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Gold Nanoshells: Contrast Agents for Molecular Imaging

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ABSTRACT

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Cancer remains a significant health concern today. It is the 2nd leading cause of death in the United States. Critical to controlling cancer-associated morbidity and mortality is early detection. Early detection strategies include detecting molecular-level changes prior to phenotypic changes, enabling a sufficient amount of time for effective therapies to be implemented. Not only is early detection critical, but issues such as patient safety and cost should be considered when implementing these strategies. This thesis examines work using nanoshell-based optical contrast agents for early cancer detection using scattering-based optical imaging systems.

Metal nanoshells are a novel class of optically-tunable nanoscale material that are composed of a dielectric core (usually silica) surrounded by a metallic shell (usually gold). By systematically varying the ratio between core
diameter and shell thickness, the absorption and scattering maxima can be tuned to different wavelengths including those in the visible and near infrared (NIR).

Specific Aim 1 addresses the fabrication of NIR scattering nanoshells for use as optical contrast agents to enable scatter-based cellular imaging. Specific Aim 2 focuses on using dual NIR absorbing/scattering nanoshells for a nanoshell-based integrated cancer imaging and therapy application. Finally, Specific Aim 3 addresses the diagnostic capabilities of gold nanoshells \textit{ex vivo} using reflectance confocal microscopy (RCM).
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DEDICATION

TO:

GOD, MY GUARDIAN ANGEL (MOM), DAD, AND NAT.
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CHAPTER 1
BACKGROUND

Introduction: For hundreds of years, optical imaging at both macroscopic and microscopic levels has been used as a tool to aid clinicians in establishing a diagnosis. Pathologists routinely use a simple compound microscope to examine stained and sectioned tissue at the microscopic level to definitively determine a diagnosis of cancer. At a macroscopic level, clinicians often rely on observed colors as indicators of physiologic status, for instance, associating yellow skin with jaundice, blue or purple hues with cyanosis, or red areas with inflammation. In each of these examples, the human eye is used to gather qualitative information about the patient’s status based on either the gross visual appearance of tissue or microscopic evaluation.

1 Adapted from:
of stained tissue sections or cytologic samples. Despite the clear importance of such qualitative optical approaches in current medical practice, these strategies are sensitive only to a highly limited subset of the diverse array of optical events that occur when light interacts with biologic tissue. There is a compelling need for the development of more quantitative optical imaging strategies, which can probe tissue physiology in vivo in real time with high resolution and relatively low costs. This chapter describes emerging technologies for quantitative optical imaging and the use of these technologies to diagnose and monitor breast disease.

Over the past decade, advances in fiber optics, light sources and detectors, imaging, and computer-controlled instrumentation have stimulated a period of unprecedented growth in the development of quantitative photonic technologies for a wide variety of diagnostic and therapeutic clinical applications. The use of noninvasive optical techniques for the detection, diagnosis, and monitoring of breast disease is one of the most rapidly emerging areas within the field of biophotonics. In general, optical imaging
strategies rely on changes in how light is scattered, absorbed, or emitted to provide image contrast. These changes may be due to either endogenous chromophores within the tissue or to the addition of exogenous optical contrast agents. In this chapter, we discuss some of the most promising new optical imaging strategies that enable functional and molecular imaging of breast cancer without the use of ionizing radiation. As emerging optical technologies mature, they may be used as clinical tools for early detection, establishing sensitive and specific diagnosis, identifying optimal treatment strategies, and monitoring the efficacy of therapeutic interventions. Although the results of definitive clinical trials are still several years away, we believe that optical imaging will play a vital role in breast cancer diagnosis in the future.

**Theory of Light Transport:** Understanding light propagation in tissue requires a basic understanding of transport theory which we derive below. In the following equations, $I(\vec{r},\hat{s})$ will represent the specific intensity at position $\vec{r}$ in space pointing in the direction $\hat{s}$ as shown in Figure 1.1. The coordinate system used for light intensity and propagation is solid angle
volume elements which utilize the unit steradian. To examine light transport, we treat light as a particle phenomenon in transport theory.

![Diagram of light propagation in tissue using transport theory](image)

**Figure 1.1:** Derivation of light propagation in tissue using transport theory. (Chang et al.).

The radiative equation of transfer for transport theory is (light specific)\(^1\):

\[
\frac{dI(\bar{r}, \hat{s})}{ds} = \nabla \cdot [I(\bar{r}, \hat{s}) \hat{s}] = -\rho \sigma_t I(\bar{r}, \hat{s}) + \frac{\rho \sigma_s}{4\pi} \int_{\Omega} p(\hat{s}, \hat{s}') I(\bar{r}, \hat{s}') d\omega' + \varepsilon(\bar{r}, \hat{s})
\]

\[(1)\]

where \(\rho\) is the particle density, \(\sigma_t\) is the total attenuation coefficient which is the sum of the scattering and absorbing coefficient, \(p\) is the scattering phase function, \(d\omega\) is the solid angle volume element, and \(\varepsilon\) is the source or sink term.
Thus, Equation 1 is another representation of the Continuity Equation written for electromagnetic flux. The change in intensity with respect to a volume element ($ds$) is equal to change in intensity by absorption and scattering ($1^{st}$ term) plus the incident flux from all directions $\hat{s}'$ ($2^{nd}$ term) plus any source or sink of intensity within the volume element ($3^{rd}$ term)$^2$. The goal of this section is to derive the radiative equation of transfer above that defines light propagation.

**Figure 1.2:** Derivation of the radiative equation of transfer. (Chang et al.).
As shown in Figure 1.2, for a given volume element, one can see that the specific incident intensity from the incoming and outgoing light is given by the following conservation of energy expression:

\[ dI = dI_{\text{outgoing}} + dI_{\text{incoming}} + dI_{\text{source}} \]

(2)

Approaching this derivation using the differential form, outgoing intensity, the first term on the right side of Equation 2, represents the change in intensity by absorption and scattering. It is derived from the product of incoming intensity, the degree of scattering and absorption \((\sigma_a + \sigma_s)\), and the number of particles scattering/absorbing the incoming flux \((\rho \, ds)\).

\[ dI_{\text{outgoing}} (\hat{r}, \hat{s}) = -\rho \, ds \, (\sigma_a + \sigma_s) \, I = -\rho \, ds \, (\sigma, I) \]

(3)

Incoming intensity, the second term on the right side of Equation 2, represents the total incident flux from all directions \(\hat{s}'\). First, let us define the scattering amplitude as a function \(f(\hat{s}, \hat{s}')\) which dictates the behavior of the scattered wave determined experimentally. To determine the total flux in all
directions, we first examine a wave incident on a single particle. We can define the incident flux density per particle through a small solid angle as:

\[ S_i = I(\hat{r}, \hat{s}) \, d\omega' \]

\[ (4) \]

Then, the flux density incident on particles on a volume \( ds \) is given by \( S_i \, \rho \, ds \).

Recall that the power flux density field of a scattered wave a distance \( R \) from the particle is defined (similar to an electric field equation) as\(^1\):

\[ S_r = \left( \frac{|f(\hat{s}', \hat{s})|^2}{R^2} \right) S_i \]

\[ (5) \]

To find the flux, we simply multiply \( S_i \) by the square of the distance \( R \).

The substitution of the incident flux density (Equation 4) into Equation 5 yields the equation for scattered flux\(^1\):

\[ S_r R^2 = |f(\hat{s}', \hat{s})|^2 S_i = |f(\hat{s}, \hat{s}')|^2 I(\hat{r}, \hat{s}) \, d\omega \]

\[ (6) \]
The incident flux throughout the medium can be found by summing the effects of scattered flux on $\rho \, ds$ particles by integrating over all directions $\omega'$ of the entire sphere (4\pi steradians):

$$\text{Incident flux} = \int_{4\pi} \rho \, ds \left| f(\hat{s}, \hat{s}') \right|^2 I(\hat{r}, \hat{s}') \, d\omega'$$

(7)

This expression simplifies further since the phase function is defined as$^1$:

$$p(\hat{s}, \hat{s}') = \frac{4\pi}{\sigma_t} \left| f(\hat{s}, \hat{s}') \right|^2$$

(8)

Solving for the scattering function and substituting into the incident flux (Equation 7) throughout the medium yields the second term of Equation 1:

$$dI_{\text{incoming}} = \frac{\rho \sigma_t}{4\pi} \int_{4\pi} p(\hat{s}, \hat{s}') I(\hat{r}, \hat{s}) \, d\omega' \, ds$$

(9)
Finally, the third term of the Transfer Equation (Equation 1) represents a source or sink at position $\vec{r}$ in space emitting in direction $\hat{s}$ within the volume element $ds$. It is simply defined as:

$$dI_{source} = \varepsilon(\vec{r}, \hat{s}) \, ds$$

(10)

To represent a point source located at $\vec{r}_0$ that radiates power $P_0$ (W/Hz) uniformly in all directions, $\varepsilon(\vec{r}, \hat{s})$ is defined as:\n
$$\varepsilon(\vec{r}) = \frac{P_0}{4\pi} \delta(\vec{r} - \vec{r}_0)$$

(11)

Summing all the intensity differentials ($dI_{outgoing}, dI_{incoming}, dI_{source}$) and dividing through by the volume element $ds$ yield the Transfer Equation (Equation 1). This equation dictates the propagation of light in a medium such as tissue. For more complex geometries, the integral formulation for deriving the equation of transfer is used instead. Solving the transport equation for complex geometries requires numerical techniques since boundaries become
too complicated to solve the equation analytically. This process is computationally intensive but manageable on high speed personal computers today.

**Non-Dimensionalization of the Equation of Transfer:** The equation of transfer derived in this paper can be non-dimensionalized by converting the units into optical distance ($\tau$). The purpose of non-dimensionalizing is to develop a scalable general solution that is independent of units. To non-dimensionalize the equation, we utilize the optical distance $\tau$ defined as$^1$:

$$\tau = \int\rho \sigma_i ds$$

(12)

This term is used to convert the equation of transfer to dimensionless form. An optical distance of $\tau = 1$ implies that, over this distance, the power flux diminishes to *incident flux* by scattering and absorption. It is synonymous to the time constant in an RC circuit. By applying the following relations

$$\frac{dl}{d\tau} = \frac{dl}{ds} \frac{ds}{d\tau} \quad \text{and} \quad \frac{ds}{d\tau} = \frac{1}{\rho \sigma_i}$$

(13 a, b)
and multiplying the equation of transfer (Equation 1) by $ds/d\tau$ (Equation 13b) to obtain $dl/d\tau$, the resulting dimensionless representation is:

$$\frac{dl(\vec{r}, \hat{s})}{d\tau} = -I(\tau, \hat{s}) + \frac{1}{4\pi} \int_{4\pi} p(\hat{s}, \hat{s}') I(\tau, \hat{s}) d\omega' + \frac{\varepsilon(\tau, \hat{s})}{\rho \sigma_t}$$

(14)

This representation is known as the Boltzmann transport equation, which is also used in neutron transport calculations. Equation 14 can be applied to the propagation of both visible or infrared light in tissue\(^1\).

For an incident light, no diffuse radiation is entering the medium at the surface since diffuse radiation is often generated by scattered photons. To model this effect, the standard boundary conditions for the transport equation should not allow a surface source of photons since no photons are scattered at the surface. Figures 1.3 and 1.4 illustrate the scattering of light within medium such as tissue.
Figure 1.3: Scattering of light within tissue. (Chang et al.).

Figure 1.4: Scattering of light within tissue. (Chang et al.).
First Order Multiple Scattering Solution: First order multiple scattering is a model that states incident light undergoes no more than a single scattering event while the remaining photons are attenuated resulting in a negligible amount of diffuse radiation ($I_d$). This model is often used for optical scattering in tissue. The results are first order because the total intensity $I$ illuminating the particle in the medium is approximately equal to the known incident intensity $I_n$ since there is no diffuse light. This assumption results in the integral form of the transport equation to be\(^1\) [Note: derivation of the integral form of Equation 1 not shown]:

\[
I_d = \int e^{-(r-n)} \left[ \left( \frac{\rho \sigma}{4\pi} \right) \int \int \int p(\hat{s}, \hat{s}') I(\hat{r}, \hat{s}') d\omega' + \varepsilon(\hat{r}, \hat{s}) \right] ds
\]

(15)

The first order assumption applies only to situations in which the density of scatterers is low resulting in an incoherent power (light rays that are
out of phase) considerably smaller than the coherent power (light rays that are in phase). This occurs in two situations:

- Plane waves incident on a medium with random particles that are mostly absorbing ($W_0 < 0.5$) and with optical distances about $< 0.4$.

The *albedo*, $W_0$, represents the degree of scattering within the tissue given by the ratio of the scattering cross section $\sigma_s$ to the total cross section $\sigma_t$:

$$W_0 = \frac{\sigma_s}{\sigma_t} = \frac{\sigma_s}{\sigma_s + \sigma_a} = \frac{1}{4\pi} \int_{4\pi} p(\hat{s}, \hat{s}) d\omega$$

(16)

- Waves are confined to a small angular region such as a narrow beam from a transmitter. This results in light penetrating greater optical distances, especially when particles in the medium is absorbing ($W_0 < 0.9$).

These models have provided insight into the various parameters that influence light propagation in tissue. By understanding how each parameter
affects the incoming and outgoing light, we are better aware of the principle advantages and shortcomings of various optical imaging modalities which are discussed in the next section.

**Optical Imaging for Breast Tumor Characterization:** Optical imaging encompasses a wide variety of diverse technologies that use light wavelengths ranging from the ultraviolet (UV) to the near infrared (NIR) to probe a tissue’s scattering, absorption, and fluorescence properties. Measurement of these fundamental optical properties can be used to quantify tissue biochemical, morphologic, and architectural features ranging from oxy- and deoxyhemoglobin levels to blood oxygen saturation to metabolic status to the size distributions of significant scatterers such as cell nuclei within the tissue. For specific clinical applications, optical methods provide a number of advantages over conventional imaging modalities such as radiography, ultrasonography, computed tomography (CT), magnetic resonance imaging (MRI), and positron emission tomography (PET). Optical methods provide new contrast mechanisms directly related to tissue physiology and do not
require the use of ionizing radiation. Optical approaches can be developed for a variety of resolution scales ranging from the micron level to full-organ imaging. Furthermore, optical methods can provide functional imaging at significantly reduced cost compared with imaging strategies such as MRI and PET and do not require the use of exogenous contrast agents.\textsuperscript{3-6}

Optical technologies can be used to image both preinvasive disease processes with lesions located in accessible epithelial tissue and to probe tumors located deep within the breast. However, the imaging methods used for superficial and deep tissue imaging differ significantly in both achievable resolution and the specific physiologic tissue parameters that may be probed. Optical techniques for superficial imaging use light in the visible portion of the electromagnetic spectrum and can provide resolution from the subcellular (1 μm) to the cellular (10–15 μm) level using imaging strategies such as confocal reflectance microscopy and optical coherence tomography. These techniques offer a mean to visualize nuclear morphology \textit{in vivo} in real time\textsuperscript{7}, providing
much of the same information content as a conventional histologic section without the need to remove, section, or stain the tissue.

Optical technologies may also be used to probe large tissue volumes. In imaging deep into a tissue, the influence of scattering becomes particularly significant, decreasing image resolution. Unlike x-ray photons, which travel in a straight line as they move through tissue, light photons scatter and diffuse as they move through tissue. In addition, light propagation through tissue is influenced by absorption from chromophores such as hemoglobin. To minimize the difficulties posed by tissue scattering and absorption, deep tissue imaging is accomplished using NIR wavelengths. At these wavelengths, tissue scattering and absorption are relatively low, and light can penetrate centimeters into the tissue at power levels that fall below American National Standards Institute (ANSI) standards\textsuperscript{3,5,6}. We begin our in-depth discussion of optical imaging by considering optical methods for deep tissue imaging because these methods were the first developed from a chronologic perspective.
The first known attempt at using an optical imaging method to detect breast cancer was a study conducted in 1929 by Cutler. Using a technique known as transillumination, a strong light source was shone through the breast, and the shadows cast by breast lesions were visualized. Transillumination was based on the idea that malignant lesions should absorb more light and, thus, appear darker owing to heightened vascularity and hemoglobin content. Transillumination does work to a certain degree but is unable to distinguish malignancies from other benign lesions, such as fibroadenomas, which also demonstrate increased absorption. Additional limitations of early transillumination work included lack of a method for quantification of tissue absorption and poor resolution of tissue architectural information owing to scattering within the tissue. During the 1970s and 1980s, wavelengths of light restricted to the red and the NIR regions were used in a similar imaging method called diaphanography. A series of clinical trials was conducted to compare diaphanography against mammography, with mixed results. Fundamentally, diaphanography encountered the same limitations seen in
transillumination: difficulty in differentiating between benign and malignant tissue. Additional limitations of diaphanography included inadequate tissue penetration, lack of a method to address distortions owing to tissue scattering, poor dynamic range, and poor spatial resolution\textsuperscript{5}. Although diaphanography did not become an accepted clinical strategy owing to insufficient sensitivity and specificity, it is worth noting that a more advanced form of transillumination imaging has recently been developed that relies on picosecond to femtosecond light pulses to create a time-gated image of breast structure that mitigate tissue scattering distortions\textsuperscript{8}, removing a fundamental limitation of previous work. The sensitivity and specificity of this more sophisticated transillumination approach have not yet been reported in the literature.

A more recent development in optical imaging of breast tissue is a technique known as diffuse optical tomography (DOT). DOT is a technology that provides three-dimensional tissue imaging via acquisition of diffuse light images at multiple projections. DOT offers several advantages over
transillumination and diaphanography. Most significantly, specific biochemical information can be accurately quantified. As an example of the type of biochemical information that can be quantified, the main absorbers of NIR light in the breast are oxy- and deoxyhemoglobin. The extinction spectra of these significant tissue chromophores are shown in Figure 1.5.

![Molar Extinction Coefficient Graph](image)

**Figure 1.5:** Plot of molar extinction coefficient as a function of wavelength for oxyhemoglobin and deoxyhemoglobin. Spectral data obtained from Prahl.9.

Measuring oxy- and deoxyhemoglobin provides information about tissue vascularity and oxygenation status, which, in turn, offer the clinician an indication of the degree of angiogenesis and hypoxia, two correlates of
malignancy. Depending on the particular technique used, DOT can uniquely quantify absorption, scattering, and fluorescence lifetimes within a tissue. Because DOT is a tomographic technique, lesion size is readily assessed. There is a potential role for DOT in breast cancer detection because it offers a unique mechanism for measuring functional characteristics that are not readily probed using other imaging methods\textsuperscript{6,10}. However, because DOT is a relatively new technology, the limits of detection, probing depths, and diagnostic potential have not been definitely established.

In DOT imaging, light at either a single or multiple wavelengths is delivered to the breast tissue. As photons of light enter the breast tissue, absorption and scattering events occur. Scattering refers to changes in the direction in which a photon is moving owing to fluctuations in tissue index of refraction; absorption refers to conversion of a photon’s energy into heat within the tissue. A complete treatment of scattering and absorption in biologic tissue may be found in Welch \textit{et al}\textsuperscript{11}. To allow three-dimensional reconstruction of tissue optical properties based on measured data, a theoretic
forward model, generally based on computationally or analytically solving the diffusion approximation, is used to predict measured optical data given a particular measurement geometry. The forward model is then used to develop an inversion approach that can be applied to the measured data. Although the details of these analysis methods are not discussed in this chapter, determining optimal mathematical approaches to reconstruction of measured DOT data is presently an area of active research\textsuperscript{5,6,12-14}.

Advances in DOT during the 1990s focused on the use of improved light sources to yield higher-resolution images. Prior to the 1990s, many DOT systems employed constant-intensity light. By using photon pulse methods, it is possible to independently sample scattering and absorption events within the tissue. The separation is possible because the temporal profile of the light source is broadened by multiple scattering processes within the tissue in a manner that can be predicted using analytic techniques. It is also possible to separate absorption from scattering using frequency-domain techniques. When light of modulated intensity propagates through a highly scattering
medium such as biologic tissue, diffuse photon density waves move through the tissue with a diffusion front. It is possible to separate absorption and scattering properties of the sample using megahertz diffuse photon density waves. It should be noted that time-domain (pulse based) optical imaging and frequency-domain imaging methods are essentially equivalent and can be mathematically related to each other through a Fourier transformation. Frequency-domain optical imaging offers specific advantages over time-domain imaging, including reduced instrumentation costs and more straightforward analysis\textsuperscript{5,15}.

When DOT is used in a diagnostic setting for breast cancer screening, it is sometimes referred to as optical mammography. The clinical goal of optical mammography is to improve on conventional mammography, increasing sensitivity and specificity through access to additional biochemical parameters. Additional advantages of DOT include the potential for increased cost-effectiveness and increased patient comfort because breast compression is not required. It is envisioned that optical mammography would initially be used
as an adjunct to traditional mammography, particularly for high-risk women, in an attempt to achieve breast cancer detection at an earlier stage. However, it must be emphasized that testing of this technology in a sufficiently large patient population to rigorously assess the diagnostic capability of the technique has not yet occurred. Although large trials of this technology are not completed, a number of research groups are actively developing optical imaging systems for breast cancer detection based on either continuous wave imaging, pulsed light imaging, or light of modulated intensity. Current research is assessing several critical issues: (1) quantifying expected variations in measured data within the normal patient population, (2) determining the extent to which it is possible to consistently discriminate between benign and malignant lesions, and (3) correlating measured optical scattering and absorption parameters to underlying physiologic processes\textsuperscript{3-6,12,16,17}.

Light propagation through tissue is a complex process impacted by multiple parameters that may vary not only with disease processes under investigation but also with patient age, menstrual cycle, and menopausal status.
Biochemical and structural features ranging from the amount of adipose tissue present to tissue water content to collagen structure, in addition to the presence of hemoglobin within the tissue, can dramatically impact measured signals\textsuperscript{3,5,18-20}. For example, the amount of water and adipose tissue in a given individual will vary and is influenced by factors including age, body mass index, stage of menstrual cycle, menopausal status, use of hormone replacement therapy, pregnancy, and lactation\textsuperscript{5}. To understand the complexities of light propagation through breast tissue, much work has focused on assessment of breast tissue properties within normal subjects\textsuperscript{17}. Further studies are then considering distinguishing normal from malignant breast tissue.

Optical imaging offers a promising new approach as an adjunctive tool in the assessment of breast and other solid tumors. NIR frequency-domain optical methods provide the ability to noninvasively image functional tissue characteristics, including total hemoglobin concentration, oxy- and deoxyhemoglobin levels, water and lipid content, and scattering parameters
correlated to cellular composition at relatively low cost and without the use of ionizing radiation. Preliminary studies have successfully demonstrated that optical measurements can be correlated to malignancy. Studies have also indicated that optical signals are keenly sensitive to other variables, such as patient age and hormonal status, suggesting that optical data will be characterized by a high degree of variability, requiring clinical trials with substantial sample sizes to thoroughly evaluate the technology. Although a number of studies involving small numbers (< 100) of patients are reported in the literature, there are no published reports documenting the diagnostic capabilities of optical imaging compared with mammography in a large screening population. However, several significant studies are under way, and more conclusive results are expected over the next 3 to 5 years.

**Optical Spectroscopy of Breast Tissue:** The previous section discussed the development of technologies using NIR optical imaging to assess breast tissue physiology in a noninvasive manner. This section considers optical spectroscopic approaches that instead use UV and visible
wavelengths to characterize the biochemical and morphologic properties of normal and pathologic breast tissue. A fundamental difference between UV and visible light and light in the NIR regions of the electromagnetic spectrum is a decrease in tissue penetration depth, which occurs at the shorter wavelengths. As a simple example of the wavelength dependence of penetration through biologic tissue, consider shining a flashlight against your hand in a dark room. The light that is transmitted through the tissue appears red in color. Wavelengths of light under approximately 600 nm do not penetrate tissue deeply owing to significant absorption from hemoglobin in the tissue. Wavelengths longer than 1,000 nm likewise do not achieve deep penetration because this light is quickly absorbed by water within the tissue. Between the wavelengths of 600 and 1,000 nm is a region of high tissue transmissivity known as the optical window. This wavelength region is exploited in the imaging applications we have described so far\textsuperscript{21}. However, in some diagnostic and therapeutic applications, it is not necessary to penetrate deeply (centimeters) into the tissue. In these instances, optical spectroscopic
approaches, including reflectance spectroscopy and fluorescence spectroscopy of UV and visible light, can be used to assess tissue physiologic status.

Fundamentals of Reflectance and Fluorescence Spectroscopy:

Spectroscopy may be defined as the study of light interactions as a function of wavelength. Diffuse reflectance spectroscopy, sometimes referred to as elastic scattering spectroscopy, is a simple, fast, and cost-effective method to probe tissue scattering and absorption characteristics. Most of the published work considering the application of reflectance spectroscopy for the assessment of breast tissue pathology has been conducted by Bigio and colleagues\textsuperscript{20}, Perelman, Backman, Feld and colleagues are responsible for significant advances in the meaningful analysis of measured spectral data\textsuperscript{22-24}. For a thorough review of diffuse reflectance spectroscopy, the reader is referred to Mourant and colleagues\textsuperscript{25}, Bigio and Mourant\textsuperscript{26}, and Wilson and Jacques\textsuperscript{27}.

The instrumentation used for reflectance spectroscopy consists of light delivery and collection fibers, a spectrophotometer, and a computer. Light is delivered to and collected from breast tissue through a series of optical fibers.
When the distance between the light source and the light collector is less than 0.1 cm, as is the case in endoscopic applications or when inserting fibers through a transdermal needle, the light detected is highly sensitive to changes in tissue morphologic structure. Light scattering within the tissue occurs owing to small-scale variations in refractive index and is sensitive to changes in nuclear size, shape, deoxyribonucleic acid (DNA) content, and chromatin texture\textsuperscript{28}. Reflectance spectroscopy is also sensitive to tissue absorption, which is dominated by the characteristic signatures of oxy- and deoxyhemoglobin. Diffuse reflectance spectra are readily measured \textit{in vivo} owing to intrinsically strong elastic scattering signals. The measured spectra plot the wavelength-dependent light intensity remitted from the tissue and may be post processed to extract quantitative features such as chromatin content or nuclear size distributions.

Although reflectance spectroscopy approaches have been applied to numerous organ sites, including the colon\textsuperscript{29}, brain\textsuperscript{30}, bladder\textsuperscript{31}, and skin\textsuperscript{32}, relatively little work has considered the application of reflectance
spectroscopy to breast disease. Bigio and colleagues have performed preliminary investigations of reflectance spectroscopy for *in vivo* breast diagnostics\(^{20}\). In their study, a pulsed xenon arc lamp was used to collect spectral data between 330 and 750 nm. An initial study of 31 women (72 histology sites) yielded a sensitivity of 69% and a specificity 85% for discrimination of malignant breast tissue from benign tissue using artificial neural network analysis. In a more extensive study recently published by Lee and his clinical collaborators, optical spectra were collected for 94 breast tissue samples\(^{33}\). Elastic scattering spectroscopy correctly diagnosed 87 of 88 normal breast tissues and 15 of 16 breast cancers using model base analysis. Additional efforts by Bigio are aimed at real-time assessment of the sentinel lymph node during surgery.

Fluorescence spectroscopy is another spectroscopy technique that probes tissue at the molecular level by monitoring the emission of light from endogenous fluorophores within the tissue. Before discussing the details of this approach, it is necessary to briefly review the physics involved in the
fluorescence generation process. Figure 1.6 displays a Jablonski diagram that shows the various electronic energy level transitions relevant to fluorescence generation. At room temperature, most molecules reside in the ground electronic energy level labeled S0 in the diagram. After absorption of electromagnetic radiation by a UV or visible excitation photon, electrons are elevated from the ground state to higher electronic levels, labeled S1 and S2 in the diagram. Because the higher excited states are not stable, the electrons will return to the ground state. The electrons first relax to the lowest vibrational energy level of the first electronic energy level, S1, via a radiationless process (internal conversion) in which the spin state does not change. The electrons can then move to the ground state by spontaneous emission of a fluorescence photon with the same spin state. Note that there are other means by which electrons may return from S1 to the ground state that are not illustrated in the figure.
Figure 1.6: The generation of fluorescence is illustrated in a classic Jablonski diagram. S0, S1, and S2 refer to the ground, first, and second electronic energy levels. At each electronic energy level, there are a number of possible vibrational states represented by the parallel lines. After a fluorophore absorbs a photon of excitation light, electrons are elevated from the ground electronic level (S0) to higher electronic energy levels (S1, S2). Fluorescence is the emission that accompanies the transition from the first electronic level (S1) to the ground energy level (S0). (Chen et al.).

Tissue fluorescence is generated due to a large number of endogenous biologic fluorophores, including the aromatic amino acids (tryptophan, phenylalanine, and tyrosine), cross-links associated with the structural proteins collagen and elastin, porphyrins, and the coenzymes nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide (FAD). Table 1.1 provides a detailed list of common biologic fluorophores and their excitation
and emission peaks. Because the pyridine nucleotides and flavins play an integral role in cellular metabolism, it is possible to indirectly assess the tissue metabolism by monitoring changes in the concentrations of these electron carriers. The reduced form of NAD (NADH) and the oxidized form of FAD are fluorescent, whereas the oxidized electron carrier NAD+ and the reduced electron carrier FADH2 are nonfluorescent. Thus, fluorescence measurements provide a useful method for noninvasively monitoring changes in metabolism. Tissue metabolic state is sometimes described by calculating the redox ratio, a quantity obtained by dividing the fluorescence of FAD by the sum of the fluorescence of FAD and NADH. The redox ratio, which typically decreases in cancer, is sensitive to changes in metabolic rate and vascular oxygen supply\textsuperscript{34,35}. 
**Table 1.1:** Endogenous fluorophores at physiologic pH

<table>
<thead>
<tr>
<th>Chromophore</th>
<th>Absorption Maxima (nm)</th>
<th>Molar Extinction Coefficient (nm)</th>
<th>Excitation Maxima (nm)</th>
<th>Emission Maxima (nm)</th>
<th>Fluorescence Quantum Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>HP, collagen, elastin</td>
<td>325</td>
<td></td>
<td>325</td>
<td>400</td>
<td></td>
</tr>
<tr>
<td>LP, collagen, elastin, Collagen powder</td>
<td>325</td>
<td></td>
<td>280, 265</td>
<td>310, 385, 390, 530</td>
<td></td>
</tr>
<tr>
<td>Elastin</td>
<td></td>
<td></td>
<td>350, 410</td>
<td>420, 500</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>450</td>
<td>520</td>
<td></td>
</tr>
<tr>
<td>Tryptophan</td>
<td>280</td>
<td>5.6 x 10^{-3}</td>
<td>280</td>
<td>350</td>
<td>0.2</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>275</td>
<td>1.4 x 10^{-3}</td>
<td>300</td>
<td></td>
<td>0.1</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>260</td>
<td>2.0 x 10^{-3}</td>
<td></td>
<td>280</td>
<td>0.004</td>
</tr>
<tr>
<td>NADH</td>
<td>260, 340</td>
<td>14.4 x 10^{-3}, 6.2 x 10^{-3}</td>
<td>290, 340</td>
<td>440, 450</td>
<td></td>
</tr>
<tr>
<td>NAD+</td>
<td>260</td>
<td>18.0 x 10^{-3}</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FAD</td>
<td></td>
<td></td>
<td></td>
<td>450</td>
<td>515</td>
</tr>
</tbody>
</table>

Adapted from Richards-Kortum and Sevick-Muraca [75]

HP = hydroxylysylpyridinoline;
LP = lysylpyridinoline;
NADH = reduced nicotinamide adenine dinucleotide;
NAD+ = oxidized nicotinamide adenine dinucleotide;
FAD = flavinadenine dinucleotide.

Fluorescence spectroscopy has been evaluated as a clinical tool in a number of organ sites, including the lung [36], cervix [37-39], colon [40,41], esophagus [42], bladder [43,44], brain [45], and skin [46,47]. See Wagnieres and colleagues for a thorough review of work in this field [48]. One of the earliest studies using fluorescence spectroscopy for breast cancer applications was conducted by Alfano and colleagues during the 1980s [36]. His group observed a significant
difference in the static and time-resolved fluorescence of normal and malignant breast tissues\textsuperscript{49}. Alfano and coworkers demonstrated that benign and malignant breast tissue could be distinguished using a 300 nm excitation source\textsuperscript{50}. More recently, the same group has investigated fluorescence imaging of the breast using an acousto-optic tunable spectroscopic imager\textsuperscript{51}. The ratio of fluorescence emission at 340 and 440 nm was used to differentiate between normal and malignant regions of breast tissue. Gupta and coworkers have completed an \textit{ex vivo}\textsuperscript{52} and an \textit{in vivo}\textsuperscript{53} study of breast autofluorescence signatures. In their \textit{in vitro} study, the group reported specificity and sensitivity in excess of 99\% using tissue from 63 patients. In this study, a 337 nm nitrogen laser was used to obtain spectra from benign fibroadenomas, ductal carcinomas, and normal tissues. Although spectral shapes were similar for all tissue types, integrated fluorescence emission intensities were significantly different, with ductal carcinomas having the highest values and benign fibroadenomas the lowest. Malignant tissue was discriminated from other categories based on the integrated fluorescence emission. The group’s \textit{in vivo}
study used a time-resolved and steady-state autofluorescence spectroscopy at 310 nm excitation. Sensitivity and specificity of 100% were reported for the discrimination of normal from malignant tissue. However, only 11 patients were examined. Again, integrated intensities were used as the discrimination feature. As indicated by the sparseness of literature reports in this area, the application of fluorescence spectroscopy to diagnosis of breast disease remains relatively unexplored. Future work must consider more systematic examination of the potential of this technique.

**Clinical Applications of Optical Spectroscopy:** Several emerging breast cancer applications rely on spectroscopy’s ability to provide real-time assessment while probing physiologic parameters that complement data obtained using conventional mammography. The first and most invasive application is the evaluation of suspicious breast lesions using small optical fibers inserted through a transdermal needle guided by a different imaging modality, such as ultrasonography. Here spectroscopy serves as an adjunct to, or perhaps eventually a replacement for, biopsy and would be used to provide
functional information to discriminate benign from malignant lesions after an abnormality warranting further evaluation was detected during mammography.

Optical spectroscopy offers the advantage of instantaneous tissue assessment, eliminating the anxiety caused as the patient waits for a biopsy result. Even if spectroscopy were not used to replace biopsy but rather to provide an initial impression, which would then be confirmed via biopsy, anxiety could be significantly reduced in the portion of the patient population provided with the negative spectroscopy result. Moreover, multiple suspicious tissue areas can be probed using spectroscopy. Furthermore, as new non-surgical treatment methods are developed and validated, spectroscopy could facilitate combined detection and treatment in a single visit for some patients, reducing health care costs and loss to follow-up. Another emerging application of optical spectroscopy is its use during surgical procedures to provide real-time detection of tumor margins or to provide intraoperative assessment of the sentinel node. Finally, spectroscopy may be implemented through the instrument channel of existing microendoscopes and could be used to provide
quantitative morphologic and biochemical evaluation of lesions discovered during ductoscopy. Initial clinical studies of optical spectroscopy for transdermal diagnosis, intraoperative tumor margin detection, and evaluation of sentinel nodes during surgery have been conducted by Bigio and colleagues with promising results\textsuperscript{20}, and trials recruiting increased numbers of patients are currently under way.

**Contrast Agents for Optical Imaging:** In general, optical imaging strategies rely on changes in how light is scattered, absorbed, or emitted to provide image contrast. These changes may be due to either endogenous chromophores within the tissue or to the addition of exogenous optical contrast agents. Exogenous agents have the advantage of providing clinicians and researchers the ability to select and know the photophysical and pharmacokinetic properties in advance. While the ability to control optical properties is ideal, issues of safety and toxicity of contrast agents need to be examined as well as determining the optimal time delay for imaging after contrast administration. As emerging optical technologies mature, they may
be useful clinical tools in determining diagnoses, identifying optimal treatment strategies, and monitoring the efficacy of therapeutic interventions. The use of contrast agents have generated considerable interest as a method to enhance existing and developing biomedical imaging modalities\textsuperscript{54}. Currently, there are already several types of contrast agents deployed for use in imaging methods such as Magnetic Resonance Imaging (MRI) and Computed Tomography (CT). Agents such as barium\textsuperscript{55}, iodine\textsuperscript{56} and barium sulphate\textsuperscript{57} have been used for many years in enhancing CT images. These agents help block out or weaken x-rays passing through organs and, thus, the organs are contrasted in the developed CT image. Typically, gadolinium-based contrast agents are commonly used in MRI\textsuperscript{58}. As MRI utilizes spin relaxation times of protons, these agents produce contrast against tissue with very different relaxation times. The region of interest can be highlighted, for example, through blood pooling in the bloodstream or by highly vascular regions such as the liver, or via targeting to tumors. Because optical imaging is a relatively new field, contrast agents are not yet routinely applied. Researchers such as Vargas and
colleagues\textsuperscript{59} are examining the effects of various agents in altering the optical properties such as in skin. Vargas and colleagues have studied agents that reduce the highly scattering properties of skin to promote better interrogation beneath the tissue optically\textsuperscript{60}. The sensitivity of many current optical imaging methods is based completely on the contrast differences between the absorption and scattering coefficients or the fluorophore levels of malignant and normal tissues. This dependence on contrast in imaging is the reason for the interest in developing exogenous contrast agents that would selectively target one type of tissue and enhance the contrast of optical data.

Recent work has demonstrated the potential of a NIR dye, indocyanine green (ICG), for breast optical imaging. Approved by the US Food and Drug Administration, ICG is a dye with excitation and emission peaks near 780 nm and 830 nm, respectively. These peaks are within the spectral region or "optical window" and are particularly useful in biologic applications because absorption is at a minimum. ICG is commonly used as an intravascular contrast agent during angiography\textsuperscript{61}. When used for tumor imaging,
preferential uptake of ICG by tumors occurs owing to leaky vasculature. Recent studies by Ntziachristos and colleagues have demonstrated that ICG enhances detection of breast tumors by DOT\textsuperscript{6,62,63}. Additional optical imaging studies have also documented favorable results based on the accumulation of ICG in tumors\textsuperscript{64,65}. ICG-assisted imaging has been used in breast cancer patients to guide sentinel node biopsy. Motomura and colleagues demonstrated that by injecting ICG near the tumor tissue, over 70% of sentinel nodes were identified using ICG-guided sentinel node biopsy\textsuperscript{66}. To further improve the accuracy of ICG imaging and decrease the false-negative rates, improvements in optical tomography instrumentation are being coupled with the use of ICG to more accurately reconstruct three-dimensional maps of the breast tissue\textsuperscript{67}. The advantages of ICG-based optical imaging over other potential imaging modalities include the decreased cost of optical imaging compared with competing approaches and sensitivity to very low levels of fluorophore concentration.
Nanoshell-based Contrast Agents: Until recently, ICG was the principal optical contrast agent available. Over the past several years, the expanding availability of a variety of nanostructures with highly controlled properties has provided a series of potential new contrast agents for optical imaging. Typically, nanostructures possess properties far superior to the molecular species they replace: higher quantum efficiencies, greater scattering or absorbance cross-sections, optical activity over more biocompatible wavelengths, and significantly increased chemical or photochemical stability.

The systematic control of nanostructure properties obtained by specific variations in particle size is in direct contrast to molecular tags, whose properties vary nonsystematically between molecular species. Examples of nanomaterials under development as optical contrast agents include gold colloid\textsuperscript{68}, metal nanoshells\textsuperscript{69,70}, and quantum dots\textsuperscript{71,72}.

Metal nanoshells are examples of nanostructures\textsuperscript{73,74} ranging in size of 75nm-300nm which are robust absorbers and scatterers in the NIR. They are composed of a dielectric silica core surrounded by a very thin metallic shell,
which is typically gold. Nanoshells possess a strong optical resonance whose wavelength can be tuned across much of the visible and infrared region of the spectrum by varying the relative size of the core and shell layer\textsuperscript{70}. By varying the absolute nanoparticle size, the relative contributions of scattering and absorption at a given wavelength of interest can be controlled\textsuperscript{69}. This extremely agile tunability of the optical resonance is completely unique to nanoshells. Gold nanoshells are capable of scattering light in the NIR, and provide appealing optical properties for use in conjunction with reflectance-based optical imaging methods. Additionally, the gold surface is biologically inert and allows proteins to be readily conjugated facilitating \textit{in vivo} use\textsuperscript{75}. Although the gold surfaces of nanoshells are generally considered to be biocompatible, stealthing polymers such as polyethylene glycol (PEG) may be attached to nanoshell surfaces to further enhance biocompatibility and improve blood circulation times\textsuperscript{76}.

Nanoshells have demonstrated promise in a variety of biomedical applications ranging from substrates for whole-blood immunoassays\textsuperscript{77} to
photothermal cancer therapy. By using magnetic resonance thermal guidance, \textit{in vitro} cancer cells were successfully ablated using gold nanoshells tuned to absorb NIR light\textsuperscript{78}. Similar use of nanoshells for photothermal ablation of tumors in mice further showed complete regression of tumors with the mice remaining healthy compared to controls\textsuperscript{79}.

Nanoshell contrast agents may be optically interrogated using noninvasive approaches and targeted to specific molecular signatures of cancer. The tunability of the nanoshells should provide a method for simultaneous imaging of multiple markers \textit{in vivo} using nanoshells of distinct colors, each targeted to particular markers via specific antibodies or peptides, and the bright optical signals produced by the nanoshells should facilitate noninvasive detection. For comparison, nanoshells provide a million fold enhancement in optical extinction over ICG: $10^{15}$ to $10^{16}$ cm\textsuperscript{2} per ICG molecule compared with $10^{9}$ to $10^{10}$ cm\textsuperscript{2} per nanoparticle (100 nm diameter).

Developing new imaging approaches for \textit{in vivo} detection of specific molecular markers of cancer is an area of research under development both by
our research team and other laboratories around the country considering the use of multiple types of nanomaterials for molecular imaging. The availability of these new nanostructures should greatly facilitate new in situ imaging methods. Further details about the optical properties of nanoshells can be found in Chapter 2. Details about nanoshell synthesis can be found in Appendix A. A review of recent advances in nanotechnology for imaging and therapy applications may be found in West and Halas.\textsuperscript{80}

**Quantum Dots as Contrast Agents:** Quantum dots are another novel type of nanoparticle useful as an optical contrast agent. Unlike nanoshells which scatter and absorb, these nanoparticles are fluorophores. They are spherical and range in size from 1 nm to $>10$ nm. The luminescent properties of quantum dots arise from these dimensions being smaller than the exciton Bohr radius which leads to a quantum confinement effect. Quantum dots are composed of a core with atoms from groups II-VI or III-V of the periodic table encapsulated in an outer shell that has a higher energy band gap; thus, the electronic excitation is confined to the core.\textsuperscript{81}
Since the luminescence of quantum dots is due to this core shell structure instead of the molecular bonds in standard fluorophores, quantum dots are highly resistant to photobleaching effects. This dependence of quantum dots on nanoparticle structure results in an extremely high photostability and quantum yield, both desirable properties in optical imaging that simple dyes have been unable to demonstrate. Quantum dots possess extinction coefficients several orders of magnitude greater than the best molecular fluorophores resulting in a much brighter luminescence$^{82,83}$. Similar to nanoshells, quantum dots also yield an optical response that is dependent upon the size of the nanoparticle core and shell thickness. By varying the relative core and shell thicknesses, the luminescent emission of the quantum dot can be tuned across a broad range of the optical spectrum spanning the visible and the near infrared spectral regions$^{81}$. Also comparable to nanoshells, the surfaces of quantum dots can be functionalized and terminated in a variety of ways for molecular targeting. This tunability and customization make
quantum dots powerful markers for targeting and for providing contrast within biological tissues.

Quantum dots can be fabricated to have emission wavelengths that lie within the “water window” of the near infrared, a region of high light transmission through tissue, which should greatly facilitate in vivo applications. The very high extinction coefficients blue-shifted to the emission wavelength make quantum dots ideal for multiphoton excitation and molecular marker targeting of multiple sites due to the ease in which one can excite multiple quantum dots with a single excitation source.

Preliminary work on quantum dots has shown universal utility in bioimaging applications from in vivo cancer imaging to use of quantum dots for photodynamic therapy. Presently, the limited information available on the properties of the luminescence signal from quantum dots restricts the ability to design optimal optical imaging systems. Further work is necessary to elucidate these properties in order to develop an optimal imaging system using targeted applications of quantum dots.
**Targeted Probes as Contrast Agents:** A final new area of contrast agent research is the development of targeted probes for molecular imaging using contrast agents activated by enzymes such as matrix metalloproteinases or cathepsins. Over the past several years, considerable effort has been invested toward development of molecularly targeted contrast agents. Studies have demonstrated conjugation of ICG and ICG analogues, in addition to other fluorophores and nanoparticles, to antibodies and peptides\(^{63,68,87,88}\). Bugaj and colleagues demonstrated that by using the peptide targeted dye cypate, an ICG analogue, the fluorescent contrast agent showed increased retention time in tumors compared with non-peptide-targeted dyes\(^ {65}\). Activated contrast agents are among the most promising probes under development because quenching prevents the probe from generating signal without targeted enzymatic activity, yielding a highly specific optical signal. Weissleder and colleagues have recently demonstrated successful *in vivo* NIR fluorescence imaging of tumors using protease-activated probes\(^ {87}\). His group imaged tumors implanted into the mammary pads of a nude mouse model using a quenched fluorescent probe
activated by cathepsin D. The increase in probe signal in tumor regions was more than an order of magnitude higher than the background signal. In another recent study conducted by Bremer and colleagues, tumor invasiveness was assessed through NIR fluorescence imaging of a probe that targeted protease expression in breast tumors\textsuperscript{89}. Although molecular optical imaging is still in its infancy, molecular imaging methods are likely to play an increasingly important role in future breast cancer imaging. Ultimately, molecular imaging strategies offer the possibility to detect molecular changes at an early point well before macroscopic anatomic indications of disease are present. A variety of molecular signatures of cancer, ranging from overexpressed surface markers to growth factors promoting vascularity to any number of genetic changes, may be directly imaged \textit{in vivo} using inexpensive optical technologies\textsuperscript{6}.

\textbf{Conclusion:} Optical imaging tools currently available offer real-time quantitative measurements of functional tissue parameters without ionizing radiation using relatively inexpensive technology. Rapid advances in the
development of optical contrast agents for molecular imaging should permit more extensive *in vivo* monitoring of molecular targets. Although optical techniques offer significant promise in breast cancer applications ranging from early detection to tumor diagnostics to planning and monitoring therapeutic interventions, translating these new technologies into clinical practice will require rigorous technology assessment, which has not yet occurred. The biologic basis of optical signals measured from the breast are not completely understood, nor are the effects of potential confounding factors such as patient age, hormonal status, and menstrual cycle variations. The sensitivity and specificity of optical approaches in both screening and diagnosis of breast cancer must be established through clinical trials with statistically justified sample sizes. Acceptance of optical technologies by patients and providers needs to be evaluated and the cost-effectiveness of optical imaging strategies investigated. Richards-Kortum and colleagues have assessed optical spectroscopy of cervical precancers at each of these levels, and a number of companies are actively working to commercialize the cervical technology\(^9\).
Similar systematic technology assessment is necessary to establish the potential and limitations of optical technologies for breast cancer detection. As this chapter goes to press, significant clinical studies to rigorously assess the potential of breast optical imaging are already under way in both the academic and industrial sectors. The results of these studies should be available over the next several years.
CHAPTER 2
NANOSHells

Overview: Metal nanoshells are a novel type of composite spherical nanoparticle consisting of a dielectric core covered by a thin metallic shell, which is typically gold. Nanoshells possess highly favorable optical and chemical properties for biomedical imaging and therapeutic applications. By varying the relative the dimensions of the core and the shell, the optical resonance of these nanoparticles can be precisely and systematically varied over a broad region ranging from the near-UV to the mid-infrared. This range includes the near-infrared (NIR) wavelength region where tissue transmissivity peaks. In addition to spectral tunability, nanoshells offer other advantages over conventional organic dyes including improved optical properties, enhanced biocompatibility, and reduced thermal susceptibility to

2 Adapted from:
chemical/thermal denaturation. Furthermore, the same conjugation protocols used to bind biomolecules to gold colloid are easily modified for nanoshells. In this chapter, the optical properties of metal nanoshells and general fabrication procedures will be described.

**Introduction:** Metal nanoshells are a new type of nanoparticle composed of a dielectric core such as silica coated with an ultrathin metallic layer, which is typically gold. Gold nanoshells possess physical properties similar to gold colloid, in particular, a strong optical absorption due to the collective electronic response of the metal to light. The optical absorption of gold colloid yields a brilliant red color which has been of considerable utility in consumer-related medical products, such as home pregnancy tests. In contrast, the optical response of gold nanoshells depends dramatically on the relative size of the nanoparticle core and the thickness of the gold shell. By varying the relative core and shell thicknesses, the color of gold nanoshells can be varied across a broad range of the optical spectrum that spans the visible and the near infrared spectral regions (Figure 2.1)\textsuperscript{69,102}. 
Figure 2.1: Visual demonstration of the tunability of metal nanoshells.
(Halas, N. and West, J. et al.).

Gold nanoshells can be made to either preferentially absorb or scatter light by varying the size of the particle relative to the wavelength of the light at their optical resonance. In Figure 2.2, a Mie scattering plot of the nanoshell plasmon resonance wavelength shift as a function of nanoshell composition for the case of a 60 nm core gold/silica nanoshell is depicted. In this figure, the core and shell of the nanoparticles are shown to relative scale directly beneath their corresponding optical resonances.
**Figure 2.2:** Optical resonances of gold shell-silica core nanoshells as a function of their core/shell ratio. Respective spectra correspond to the nanoparticles depicted beneath. (Halas, N. and West, J. *et al.*).

In Figure 2.3, a plot of the core/shell ratio versus resonance wavelength for a silica core/gold shell nanoparticle is displayed. The extremely agile “tunability” of the optical resonance is a property unique to nanoshells: in no other molecular or nanoparticle structure can the resonance of the optical absorption properties be so systematically “designed.”
Figure 2.3: Core/shell ratio as a function of resonance wavelength for gold/silica nanoshells. (Halas, N. and West, J. et al.).

Halas and colleagues have completed a comprehensive investigation of the optical properties of metal nanoshells. Quantitative agreement between Mie scattering theory and the experimentally observed optical resonant properties has been achieved. Based on this success, it is now possible to predictively design gold nanoshells with the desired optical resonant properties, and then to fabricate the nanoshell with the dimensions and nanoscale tolerances necessary to achieve these properties. The synthetic
protocol developed for the fabrication of gold nanoshells is based on principles of molecular self-assembly and colloid chemistry in aqueous solution. The method is very simple in concept:

1. grow or obtain silica nanoparticles dispersed in solution,

2. attach very small (1-2 nm) metal "seed" colloid to the surface of the nanoparticles via molecular linkages; these seed colloids cover the dielectric nanoparticle surfaces with a discontinuous metal colloid layer,

3. grow additional metal onto the "seed" metal colloid adsorbates via chemical reduction in solution.

This approach has been successfully used to grow both gold and silver metallic shells onto silica nanoparticles. Various stages in the growth of a gold metallic shell onto a functionalized silica nanoparticle are shown in Figure 2.4.

**Figure 2.4:** Transmission electron microscope images of gold/silica nanoshells during shell growth. (Halas, N. and West, J. et al.).
Figure 2.5 shows the optical signature of nanoshell coalescence and growth for two different nanoshell core diameters.

![Graphs showing extinction of gold shells at different wavelengths](image)

**Figure 2.5:** (a) Growth of gold shell on 120 nm diameter silica nanoparticle. The lower spectral curves follow the evolution of the optical absorption as coalescence of the gold layer progresses. Once the shell is complete, the peak absorbance is shifted to shorter wavelengths. Corresponding theoretical peaks are plotted with dashed lines. (b) Growth of gold shell on 340 nm diameter silica nanoparticles. Here the peak shifts are more pronounced with only the shoulder of the middle curve visible in our instrument range. (Halas, N. and West, J. *et al.*).

Based on the core/shell ratios that can be achieved with this protocol, gold nanoshells with optical resonances extending from the visible region to approximately 3 μm in the infrared can currently be fabricated. This spectral region includes the 800-1300 nm “water window” of the near infrared, a region of high physiological transmissivity which has been demonstrated as the spectral region best suited for optical bio-imaging and biosensing.
applications. The optical properties of gold nanoshells, when coupled with their biocompatibility and their ease of bioconjugation, render these nanoparticles highly suitable for targeted bioimaging and therapeutics applications. By controlling the physical parameters of the nanoshells, it is possible to engineer nanoshells which primarily scatter light as would be desired for many imaging applications, or alternatively, to design nanoshells which are strong absorbers permitting photothermal-based therapy applications. The tailoring of scattering and absorption cross-sections is demonstrated in Figure 2.6 which shows sample spectra for two nanoshell configurations, one designed to scatter light and the other to preferentially absorb light.

Because the metal layer of gold nanoshells is grown using the same chemical reaction as gold colloid synthesis, the surfaces of gold nanoshells are virtually chemically identical to the surfaces of the gold nanoparticles universally used in bioconjugate applications. The use of gold colloid in biological applications began in 1971 when Faulk and Taylor invented the
Figure 2.6: Nanoshells may be designed to be predominantly scattering or absorbing by tailoring the core and shell fabrication materials. To demonstrate this concept, the predicted scattering efficiency, absorption efficiency, and extinction are shown for two nanoshells: (A) a scattering configuration (core radius = 40 nm; shell thickness = 20 nm) and (B) an absorbing configuration (core radius = 50 nm; shell thickness = 10 nm). (Courtesy Alex Lin).
immunogold staining procedure\textsuperscript{104}. Since that time, the labeling of targeting molecules, especially proteins, with gold nanoparticles has revolutionized the visualization of cellular or tissue components by electron microscopy. The optical and electron beam contrast qualities of gold colloid have provided excellent detection qualities for such techniques as immunoblotting, flow cytometry, and hybridization assays. Conjugation protocols exist for the labeling of a broad range of biomolecules with gold colloid, such as protein A, avidin, streptavidin, glucose oxidase, horseradish peroxidase, and IgG. Successful gold nanoshell conjugation with enzymes and antibodies has previously been demonstrated.
CHAPTER 3
SPECIFIC AIM 1
SCATTER-BASED CELLULAR IMAGING OF HER2

Overview: Advances in scattering-based optical imaging technologies offer a new approach to non-invasive point-of-care detection, diagnosis, and monitoring of cancer. Emerging photonics technologies provide a cost-effective means to image tissue in vivo with high resolution in real-time. Advancing the clinical potential of these imaging strategies requires the development of optical contrast agents targeted to specific molecular signatures of disease. In this chapter, we describe the use of a novel class of contrast agents based on nanoshell bioconjugates for molecular imaging in living cells. Nanoshells offer significant advantages over conventional imaging probes including continuous and broad wavelength tunability, far greater scattering and absorption coefficients, increased chemical stability, and improved biocompatibility. We show that nanoshell bioconjugates can be

used to effectively target and image HER2, a clinically relevant biomarker, in live human breast carcinoma cells.

**Introduction:** Optical imaging tools such as reflectance confocal microscopy (RCM) and optical coherence tomography (OCT) offer the potential for non-invasive, high-resolution *in vivo* imaging at competitive costs relative to current imaging modalities. Scattering-based optical technologies rely on inherent changes in indices of refraction for image contrast. Strategies which depend only on the intrinsic optical contrast within tissue have proven clinically valuable in many screening applications including early cancer detection; however, such techniques are not sensitive enough to resolve an image based solely on the presence of biomarkers of disease. In cases of cancer, where early detection is critical to reducing morbidity and mortality, the use of molecular-specific contrast agents provides the capacity to optically sense and image abnormalities long before pathologic changes occur at the anatomic level. In addition, imaging based on molecular-specific targets enables real-time *in vivo* monitoring of treatment course and can provide
fundamental insights into cancer biology\textsuperscript{92}. A recent demonstration of scattering-based optical molecular imaging used gold colloid conjugated to antibodies to the epidermal growth factor receptor as a contrast agent in imaging cervical cancer cells and biopsy samples\textsuperscript{93}. While gold colloid conjugates are highly valuable as contrast agents for detecting superficial epithelial cancers with visible light, there is particular need for contrast agents in the near infrared (NIR) region of the spectrum. This is the spectral region where tissue is most optically transparent\textsuperscript{94}, allowing imaging of deeper tissue structures. The NIR region is also the region already exploited by RCM and OCT; thus, contrast agents would provide greatly needed enhancement wherever these imaging modalities are utilized.

In this study, highly-scattering NIR nanoshells were fabricated for optical imaging. We then demonstrate the feasibility of using these targeted nanoshell bioconjugates as contrast agents to image HER2 expression in living human breast carcinoma cells.
**Darkfield Microscopy:** Nanoshell fabrication, antibody conjugation, and cell culture are described in Appendix A. HER2-expressing SKBr3 cells were exposed to bioconjugated nanoshells (8 µg/mL), and observed under darkfield, a form of microscopy sensitive only to scattered light. Images were taken with a Zeiss Axioskop 2 plus microscope equipped with a black-white CCD camera under the same magnification and lighting conditions. Optical contrast was quantified using the Scion Image Analysis Program. Average intensity values were obtained in each darkfield image. Normality of intensity data was established through a Shapiro Wilk test prior to using a paired Student’s t-test (two-tailed) to test for significance.

**Results:** Figure 3.1 shows the optical properties for nanoshells possessing a 120 nm silica core radius and 35 nm thick shell that were used in this study. Nanoshells with these dimensions generate a scattering spectrum beginning at 700 nm and extending far into the NIR region; thus, nanoshells with these spectral characteristics are capable of facilitating imaging in both
the visible and NIR regions, enabling the nanoshell conjugates to be used as contrast agents for RCM and OCT.

![Graph showing efficiency vs. wavelength](image)

**Figure 3.1:** Mie scattering theory predictions of the scattering (black) and absorption efficiencies (gray) for nanoshells with a 120 nm silica core radius and 35 nm thick shell that were used in this study.

As shown in Figure 3.2, significantly increased optical contrast under darkfield due to HER2 expression was observed in HER2-positive cells targeted with anti-HER2-labeled nanoshells (right column) compared to cells targeted by either anti-IgG labeled nanoshells (middle column) or cells not exposed to nanoshell conjugates (left column). Images in Figure 3.2 A-C are
cross sectional slices of cells taken at the mid-focal plane at 40X magnification.

A series of darkfield images (Figure 3.2 D-F) taken at a lower magnification (10X) is included demonstrating nanoshell targeting and coating of cell surfaces (Figure 3.2 F).

![Image of images A to F showing nanoshell targeting](image)

**Figure 3.2**: High magnification darkfield images (A-C) of HER2-positive SKBr3 breast cancer cells exposed to no nanoshells (left-hand column), anti-IgG (middle column), or anti-HER2 (right-hand column) labeled nanoshells. Cross sectional images were taken at 40X magnification at the mid-focal plane. Bottom row (D-F) shows darkfield images of HER2-positive cells taken at lower magnification (10X) demonstrating nanoshell targeting and coating of the cell surface.

Histogram analysis of darkfield images showed that nanoshell targeting of the HER2 receptor resulted in significantly (p<0.05) greater average contrast values in the anti-HER2 group (142 ± 16) compared to controls (anti-
IgG 48 ± 12, no nanoshells 26 ± 4) (Figure 3.3). Significantly less contrast was measured in HER2-negative cells exposed to anti-HER2 labeled nanoshells (34 ± 5) compared to HER2-positive cells (142 ± 16) providing additional evidence that the increased contrast seen under darkfield may be specifically attributable to nanoshell targeting of the HER2 receptor. No significant differences were found between control groups.

![Graph](image)

**Figure 3.3:** Quantitative analysis of optical contrast due to HER2-expression. Contrast was quantified by obtaining average histogram intensity values of darkfield images. Contrast data quantified using a HER2-negative MCF7 cell line is shown for comparison purposes. Intensity values range from 0 (black) to 255 (white), with higher values corresponding to greater contrast. Differences in mean scattered intensity between the anti-HER2 group and all other cell groups are statistically significant (p<0.05).
**Conclusion:** Knowledge of potential molecular targets for diagnosis and therapy of disease continues to expand at a rapid rate. However, translating knowledge of potential targets into new diagnostic and therapeutic techniques requires the development of methods to image molecular targets, or the effects of therapeutic interventions on these targets, *in vivo*, in real-time, and in a cost-effective manner. Nanoshell-based molecular contrast agents offer unique advantages including NIR-tunability, size flexibility, and systematic control of optical properties. In this study, we demonstrated that nanoshell bioconjugates can provide molecular optical contrast enhancement both qualitatively and quantitatively. Our findings collectively show that gold nanoshells can be used to target specific cancer markers and allow *in vitro* cell level molecular imaging using a scattering-based optical approach. A darkfield microscope was used in this study to demonstrate the feasibility of nanoshell bioconjugates for molecular imaging in living cells. While darkfield microscopy is appropriate for *in vitro* imaging applications, use of nanoshell conjugates *in vivo* will require more sophisticated imaging techniques. Our
current results encourage future work assessing nanoshell-based contrast agents \textit{in vivo} using RCM and OCT. The combination of targeted nanoshells and the field of biophotonics have the potential to play a vital role in the future of cancer screening and diagnosis, in designing and monitoring therapeutic interventions, and in fundamental studies of carcinogenesis.
CHAPTER 4
SPECIFIC AIM 2
INTEGRATED CANCER IMAGING AND THERAPY

Overview: Nanoshells can be designed to scatter and/or absorb light over a broad spectral range including the near infrared (NIR), a wavelength region which provides maximal penetration of light through tissue. The ability to control both wavelength-dependent scattering and absorption of nanoshells offers the opportunity to design nanoshells which provide, in a single nanoparticle, both diagnostic and therapeutic capabilities. Here, we demonstrate a novel nanoshell-based all-optical platform technology for integrating cancer imaging and therapy applications. Immunotargeted nanoshells are engineered to both scatter light in the NIR enabling optical molecular cancer imaging and to absorb light allowing selective destruction of targeted carcinoma cells through photothermal therapy. In a proof of principle experiment, dual imaging/therapy immunotargeted nanoshells are used to

4 Adapted from:

detect and destroy breast carcinoma cells that overexpress HER2, a clinically relevant cancer biomarker.

**Introduction:** Nanoshells designed to have a high scattering optical cross-section are potentially valuable contrast agents for photonics-based imaging modalities such as reflectance confocal microscopy (RCM)\(^95\) and optical coherence tomography (OCT)\(^91\) which offer high resolution approaches to early cancer detection. To image deeper tumors, other methods such as frequency-domain photon migration may be used to enable optical visualization of scatter-based contrast\(^96\). Alternatively, nanoshells can be designed to strongly absorb NIR light providing a novel means to mediate photothermal ablation of cancer cells\(^78,79\). Of particular interest is the possibility of engineering nanoshells with optical properties suitable for combined imaging and therapy. Past attempts to develop combined approaches to imaging and therapy have relied on methods such as the use of radio-immunoconjugates\(^97\) whose clinical effectiveness is limited by factors including low tumor uptake, dose-limiting toxicity, and the necessity to expose
patients to ionizing radiation\textsuperscript{98}. Nanoshells provide an alternative means to enable dual imaging/therapy applications as they can be engineered to simultaneously provide both scattering and absorption properties at specific frequencies. Selective accumulation of nanoshells may be achieved via passive extravasation based on the enhanced permeability and retention (EPR) of small particles (< 400 nm) associated with the leaky tumor vasculature\textsuperscript{99}, with further targeting possible using antibodies targeted against oncoproteins overexpressed on cell surfaces. In principle, upon accumulation within tumors, nanoshells may provide both molecular-specific image contrast, and when clinically indicated, mediate cancer treatment based on NIR thermal ablation therapy. Here, we provide an \textit{in vitro} demonstration of the dual imaging/therapy approach first detecting and then thermally ablating human breast cancer cells which overexpress HER2 using immunotargeted nanoshells which have been designed to both scatter and absorb light within the NIR.
Figure 4.1: Spectral characteristics and SEM image of NIR scattering/absorbing nanoshells. Spectrum obtained by Mie scattering theory shows the dual scattering/absorbing NIR nature (~800 nm) of nanoshells with dimensions consisting of a 120 nm diameter silica core and 10 nm thick shell. Extinction curve, the sum of the relative contribution of absorption and scattering efficiencies, was confirmed with a UV-Vis spectrophotometer (not shown). SEM image of nanoshells with an overall diameter of 140 nm is included. Scale bar = 200 nm.

Incubation of Nanoshell Bioconjugates with Cells: Nanoshells with dimensions providing peak optical scattering and absorption efficiencies in the NIR (~800 nm) were fabricated and labeled with antibodies as described in Appendix A. Figure 4.1 shows the spectral characteristics and an SEM image of nanoshells possessing a 10 nm thick shell that were used in this study.
Concentrated 10X McCoy’s media (free of FBS and antibiotics to eliminate non-specific interactions with nanoshells) was quickly added to the nanoshells at a volumetric ratio of 1:9. 500 µl of this McCoy’s-nanoshell suspension was placed on HER2-positive SKBr3 cells followed by 1 hr incubation. McCoy’s media containing FBS and antibiotics was added following rinsing of unbound nanoshells.

**Molecular Imaging of HER2 Expression and *in vitro* Photothermal Therapy:** Cells were imaged under a darkfield microscope sensitive to scattered light. Images were taken with a Zeiss Axioscope2 microscope equipped with a black/white CCD camera. All images were taken at the same magnification under the same lighting conditions. Immediately following imaging, cells were exposed to NIR irradiation (820 nm, 9 mW/m² for 7 min). The overlap of peak nanoshell absorbance with the emission wavelength of the laser source promoted optimal laser-induced nanoshell heating. Cells were stained for viability using calcein AM. Stained cells were examined under fluorescence and phase contrast microscopy with a Zeiss Axiovert 135
microscope. Silver staining was then performed to assess the presence of nanoshell binding on cell surfaces. Antibody blocking experiments and HER2-negative control experiments were performed to confirm specificity of therapy.

**Results:** Figure 4.2 presents results from combined imaging and therapy of SKBr3 breast cancer cells using nanoshells targeted against HER2 (right column). In addition, control images of cells taken without nanoshells (left column) and of cells incubated with non-specifically labeled nanoshells (middle column) are presented. Significantly increased scatter-based optical contrast due to nanoshell binding was observed in cells incubated with anti-HER2 nanoshells (top row, right column) as compared to the two control cell groups (top row, left & middle columns). After photothermal therapy, cell death was only observed in cells treated with NIR laser following exposure to anti-HER2 nanoshells (middle row, right column). This effect was not observed in cells treated with either nanoshells conjugated to a non-specific antibody or NIR light alone (middle row, left & middle columns). Greater silver staining intensity was seen in cells exposed to anti-HER2 nanoshells
(bottom row, right column) compared to controls (bottom row, left & middle columns) suggesting enhanced nanoshell binding to cell surfaces overexpressing HER2.

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<th>Scatter-based imaging</th>
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<td>Silver stain</td>
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**Figure 4.2:** Combined imaging and therapy of SKBr3 breast cancer cells using HER2-targeted nanoshells. Scatter-based darkfield imaging of HER2 expression (top row), cell viability assessed via calcein staining (middle row), and silver stain assessment of nanoshell binding (bottom row). Cytotoxicity was observed in cells treated with a NIR-emitting laser following exposure and imaging of cells targeted with anti-HER2 nanoshells only. Note increased contrast (top row, right column) and cytotoxicity (dark spot) in cells treated with a NIR-emitting laser following nanoshell exposure (middle row, right column) compared to controls (left & middle columns).

In order to establish that anti-HER2 nanoshells alone do not induce cytotoxicity, we have incubated cells with anti-HER2 nanoshells over a range
of concentrations and incubation times. Figure 4.3 shows calcein AM staining in which green fluorescence indicates live cells of SKBr3 cells that were exposed to HER2-targeted nanoshells \((3 \times 10^9 \text{ nanoshells/ml})\). In statistical analysis, no significant differences \((p>0.05)\) in cytotoxicity were observed comparing cells incubated with targeted nanoshells \((96\% \pm 3.0 \text{ cell viability})\) and control cells not exposed to nanoshells \((98\% \pm 1.5 \text{ cell viability})\).

![Viability of cells incubated with anti-HER2 nanoshells](image)

**Figure 4.3:** Viability stain (green = live cells) of HER2-positive SKBr3 cells incubated with anti-HER2 nanoshells without NIR photothermal therapy. No cytotoxicity is observed.

**Conclusion:** Currently, distinct diagnostic and therapeutic modalities are employed for the diagnosis and treatment of cancer. Furthermore, in most cases, standard of care treatment requires invasive surgical procedures or other therapies associated with significant side effect profiles, high cost, and poor
clinical outcome. A single technology providing both diagnostic and therapeutic capabilities would potentially yield significant savings in time, cost, and patient discomfort associated with diagnosing and treating many cancers today. Nanoshells offer unique properties which facilitate an integrated imaging/therapy approach including systematic control of both optical scattering and absorption, tunability throughout the NIR where tissue penetration is highest, and a particle size conducive to passive extravasation from the tumor vasculature. We showed that immunotargeted nanoshells can provide scattering contrast for imaging while also exhibiting sufficient absorption to enable effective photothermal therapy. This is the first demonstration of coupling a bioimaging application to a cancer therapy application using nanoshells targeted against a clinically relevant biomarker. Future studies will extend the in vitro concept demonstrated here to in vivo animal experiments.
**CHAPTER 5**

**SPECIFIC AIM 3**

**SCATTER-BASED IMAGING EX VIVO**

**Overview:** Scattering-based optical imaging technologies such as reflectance confocal microscopy (RCM) offer a unique non-invasive approach to the detection, diagnosis, and monitoring of cancer. RCM provides a cost-effective means for high-resolution real-time imaging of tissue *in vivo*. Realizing the clinical potential of these imaging strategies requires the use of near infrared (NIR) tuned molecular-specific optical contrast agents. In this chapter, we describe the use of targeted NIR-tunable nanoshell-based contrast agents for molecular imaging *ex vivo*. Nanoshells offer significant advantages over conventional imaging probes including NIR-tunability, enhanced scattering coefficients, biocompatibility, and ease of antibody targeting. We demonstrate using NIR RCM that nanoshells can be used to target and image the clinically-relevant breast cancer marker HER2 *ex vivo*.

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**Introduction:** Scatter-based optical imaging offers a novel approach to biomedical imaging. Imaging modalities such as reflectance confocal microscopy (RCM) offer the potential for non-invasive high-resolution, real-time imaging at competitive costs. These techniques, however, have limited sensitivity since contrast relies on inherent changes in refraction\textsuperscript{91}. Imaging based on the presence of disease specific molecular markers may prove highly beneficial in the area of early cancer detection\textsuperscript{92}. Contrast agents possessing inherent optical properties and targeted to these markers would improve the signal-to-background ratio that would otherwise be normally undetectable. Moreover, the near infrared (NIR) region is of particular interest for biomedical imaging applications because this is the spectral region where tissue is most optically transparent, allowing for deeper structures to be imaged\textsuperscript{94}. Tunable NIR contrast agents, targeted to disease-specific molecular markers would be greatly beneficial whenever these optical imaging strategies are employed. Here, we describe the use of nanoshells, with optical properties
tunable into the NIR, targeted to disease-specific molecular markers for NIR RCM imaging in tissues.

**Methods:** Nanoshells with dimensions providing peak optical scattering properties in the NIR (~800 nm) were fabricated and targeted with antibodies as described in Appendix A. HER2-expressing SKBr3 and HER2-negative MCF7 breast cancer cells were grown at 37°C and 5% CO₂ in 75 cm² tissue culture flasks until confluent, and were harvested using 0.25% trypsin-EDTA. Cells were centrifuged at 150 G for 5 min, excess media was aspirated and resuspended in cell culture media. Cells were then exposed to nanoshell bioconjugates for 1 hr. Cells were washed by centrifugation and resuspension in media. Nanoshell-labeled cells were placed on a gelatin-coated glass slide in order to eliminate background scattering from the glass substrate.

**Preparation of Fresh Cervical Biopsies:** Histologically normal and abnormal (invasive carcinoma) fresh frozen breast tissue samples were obtained from the Cooperative Human Tissue Network and were stored at
-80°C. Protocol approval was obtained by the Rice University Institutional Review Board. HER2-status was obtained from accompanying pathology reports. Samples were brought to room temperature, thin tissue sections were made, and reflectance confocal images were taken prior to nanoshell incubation. Following imaging, sections were injected with labeled nanoshells using a 3 ml, 25 gauge syringe with a 1.5 inch needle in order to ensure equal nanoshell distribution. Sections were then washed with PBS and imaged. Remaining samples were immediately frozen in liquid N₂. In order to assess RCM findings with established tissue staining procedures, samples were subjected to hematoxylin and eosin (H&E) and HER2 staining at the Baylor College of Medicine Breast Cancer Center.

**Confocal Microscopy:** Confocal images were acquired with a Lucid inverted reflectance laser scanning confocal microscope equipped with a diode laser and detector with an emission wavelength at 830 nm. All images were taken at the same magnification and power. Images of H&E and HER2-
stained tissues were taken with a Zeiss Axiovert 135 inverted brightfield microscope.

**Results:** Figure 5.1 shows the results of RCM imaging of HER2 *in vitro*. HER2-expressing SKBr3 (top row) and HER2-negative MCF7 cells (bottom row) were incubated with nanoshells conjugated with antibodies against either HER2 (right column) or a non-specific antibody (middle column). A no nanoshell control is included in the left column for comparison purposes. Greater contrast was observed in HER2-positive cells labeled with HER2-targeted nanoshells (top row, right column) compared to controls, an effect similar to what was observed in previous studies\textsuperscript{100,101}.

RCM imaging of HER2 expression *ex vivo* is shown in Figure 5.2. Middle and right-hand columns are of HER2-positive invasive carcinoma tissues. The left-hand column shows normal tissue. Top and bottom rows show tissues before and following nanoshell addition, respectively. Samples were exposed to either IgG (middle column) or HER2-labeled nanoshells (right-hand column). Compared to controls, greater contrast was observed in
HER2-targeted tissues following addition of HER2-labeled nanoshells (bottom row, right-hand column). In order to compare findings observed under RCM to established staining procedures, samples underwent H&E and HER2 staining (Figure 5.3). Greater intensity was observed in HER2-positive tissues further corroborating the contrast increases in Figures 5.1 and 5.2. Normal tissues were negative for HER2 staining. Contrast increases under RCM are attributable to nanoshell targeting of the HER2 receptor.
Figure 5.1: Reflectance confocal imaging of HER2 expression *in vitro*. HER2-positive SKBr3 (top row) or HER2-negative MCF7 (bottom row) cells were incubated with nanoshells labeled against HER2 (right column). A no nanoshell control and non-specific antibody control group are included for comparison purposes (left & middle columns). Note the greater scatter-based contrast during imaging in HER2-targeted cells (right column) compared to controls. Note also no differences between HER2-negative cells targeted with either HER2-labeled or non-specifically labeled nanoshells (bottom row, middle & right columns). 30x magnification. Field of view is 0.5 mm.
Figure 5.2: Reflectance confocal imaging of HER2 expression ex vivo. Histologically normal (left column) and HER2-positive breast cancer tissue (middle & right columns) before and after nanoshell addition (top & bottom rows, respectively). Scale bar is ~125 μm.

Figure 5.3: H&E (left) and HER2 staining (right) of histologically abnormal tissue. Scale is ~200 μm. 20x magnification.
Conclusion: Translation of knowledge of current molecular markers into novel diagnostic and therapeutic techniques requires the development of novel agents and methods capable of imaging these targets \textit{in vivo}, in real-time, and in a cost-effective manner. Nanoshells offer unique advantages including tunability into the NIR, enhanced optical properties, biocompatibility, and easy targeting of antibodies. In this study, we demonstrated that NIR-tunable, HER2-targeted nanoshell bioconjugates can provide molecular-specific optical contrast enhancement in breast tissue biopsies using a novel \textit{in vivo} NIR scattering-based optical imaging technology. Current work involves assessing targeted NIR-tuned nanoshells as contrast and cancer therapy agents \textit{in vivo} using OCT.
CHAPTER 6
DISCUSSION AND FUTURE DIRECTIONS

The area of biomedical optics continues to develop at a rapid pace. Several key areas that will be the focus of intense research in the future include the development of nanoparticle-based contrast agents for the field of molecular imaging, and the development of these agents for novel optical systems such as RCM and OCT.

The field of molecular imaging will be extremely important due to its ability to detect subcellular changes including changes in receptor expression prior to any gross phenotypic changes. This inherent ability to probe at levels below what conventional modalities are capable of currently holds enormous potential since timing is of the essence in the clinical management of cancer. An early diagnosis and subsequent treatment almost minimizes any chances for morbidity and mortality.

In order to realize the full potential that molecular imaging holds, contrast agents will need to possess: extremely high signal-to-background ratios, whose signals can be easily separated between normal and diseased
states and will be present for extended periods of time, optical “tunability” so that the operating wavelengths can be systematically designed and controlled in a way that is predictable and feasible, biocompatibility, easy fabrication, extremely low cost, and easy integration into existing as well as developing high level optical systems.

Nanoshells are ideal agents since they possess “tunable” optical resonances, are easy to fabricate, and are extremely biocompatible. Additionally, they can be easily targeted to specific molecular markers of disease, and can make their way into tumors without difficulty due to their small size and the leaky vasculature. The challenge with using gold nanoshells is being able to retain and sufficiently amplify the reflectance signal relative to background, and separating that signal from diseased as opposed to normal cells and tissues.

One strategy is to use technologies capable of harnessing the scattering optical properties of nanoshells. Two optical imaging modalities currently being used in conjunction with nanoshells are RCM and OCT. Studies of the
optical effects of nanoshells with OCT have shown that the scattering behavior of the nanoshells can be harnessed for these imaging technologies. These studies have progressed into higher level systems into tissues and into mice using both non-targeted and targeted nanoshells, with extremely promising results. Furthermore, studies are currently underway taking the integrated cancer imaging and therapy concept described in Chapter 3 into mice using targeted nanoshells. These studies utilizing the optical effect of gold nanoshells show the potential for both absorption and scattering-based diagnostics and treatment.

Developing RCM and OCT and incorporating them into clinically useful instruments remains a lofty but extremely viable goal. Ultimately, these technologies will have high sensitivities and specificities, can be used practically anywhere in the clinic and operating room settings, and will be cost effective. Other avenues to be further explored will be to develop these technologies at the microscale-level including implementing them into devices such as probes. These devices will be connected to computer-based systems
that are capable of rapidly gathering, sorting, and analyzing complex data in real-time.

Debate continues over the use of fluorescence-based versus reflectance-based agents. Quantum dots possess very high signal-to-background ratios, and do not photobleach. The signals from diseased and non-diseased states can easily be distinguished from each other. Similar to nanoshells, they are optically "tunable", can be easily targeted to molecular markers, possess dimensions that are on the nanoscale, and are easy to fabricate. The challenge with quantum dots is that they are composed of heavy metals such as cadmium and selenium.

Quantum dots are a very intriguing class of nanoparticle to study. A large amount of time exists between moving quantum dots from the laboratory into human clinical trials. Future directions for quantum dots include the development of targeted enzyme-activated probes, simultaneous imaging of multiple markers using targeted quantum dots tuned to completely different
optical wavelengths, and the development of safer, more biocompatible quantum dot species.

Other issues to be further explored include biodistribution, pharmacokinetic, and pharmacodynamic studies. Ultimately, the future of biomedical optics and medicine will likely steer toward the areas of nanotechnology and biotechnology given the huge potential these areas hold for clinical medicine. These newer nanobiotechnological advancements will not immediately replace existing means, if at all, but will serve as adjuncts to current modalities, and be used to further enhance their clinical reliability and effectiveness. The world of nanobiotechnology will be a world governed by nanoscale particles and devices, that can perform multiple complex tasks extremely rapidly and in real-time, that are cost effective, and that ultimately benefit society by being able to save lives.
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APPENDIX A
METHODS AND MATERIALS

The methods used throughout Specific Aims 1-3 will be described in Appendix A.

**Gold Nanoshell Fabrication:** Cores of silica nanoparticles were fabricated as described by Stober *et al.*\(^{105}\) in which tetraethylorthosilicate (TEOS) was reduced in NH\(_4\)OH in ethanol. Particles were sized with a Philips XL30 scanning electron microscope. Polydispersity of <10% was considered acceptable for nanoshells used in imaging or therapy applications. Next, the silica surface was aminated by reaction with aminopropyltriethoxysilane (APTES) in ethanol. Gold shells were grown using the method of Duff *et al.*\(^{106}\). Briefly, small gold colloid was adsorbed onto the aminated silica nanoparticle surface. More gold was then reduced onto these colloid nucleation sites using potassium carbonate and HAuCl\(_4\) in the presence of formaldehyde. Gold nanoshell formation and dimensions were assessed with a UV-VIS spectrophotometer and scanning electron microscopy (SEM).
**Antibody**  |  **Conjugation:** Ortho-pyridyl-disulfide-\(n\)-hydroxysuccinimide polyethylene glycol polymer (OPSS-PEG-NHS, MW=2000) was used to tether antibodies onto the surfaces of gold nanoshells.

Using NaHCO\(_3\) (100 mM, pH 8.5) OPSS-PEG-NHS was re-suspended to a volume equal to that of either HER2 (specific) or IgG (non-specific) antibodies. At this concentration, the concentration of polymer was in molar excess to the amount of HER2 or IgG antibody used. The reaction was allowed to proceed on ice overnight. Excess, unbound polymer was removed by membrane dialysis (MWCO=10,000). Different volumes of PEGylated antibody (0.67 mg/mL) were added to nanoshells (2\(*10^9\) nanoshells/mL) for 1 hr to facilitate targeting, and to determine the optimal nanoshell bioconjugate concentration. Unbound antibody was removed by centrifugation at 650 G, supernatant removal, and resuspension in potassium carbonate (2 mM).

Following antibody conjugation, nanoshells surfaces were further modified with PEG-thiol (MW=5000, 1 \(\mu\)M) to block non-specific adsorption sites and to enhance biocompatibility.
Cell Culture: HER2-positive SKBR3 human breast cancer cells were cultured in McCoy’s 5A modified medium supplemented with 10% FBS and antibiotics. HER2-negative MCF7 human breast cancer cells were cultured in Eagle’s minimum essential medium supplemented with 10% FBS, 0.01 mg/ml of bovine insulin, and antibiotics. Cells were maintained at 37°C and 5% CO₂.