A NOVEL OPTICAL APPROACH TO THE INTRAOPERATIVE DETECTION OF PARATHYROID GLANDS

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CHAPTER I

INTRODUCTION

Overview of Thyroid and Parathyroid Glands

Anatomy of the Neck

Endocrine surgery involves exploration of the neck in order to visualize vital tissues for benign and malignant thyroid and parathyroid conditions. The thyroid gland is positioned anterolateral to the larynx and trachea. Generally, there are four parathyroid glands which tend to lie symmetrically on the two sides of the neck. Each side consists of a superior and inferior gland based on the position in the neck. Incidence of a fifth parathyroid is 13% and that of only three glands is 3% (Bliss, Gauger and Delbridge). Important nerves pass close to the capsule of the thyroid gland including the superior laryngeal nerves and recurrent laryngeal nerves (Bliss, Gauger and Delbridge; Miller). The neck also contains an abundance of lymphatic tissue and adipose tissue. The general anatomy of this region is illustrated in Figure 1.

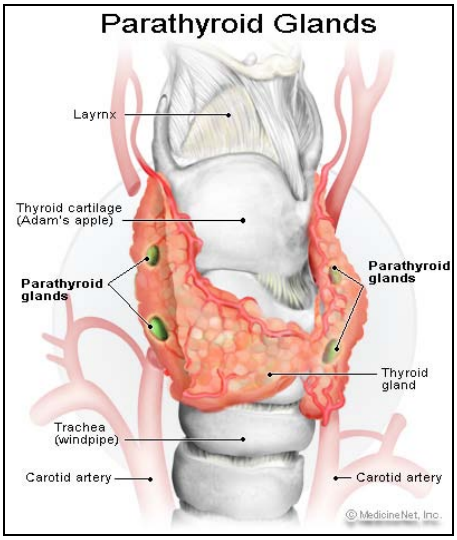

Figure 1: Anatomy of the neck region emphasizing relative position of the parthyroid glands (Medicinenet)

The parathyroid glands are approximately 6 to 8 mm in size and bean shaped with a yellow-tan to caramel color (size of a grain of rice) (Miller; Shaha and Jaffe). Most glands are suspended by a small vascular pedicle and enveloped by a pad of fatty tissue (Le and Norton). Most inferior parathyroid glands lie within the thyroid capsule on the surface of the inferior pole of the thyroid and may give the impression of being "intrathyroidal" when found within the clefts of thyroid tissue. The inferior parathyroid glands descend along with the developing thymus. This long line of migration causes variability in their position and the glands can be carried to the anterior mediastinum or the pericardium, or they can be left behind high in the carotid sheath. Comparatively, the superior parathyroids have a short line of embryologic descent. The glands are closely associated with the developing lateral lobes of the thyroid and remain close along the posterior capsule in the region of the inferior thyroid artery (ITA). Even with the variability in anatomy of the parathyroid glands, there tends to be symmetry between the

positions of the glands on the two sides of the neck. The inferior and superior glands are symmetrically positioned in about 70% and 80% of cases respectively(Bliss, Gauger and Delbridge).

Parathyroid glands are comprised of densely packed cells that fall into one of three main types: chief, oxyphil and adipose cells. The glands primarily consist of chief cells which contain cytoplasmic fat droplets and it is these cells that are responsible for the production of parathyroid hormone (PTH) (Elgazzar). Parathyroid hormone is an 84-amino acid polypeptide, secretion of which is responsible for the release of calcium and phosphate from bone matrix, calcium reabsorption by the kidney, and regulating renal production of calcitriol, which increases calcium absorption in the intestine. The final effective result of PTH secretion is an increase in plasma calcium concentration (Elgazzar). Thus, the parathyroid glands maintain the range of calcium concentration in the body important for normal function.

The thyroid gland is the largest endocrine gland in the body weighing 10 to 20 g and consists of right and left lobes joined anteriorly by the isthmus commonly positioned between the second and third tracheal rings (Bliss, Gauger and Delbridge) (Panza and Mansi). The thyroid's two lateral lobes are roughly conical in shape, approximately 5 cm long and 2 to 3 cm in the transverse and anterioposterior dimensions. There may also be a pyramidal lobe, a superior extension near the midline of the gland. Histologically, the functional unit of the thyroid is the follicle, a group of cells spherically arranged around colloid, a structure rich in the glycoprotein thyroglobulin (Whitehead). Thyroglobulin is synthesized in the follicular cells and then secreted into the colloid where thyroid hormones are formed (Panza and Mansi). The thyroid produces multiple hormones which

have diverse and widespread effects throughout the human body (Whitehead). Histology can be used to identify the various normal tissues discussed previously. Figure 2 is a histologic section showing the transition between thyroid and parathyroid tissue. The blue arrows in the figure highlight the thyroid tissue where follicles surrounding the large colloids are evident. Yellow arrows mark parathyroid tissue containing the chief and oxyphil cells.

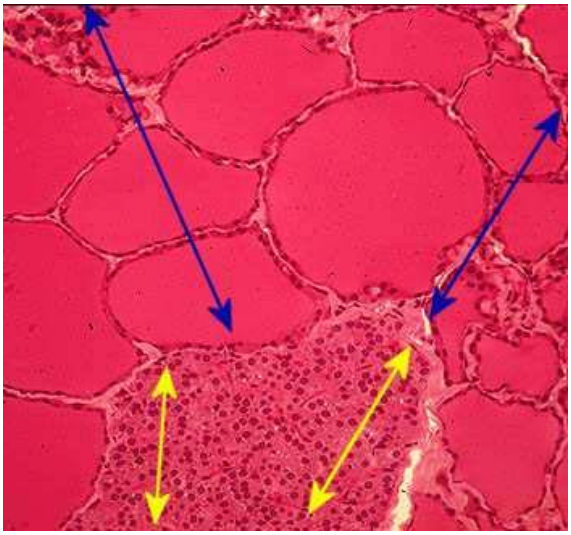

Figure 2: Histological slide of thyroid (blue arrows) and parathyroid (yellow arrows) tissue

Other important structures found in this region include the superior and inferior laryngeal nerves and lymph nodes. The superior laryngeal nerve (SLN) descends inferiorly to the carotid system and splits into an internal and external branch posterior to the internal carotid artery. The external branch runs deep to the superior thyroid artery and serves as the motor nerve to the cricothyroid muscle important to tensing vocal chords. The internal branch along with the superior laryngeal artery descends to supply

sensory innervation (Bliss, Gauger and Delbridge; Miller). The inferior, or recurrent, laryngeal nerve is a branch of the vagus nerve which turns back on itself in the chest and runs superiorly back into the neck serving as a motor nerve to the intrinsic muscles of the larynx. The nerve passes close to the capsule of the thyroid glands and may even appear to be within the parenchyma (Bliss, Gauger and Delbridge). In a normal adult there is an abundance of lymphatic tissue found throughout the region. Lymph nodes can vary in size averaging around 2 to 5 mm in diameter but can reach 20 mm by 10 mm helping to trap pathogens and contain white blood cells as part of the lymphatic system (Senchenkov and Staren).

Diseases in the Neck

Primary *hyperparathyroidism* (HPT) is a relatively common condition with annual incidence rates of 25-28 cases per 100,000 people (Kim; Sosa et al.). The rate is higher in Caucasian women above 60 years old, approaching 190 cases per 100,000 annually (Sosa et al.). Typically, HPT is characterized by excessive secretion of PTH, which in turn results in elevated levels of plasma calcium. In 80-90% of cases, primary HPT is caused by *parathyroid adenoma,* a benign tumor that is usually caused by a genetic condition; in about 15% of these cases, more than one gland is involved(Scott-Coombes). Surgical excision of abnormal glands is advocated for patients with primary HPT, especially those with symptoms such as muscle weakness, bone pain, nephrolithiasis, and peptic ulcers. Two of the main disadvantages of the procedure include a relatively long operative time and post-operative hospitalization (Ahuja et al.).

Thyroid disease occurs when the thyroid gland does not supply the appropriate amount of thyroid hormone needed. Currently, about 20 million Americans have some

form of thyroid disease. People of all ages and races can get thyroid disease affecting both genders; however, women are five to eight times more likely than men to have thyroid problems. If the thyroid is overactive, it releases too much thyroid hormone into the bloodstream, resulting in *hyperthyroidism*. An underactive thyroid produces too little thyroid hormone, resulting in *hypothyroidism***.** Both conditions can result in the thyroid becoming larger than normal. When it is large enough to see easily, it's called a *goiter*. *Graves disease*, an autoimmune disease, is the most common cause of hyperthyroidism wherein increased abnormal antibodies result in increased production of thyroid hormone causing the thyroid gland to enlarge eventually. When the condition cannot be controlled with medication, surgery is often performed to remove part of the thyroid. As the gland enlarges, there is an increased tendency for nodules to form.

Thyroid nodules can sometimes occur in a normal working thyroid and while most nodules are benign, some may lead to thyroid cancer. In 2007, approximately 33,550 new cases of thyroid cancer were diagnosed in the United States. The disease is most common in young people, with nearly two-thirds of diagnosed cases in people between the ages of 20 and 55. Since 1997, there has been a 6% yearly increase in the likelihood of being diagnosed with thyroid cancer which may be due to the increasing use of ultrasound to detect small thyroid nodules. The main treatment of thyroid cancer is a thyroidectomy, or surgical removal of all or part of the affected thyroid gland (Society). Thyroid surgery is considered one of the safest surgical procedures; however, it involves careful dissection of the thyroid due to its proximity to important structures, including the parathyroid (Lin et al.).

Motivation

There are several possible complications related to thyroid surgery, two of the major problems are postoperative hypocalcaemia and hypoparathyroidism (Shaha and Jaffe). Within 2 to 5 days after total or subtotal thyroidectomy, a decrease of serum calcium, a condition known as hypocalcaemia, is reported to occur in 1.6% to more than 50% of operations. The most probable cause is hypoparathyroidism due to trauma, devascularization, or inadvertent removal of one or more parathyroid gland(s) during surgery (Pattou et al.). This condition is categorized as either transient or permanent. In the case of transient hypocalcaemia, within a few months serum calcium levels normalize as function of the parathyroid is recovered. Permanent hypocalcaemia lasts more than 6 months and is associated with significant impairment of quality of life. Chronic gastrointestinal discomfort, changes in bone metabolism and development of cataracts are a few of the possible resulting symptoms (Frilling and Weber). A patient with permanent hypoparathyroidism requires calcium and vitamin D supplements for the remainder of their life and represents a significant source of morbidity to the patient (Shaha and Jaffe). Hypocalcaemia is the most common cause of malpractice litigation after endocrine surgery (Pattou et al.). Accordingly, effective management of thyroid disease is dependent on parathyroid preservation during thyroidectomy (Shaha and Jaffe). In the literature, the incidence of inadvertent parathyroidectomy ranges from 8% to 19% of patients undergoing total thyroidectomy (Sakorafas et al.). Complication rates have been shown to be directly proportional to the extent of the thyroidectomy, and inversely proportional to operating surgeon's experience level. The rate is also related to the extent of the invasion of the thyroid cancer. Consequently, the surgeon's familiarity of the

parathyroid glands' anatomy and blood supply is imperative to tissue removal (Shaha and Jaffe). Further complicating the situation, thyroidectomies and parathyroidectomies are routinely performed by general surgeons (particularly in centers/hospitals without a division of endocrine surgery). In these cases, the level of experience in finding the parathyroid may be further reduced.

There is a need for a tool that is sensitive and fast to help identify parathyroid glands intraoperatively. Current technology relies on histopathology or post-operative diagnosis of symptoms to determine if the parathyroid was accidentally or incompletely removed. Recently the use of imaging techniques, such as sestamibi imaging (nuclear medicine imaging) or ultrasonography, has been applied preoperatively to localize abnormal parathyroid glands (Kim). However, these techniques are not applicable intra-operatively and are not as effective when multiple glands are involved. Intact PTH (iPTH) assay has been used for this purpose; however it is an expensive technique that is available at only a limited number of centers (Kim). This report is focused on the development of imaging technology specifically, optical imaging and spectroscopy as it pertains to tissue identification. Imaging modalities cover a wide range of topics; within this area, optical techniques deal with the application of light from the ultraviolet to the infrared for identification and visualization of relevant structures. Near-infrared fluorescence is examined as a potential method to detect parathyroid tissue in real-time.

Optical Spectroscopy

Existing methods for idenifying parathyroid glands are limited in their applicability and sensitivity and are, thus, not adequate enough to prevent surgical complications

(Prosst, Gahlen et al.). Primary means include ultrasound, sestamibi scintigraphy, CT, MRI and intact iPTH assay. Ultrasound is one of the most common techniques for imaging the neck and has sensitivity ranging from 27 – 85% (Ahuja et al.; Fakhran, Branstetter and Pryma). The normal parathyroid gland is not typically visualized because of its deep location and small size; ultrasound is mainly used to locate parathyroid adenomas larger than 1 cm. It has the advantages of being fast cheap and relatively harmless but yields suboptimal results patients with a short thick neck requiring a lower frequency transducer which decreases spatial resolution and adenomas located in "silent," low contrast, US areas of the neck (Ahuja et al.; Fakhran, Branstetter and Pryma). Thyroid complications often occur simultaneously with parathyroid disease which further restricts the use of US because in patients with multi-nodular thyroid disease, nodules can mimic or mask adenomas. Lymph nodes can also easily be mistaken for adenomas.

Nuclear imaging is based on different radiotracer uptake patterns and kinetics between the thyroid gland, normal parathyroid and abnormal parathyroid. Specifically, radioiodine is taken up and organified by the thyroid (which uptakes iodine normally) whereas blood flow tracers such as ^{201}thallous chloride and ^{99m}Tc sestamibi are used to identify both the thyroid and enlarged parathyroid glands. The most common use is the injection of Technetium ^{99m}Tc labelled 2 –methoxy-isovutyl-isonitrile (sestamibi) and is often considered the gold standard for pre-operative localization of hyperfunctioning parathyroid tissue. Overactive parathyroid glands tend to absorb the tracer more than the surrounding tissue. Patients are imaged using single photon emission computed tomography (SPECT) after the tracer is administered. Sestamibi scintigraphy can detect abnormally located parathyroid adenomas with more than 90% accuracy but requires

administration of a radiopharmaceutical, use of sophisticated scanning equipment and well-trained operators. Due to the small size of the parathyroid gland the sestamibi scan can give false-negatives or recognize some thyroid diseases as a false-positive due to uptake of the tracer (Ahuja et al.; Fakhran, Branstetter and Pryma). As a result, a second image is usually taken hours after the initial image because adenomas should display delayed washout of the tracer due to their hyperactivity.

Other imaging modalities are used to supplement US and sestamibi scans. Thin-section, contrast-enhanced CT has been used with reported sensitivity ranging from 46 – 87%. CT is most often used in addition to ultrasound in order to find abnormal glands in nonresponsive areas. It is also used to agree with sestamibi findings. CT is better at detecting harder to find parathyroid adenomas over ultrasound but is susceptible to movement artifacts during imaging and exposes the patient to ionizing radiation. As in ultrasound, lymph nodes can also be mistaken for adenomas. (Ahuja et al.; Fakhran, Branstetter and Pryma). Similarly, MRI has been used in recent years with a sensitivity of 65 – 80%. MR is another option that is used to confirm results rather than a first line technique. Adenomas can appear much more intense in T2-weighted images but only 40% of masses exhibit this effect. Due to limited availability, high cost and long examination time, MRI is still not widely used (Ahuja et al.).

The inherent problem with all preoperative methods is that they are only images. They are reporting information about the anatomy, however, when the neck is opened the anatomy is not as clear and parathyroid glands can be obscured by the thyroid gland, fat and blood. Additionally, in the cases of CT and sestamibi imaging, a patient who may already have cancer is exposed to doses of radiation. Except for ultrasound, each method

requires expensive equipment and additional technicians to operate adding to the price of the surgery. Finally, every method is only applicable when the parathyroid gland is enlarged or hyperactive and susceptible to false positives from concurrent thyroid disease or lymph nodes. There is the need to guide surgery in real-time with high accuracy.

Current intraoperative techniques include iPTH and radio-guided parathyroidectomy. Intra-operative assays are a measure of the levels of parathyroid hormone in the blood. Once the hyperfunctioning gland is removed, the amount of PTH will return to normal. However, PTH starts to degrade around four minutes so the samples must be rushed to the testing lab which is located outside the OR. Additionally, the assays are expensive and are only available at centers that perform a high volume of parathyroidectomies (Kim). Radio-guided parathyroidectomy involves the intravenous administration of technetium-99m-sestamibi 1-2 hours before surgery. A hand-held gamma probe is used to localize the abnormal glands, however, the radiation background is unvalidated and the technique is susceptible to non-selective uptake of the radionucleotide as in the preoperative method (Ahuja et al.; Kim).

Fluorescence Spectroscopy

An accurate, automated diagnostic method could allow faster, more effective patient management. The application of optical spectroscopy is suggested because it can detect differences in tissue architecture and biochemical composition. In particular, fluorescence spectroscopy has been of considerable interest in the development of new clinical diagnostic tools. Fluorescence measurements of human tissue can be made in real-time, without tissue removal and diagnosis based on tissue fluorescence can be easily automated. Fluorescence spectroscopy is the most commonly tested optical technique for

the *in vivo* detection of diseases. Fluorescence imaging can reveal the localization and measurements of intracellular molecules, sometimes at the level of single-molecule detection. Fluorescence is now a dominant methodology used extensively in biotechnology, flow cytometry, medical diagnostics, DNA sequencing and forensics to name just a few (Lakowicz "Introduction to Fluorescence"). Fluorescence spectroscopy of both exogenous and endogenous fluorophores has been successfully used to identify neoplastic cells and tissues in a variety of organ systems (Ramanujam). Studies have successfully demonstrated the potential of fluorescence to improve diagnosis in various organ systems (Andersson-Engels et al.; Baumgartner et al.; Hung et al.; Ingrams et al.; Lohmann et al.; Panjehpour et al.; Ramanujam et al.; Schomacker et al.; Tang, Pradhan and Alfano). Intrinsic tissue fluorescence (autofluorescence) has been used to differentiate normal and non-normal tissues in the human breast and lung (Alfano et al.), brain (Hung et al.), oral mucosa (Ingrams et al.) and cervix (Lohmann et al.).

Application of optical spectroscopy to endocrine surgery is limited to disease detection. Several groups have applied autofluorescence spectroscopy with excitation in the ultraviolet and visible wavelengths as well as Raman spectroscopy for the discrimination of laryngeal and thyroid cancers from normal tissues (Arens et al.; Giubileo et al.; Liu et al.; Medina-Gutierrez et al.; Pitman et al.; Prosst, Willeke et al.; Z.V. Jaliashvili). Two groups demonstrated the use of 5-aminolevulimic acid (ALA) to guide parathyroidectomies due to hyperparathyroidism. Increased ALA fluorescence with HPT resulted in strong fluorescence contrast of (hyper)parathyroid tissue compared to background soft tissues and thyroid demonstrating the potential of using 5-ALA to guide dissection in parathyroidectomies(Asher et al.; Prosst, Gahlen et al.; Prosst,

Willeke et al.). Stone et al. used NIR Raman spectroscopy for *ex-vivo* diagnosis of adenoma and hyperplasia in parathyroid tissue in patients undergoing parathyroidectomies. Their results showed a detection sensitivity of 95% for parathyroid adenomas and 93% for hyperplasia (Das et al.). However, no papers were found that applied optical methods for anatomic guidance of endocrine surgery rather than disease detection.

Near Infrared Wavelengths

Biological fluorophores typically exhibit fluorescence in the UV/VIS wavelengths. As excitation wavelengths become longer, autofluorescence decreases (Lakowicz "Fluorophores"). Thus, there are no published accounts of near infrared autofluorescence being observed in tissues; near infrared wavelengths are attractive due to their increased penetration depth in biological tissues. Research in near-infrared fluorescence has mostly involved exogenous contrast agents, the most common of which are polymethines. In particular, indocyanines, such as indocyanine green, and cardio-green has been used extensively as contrast agents for many applications. Inorganic fluorescent semiconductor nanocrystals (quantum dots) solve many instability problems of organic fluorophores and have been used to help identify esophageal sentinel lymph nodes (Frangioni; Parungo et al.). However, application of contrast agents is associated with many problems such as potential toxicity, photobleaching and localization. Autofluorescence uses biological fluorophores that occur naturally in tissues and thus negate the need for the introduction of foreign agents that may be toxic. Preliminary studies show strong autofluorescence past 800 nm by parathyroid tissues in vitro as well

as *in-vivo*. This method has the advantages of intrinsic fluorescence and avoids the problems associated with exogenous contrast agents.

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CHAPTER II

A NOVEL OPTICAL APPROACH TO THE INTRAOPERATIVE DECTION OF PARATHYROID GLANDS

Introduction

Complications with the parathyroid and thyroid glands are common with 25-28 cases per 100,000 of hyperparathyroidism and approximately 20 million affected with some sort of thyroid disease (Kim; Sosa et al.). Surgical means are used to remove the affected gland(s) when the disease cannot be treated by other methods (Doherty). Endocrine surgeries have traditionally involved meticulous dissection and resection of diseased glands while leaving the normal glands intact. Inadvertent removal of parathyroid glands is a recognized complication of this procedure. The incidence of inadvertent parathyroidectomy ranges from 8% to 19% out of patients undergoing total thyroidectomy (Lin et al.). Such inadvertent removal or accidental injury of the parathyroid may lead to complications such as postoperative hypocalcaemia and hypoparathyroidism that could have consequences on the long-term regulation of calcium homeostasis post-operatively

The thyroid gland, parathyroid glands, nerves, adipose tissue, and lymph nodes are closely positioned in the neck region. Due to their close proximity and tendency to blend into each other, many of these structures, specifically the parathyroid glands, are difficult to distinguish visually during endocrine surgery. The situation is further complicated by the parathyroid's small size and variability in position. Existing methods for identifying parathyroid

glands rely on histopathology or post-operative diagnosis of symptoms to determine if the parathyroid was accidentally or incompletely removed which can lead to complications(Frilling and Weber; Prosst, Gahlen et al.). Surgeons must ultimately rely on visual inspection to identify the different tissues, which can be subjective and often inconclusive (Bliss, Gauger and Delbridge; Miller). Complications occur when the parathyroid is accidentally injured or removed during thyroidectomies or only partially removed in the case of parathyroidectomies(ATA). An accurate, automated diagnostic method could allow faster, more effective patient management (Ahuja et al.; Fakhran, Branstetter and Pryma).

Current intraoperative techniques include intact parathyroid hormone (iPTH) assay and radio-guided parathyroidectomy(Ahuja et al.; Kim). Intra-operative assays are a measure of the levels of parathyroid hormone in the blood. Once the hyperfunctioning gland is removed, the amount of PTH will return to normal. However, PTH starts to degrade around four minutes so the samples must be rushed to the testing lab which is located outside the OR. Additionally, the assays are expensive and are only available at centers that perform a high volume of parathyroidectomies (Kim). Radio-guided parathyroidectomy involves the intravenous administration of technetium-99m-sestamibi 1-2 hours before surgery. A hand-held gamma probe is used to localize the abnormal glands. However, the radiation background is unvalidated and the technique is susceptible to non-selective uptake of the radionucleotide(Ahuja et al.; Kim). The major drawback of these techniques is that they are only applicable in cases of hyperfunctioning parathyroid tissue and in the case of iPTH the gland must be found by the surgeon anyways. Therefore, there is a continued need for a tool to find the parathyroid intraoperatively.

Optical spectroscopy can detect differences in tissue architecture and biochemical composition; in particular, fluorescence spectroscopy has been of considerable interest in the development of new clinical diagnostic tools. Fluorescence measurements of human tissue can be applied in real-time, without tissue removal and diagnosis based on tissue fluorescence can be easily automated (Ramanujam et al.). Auto and dye induced fluorescence have been applied for the detection of atherosclerosis and various types of cancers. Exogenous fluorescent dyes have also been shown to selectively collect in tumor tissue and have been used for enhancement of fluorescent contrast between normal and neoplastic tissue in the human lung, brain and colon(Ramanujam). Auto-fluorescence spectra of normal and diseased tissues have been measured from several organ sites, both *in vitro* and *in vivo(Ramanujam)*. Application of optical spectroscopy to endocrine surgery is limited to disease detection. Two groups demonstrated the use of 5-aminolevulimic acid (ALA) to guide parathyroidectomies due to hyperparathyroidism. Increased ALA fluorescence with HPT resulted in strong fluorescence contrast of (hyper)parathyroid tissue compared to background soft tissues and thyroid demonstrating the potential of using 5-ALA to guide dissection in parathyroidectomies(Asher et al.; Prosst, Gahlen et al.; Prosst, Willeke et al.). Several groups have applied autofluorescence spectroscopy with excitation in the ultraviolet and visible wavelengths as well as Raman spectroscopy for the discrimination of laryngeal and thyroid cancers from normal tissues (Arens et al.; Giubileo et al.; Liu et al.; Medina-Gutierrez et al.; Pitman et al.; Prosst, Willeke et al.; Z.V. Jaliashvili). Stone et al. used NIR Raman spectroscopy for *ex-vivo* diagnosis of adenoma and hyperplasia in parathyroid tissue in patients undergoing parathyroidectomies. Their results showed a detection sensitivity of 95% for parathyroid adenomas and 93% for hyperplasia (Das et al.). However, no

papers were found that applied optical methods for anatomic guidance of endocrine surgery rather than disease detection.

Tissue typically exhibits fluorescence signal in the UV/VIS wavelengths, or about 400 – 700 nm(Lakowicz "Fluorophores"). As excitation wavelengths become longer, autofluorescence decreases making NIR wavelengths attractive due to their increased penetration depth in biological tissues(Lakowicz "Fluorophores"). There have been recent efforts to use NIR wavelengths for fluorescence spectroscopy in the diagnosis and detection of disease. One group took advantage of NIR autofluorescence in conjunction with cross-polarized light scattered images to detect breast cancer, but this work was on the edge of the NIR window using 632.8 nm excitation(Demos et al.). Another group demonstrated NIR autofluorescence to detect melanin distribution in the skin(Han et al.).

Our goal was to develop an optical method to discriminate parathyroid tissue from all other anatomical structures in the neck. We applied a method based on intrinsic NIR autofluorescence for identification of parathyroid tissue regardless of disease state for direct clinical application in endocrine surgery. Data was collected from 21 patients undergoing surgery *in vivo* in real-time. In every patient, parathyroid tissue exhibited more intense autofluorescence above 800nm allowing us to distinguish it from the surrounding tissue.

Methods

Measurements were performed at the Vanderbilt University Medical Center under approval of the Vanderbilt Institutional Review board. Twenty-one patients ages 18-99 regardless of race and gender were included in the study under informed written consent. All patients with primary thyroid or parathyroid pathophysiology undergoing thryoidectomy or

parathyroidectomy were considered. An initial evaluation was conducted by the participating endocrine surgeon (Dr. John Phay) while seeing the patients at the Vanderbilt Clinic and final eligibility was determined in the preoperative evaluation based on the clinical condition and safety of the patient.

Tissue was excited with a 785 nm diode laser (Innovative Photonic Solution, Monmouth Junction, NJ) supplying 80 mW of power at the tissue. Clinical spectra were recorded with an Ocean Optics (Ocean Optics, Dunedin, FL) S2000-FL fluorescence spectrometer with a spectral resolution of approximately 10.5 nm (FWHM) using a custom program in LabVIEW (National Instruments, Austin, TX). An additional Schott color glass filter (RG-830, CVI Melles Griot, Albuquerque, NM) was used to reduce signal from the reflected laser light and visible wavelengths. A sterilized fiber optic probe with a 400 μm diameter excitation spot size was used for measurements. Initial background measurements were recorded with the laser off. Six measurements were taken at each tissue site at an integration time of 300 ms each by touching the probe to the tissue of interest; the tissue type was noted, along with physician's confidence in the investigated sites' histological identity. If excised, specimens were processed and analyzed by a pathologist and spectral results were validated with histology. The fluorescent operating room lights were turned off during spectral measurements.

Spectra obtained in the clinic were processed using MATLAB (Mathworks Inc., Natick, MA). First the background was subtracted from each sample; any negative values resulting from the subtraction were considered to be noise and set to zero. The data was then corrected for the wavelength dependent response of the system with a NIST calibrated light source. Six measurements for each site were averaged together. Finally, all spectra were normalized to the maximum intensity of the mean thyroid spectrum from that patient. Clinical spectra were

smoothed with an averaging filter of size 10. A right-tailed student's T-test was used to test for the significance of the increased parathyroid signal over the 21 patients. Values of $P \leq 0.05$ were considered to be significant.

Imaging studies were performed by exciting tissue with the same 785 nm diode laser, defocused to provide a ~6 cm diameter spot size. Images were obtained using a Aspherical HF 23-80 mm f/3.5-5.6 macro lens (Sigma, Ronkonkoma, NY) with a PhotonMAX 512 (Princeton Instruments, Trenton, NJ) charge-coupled device (CCD) camera. A notch filter was placed in front of the lens to block reflected laser light. Images were recorded using Winview software (Princeton Instruments). Frozen samples of thyroid and parathyroid tissue were obtained and thawed and at room temperature in phosphate-buffered saline in a petri dish. A non-reflective, non-fluorescent layer was placed between the samples and the dish. The fluorescent room lights were turned off and the diode laser was used to illuminate both tissue samples equally. The light was delivered at a slight angle such that background specular reflection from the tissue was minimized and the camera was placed 7 inches above the tissues and focused. Images were taken with a 200 ms acquisition time using Winview. The images were processed using MATLAB to remove speckle by applying a median filter.

Optical property measurements were performed using human tissue samples of parathyroid tissue from pathology. An excitation-emission matrix was obtained on a Fluorolog-3 FL3-111 Spectrofluorometer (HORIBA Jobin Yvon Inc., Edison, NJ). A portion of the tissue was placed in a quartz cuvette for measurements. Excitation wavelengths were scanner from 650 – 800 nm in 5 nm increments and emission wavelengths were scanned from 810 – 850 nm in 2 nm increments. Fluorescence measurements were collected at .5s acquisition time. Measurements within 10 nm and of the excitation wavelength and beyond its harmonic were set

to zero. An absorption spectrum was taken with a Varian Cary 5000 Spectrophotometer (Varian, inc., Walnut Creek, CA). The tissue sample was placed between two glass slides with a pinhole slit. Wavelengths were scanned from 1000 – 375 nm in 2 nm intervals at an acquisition time of 1s.

Results

Participants and study

Clinical measurements were performed under Vanderbilt Institutional Review Board (IRB) approval at the Vanderbilt University Medical Center. Twenty-one patients regardless of age, race and gender, were included in the study under informed written consent. All patients with primary thyroid or parathyroid pathophysiology undergoing thryoidectomy or parathyroidectomy were considered. An initial evaluation was conducted by the participating endocrine surgeon while seeing the patients at the Vanderbilt Clinic. Final eligibility was determined in the preoperative evaluation based on clinical condition and safety of the patient.

A standardized protocol for all fluorescence measurements from patients *in vivo* was followed. During each surgery, the sterilized optical probe was placed in contact with various tissues in the exposed neck area and spectral measurements were acquired from each of those sites. The tissue type was noted, along with the physician's confidence in the investigated sites' histological identity. Spectra were collected using a 300 ms signal collection time. In all cases, the overhead fluorescent lights were turned off during the measurements and any luminescent lights left on were turned away from the measurement site as they contain spectral components that can interfere with the results. Following surgical resection, investigated sites were collected

for histological identification. All specimens collected were processed and analyzed by a pathologist.

Fluorescence characterization

Individual spectra from each patient were examined and the fluorescence from the parathyroid was compared to the thyroid and other tissues in the neck. Figure 1 depicts the spectra acquired from a typical patient.

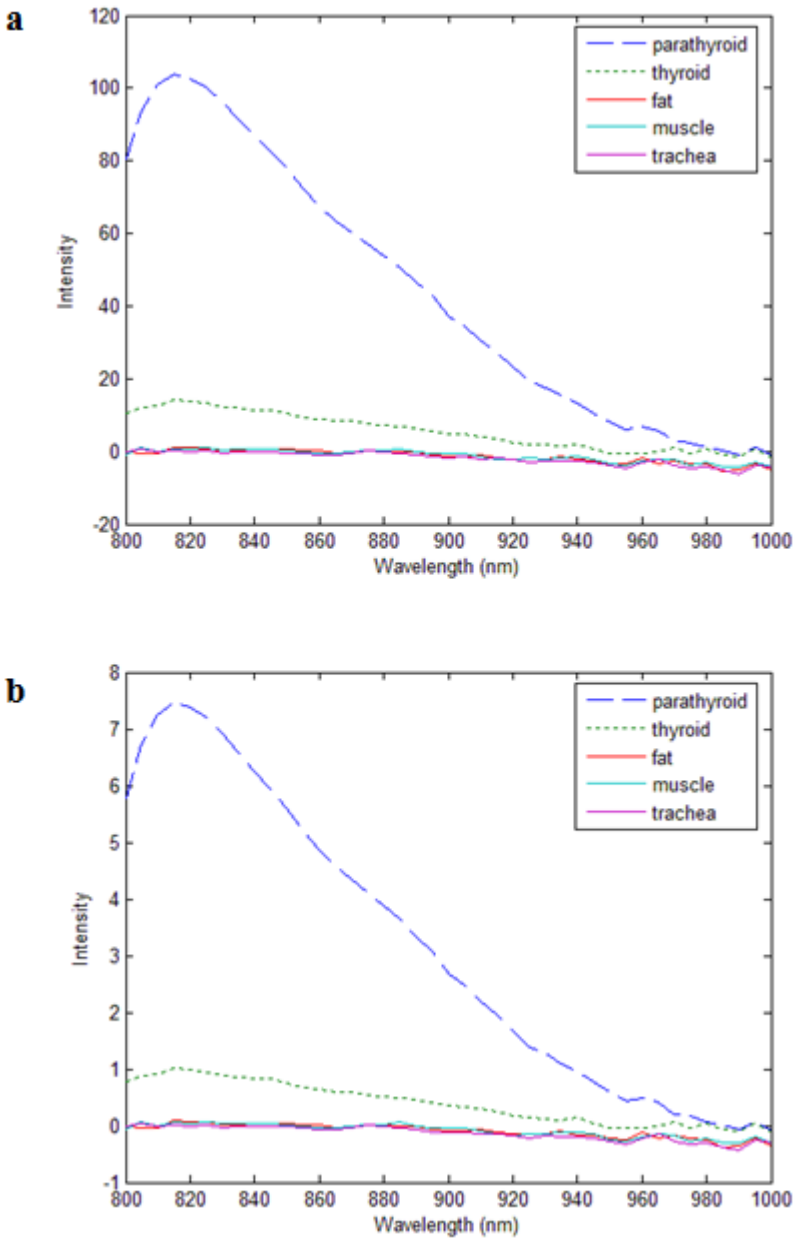

Figure 1: Typical NIR spectra (A) Signal from parathyroid (dashed line), thyroid (dotted line) and fat, muscle and trachea. Each spectra is taken as the average of 6 measurements at the site of investigation. (B) Normalized signal from parathyroid tissue (dashed line), two thyroid measurements (dotted line) and fat muscle and trachea. The parathyroid signal is significantly stronger than the anything else in the neck. It is seven times greater than the thyroid and its peak intensity.

The signal from the parathyroid gland is observed to have the highest peak intensity and is easily distinguishable from the surrounding tissues. Further, thyroid fluorescence is stronger than surrounding muscle and fat but weaker than the parathyroid. Figure 2 shows the average peak intensity of the parathyroid normalized to the thyroid fluorescence of a particular patient, for all 21 patients. Parathyroid fluorescence can be seen to be consistently greater than that of the thyroid and other tissues with a p-value of .0001. Furthermore, parathyroid is 2 – 11 times more fluorescent than all other tissues found in the neck across all patients *in vivo*. Power analysis of the peak intensity values shows that the parathyroid exhibits more intense fluorescence with a power over 90% with a confidence of 99%. This phenomenon was also observed *in vitro*.

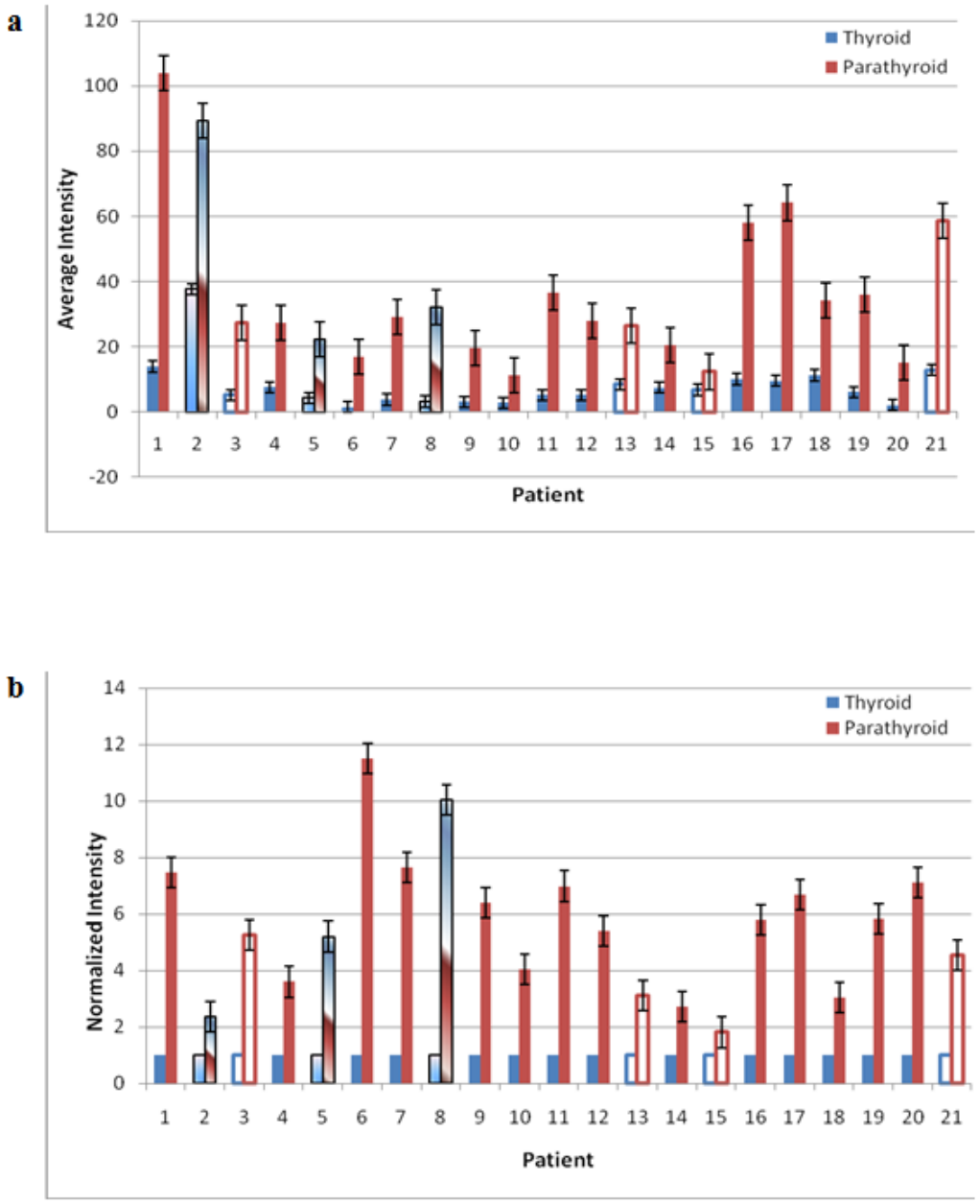

Figure 2: Parathyroid tissue consistently exhibits a stronger signal than thyroid tissue. (A) Average peak intensity from parathyroid and thyroid measurements within each patient as recorded by the NIR system. (B) Normalized peak intensity from parathyroid and thyroid measurements within each patient. Each measurement is normalized to the average peak of all thyroid measurements. Thyroid disease, parathyroid disease and concurrent thyroid and parathyroid diseases are represented by solid bars, outlined bars and a gradient respectively.

In vitro imaging of tissue

Detection using a probe based NIR fluorescence system provides a fast and accurate way to detect parathyroid glands. This method is particularly useful when the parathyroid is located deep in the neck and is not necessarily exposed. However, in most cases, an imaging system would provide more spatial information to the surgeon improving upon the guidance using the probe based system. An NIR imaging setup was assembled to assess the feasibility of imaging the parathyroid. Samples of thyroid and parathyroid tissues were placed side by side and illuminated with the 785 nm laser used for *in vivo* measurements. A filter was used to block all reflected light from the laser. Figure 3 demonstrates the fluorescent image of the samples. The parathyroid fluoresces just over two times stronger than the thyroid illustrating that not only is it possible to capture the intrinsic fluorescence with a camera but the parathyroid exhibits stronger fluorescence *in vitro* as well as *in vivo*.

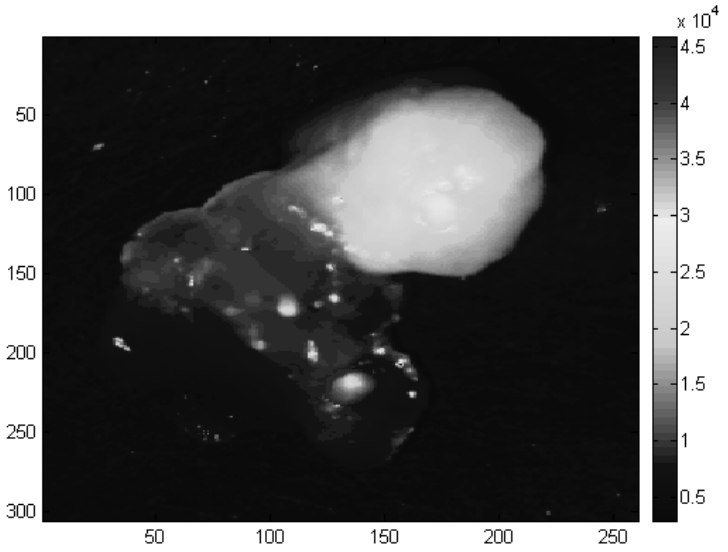

Figure 3: *In vitro* NIR fluorescence of parathyroid and thyroid samples obtained from pathology. The parathyroid located on the right exhibits approximately two times stronger overall fluorescence than the thyroid.

Optical property measurements

There are no known biological fluorophores that autofluoresce above 800 nm. In order to isolate the responsible fluorophore, experiments were performed to determine the optical properties of parathyroid tissue. First, the absorption spectrum was recorded using a spectrophotometer. As shown in figure 4, there are no distinct features in the 700-800 nm range. The large jump in the spectrum is due the machine changing from the NIR to the UV/VIS detector. Above 800nm, there is a large broad peak between 800 and 1000 nm possibly correlating to the peak emission detected at 820 nm.

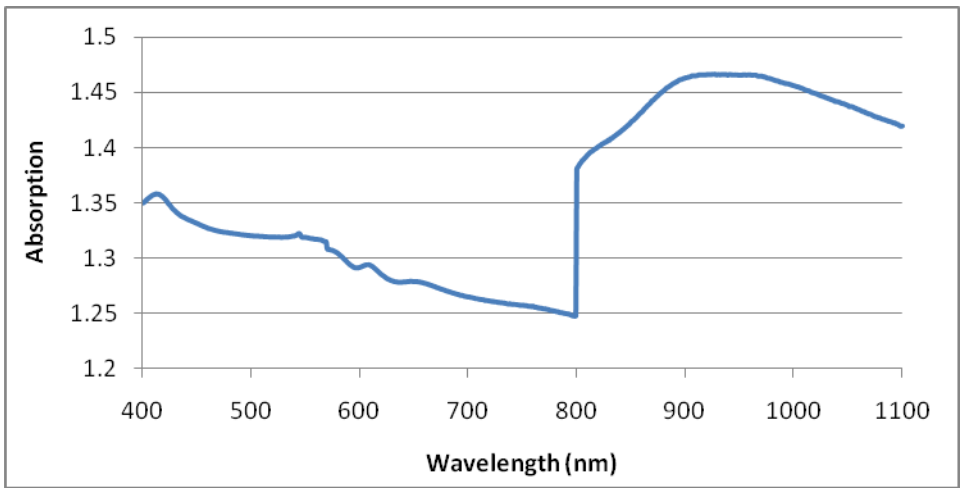

Figure 4: Absorption spectrum of parathyroid tissue

The fluorescence peak was examine by creating an excitation-emission matrix centered around the detected peak at 820 nm. Excitation wavelengths were scanned from 650 – 800 nm and emission wavelengths were recorded from 800 – 850 nm. Figure 5 shows the resulting matrix with a peak around 822 nm. The peak is very broad stretching from 680 nm to 800 nm. These results confirm the peak that is being detected *in vivo*.

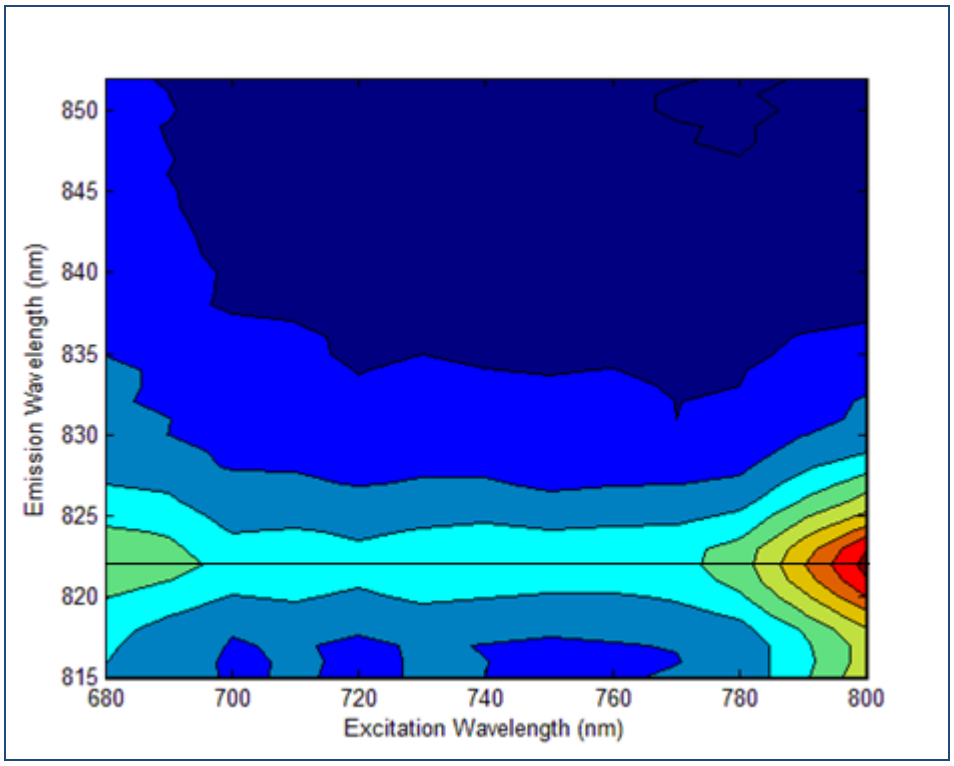

Figure 5: Excitation-emission matrix or parathyroid tissue. The matrix shows a broad peak around 822 nm shown by the line.

Discussion

Results presented here show that NIR fluorescence spectroscopy can successfully detect parathyroid tissue *in vivo*, in real-time and non-intrusively during endocrine surgery. In each patient, the parathyroid signal is greater than the thyroid signal and other tissues in the neck. Moreover, the standard error and p-value show that the parathyroid can be classified with a statistically significant difference each time. This success is very promising for endocrine surgery. Not only can the system discriminate parathyroid glands from the surrounding tissue but it does so with high accuracy. Near infrared fluorescence is quick and relatively cheap to implement whereas other intraoperative localization methods such as sestamibi scan can be very

time consuming and expensive(Ahuja et al.; Fakhran, Branstetter and Pryma; Kim). This method improves upon the accuracy and sensitivity of visual recognition: a highly subjective measure dependent on the experience of the surgeon. Further complicating the situation, thyroidectomies and parathyroidectomies are typically performed by general surgeons (particularly in centers/hospitals without a division of endocrine surgery). In these cases, the level of experience in finding the parathyroid may be further reduced pointing to the clinical need for the proposed method.

Preliminary imaging studies show that it is possible to image the NIR fluorescence from thyroid and parathyroid tissue. Further, as in the probe based measurements, the parathyroid fluoresces at least two times stronger than the thyroid providing the same guidance with improved spatial information. Imaging could improve the utility of NIR fluorescence in the clinic by providing real-time feedback in the OR while removing the need for the surgeon to interact with the device. Such an approach would free up the hands of the surgeon and provide spatial context to correlate the actual field with spectral information and therefore the location of the parathyroid.

Near infrared wavelengths are attractive in biomedical applications due to their increased penetration depth and decreased scattering and absorption in biological tissues. Research in NIR fluorescence has mostly involved exogenous contrast agents, the most common of which are polymethines. In particular, indocyanines, such as indocyanine green (cardio-green) have been used extensively as contrast agents for many applications. Inorganic fluorescent semiconductor nanocrystals (quantum dots) solve many instability problems of organic fluorophores and have been used to help identify esophageal sentinel lymph nodes (Frangioni; Parungo et al.). However, contrast agents are difficult to translate to the clinic typically due to potential problems

such as toxicity, photobleaching and localization. Autofluorescence uses biological fluorophores that occur naturally in tissues and thus negate the need for the introduction of exogenous agents that may be toxic. Our studies show strong autofluorescence past 800 nm by parathyroid tissues *in vitro* as well as *in vivo*. This method has the advantages of NIR wavelengths and avoids the problems associated with exogenous contrast agents.

No known intrinsic biological fluorophores have been reported to exhibit fluorescence around 800 nm of the NIR region(Lakowicz "Introduction to Fluorescence"). However, this paper clearly demonstrates the consistent presence of autofluorescence at 820 nm peak emission in parathyroid and thyroid tissues. Das et al. have used Raman to examine parathyroid pathology but used 830 nm excitation missing the fluorescence peak(Das et al.). The biological basis for this NIR fluorescence is presently unknown. One possible candidate is the presence of Parathyroid Hormone (PTH) in the parathyroid being responsible for the signal. However, PTH exhibits no fluorescence in this region (unpublished data). Furthermore, hyperfunctioning parathyroid glands show no increase in signal intensity. Thyroid tissue also exhibits similar but reduced fluorescence and PTH is not known to be present in these glands, therefore, PTH is not responsible. We expect that potential candidates are present in the thyroid and parathyroid but in greater amount or concentration in the parathyroid or that the fluorescence is somehow quenched in the thyroid but not in the parathyroid. Perhaps the fluorescence is due to the innate optical properties of glandular tissues explaining why it is not seen in the surrounding tissues in the neck. Tissue samples can be thawed and refrozen with no immediate impact on the fluorescent signal. Detailed analysis of the parathyroid and thyroid needs to be performed to identify the primary constituent responsible for the fluorescence.

Initial optical property experiments provided some interesting possible findings but indicate that more in depth analysis is required. The spectrophotometer only provided transmission measurements. To accurately determine the optical properties, tissue samples need to be examined using a double-integrating sphere setup that can determine transmission and diffuse reflectance. This data could then be modeled using inverse adding-doubling to calculate the absorption and reduced scattering coefficients (μ_a and μ_s'). The excitation-emission matrix confirmed the peak that was detected *in vivo*. Interestingly, the peak detected by the spectrofluorometer was much broader than a typical fluorescence peak; however, the experiment has a potential flaw because the peak of interest is near the edge of the detection capability of the detector. Repeat experiments need to be performed on a machine with a wider range of wavelengths in order to remove any effects introduced by the detector.

The results obtained show that the increased signal in the parathyroid is consistent across all patients and disease states. Moreover, the variability in ratio is not related to disease. Figure 2 demonstrates that thyroid disease (solid bars), parathyroid disease (outlined bars) and concurrent parathyroid and thyroid disease (gradient filled bars) have no consistent trend. The type of disease does not appear to be related to the signal strength that is recorded from the thyroid or parathyroid. Parathyroid tissue produces a much stronger signal in all diseases making it applicable across all endocrine surgeries as opposed to current intra-operative localization methods which are restricted to cases of hyperparathyroidism where the parathyroid glands are enlarged and/or hyperactive(Ahuja et al.). Additionally, the unique signal found in thyroid and parathyroid tissue allows detection of infiltrating cells in the surrounding tissues. Cancerous thyroid or parathyroid tissue that has spread to the lymph or surrounding tissue result

in increased fluorescence in non-fluorescent tissues.. This can, in fact, be used to detect the presence of thyroid or parathyroid cancer metastasis outside the patients' glands.

This paper presents the potential of using NIR fluorescence for the real-time guidance of endocrine surgery. Even though the basis for this fluorescence is not understood, NIR fluorescence provides consistent and accurate detection of the parathyroid intra-operatively that any surgeon can use regardless of experience. Translation of this technology to practice would reduce the rate of complications due to accidental or incomplete removal of parathyroid tissue. Successful anatomical guidance would also decrease the time necessary for surgery especially during lengthy parathyroidectomies where the surgeon must search for parathyroid glands. Successful translation of NIR imaging in the OR would simplify the implementation of this technology into clinical practice.

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CHAPTER III

FUTURE DIRECTIONS

Near infrared fluorescence was successfully implemented *in vivo* during endocrine surgery. Moreover, the results obtained at the Vanderbilt University Medical Center indicate that the parathyroid produces a distinctly stronger fluorescence signal than any other tissue in the neck. A larger patient database is required to gain a better understanding of the different fluorescence intensities between patients. Specifically, a research plan will be developed for my Ph.D. dissertation to continue taking data on over 120 patients to analyze spectral differences over several factors including: disease state, gender and age. We will also compare the fluorescence from diseased and non-affected tissue of the same type from within the same patient. Further case work will help to statistically establish the effects of various aspects on the signal. This knowledge will help predict when the method might fail and, more importantly, help develop a detection algorithm to automate detection of parathyroid glands in the operating room.

In vitro experiments have shown that is possible to image the fluorescence produced by the parathyroid and thyroid glands. Moreover, as in the spectroscopic studies, the parathyroid exhibits overall fluorescence that is at least twice as strong as the thyroid indicating that imaging has the potential to differentiate the glands as well. An imaging system would provide increased spatial information to the surgeon providing a more intuitive view of the anatomy. An imaging system would be particularly useful because thyroidectomies and parathyroidectomies are commonly performed by general

surgeons with limited experience in locating parathyroid glands. The next logical step is the development of an imaging system which can be implanted in the OR. Imaging can be performed using any detector that is sensitive in the near-infrared region of the spectrum above 800 nm. Preliminary studies have already shown the parathyroid exhibits more intense fluorescence using two different imaging systems. These systems will be developed to optimize their implementation and output in order to provide anatomical guidance to the surgeon. Specifically, the imaging system can be made to integrate with the surgeon's headlight using the same mount and power source. Simple false color mapping would increase the inherent contrast produced by the parathyroid.

The endogenous fluorophore in the thyroid and parathyroid glands remains unknown. Further work needs to be performed to identify the source of the autofluorescence. This will be specifically addressed in an aim of my PhD dissertation. Protein analysis needs to be performed on the glands to isolate the possible sources of fluorescence. The hypothesized fluorophore is the calcium-sensing receptors present in both parathyroid and thyroid tissues. The receptor is also found in the kidney and colon. Experiments on these tissues show autofluorescence exhibited above 800nm indicating that CaR is a good candidate for the responsible fluorophore. Discovering the source of the fluorescence would be an immensely beneficial to the project. It would help ensure optimal excitation and collection schemes and help predict where if at all the method will fail. Additionally, it would elucidate the mechanism for biological NIR fluorescence which could point to other fluorophores throughout the body.

The system has already been used in a clinical setting so a translational to an accepted instrument requires only automation. Successful translation of this technology

to clinical use would reduce the rate of complications from accidental or incomplete removal of parathyroid tissue. Anatomical guidance would also decrease operative time especially during lengthy parathyroidectomies where the surgeon must search for parathyroid glands.