analyzed. If $n^*(k)$ represents the number of photons observed in pixel $k$ then

$$n^*(k) = \sum_j n(j, k) \quad (5.4)$$

where $n(j, k)$ represents the number of photons that originated in pixel $j$ but were observed in pixel $k$. Therefore, the following equation must hold:

$$p(j, k) = \frac{n(j, k)}{N_f} \quad N_f \to \infty \quad (5.5)$$

It can now be seen that:

$$\lambda^*(k) = E n^*(k) = \sum_j \lambda(j)p(j, k) = \sum_j \lambda(j, k) \quad (5.6)$$

where $E$ is the expected value and $\lambda(j, k)$ is the mean number of photons going from pixel $j$ to pixel $k$. A similar derivation of this was explained by Shepp et al. in terms of positron emission tomography [48].

A vector of all the mean values from the observed data can be represented by

$$\lambda^* = (\lambda^*(1), \lambda^*(2), \ldots, \lambda^*(N_k)) \quad (5.7)$$

where $N_k$ is the number of pixels in the observed data. Likewise, a vector of all the mean values from the complete data can be represented by

$$\lambda = (\lambda(1), \lambda(2), \ldots, \lambda(N)) \quad (5.8)$$

where $N$ is the number of pixels in the complete data.

Each of the Poisson random variables associated with the pixels in the sample space $\mathbb{J}$ can be identified as $j_j$, where $j \in \mathbb{J}$. The Probability Mass Function (PMF) of each of these random variables is represented by

$$f_{j}(n(j)) = \frac{\lambda(j)^n(j)e^{-\lambda(j)}}{n(j)!}, \quad \forall j \in \mathbb{J} \quad (5.9)$$
where \( n(j) \) is the number of photons occurring at pixel \( j \). All random variables \( j \) are \textit{mutually independent} of each other because an event occurring for any one of these random variables is not dependent on events occurring for any of the other random variables. Therefore, if a random vector comprising all these variables is denoted as

\[
J = (j_1, j_2, \ldots, j_{N_j})^T
\]  

(5.10)

where \( N_j \) is the total number of pixels in the sample space \( J \), then the joint probability mass function of this random vector is

\[
f_J(j_1, j_2, \ldots, j_{N_j}) = f_{j_1}(n(1)) \cdot f_{j_2}(n(2)) \cdots f(n(j_{N_j}))
\]

\[=
\prod_{j=1}^{N_j} \frac{\lambda(j)^{n(j)}e^{-\lambda(j)}}{n(j)!}
\]  

(5.11)

Similarly, each of the Poisson random variables associated with the pixels in the sample space \( K \) can be identified as \( k_k \), where \( k \in K \). The PMF of each of these random variables is represented by

\[
f_{k_k}(n(k)) = \frac{\lambda(k)^{n(k)}e^{-\lambda(k)}}{n(k)!}, \ \forall k \in K
\]

(5.12)

where \( n(k) \) is the number of photons observed at pixel \( k \). It can be deduced that all random variables \( k \) are mutually independent of each other by observing Equation (5.4) and knowing that each of the random variables \( j \) are mutually independent. Therefore, a random vector comprising all the random variables \( k \) can be identified as

\[
K = (k_1, k_2, \ldots, k_{N_k})^T
\]  

(5.13)

where \( N_k \) is the total number of pixels in the sample space \( K \). The joint probability mass function of this random vector is

\[
f_K(k_1, k_2, \ldots, k_{N_k}) = f_{k_1}(n(1)) \cdot f_{k_2}(n(2)) \cdots f(n(k_{N_k}))
\]

\[=
\prod_{k=1}^{N_k} \frac{\lambda(k)^{n(k)}e^{-\lambda(k)}}{n(k)!}
\]  

(5.14)

### 5.2 The EM Algorithm

Many deconvolution algorithms exist that attempt to restore \( f(k) \) in Equation (5.1) after knowing some information about \( g(k) \), \( h(k) \) and \( \eta(k) \). A good overview of some of these algorithms is presented in a paper by Sarder and Nehorai [46]. The EM algorithm can be used to act as an iterative deconvolution algorithm. It was chosen for this thesis primarily because it has had favorable image restoration outcomes when compared to other techniques [46, 62]. Additional benefits will be discussed in subsequent sections of this thesis. The implementation of the EM algorithm, as discussed in this section, results in the same equation as the Richardson-Lucy deconvolution algorithm [38][44]. Unlike the EM algorithm, the Richardson-Lucy deconvolution algorithm was derived from Bayesian estimation.

Dempster \textit{et al.} were the first to coin the name EM algorithm in a well known paper published in 1977 by the name of \textit{Maximum Likelihood from Incomplete Data via the EM
Algorithm [13]. This paper presented the most general form of the EM algorithm. Various specific applications of this algorithm had been published prior to 1977 [13].

The EM algorithm uses an iterative method to obtain an approximation by adopting maximum likelihood estimation. There must exist two sample spaces of data and a mapping function between them for the EM algorithm to be applicable. The first space is comprised of observed data. The second space is comprised of complete data that can be seen through the observed data by means of the mapping function. The signal model discussed in Section 5.1 meets prerequisites for using the EM algorithm. The observed data space is comprised of the sample space \( K \) that represents the pixels in the captured two-photon image. The complete data space is comprised of the sample space \( J \) that represents the unobserved image. The mapping from space \( J \) to \( K \) is described by the PSF.

Two steps exist for each iteration of the EM algorithm. The first step is the expectation step (E-step). This step seeks to estimate the complete data sufficient statistics \( t(x) \) by finding

\[
t^{(p)}(p) = E(t(x)|y, \phi^{(p)})
\]

where \( t^{(p)} \) is an array of sufficient statistics at iteration \( p \), \( y \) is the observed data and \( \phi^{(p)} \) is the current estimate. The second step in the EM algorithm is the maximization step (M-step). This step can be represented by

\[
E(t(x)|\phi) = t^{(p)}
\]

where \( \phi \) is the complete data. The objective of the M-step is to solve Equation (5.16) for \( \phi \) in order to estimate \( \phi^{(p+1)} \). Equations (5.15) and (5.16) were written in the most general form of the EM algorithm (see Dempster et al. [13]).

As implied in Section 5.1, the complete data we wish to restore is the image \( f(k) \) in Equation (5.1). Therefore, the pixels in \( f(k) \) make up the complete data sample space \( J \) that was mentioned in Section 5.1. The intensity value at each of the pixels is \( \lambda(j), \forall j \in J \).

The Fisher-Neyman Factorization Theorem can be used to show that \( n(j) \) is a sufficient statistic for \( \lambda(j) \), for any \( j \). Consider \( s \) to represent a random sample of \( N_f \) trials such that

\[
s = \{n_1(j), n_2(j), \ldots, n_{N_f}(j)\}, \forall j \in J
\]

Therefore, the Fisher-Neyman Factorization Theorem implies that the following equation must hold if \( n(j) \) is a sufficient statistic for \( \lambda(j) \), for any \( j \):

\[
P_{\lambda_j}(s) = a(s) \cdot b_{\lambda_j}(n(j)), \forall j \in J
\]

where \( P_{\lambda_j}(s) \) is the probability of obtaining an arbitrary random sample \( s \) and \( a \) and \( b \) are two factors. Equation (5.9) shows the PMF for any pixel \( j \). Therefore, an arbitrary random sample \( s \) has a joint PMF that can be represented by:

\[
P_{\lambda_j}(s) = \prod_{i=1}^{N_f} f_{\lambda_i}(n_i(j))
\]

\[
= \frac{\lambda(j)\sum_{i=1}^{N_f} n_i(j) e^{-N_f \lambda(j)}}{\prod_{i=1}^{N_f} n(j)!}
\]

\[
= a(s) \cdot b_{\lambda_j}(j), \forall j \in J
\]
where,
\[ n(j) = \sum_{i=1}^{N_f} n_i(j), \quad a(s) = \frac{1}{\prod_{i=1}^{N_f} n(j)_i!}, \quad b_{\lambda_j}(j) = e^{-N_f \lambda(j)} \]

Hence, Equation (5.2) proves that \( n(j) \) is a sufficient statistic for \( \lambda_j \), for any \( j \). The concept of a sufficient statistic and the Fisher-Neyman Factorization Theorem is explained well by Scharf [47].

A vector of sufficient statistics for \( \lambda_j \), \( \forall j \in J \) can be denoted as
\[ n_j = (n(j_1), n(j_2), \ldots, n(j_{N_j}))^T \quad (5.20) \]

where \( N_j \) is the number of pixels in sample space \( J \). It can now be observed that \( n_j \) is a sufficient statistic for the random vector \( J \).

The variables in Equations (5.15) and (5.16) can now be written in terms of the variables used in the microscope signal model that is used for this thesis. The E-step can now be represented as
\[ n^{(p)} = E(n_j | \lambda^*, \lambda^{(p)}) \quad (5.21) \]

where \( n^{(p)} \) is a vector of sufficient statistic estimates at iteration \( p \), \( n_j \) is a vector of sufficient statistics for the random vector \( J \), \( \lambda^* \) is a vector of mean values of the observed data and \( \lambda \) is the estimate of the mean values of the pixels from the complete data image at iteration \( p \).

The M-step can now be represented as
\[ E(n_j | \lambda) = n^{(p)} \quad (5.22) \]

where \( \lambda \) is a vector of the mean values of the pixels from the complete data image. Equation (5.22) can be reduced since the expected value of the number of number of photons arriving at each pixel \( j \in J \) is simply the mean number of photons arriving at each of those pixels. In other words the probability of a particular number of photons arriving is maximized at the mean number of photons arriving. This holds true because the random variables \( j_j \), \( \forall j \in J \) are Poisson (see Section 5.1). Therefore, Equation (5.22) can be reduced to
\[ \lambda = n^{(p)} \quad (5.23) \]

and the E-step and M-step can be combined to form the equation
\[ \lambda = E(n_j | \lambda^*, \lambda^{(p)}) \quad (5.24) \]

Now, we can take note that \( \lambda \) is our new estimate for \( \lambda(j) \), \( \forall j \in J \). Therefore, Equation (5.24) becomes
\[ \lambda^{(p+1)} = E(n_j | \lambda^*, \lambda^{(p)}) \quad (5.25) \]

As mentioned in Equation (5.26), \( n_j \) is a column vector of sufficient statistics comprised of \( n(j_1), n(j_2), \ldots, n(j_{N_j}) \). This vector can therefore be alternatively represented as
\[ n'_j = (n(j_1, \cdot), n(j_2, \cdot), \ldots, n(j_{N_j}, \cdot))^T \quad (5.26) \]

where \( n'_j \) is the alternative representation for \( n_j \) and \( n(j_1, \cdot), \ldots, n(j_{N_j}, \cdot) \) are the number of photons that originate from a location in the complete data that end up at any location in the observed data. Therefore, Equation (5.25) can be represented as
\[ \lambda^{(p+1)}(j) = \sum_{k=1}^{N_k} E(n(j, k) | \lambda^*, \lambda^{(p)}), \quad \text{for } j = 1 \ldots N_j \quad (5.27) \]
where \( n(j, k) \) is the number of photons going from pixel \( j \) to \( k \). The vector notations \( \lambda^{(p+1)} \) and \( n'_j \) were removed for convenience. The representation for the sum of expected values is valid because, as shown in Section 5.1, the Poisson random variables are mutually independent.

It can now be seen that Equation (5.27) can be represented as

\[
\lambda^{(p+1)}(j) = \frac{\sum_{k=1}^{N_k} n^*(k) \lambda^{(p)}(j, k)}{\sum_{j'=1}^{N_{j'}} \lambda^{(p)}(j', k)}
\]

(5.28)

because the conditional expectation in Equation (5.27) is for Poisson random variables that are mutually independent. If \( X_i \) are independent Poisson random variables with mean \( a_i (i = 1, \ldots, m) \), then the conditional distribution of \( X_j \), given \( \sum X_i = x^* \), is binomial \( (x^*, a_j / \sum a_i) \), so \( E[X_j | \sum X_i = x^*] = x^* a_j / \sum a_i \) [64].

Using Equation (5.6), Equation (5.28) becomes

\[
\lambda^{(p+1)}(j) = \lambda^{(p)}(j) \frac{\sum_{k=1}^{N_k} n^*(k) p(j, k)}{\sum_{j'=1}^{N_{j'}} \lambda^{(p)}(j') p(j', k)}
\]

(5.29)

An equation similar to Equation (5.29) has been derived in the paper *A Statistical Model for Positron Emission Tomography* written by Vardi et al. [64]. This paper uses the EM algorithm for images taken from positron emission tomography. Much of the EM algorithm derivation approach mentioned above uses the same approach as Vardi et al. A more rigorous approach in using the EM algorithm to estimate Poisson intensities is discussed by Snyder et al. in the book *Random Point Processes in Time and Space* [53]. The approach by Vardi et al. was chosen for this thesis because it is a quicker derivation.

As mentioned in Section 5.1, the PSF behaves like a mapping function going from sample space \( J \) to sample space \( K \). It can also be seen in Equation 5.29 that \( p(j, k) \) and \( p(j', k) \) also behave like a mapping between \( J \) and \( K \). Therefore, Equation (5.30) can be represented as

\[
\lambda^{(p+1)}(j) = \lambda^{(p)}(j) \frac{\sum_{k=1}^{N_k} n^*(k) h(j - k)}{\sum_{j'=1}^{N_{j'}} \lambda^{(p)}(j') h(j' - k)}
\]

(5.30)

where \( h(j - k) \) and \( h(j' - k) \) are the PSF.

### 5.3 Application of the EM Algorithm

Section 5.2 describes how the mapping from space \( J \) to \( K \) is described by the PSF. The PSF used in the algorithm can be taken from any of the candidate nanoprobes discussed in Chapter 3. The deconvolution result contains less artifacts when using an experimental PSF that is extracted from the raw image data by framing a cube around the PSF rather than by using the automatic segmentation discussed in Section 4.1. The boundaries of the cube are chosen such that the peripheral areas of the PSF cannot be distinguished from the nearby signal intensity that does not decrease with respect to the distance from the centroid of the PSF. The PSF obtained from the automatic segmentation is hypothesized to not perform as well because the automatic segmentation methodology is not able to capture the peripheral pixels of the PSF that have very low intensities. Figure 5.23 shows how the middle x-y slice of PSF six in Table 1 looks after it has been segmented automatically.
and manually. It can be seen that the manual segmentation includes a few additional low intensity pixels that the automatic segmentation does not include. The distance in the horizontal direction is 4 \( \mu m \) while the distance in the vertical direction is 3.27 \( \mu m \).
All of the automatically detected experimental PSFs mentioned in Chapter 3 were segmented a second time. They were segmented by framing a cube around the raw image data before being used in the deconvolution algorithm. They were then normalized so the sum of their voxels equals 255. The value of 255 was used to coincide with the theoretical statements made in Sections 5.1 and 5.2. In other words, a voxel value of 255 represents a probability of one for a photon being detected. Therefore, the sum of all the voxels in the PSF should be 255 since the PSF represents a probability distribution function.

As mentioned in Section 5.2, the EM algorithm is an iterative algorithm. The maximum likelihood solution converges as the number of iterations increases. However, the EM algorithm may converge to a suboptimal solution because the experimental PSF is only an approximation of the ideal PSF. This means the most accurate PSF should be taken from the candidate PSFs discussed in Chapter 3 so that the EM algorithm will converge in a direction that yields the most accurate results.

The EM algorithm in Equation (5.30) was slightly modified before being used in the computations. Kempen et al. have shown it is beneficial to have the average intensity of the background noise to be inserted within the denominator in Equation (5.30) [63]. This modification is shown in the following equation that was used in the computations:

\[
\lambda^{(p+1)}(j) = \lambda^{(p)}(j) \sum_{k=1}^{N_k} n^*(k) h(j - k) \sum_{j' = 1}^{N_{j'}} h(j' - k) \lambda^{(p)}(j') + B
\]  

(5.31)

where \( B \) is the average background noise found in Section 3.2 and the remaining parameters are the same as in Equation (5.30). The use of \( B \) acts as a regularization to compensate for background noise. See Appendix A.1 for how the EM algorithm was implemented in MATLAB.

In this work, we assume that the voxels transitioning from a dark intensity to an unwanted, noticeably high intensity after applying the EM algorithm can be considered as artifacts. Artifacts in the resulting image data, using this definition, can be observed as the iterations of the EM algorithm continue, and are assumed to be a result of the EM algorithm starting to converge to a suboptimal solution with non-physical high frequency components. The type of artifact previously mentioned can be observed in Figure 5.28 after 50 iterations. An automated artifact counting methodology was created to assess the EM algorithm’s performance quantitatively. This methodology is described in the following procedure:

**Artifact Detection Procedure:**

1. Locate a voxel in the raw image which is located in a dark region near the surface of skin. This voxel should have a higher intensity value than the other dark voxels in the dark region. The dark region should not have intensities greater than 50.

2. Consider all voxels having an intensity value that is less than or equal to the selected voxel to be ”dark voxels”.

3. Consider an artifact to be detected when the output of the EM algorithm contains a dark voxel that has increased in value by an amount that is twice the threshold used to designate dark voxels.
The artifact detection procedure above was designed to track a large sampling of voxels which are not expected to significantly increase. This procedure was used to determine which of the candidate PSFs in Chapter 3 is likely to be the most accurate representation of the best experimental PSF suitable for the imaging environment. It is of course possible that the ideal PSF could change depending where the focal point is inside skin. Therefore, the best experimental PSF for the imaging environment can be defined to be the PSF yielding the best results for the image as a whole.

<table>
<thead>
<tr>
<th>PSF Id</th>
<th>Number of Artifacts Detected</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4210963</td>
</tr>
<tr>
<td>2</td>
<td>6844532</td>
</tr>
<tr>
<td>3</td>
<td>2580387</td>
</tr>
<tr>
<td>4</td>
<td>3542092</td>
</tr>
<tr>
<td>5</td>
<td>2278696</td>
</tr>
<tr>
<td>6</td>
<td>1915191</td>
</tr>
<tr>
<td>7</td>
<td>3414974</td>
</tr>
<tr>
<td>8</td>
<td>6099169</td>
</tr>
<tr>
<td>9</td>
<td>5482405</td>
</tr>
<tr>
<td>10</td>
<td>4416204</td>
</tr>
<tr>
<td>11</td>
<td>3308807</td>
</tr>
<tr>
<td>12</td>
<td>6869332</td>
</tr>
<tr>
<td>13</td>
<td>3341426</td>
</tr>
<tr>
<td>14</td>
<td>2707110</td>
</tr>
<tr>
<td>15</td>
<td>6263125</td>
</tr>
<tr>
<td>16</td>
<td>4112226</td>
</tr>
<tr>
<td>17</td>
<td>3078950</td>
</tr>
<tr>
<td>Avg</td>
<td>1517657</td>
</tr>
</tbody>
</table>

Table 3 shows the number of detected artifacts for each of the candidate PSFs mentioned in Section 4.1. The number of artifacts detected represents the addition of all the artifacts counted for all iterations between one and 50. The PSF with the "Avg" Id represents the all the candidate PSFs averaged together after they have been normalized. As a reminder, all PSFs were obtained by manually extracting them from the raw imaging data.
Figure 5.23: Artifacts detected as a function of deconvolution iterations. The PSF yielding the least number of artifacts is the best performing PSF (the averaged PSF). The worst performing PSF is the one yielding the most number of artifacts (PSF with Id 12 in Table 1). Performance of other PSF is not shown due to space constraints.

Table 3 suggests that the best performing PSF is the averaged PSF and the worst performing PSF is PSF 12. Figure 5.23 shows how these best and worst performing PSFs vary for each of the 50 iterations. There is a noticeable transition between 5 and 20 iterations where the number of artifacts detected changes a lot. Figure 5.24 shows a z-slice of the raw image as well as the EM algorithm output after 20 iterations using the averaged PSF and PSF 12. Each image is 275.3 $\mu$m in the horizontal direction and 224.2 $\mu$m in the vertical direction. An abundance of artifacts can be seen in the dark areas of the PSF 12 image when compared to the averaged PSF image. It can also be seen that the region of high signal intensity in the upper-left part of the PSF 12 image appears to cover up the ”dots” in that region which are shown in the raw image. Comparatively, the averaged PSF image tends to retain more of those ”dots” and make them brighter. It would seem that the artifact detection procedure mentioned above is an adequate means to assess performance of the EM algorithm in this application.
Figure 5.24: Comparison of deconvolution artifacts.
Figure 5.24: Comparison of deconvolution artifacts. (cont.)
As previously discussed, the averaged PSF is the best PSF to use for the EM algorithm. An assessment for the optimal number of EM algorithm iterations will now be performed when the averaged PSF is used. Figure 5.27 shows the same z-slice as in Figure 5.24, but when the number of iterations are 1, 10, 20, 30, 40 & 50. It can be seen that a large number of artifacts are present in the previously dark regions of the 50 iterations image. By comparison, the image with one iteration looks nearly identical to the raw image, except that much of the existing data is noticeably brighter. By visual assessment, it would appear that a reasonable number of iterations is 10 because there are a limited number of artifacts seen and the nanopores are well emphasized and less blurry. Figure 5.28 shows the cumulative number of voxel intensity changes for each of the iterations. The trend of voxel intensity changes appears to level off at 10 iterations.
Figure 5.25: Varying the number of deconvolution iterations when using the averaged PSF.
Figure 5.25: Varying the number of deconvolution iterations when using the averaged PSF. (cont.)
Figure 5.26: Varying the number of deconvolution iterations when using the averaged PSF.
(cont.)
Figure 5.27: Varying the number of deconvolution iterations when using the averaged PSF. (cont.)
Figure 5.28: Number of intensity changes for each deconvolution iteration. Deconvolution was performed using the averaged PSF.
CHAPTER VI

RESULTS AND FURTHER ANALYSIS

This Chapter contains additional results supporting that the implementation of the EM algorithm is working when using the data collected for this thesis. Overall conclusions are discussed at the end of this Chapter.

6.1 Deconvolution Without Nanoprobes Inside Skin

Additional experimentation was performed to image a skin sample that was not subjected to the sonophoresis procedure discussed in Chapter 3.1.1. The imaging of this skin sample was identical to the skin imaging procedure in Chapter 3.1.1, except that the number of frames averaged was 10 instead of 20.

The purpose of this new experiment is to assess how skin looks without nanoprobes inside as well as determine how the EM algorithm performs without nanoprobes inside skin. The number of EM algorithm iterations chosen was 10. The experimental PSF used in the EM algorithm was the averaged PSF, as discussed in Section 5.3. As seen in Figure 6.31, 10 iterations shows significant improvement to the image with nanoprobes inside skin.

Figure 6.30 shows raw image data at approximately 20 \( \mu m \), 40 \( \mu m \) and 60 \( \mu m \) inside the skin sample that was not subjected to sonophoresis. Varying skin depths were chosen to assess what the skin looks like at more than one location. As you can see, all three of the skin depth locations show little to no signal strength. The voxel values were multiplied by three in Figure 6.30 to elevate the dark level for visual inspection. It is with high certainty that the darkness of the images in Figure 6.30 was not caused by experimental error. Figure 6.32 shows the histogram of the voxels at 20 \( \mu m \), 40 \( \mu m \) and 60 \( \mu m \) into skin. It can be seen that some signal strength is present, and it decreases with depth into skin, as expected.

Figure 6.29 shows further evidence supporting no experimental error. It can be seen that the surface of the skin shown in Figure 6.29 has areas of high signal strength. The same coverslip used from the procedures explained in Chapter 3.1.1 was used to image the sample of skin that has not undergone sonophoresis. The coverslip was washed, but there were some residual quantum dots that remained and became affixed to the new skin sample. This explains the bright dots seen in Figure 6.29.

The right column in Figure 6.30 shows the output of the EM algorithm when the input was the raw image of the skin sample that has not undergone sonophoresis. It can be seen that tiny, speckle-like, artifacts can be identified. These speckle-like objects appear to decrease in signal strength with respect to skin depth and somewhat resemble the low-level speckle-like objects seen from the raw data, as shown in the left column of Figure 6.30. Therefore, the speckle-like artifacts can be the outcome of low-intensity voxel values being brightened by the EM algorithm, which is undesired.
Figure 6.29: Surface of skin sample that has not undergone sonophoresis.

The left column in Figure 6.31 shows the raw images of the skin sample that has undergone the sonophoresis procedure mentioned in Chapter 3.1.1. Similarly to Figure 6.30, the data at approximately 20 μm, 40 μm and 60 μm inside the skin is shown. However, it can be seen that there is much more signal strength in these raw images when compared to the raw images of the skin sample that has not undergone sonophoresis. As mentioned in Section 2.2, the quantum dot nanoprobes are estimated to be approximately $11.8 - 13.8$ nm in diameter, which is far below the imaging resolution of the Keck microscope. Therefore, it would seem reasonable to believe that most of the signal shown in the left column of Figure 6.31 is caused by lots of quantum dots inside skin.

The right column in Figure 6.31 shows the images from the output of the EM algorithm when the input is the raw image data of the skin sample that has undergone sonophoresis. The EM algorithm was iterated 10 times to get these images. In comparison to the right column in Figure 6.30, much of the signal intensity is caused by brightening the quantum dots shown in the left column. This brightening phenomenon is a desired feature so the quantum dot nanoprobes can be more easily distinguished and perhaps segmented in other applications. Figure 6.32 shows the histograms of each of the images shown in Figures 6.30 and 6.31. It can be seen that the histograms before and after deconvolution are mostly comparable.
Figure 6.30: Results for human skin without nanoprobes. Computed data is the result of 10 iterations of the EM algorithm. The lack of nanoprobes produces nearly no signal, therefore the data was multiplied by three before being shown in the images above.
Figure 6.31: Deconvolution results involving human skin with nanoprobes. Computed data is the result of 10 iterations of the EM algorithm.
Figure 6.32: Histograms of images shown in Figures 6.30 and 6.31. The histogram figures show histograms before and after deconvolution was applied.
Figure 6.32: Histograms of images shown in Figures 6.30 and 6.31. The histogram figures show histograms before and after deconvolution was applied.
Figure 6.32: Histograms of images shown in Figures 6.30 and 6.31. The histogram figures show histograms before and after deconvolution was applied.
6.2 Qualitative Assessment

The images discussed in this section are images resulting from 10 iterations of the EM algorithm. As previously discussed in Section 5.3, 10 iterations was chosen based on our results as a reasonable number of iterations. The raw image data was taken from the data discussed in Chapter 3. Furthermore, the raw data is composed of 20 frames averaged for the reasons discussed in Section 3.1.2. None of the image shown in this section were scaled. The experimental PSF used in the EM algorithm was the averaged PSF discussed in Section 5.3.

Figure 6.33 shows a before and after image of the skin surface. Corneocytes on the stratum corneum can easily be distinguished by the large, edgy structures in the center portion of the images. The bottom image shows how the deconvolution EM algorithm deblurs the raw data. The nanoprobe point-like objects become brighter and easier to see in the image after deconvolution. The distance in the horizontal and vertical directions is $75.2 \, \mu\text{m} \text{ and } 108.3 \, \mu\text{m}$, respectively.

![Figure 6.33: Nanoprobes on surface of skin.](image)

(a) Raw data.

(b) After 10 iterations.
The before and after images in Figure 6.34 show nanoprobes deep in the skin sample at 76 μm into skin. The signal strengths from the nanoprobes are very weak and individual point-like objects depicting the nanoprobes are hard to see, or cannot be seen at all in the raw image data. However the image data after deconvolution shows point-like objects showing where the nanoprobes likely are. The distance in the horizontal and vertical directions is 71.9 μm and 50.0 μm, respectively.

Figure 6.34: Nanoprobes at 76 μm inside skin.

Figure 6.35 shows images from a location at a depth of 50 μm inside skin. The high signal strength and close proximity of the nanoprobes makes it nearly impossible to distinguish individual nanoprobes from each other in the raw image. But, the image shown after deconvolution breaks apart the overlapping nanoprobes into easily distinguishable point-like sources. The distance in the horizontal and vertical directions is 70.2 μm and 50.5 μm, respectively.

Figure 6.35: High signal strength at 50 μm inside skin.
The images in Figure 6.36 depict nanoprobes clinging to the surface of a skin cell that is 46 µm into skin. It can be seen that the boundary of the cell is more distinguishable after deconvolution is applied. The distance in the horizontal and vertical directions is 53.7 µm and 38.8 µm, respectively.

![Figure 6.36](image.png)

(a) Raw data.  (b) After 10 iterations.

**Figure 6.36:** Cell at 46 µm inside skin.

### 6.3 Concluding Remarks

Delivering quantum dot nanoprobes QSH550 into human skin has been accomplished by using low frequency ultrasound having a frequency of 28 kHz. Referenced literature in Section 2.4 suggests rapidly collapsing cavitation bubbles caused by low frequency ultrasound is the likely reason why particles are able to be delivered into skin. The skin preparation procedure discussed in Section 3.1.1 describes the methodology developed to apply sonophoresis. Section 6.1 shows convincing evidence that quantum dot nanoprobes have penetrated deep into skin by comparing images from skin samples with and without sonophoresis applied. Moreover, the analysis in Section 4.2 supports that the experimental PSF has been obtained from within human skin. The experimental PSF results from imaging particles that are sufficiently far below the resolution limit of the microscope. Quantum dot nanoprobes QSH550 are expected to be observed as PSFs when imaged. Section 2.2 mentions that these quantum dots can be estimated to be only 3.8 nm in diameter.

The analysis in Chapter 3 shows that candidate experimental PSFs can be obtained from the raw image data by the aid of an automated nanoprobe detection procedure. This procedure detects nanoprobes that would be good candidates to serve as an experimental PSF. It has been found that the devised sonophoresis procedure mentioned in Section 3.1.1 distributes a large quantity of nanoprobes in a scattered fashion to depths near the dermis-epidermis junction. This procedure has the benefit of delivering a sufficiently large amount of nanoprobes that could possibly be used for diagnosis while also being able to obtain the experimental PSF from several different locations.

The analysis in Sections 5.1 and 5.2 shows the theory how the EM algorithm can be used to deconvolve three-dimensional raw image data obtained from the Keck microscope. Section 5.3 suggests that the best PSF to use with the EM algorithm is the average of the candidate PSFs discussed in Chapter 3.

Sections 5.3, 6.1 and 6.2 together prove that the EM algorithm can successfully be applied to two-photon images depicting quantum dot nanoprobes inside human skin. The qualitative assessment in Section 6.2 concludes that the EM algorithm can help distinguish individual nanoprobes in cases where it is difficult to distinguish these nanoprobes in the raw data. The results shown in Section 6.1 indicate that little to no signal strength is
observed inside skin when using an imaging environment similar to what was discussed in Chapter 3.

The conclusions drawn from this thesis suggest that quantum dot nanoprobes can be delivered deep into human skin by using the procedure mentioned in Chapter 3. Moreover, an experimental PSF obtained from the raw imaging data depicting nanoprobes inside skin can be successfully used in the EM deconvolution algorithm to produce three-dimensional restored images showing nanoprobe locations with better accuracy.

Future research could involve experimentation that would test for the furthest depth the nanoprobes have penetrated subsequent to performing the sonophoresis procedure. Such an experiment could involve slicing the skin sample perpendicular to the skin’s surface after sonophoresis. The inner-side of the skin would then be placed on the coverslip so that it is facing the objective. Imaging in this orientation would allow for observation of nanoprobes at all depths in skin. Additional future experimentation may involve imaging in CRM mode subsequent to imaging in two-photon mode. The information obtained from CRM mode will give useful cellular images that could be overlaid on the two-photon images. Cell health and physical characteristics can be obtained from a CRM image.
APPENDIX A

SPECIFICATIONS

A.1 Quantum Dots

Technical Specification of Quantum Dots with Carboxylic Acid Group

Description: QSH is a group of water soluble CdSe/ZnS quantum dot nanocrystals with amphiphilic polymer coating. Their surface functional group is carboxylic acid. The zeta potential of QSH is from -30mV to -50mV. Their organic layers consist of a monolayer of Octadecylamine and a monolayer of amphiphilic polymer. The thickness of the total organic layers is about 4 nm. The hydrodynamic size of the nanocrystals is about 8-10 nm larger than their inorganic core size measured by TEM.

QSH is very stable in most buffer solutions in the pH range of 3-14.

QSH can be conjugated to protein, peptide and DNA by following our Standard Conjugation Protocol. Ocean NanoTech also offers QSH-Protein Conjugation Kit which includes all the crosslinking agents and buffer solutions. If you need to perform QHP-protein conjugation, we recommend that you remove your original buffer solutions and use our Activation Buffer to disperse your protein for the conjugation. Otherwise, precipitation may occur. If it's your first time to perform this conjugation, you may use BSA as model protein to get familiar with the whole process.

Catalog number: QSH
Product name: CdSe/ZnS core/shell QDs with carboxylic acid group.
Solvent: Water
Surface group: Carboxylic acid
Storage: 4°C; Do not freeze.
pH stability: 4-10
Buffer stability: Borate, Tris, HEPES, PBS, MES, etc.
Shelf life: 12 months
Concentration: 8.0 uM

<table>
<thead>
<tr>
<th>Emission Peak (nm)</th>
<th>QSH530</th>
<th>QSH550</th>
<th>QSH580</th>
<th>QSH600</th>
<th>QSH620</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peak Tolerance (nm)</td>
<td>&lt;35</td>
<td>&lt;35</td>
<td>&lt;25</td>
<td>&lt;25</td>
<td>&lt;25</td>
</tr>
<tr>
<td>FWHM* (nm)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Emission Efficiency**</td>
<td>&gt;50%</td>
<td>&gt;50%</td>
<td>&gt;50%</td>
<td>&gt;50%</td>
<td>&gt;50%</td>
</tr>
<tr>
<td>Surface Coating</td>
<td>Polymer</td>
<td>Polymer</td>
<td>Polymer</td>
<td>Polymer</td>
<td>Polymer</td>
</tr>
</tbody>
</table>

*FWHM: Full Width of Half Maximum
**Emission efficiency was measured by integrating sphere.
Spectra:

Absorbance (a.u.) vs. Wavelength (nm)

Abs of QD530

Abs of QD550

Abs of QD580

PL of QD530

PL of QD550

PL of QD580

Absorbance (a.u.)

Intensity (a.u.)
For R&D only. Not intended for food, drug, household, agricultural, or cosmetic use.
Ocean NanoTech, LLC shall not be held liable for any damage resulting from handling or contact with the above product.
A.2 DTE Scaler

1. The installation and components of equipment

1.1 Instruction
Guilin Woodpecker Medical Instrument Co., Ltd. is a professional manufacturer in researching, developing and producing ultrasonic piezo scalers. The product is mainly used for teeth cleaning and is also an indispensable equipment for tooth disease prevention and treatment. The new product, D1 ultrasonic piezo scaler, has scaling function. It contains the following features:

1. Automatic frequency tracking ensures that the machine always works on the best frequency and performs more steadily.
2. Singlechip controlled, easy operation and more efficient for scaling.

1.2 Components
1.2.1 The components of the machine are listed in the packing list.
1.2.2 Product performance and structure
Ultrasonic piezo scaler is composed of electrocircuit, water way and ultrasonic transducer.
1.2.3 Scope of application
Ultrasonic piezo scaler D1 is used for the dental calculus elimination.

1.3 The main technical specifications
   a) Adapter Input: 100V to 240V~ 50Hz/60Hz  1.2A (Max)
   b) Main unit input: 30VDC  1.3A
   c) Output primary tip Vibration excursion: ≤100μm
   d) Output half-excursion force: <2N
   e) Output tip Vibration frequency: 28kHz ± 3kHz
   f) Output power: 3W to 20W
   g) Main unit fuse: 250VT  1.6AL
   h) Adapter fuse: 250VT  2.0AL
   i) Water pressure: 0.1bar to 5bar (0.01MPa to 0.5MPa)
   j) Weight of main unit: 0.62kg
   k) Weight of adapter: 0.3kg
   l) Operating mode: Continuous operation
   m) Type of protection against electric shock: Class II equipment

01
function eIm=deconvem(psf,cIm,b,iterations,outDir,avgId)
%
%Variables
%psf = 3D point spread function that is the size of cIm
%b = average background intensity
%cIm = 3D captured image
%eIm = 3D estimated image

imSize=size(cIm);

eIm=cIm;
eIm=double(eIm);
cIm=double(cIm);
otf = psf2otf(psf,imSize);

for n=1:iterations
    disp(strcat('starting iteration:',num2str(n)));
    c_psfEImb = real(ifftn(otf.*fftn(eIm)))+b;
    convTerm=cIm./c_psfEImb;
    c_mult=real(ifftn(otf.*fftn(convTerm)));
    eIm=eIm.*c_mult;
end

eIm=uint8(eIm);
REFERENCES


