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Label-free Imaging of Thyroid and Parathyroid Glands Using Coherent Anti-Stokes Raman Scattering (CARS) Microscopy

by

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ABSTRACT

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Thyroid and parathyroid glands play a vital role in regulating the body's metabolism and calcium levels. Surgical removal of the glands is the main treatment for both thyroid cancer and parathyroid adenoma. In thyroidectomy and parathyroidectomy, it is very important to differentiate thyroid, parathyroid, and the other tissues around the neck. Here, we applied the emerging CARS technique to image both thyroid and parathyroid tissues, which has potential to be used in real-time *in vivo* examination of different structures. We also developed algorithms to differentiate different cellular structures based on CARS images. When incorporated with a fiber optic endoscope in the future, CARS imaging technique can help surgeons identify cancerous thyroid tissue intraoperatively, preserve good parathyroid glands during thyroidectomy and find parathyroid adenoma during parathyroidectomy.
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# Contents

Acknowledgments ........................................................................................................ iii

Contents ......................................................................................................................... iv

List of Figures .................................................................................................................. vi

List of Tables ................................................................................................................... viii

List of Equations ........................................................................................................... ix

Nomenclature .................................................................................................................. x

Chapter 1 ......................................................................................................................... 1

Introduction ..................................................................................................................... 1

1.1. Basics of thyroid and parathyroid glands ................................................................. 1

1.2. Thyroid diseases and FNA ....................................................................................... 4

1.3. Parathyroid diseases and sestamibi scan ................................................................. 6

Chapter 2 ......................................................................................................................... 9

CARS Microscopy ........................................................................................................... 9

2.1. Fluorescence and Raman spectroscopy ................................................................... 9

2.2. CARS principles ..................................................................................................... 11

Chapter 3 ......................................................................................................................... 15

Materials and Methods ................................................................................................. 15

3.1. Tissue sample preparation ....................................................................................... 15

3.2. Optical imaging system ............................................................................................ 16

3.3. Imaging processing and analysis ............................................................................. 17

Chapter 4 ......................................................................................................................... 20

Results ............................................................................................................................. 20

4.1. CARS images of normal thyroid and benign thyroid tumor .................................... 20

4.2. Separating normal thyroid and benign thyroid tumor ........................................... 22

4.3. Parathyroid adenoma .............................................................................................. 29

Chapter 5 ......................................................................................................................... 34

Discussion ....................................................................................................................... 34
List of Figures

Figure 1.1 - (A) Anatomy of the neck. (B) Parathyroid glands on the back side of thyroid gland. (C) Potential locations of left parathyroid glands. Image courtesy of National Cancer Institute and Norman Parathyroid Center. ............ 2

Figure 1.2 - H&E image of follicles in normal thyroid tissue. ........................................... 3

Figure 1.3 - Frequency of papillary thyroid cancer in children following the Chernobyl disaster of 1986 in Belarus[8]. ................................................................. 5

Figure 1.4 - Sestimibi scan. (A, B) Parathyroid adenoma hiding behind left thyroid lobe. (C, D) Ectopic parathyroid tumor pointed out by arrow. Image courtesy of Norman Parathyroid Center......................................................... 8

Figure 2.1 - Energy diagram of CARS................................................................. 13

Figure 3.1 - Set-up of CARS imaging system...................................................... 17

Figure 4.1 - (A) Normal thyroid tissue. (B) Benign thyroid tumor. ...................... 21

Figure 4.2 - Number of follicular cells on different follicles. ................................. 23

Figure 4.3 - Comparison of nuclei center detection tools..................................... 24

Figure 4.4 - Nuclei detection and segmentation on normal thyroid and benign thyroid tumor H&E images................................................................. 25

Figure 4.5 - Confidence interval of the mean values of the distance between the neighboring follicular cells in normal thyroid and benign thyroid tumor. ....... 26

Figure 4.6 - Confidence interval of the mean values of the distance between the neighboring follicular cells in benign thyroid tumor, papillary non-tumor and adenoma non-tumor......................................................... 27

Figure 4.7 - (A) Papillary thyroid carcinoma. (B) Papillary thyroid carcinoma with papillary architecture. ................................................................. 29

Figure 4.8 - (A) H&E representation of the transition from thyroid to parathyroid glands. Image courtesy of Deltagen Inc. (B) CARS image of parathyroid adenoma tissue................................................................. 30
Figure 4.9 - CARS image of parathyroid adenoma tissue with increased FOV by stitching 18 images together.................................................................31

Figure 4.10 - Nuclei segmentation on CARS image of parathyroid adenoma tissue........................................................................................................32

Figure 4.11 - CARS image of adipose tissue.................................................................33

Figure 5.1 - (A) Original H&E image of a benign thyroid tumor tissue (cropped). (B) Nuclei segmentation using Otsu’s method. (C) Direct calling of watershed function in MATLAB. (D) Watershed regions obtained by watershed function after preprocessing. (E) Our proposed algorithm result. .................................38
List of Tables

Table 1 - Distance between the neighboring follicular cells in normal thyroid and benign thyroid tumor. ................................................................. 25

Table 2 - Distance between the neighboring follicular cells in benign thyroid tumor, papillary non-tumor and adenoma non-tumor. .............................................. 27
List of Equations

Equation 2.1 - Dipole moment induced by incident field............................................. 10
Equation 2.2 - Polarizability approximated by a Taylor expansion......................... 10
Equation 2.3 - Dipole moment in Raman process...................................................... 11
Equation 2.4 - CARS intensity.................................................................................. 12
# Nomenclature

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>CARS</td>
<td>Coherent anti-Stokes Raman Scattering</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>Hematoxylin and eosin</td>
</tr>
<tr>
<td>FNA</td>
<td>Fine needle aspiration</td>
</tr>
<tr>
<td>OPO</td>
<td>Optical parametric oscillator</td>
</tr>
<tr>
<td>FOV</td>
<td>Field of view</td>
</tr>
<tr>
<td>STD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>GVF</td>
<td>Gradient vector field</td>
</tr>
<tr>
<td>PMT</td>
<td>Photomultiplier tubes</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
</tr>
<tr>
<td>ITCN</td>
<td>Image-based tool for counting nuclei</td>
</tr>
<tr>
<td>PDF</td>
<td>Probability density function</td>
</tr>
<tr>
<td>FWHM</td>
<td>Full width at half maximum</td>
</tr>
<tr>
<td>T4</td>
<td>thyroxine</td>
</tr>
<tr>
<td>T3</td>
<td>triiodothyronine</td>
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1.1. Basics of thyroid and parathyroid glands

The thyroid is a butterfly-shaped gland that sits in front of larynx and trachea. The anatomy of the neck is shown in Figure 1.1 (A). The thyroid has two side lobes connected by the isthmus in the middle. There are some important nerves passing through the region near the thyroid, including the superior and recurrent laryngeal nerves[1]. This region also contains lymph nodes and adipose tissues.
Parathyroid glands are normally the size of a grain of rice (6-8 mm long)[1]. There are four parathyroid glands located behind the thyroid, as shown in Figure 1.1 (B). However, the actual locations of parathyroid glands are unpredictable. They can be anywhere in the neck, from just below the jaw all the way down to the chest, which makes them very difficult to find during surgery. In Figure 1.1 (C), the black dots delineate the potential locations of the left parathyroid glands.

Thyroid and parathyroid glands have different functions even though they are located close together. Thyroid is the largest endocrine gland in the body. It secretes thyroxine (T4) and triiodothyronine (T3) that act throughout the body, influencing the body’s metabolism, growth, and temperature. Parathyroid glands monitor and control the amount of calcium in our blood and bones by releasing parathyroid hormone. Calcium is an essential signaling molecule for the nervous and muscular systems, and it also strengthens our skeletal system. In fact, calcium is the only
element that has its own regulatory system. If the calcium level goes down, the parathyroid glands recognize it and in response make parathyroid hormone that goes into the bones and releases some calcium in order to keep the blood calcium level within a normal and narrow range.

![Diagram of follicles in normal thyroid tissue](image)

**Figure 1.2 - H&E image of follicles in normal thyroid tissue.**

The functional unit of the thyroid is called the follicle. Figure 1.2 represents a normal thyroid H&E image, where a lot of follicles can be seen. The follicle consists of a group of follicular cells spherically arranged around colloid. Follicular cells produce thyroglobulin and release it into colloid where T3 and T4 are produced. Parafollicular cells (C cells) are located near the follicular cells in the connective tissue. The primary function of C cells is to release calcitonin to reduce blood calcium level, as opposed to parathyroid hormone trying to increase the blood calcium level.
1.2. Thyroid diseases and FNA

Thyroid cancer is the most common malignancy of the endocrine system. It happens 3 x more frequently in females; in 2013, there were about 45,000 women and 15,000 men diagnosed. Thyroid cancer can be treated successfully if diagnosed early; the ten-year survival rate is over 90%[2]. Thyroid cancer can be classified as papillary, follicular, medullary, or undifferentiated (or anaplastic) carcinoma based on morphology and clinical features[3]. Papillary thyroid carcinoma comprises about 80% of thyroid malignancies. Figure 1.3 shows that there is a striking increase in papillary thyroid cancer in children following the Chernobyl disaster of 1986 in Belarus[4], which suggests that papillary carcinoma is closely linked to certain forms of radiation[5]. The frequency of follicular carcinoma is greater in geographic regions of iodine deficiency compared to regions of normal dietary iodine intake[6]. Medullary carcinoma is often caused by familial nature[7].
The other two major thyroid diseases are hyperthyroidism and hypothyroidism. Hyperthyroidism is associated with excessive thyroid hormone production, and hypothyroidism occurs when the thyroid gland fails to produce enough thyroid hormone. Hyperthyroidism and hypothyroidism can both result in the thyroid gland becoming larger than normal, which may lead to thyroid nodules. It is estimated that about 50% of the population will develop a small thyroid nodule at some time in their life. Most nodules (90%) are benign and unnoticeable[8].

The presence of thyroid nodules is a sign of an underlying problem in the thyroid gland[9]. The traditional diagnosis of thyroid nodules has evolved from palpation to ultrasound guided fine needle aspiration (FNA)[10]. Currently, ultrasound guided
FNA biopsy is the most common method for the diagnosis and management of nodules, lumps, and enlargement of the thyroid gland. It is performed under local anesthesia using a long and thin needle to draw out fluid and cells for analysis. Surgery is the recommended treatment for lesions classified as malignant because chemotherapy is rarely used for thyroid cancer. However, one of the most frustrating challenges facing thyroid patients is when an FNA on a suspicious nodule is found to be "inconclusive" or "indeterminate." In up to 30% of FNA the pathological assessment cannot rule out cancer, which affects about 135,000 patients in the US each year[11]. In these cases, the recommended follow-up is either re-biopsy or surgical examination. The crisis is that only 20% to 30% of inconclusive nodules turn out to be malignant, which indicates that as many as 100,000 patients in the US each year could end up suffering an unnecessary thyroidectomy[11, 12]. In some cases, the suspected nodules have to be surgically removed for further pathological examination, resulting in a turnaround time ranging from hours to days. Also, it is important but difficult to conserve parathyroid glands during thyroid surgery because they are located very close to each other. Surgeons cannot damage or even touch the normal parathyroid glands when they are dealing with thyroid gland.

### 1.3. Parathyroid diseases and sestamibi scan

The United States has about 100,000 cases of hyperparathyroidism per year, mostly due to the effects of calcium and vitamin D in the diet. In 91% of cases, one of the four parathyroid glands becomes tumor and grows large[8]. Hyperparathyroidism is
A destructive disease that can result in redundant release of parathyroid hormone, which may cause high blood calcium level and eventually leads to osteoporosis or other serious health problems. Hyperparathyroidism can be cured in most patients by parathyroid tumor removal. The most common cause of unsuccessful parathyroid operation is that surgeons could not find the parathyroid tumor, as parathyroid glands are very small and they have unpredictable locations in the neck. The other cause is that surgeons remove only one tumor but fail to find the second one.

Inappropriately high level of secretion of parathyroid hormone is an indication of parathyroid adenoma. The preferred method of imaging parathyroid glands is sestamibi scan. Sestamibi scan is a nuclear imaging technique that uses radiopharmaceutical technetium-99[13]. It is currently the best scan to find parathyroid adenoma, but it only has about 50% accuracy[14]. Sometimes the tumor is present, but the scan does not have the resolution to locate it. Sometimes the parathyroid adenoma is hiding behind the thyroid, as seen in Figure 1.4 (A) and (B). Even though the sestamibi scan shows one parathyroid adenoma, up to 15% of people will have a second tumor that is being suppressed and does not show up. The main purpose of taking a sestamibi scan is to check if the patient has an ectopic parathyroid tumor[15], as shown in Figure 1.4 (C) and (D). However, sestamibi scan is still not a perfect way to locate all the parathyroid adenoma.
Old-fashioned parathyroid surgery relies too much on the surgeon's head and neck operative experience. Since parathyroid disease is quite unusual, many endocrine surgeons do not perform parathyroid surgery on a frequent basis. They would have to make a big incision in the neck and explore for all parathyroid glands, which will usually take 3-8 hours until every gland is found and sent to a pathologist to determine if the gland is good or bad. Another challenge is that it is difficult to differentiate parathyroid glands from the surrounding adipose tissues by visual examination. The pathologist has to look under the microscope to determine the amount of adipose tissues in the samples. Parathyroid tumor usually has less adipose tissues than normal parathyroid glands.

Figure 1.4 - Sestimibi scan. (A, B) Parathyroid adenoma hiding behind left thyroid lobe. (C, D) Ectopic parathyroid tumor pointed out by arrow. Image courtesy of Norman Parathyroid Center.
2.1. Fluorescence and Raman spectroscopy

A variety of optical imaging techniques have been explored to complement the traditional diagnostic approaches. Stained histopathology remains the gold standard for diagnosing disease in tissue and relies on experienced pathologists to assess subtle architectural features of labeled biological structures at the subcellular, cellular, and tissue levels. However, it is a subjective practice that takes from hours to days. Fluorescence imaging has been demonstrated as a useful tool in a wide range of applications[16-19]. Although fluorescence imaging provides relatively high signal-to-noise ratio, the fluorophores must be approved by FDA before clinical use. During the process, most contrast agents fail due to toxicity. There is a strong need for a label-free and rapid histopathologic approach to give pathologists more powerful tools for disease diagnosis, especially for in vivo imaging applications[20].
Raman spectroscopy identifies specific chemicals by capturing intrinsic chemical features in the sample without the use of external labels. The Raman effect can be understood as follows[21]. An incident electromagnetic wave induces a dipole moment during the light-material interaction, which is given by

\[ P = \alpha E_0 \cos(2\pi\nu_0 t) \]

**Equation 2.1 - Dipole moment induced by incident field.**

where \( \alpha \) is the polarizability and \( \nu_0 \) is the frequency of the incident field. The polarizability is a function of the instantaneous position of the atoms, and the atoms are confined to quantized vibrational energy levels. The physical displacement \( dQ \) of the atoms about their equilibrium position can be expressed as

\[ dQ = Q_0 \cos(2\pi\nu_{\text{vib}} t) \]

where \( Q_0 \) is the maximum displacement the atoms can reach and \( \nu_{\text{vib}} \) is the frequency of the vibrational mode. If the displacements are small, the polarizability can be approximated by a Taylor expansion

\[ \alpha = \alpha_0 + \frac{\partial \alpha}{\partial Q} Q_0 \cos(2\pi\nu_{\text{vib}} t) \]

**Equation 2.2 - Polarizability approximated by a Taylor expansion.**
Substituting Equation 2.2 into Equation 2.1, the final dipole moment can be expressed as

\[ P = \alpha_0 E_0 \cos(2\pi \nu_0 t) + \left( \frac{\partial \alpha}{\partial Q} \frac{Q_0 E_0}{2} \right) \{ \cos[2\pi(\nu_0 - \nu_{\text{vib}})t] + \cos[2\pi(\nu_0 + \nu_{\text{vib}})t] \} \]

**Equation 2.3 - Dipole moment in Raman process.**

Equation 2.3 demonstrates that the induced dipole moments are created at three different frequencies, where \( \nu_0 \) corresponds to the Rayleigh elastic scattering, \( \nu_0 - \nu_{\text{vib}} \) refers to the Stokes shift, and \( \nu_0 + \nu_{\text{vib}} \) refers to the anti-Stokes shift.

However, spontaneous Raman effect is typically very weak; therefore, the data acquisition time is extremely long, preventing its applications from the study of living systems[22-24].

### 2.2. CARS principles

Coherent anti-Stokes Raman Scattering (CARS) is a label-free nonlinear four-wave mixing Raman process based on molecular vibrational spectroscopy[25-27]. The intrinsic molecular vibrational contrast provides a noninvasive characterization of the sample without using external labeling or staining. The energy diagram of the CARS process is shown in Figure 2.1. CARS signal is resonantly enhanced when the frequency difference between the pump and Stokes beams matches the frequency of a molecular vibration. If the pump and probe beam are the same, the resulting anti-Stokes signal is at \( \omega_{\text{as}} = 2\omega_p - \omega_s \), where \( \omega_p \) is the pump frequency and \( \omega_s \) is the
Stokes frequency. Assuming plane pump and Stokes waves, one obtains the CARS signal intensity by solving the wave equation

$$I_{CARS} \propto |\chi^{(3)}|^2 I_P^2 I_s \left( \frac{\sin(\Delta k l / 2)}{\Delta k / 2} \right)^2$$

**Equation 2.4 - CARS intensity.**

where $\chi^{(3)}$ is the third-order susceptibility, $I_P$ is the pump intensity, $I_s$ is the Stokes intensity, $l$ denotes the interaction length between the sample and the light fields, $\Delta k = k_{as} - (2k_p - k_s)$ is the wave-vector mismatch. CARS signal intensity is maximized when $\Delta k l$ is close to zero, i.e., $k_{as} = 2k_p - k_s$. This is regarded as the phase-matching condition for CARS, which determines the direction of the anti-Stokes wave. By using a high NA objective, the phase match condition is relaxed because the interaction length between the three beams is so short that the waves are unable to run out of phase[26]. The complex coefficient $\chi^{(3)} = \chi_{NR}^{(3)} + \chi_R^{(3)}(\omega)$ has two terms[25], resonant $\chi_R^{(3)}(\omega)$ and non-resonant $\chi_{NR}^{(3)}$. The non-resonant part is purely due to electronic response, where the electrons will oscillate at the beat frequency $\omega_p - \omega_s$ when they are driven by the two incident fields regardless of the presence of vibrational modes. Such oscillation will modulate the refractive index of the material. A changing refractive index will modulate the third light (probe beam) at the non-resonant beating frequency[28]. Various methods have been reported for suppressing the background, for example, polarization sensitive detection[29, 30],
interferometric mixing[31, 32] and frequency modulation[33]. The resonant part, on the other hand, depends on molecular vibration in nature. The presence of vibrational modes perturb the polarizability of the molecule's electrons and enhance their oscillation whenever the difference frequency \( \omega_p - \omega_s \) matches the molecular vibrational frequency.

![Energy diagram of CARS](image)

**Figure 2.1 - Energy diagram of CARS.**

CARS signal is orders of magnitude stronger than spontaneous Raman due to the following reasons[28, 34]. First, from Equation 2.4 we know that CARS signal scales with three incident fields, while for spontaneous Raman the signal scales linearly with the incident field. Second, spontaneous Raman is an incoherent process because the molecules are oscillating at random phases. In contrast, because the molecules in CARS are driven by incident fields with strong peak powers, their electron motions are phase correlated as they all move in step. The coherent nature
also makes CARS signal scale quadratically with the molecular density, while for spontaneous Raman the signal scales linearly with the molecular density. Another important practical advantage of coherence is that CARS signal propagates in a well-defined direction, which makes it easy to collect with a collimating lens. Lastly, since CARS signal is generated from a small excitation volume ($<1\ \mu m^3$), it is suitable for 3D sectioning imaging[35, 36].
Chapter 3

Materials and Methods

3.1. Tissue sample preparation

Fresh human thyroid tissues were obtained from patients with benign thyroid tumors and patients with papillary carcinoma. Parathyroid tissues were obtained from patients with parathyroid adenoma. The acquisition of these tissues was approved by Houston Methodist Hospital. The excised tissues were immediately snap-frozen in liquid nitrogen for storage. A total of nine patients were enrolled in this study, including 4 cases of benign thyroid adenoma, 4 cases of papillary thyroid carcinoma, and 1 case of parathyroid adenoma. Adenoma non-tumor and papillary non-tumor tissue samples were also collected from thyroid patients. Frozen tissue samples were passively thawed for 30 minutes at room temperature before image acquisition. After imaging, all samples were marked to indicate the imaged
locations, fixed with 4% formaldehyde, paraffin-embedded, sectioned through imaged locations, and stained with hematoxylin and eosin (H&E).

### 3.2. Optical imaging system

We utilize a tunable picosecond optical parametric oscillator (OPO, Levante, APE, Berlin) and a mode-locked Nd:YVO4 laser (High-Q Laser, Hohenems, Austria) as our optical source system. The laser provides a 7-ps, 76-MHz pulse train at 1064 nm, and a frequency-doubled pulse train at 532 nm. The 1064 nm pulse train is used as the Stokes beam. The 532 nm pulse train is used to pump the OPO to generate the 5-ps pump beam at 817 nm. The resulting CARS signal is at 663 nm which corresponds to CH$_2$ stretch vibration mode (2845 cm$^{-1}$). The Stokes and pump laser beams are spatially and temporally overlapped in the microscope, and tightly focused by a 1.2-NA water immersion objective lens (60×, IR UPlanApo, Olympus, Melville, NJ), yielding a CARS resolution of 0.4 μm in the lateral plane and 0.9 μm in the axial direction, respectively[37]. The resolution is measured by imaging various size-calibrated polystyrene beads (103 nm, 200 nm, 509 nm, 723 nm) on a coverslip and making a Gaussian fit to the CARS intensity profile to render the FWHM values. A band-pass filter (hq660/40m-2p, Chroma Inc.) is placed before the photomultiplier tube (PMT, R3896, Hamamatsu, Japan) to filter out other photons except the CARS signal. The upright microscope is modified from an FV300 confocal laser scanning microscope (Olympus, Japan) adopting a 2D galvanometer. A dichroic mirror is used in the microscope to separate emission signal from excitation laser beams. The acquisition time is about 4 seconds per imaging frame of 512 × 512
pixels. Image is displayed with the Olympus FluoView v5.0 software. The field of view (FOV) that we can normally achieve is $236 \times 236 \, \mu m$. An auto-stage (PS3J100, Prior Scientific) is installed to automatically collect a series of images in order to obtain a bigger field of view by stitching all the images together. Bright-field images of the H&E slides are examined with an Olympus BX51 microscope as a standard control. The overall set-up is shown in Figure 3.1.

![CARS Imaging System Diagram]

**Figure 3.1 - Set-up of CARS imaging system.**

### 3.3. Imaging processing and analysis

We applied two approaches towards nuclei segmentation. The first method consists of five steps\cite{38,39}, which were used to process H&E images: 1) Convert H&E images to gray-scale images. 2) Take a background correction to correct the uneven illumination in the background\cite{40}. The correction works by fitting a smooth cubic
B-spline surface to the image which produces a better and better estimate of the intensity variations of the background. All image pixels that are brighter than a constant number (we use 1.7) of standard deviation from the background estimate are considered to belong to the foreground and are masked away. The iterative estimate of the background converges when the average change in pixel value between two successively calculated backgrounds is less than a certain threshold[41].

3) Use Gaussian filtering[42] to suppress noise and generate a unique local intensity maximum inside each nucleus, which can be used to represent the position of the nucleus as well as the seed point. After Gaussian filtering the detection of nuclei is reduced to the local intensity maxima detection problem. 4) Implement a gradient vector field (GVF) on the filtered images to detect the central points[43, 44]. The pseudo-code for nuclei detection with GVF can be found in Fuhai Li et al. [45]. In the gradient vector field, all the gradient vector lines are pointing toward the local maximum. The pixels will move along the gradient vector lines and stop at the central points inside the nucleus. Thus the nuclear center can be defined by finding the point where a significant number of pixels are gathered together. 5) Apply a seeded watershed segmentation algorithm to estimate the nuclear boundary[46, 47]. In the seeded watershed segmentation algorithm, we envision each gray-scale image as a topological surface. There are three types of points in topological images: regional minimum, catchment basins, and watershed lines. Regional minimum is defined by Gaussian filtering and gradient vector field. Catchment basins are points at which a drop of water will fall into a single regional minimum. Watershed lines are points at which a drop of water will be equally likely
to fall into more than one regional minimum. Watershed lines are delineated by a flooding process [46]. Imagine we punch a hole at each seed of the catchment basins and flood the area with water. The water will rise until it reaches a point where two or more floods coming from different seeds may merge. We build dams on these points to avoid merging. These dams define the watershed lines of the topological surface, which separate one catchment basin from the next one.

The second method is a semi-automatic segmentation algorithm which consists of one manual step and four automatic steps to precisely delineate boundaries of cell nucleus [48]: 1) Manually select a point inside each cell nucleus. 2) Crop an image patch centered at the selected point with predefined size. 3) Apply a seeded watershed algorithm on the image patch to obtain a rough estimation of the nuclear boundary. 4) Use intensity threshold to identify another rough nuclear region. 5) Fit the overlapped watershed and thresholding results (step 3 and 4) with an ellipse using least square fitting criterion [49] to obtain a refined nuclear boundary. An additional ellipse-fitting algorithm is developed to overcome the situations when the semi-automatic approach does not work well. In this algorithm, the user needs to select four points on the nuclear boundary in order to generate an accurate result. All the above mentioned code can be found in the supporting materials.
4.1. CARS images of normal thyroid and benign thyroid tumor

Using CARS imaging, we were able to obtain images of both normal thyroid and benign thyroid tumor with sufficient level of contrast to allow identification of cellular structures and comparison to those identified from H&E images. We used the combination of a half-waveplate and a polarizer to change the laser intensities for both the pump and Stokes beams. Here, the pump was tuned to 200 mW, and the Stokes was tuned to 100 mW. Each CARS image was acquired within 4 seconds. Figure 4.1 (A) illustrates representative CARS and H&E images of a normal thyroid tissue, where many follicles can be observed. Not only can we see the follicular cells surrounding the colloid, but also the nearby C cells. These structures are closely correlated with the H&E picture from the same sample. In order to show the capability of 3D sectioning of CARS imaging, we took multiple CARS images as we
moved the focus point every one-micron deeper into the tissue. The resulting movie can be found in the supporting materials. It should be noticed that the depth of penetration is limited by the scattering of photons inside the tissue which will overwhelm the CARS signal if the focus point goes beyond 0.5 mm deep[28].

Figure 4.1 - (A) Normal thyroid tissue. (B) Benign thyroid tumor.
Unlike normal thyroid tissues, CARS and H&E images of benign thyroid tumor in Figure 4.1 (B) look different in terms of the follicle sizes. Benign thyroid tumor seems to have smaller colloid than those in normal thyroid tissues. The biological reason for this phenomenon is largely due to the rapid reproduction of follicular cells in benign thyroid tumor tissues, which may have invaded into the nearby colloid.

4.2. Separating normal thyroid and benign thyroid tumor

The distinctive follicular features discussed in section 4.1 can help surgeons differentiate normal thyroid tissue from benign thyroid tumor. We collected the number of follicular cells on different follicles with different sizes in both normal thyroid and benign thyroid tumor tissues. We used weighted least-square fitting curves to represent the tendency of the data points. The result in Figure 4.2 proves that benign thyroid tumors tend to have more follicular cells arranged around colloid than those in normal thyroid tissues, which suggests a way of separating benign thyroid tumor and normal thyroid tissues.
Moreover, we investigated this phenomenon by measuring the specific distance between the neighboring follicular cells in both normal thyroid and benign thyroid tissues. A nuclei detection and segmentation algorithm (discussed in methods) was developed in order to find the center of each follicular cell nucleus. We tested this algorithm by comparing it with the cell counting tool called ITCN in ImageJ. ITCN plugin uses Laplacian of Gaussian filtering as the nuclei center detector[39]. However, ITCN is very sensitive to the input estimated value of the cell diameter as well as the background noise. As indicated in Figure 4.3, our algorithm works better than ITCN in locating the centers of the nuclei especially when the nuclei have various sizes and shapes.

**Figure 4.2 - Number of follicular cells on different follicles.**
We then applied the proposed algorithm on the H&E images of both normal thyroid and benign thyroid tumor. The results shown in Figure 4.4 clearly reveal all the centers of the nuclei. Next, we measured the distance between the neighboring follicular cells using ImageJ. The statistical results are shown in Table 1.
Figure 4.4 - Nuclei detection and segmentation on normal thyroid and benign thyroid tumor H&E images.

<table>
<thead>
<tr>
<th></th>
<th>Benign thyroid tumor</th>
<th>Normal thyroid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Minimum/μm</td>
<td>5.13</td>
<td>5.13</td>
</tr>
<tr>
<td>Median/μm</td>
<td>8.97</td>
<td>13.46</td>
</tr>
<tr>
<td>Maximum/μm</td>
<td>31.09</td>
<td>50.00</td>
</tr>
<tr>
<td>Mean/μm</td>
<td>9.58</td>
<td>16.49</td>
</tr>
<tr>
<td>STD/μm</td>
<td>2.69</td>
<td>9.36</td>
</tr>
</tbody>
</table>

Table 1 - Distance between the neighboring follicular cells in normal thyroid and benign thyroid tumor.
We found that the mean value of the distance could be a promising feature to separate normal thyroid and benign thyroid tumor tissues. The 95% confidence interval for benign thyroid tumor is $9.58\pm0.32 \, \mu m$, and $16.49\pm1.92 \, \mu m$ for normal thyroid, as shown in Figure 4.5. The wider range of 95% confidence interval for normal thyroid is due to the smaller sample size.

**Figure 4.5 - Confidence interval of the mean values of the distance between the neighboring follicular cells in normal thyroid and benign thyroid tumor.**

We further collected more than 1400 data points to verify the above findings. Notice that the previous normal thyroid tissues were obtained from a patient with papillary carcinoma, so we called them “papillary non-tumor”. We also got some normal thyroid tissues from a patient with thyroid adenoma, which we named “adenoma non-tumor”. Although they are both normal tissues, they differ in cellular structures as indicated by the different mean values of the distance between the
neighboring follicular cells, shown in Table 2. The 95% confidence intervals in Figure 4.6 of the three groups are different, which can serve as a reference data set to help surgeons identify different classifications of thyroid tissues.

<table>
<thead>
<tr>
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<th>Benign thyroid tumor</th>
<th>Papillary non-tumor</th>
<th>Adenoma non-tumor</th>
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</thead>
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<tr>
<td>Minimum/μm</td>
<td>4.49</td>
<td>5.13</td>
<td>4.81</td>
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<tr>
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<td>31.41</td>
<td>50.00</td>
<td>33.97</td>
</tr>
<tr>
<td>Median/μm</td>
<td>8.01</td>
<td>13.46</td>
<td>9.29</td>
</tr>
<tr>
<td>Mean/μm</td>
<td>8.51</td>
<td>16.49</td>
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<tr>
<td>STD/μm</td>
<td>2.33</td>
<td>9.36</td>
<td>3.75</td>
</tr>
</tbody>
</table>

Table 2 - Distance between the neighboring follicular cells in benign thyroid tumor, papillary non-tumor and adenoma non-tumor.

Figure 4.6 - Confidence interval of the mean values of the distance between the neighboring follicular cells in benign thyroid tumor, papillary non-tumor and adenoma non-tumor.
Figure 4.7 (A) shows the imaging results of papillary thyroid carcinoma. We can find a number of dense cancerous cells gathering together in papillary thyroid carcinoma tissues, without evident presence of thyroid follicles. The disappearance of follicles is mainly due to the damage on follicular epithelium caused by radiation to the neck[2]. The images in Figure 4.7 (B) were taken from another papillary thyroid carcinoma tissue. In this CARS image, we can see abundant cells grouped in papillary architecture, revealing a branching pattern with a regular external contour[50]. Therefore, CARS images can be used to differentiate normal thyroid tissue from papillary thyroid carcinoma by identifying the presence of follicles.
4.3. Parathyroid adenoma

Although parathyroid glands and thyroid gland are close with each other, they have different cellular structures under the microscope. Figure 4.8 (A) shows the H&E
image of the transition from thyroid gland to parathyroid gland. The parathyroid gland cells are very dense, and no follicle can be seen. This observation is approved by the CARS image of parathyroid adenoma tissues shown in Figure 4.8 (B).

Small field of view (FOV) of CARS imaging has long been an issue, as surgeons would be more confident to make diagnosis if they could see a larger area of the sample. By using a programmable auto-stage, we were able to collect a series of CARS images at different preset sampling locations. After that, we stitched them together using MATLAB in order to increase the FOV. In the example shown in Figure 4.9, we stitched 18 CARS images to reach $972 \times 636 \, \mu m^2$ FOV. The dark parts in the image are because of the uneven background that CARS images normally have, but do not affect diagnosis.
We again used a seeded watershed algorithm to extract the cell nuclei of the parathyroid adenoma tissue. The program shown in Figure 4.10 was designed for CARS images and very convenient to use[48]. The red boundary will automatically show up after selecting one point inside each cell nucleus. If the cell boundary is not very clear, the manual-ellipse method is preferred in which the user needs to select four points on the nuclear boundary in order to get an accurate result. We used this
program to extract the cell nuclei from the CARS images of parathyroid adenoma tissue, normal thyroid tissue, and benign thyroid tumor.

![Image of CARS image with nuclei segmentation](image)

**Figure 4.10 - Nuclei segmentation on CARS image of parathyroid adenoma tissue.**

We then measured the nuclear sizes in the three cases. The average nuclear area in parathyroid adenoma tissue is 14.189 um², which is smaller than those in normal thyroid tissue and benign thyroid tumor. The normal thyroid tissue and benign thyroid tumor have similar cell nuclear size, 23.932 um² and 23.849 um², respectively. Therefore, extracting and measuring the cell nuclear size can help us differentiate parathyroid adenoma tissue from normal thyroid tissue or benign thyroid tumor.
In addition, we used CARS to look at the adipose tissue located near the parathyroid adenoma tissue. The CARS image of adipose cells shown in Figure 4.11 looks very different as compared to parathyroid adenoma. This could be potentially useful when surgeons are not able to separate parathyroid adenoma tissue and the surrounding adipose tissue during surgery.

Figure 4.11 - CARS image of adipose tissue.
5.1. Image acquisition

Unlike fluorescence emission and spontaneous Raman scattering where the signal is emitted in all directions, the radiation pattern in CARS microscopy is dependent on the size, shape, and nonlinear susceptibilities of the scattering objects\[26\]. It was previously believed that strong CARS signal could be generated mostly in the forward direction because of phase matching, whereas the epi-CARS signal was severely suppressed due to destructive interference of the CARS photons. However, a dominant epi-CARS signal can be detected in thick samples such as thyroid and parathyroid tissues because up to 45% of the forward-propagating CARS signal is backscattered by multiple scattering events due to the turbidity of the specimen\[27\]. Collecting CARS signal in the epi-direction has become a routine method for tissue imagine.
With the collinear beam geometry, CARS images can be recorded by either raster scanning the sample plane or the incident laser beams. In the sample-scanning scheme, the imaging speed is primarily limited by the scanning rate and the photon counting rate. The acquisition time for a cell image of 512 × 512 pixels was reported to be around 10 minutes[26]. In our experiment, by using a laser-scanning microscope with a pair of galvanometer mirrors and two near-IR picosecond laser beams of high repetition rate, we were able to acquire a CARS image (512 × 512 pixels) within a few seconds. The pixel dwell time for the scanning was about 15.3 µs/pixel.

The beating frequency was tuned to match symmetric CH$_2$ stretching bonds which are abundant in lipid. The cell nuclei appear as dark spots in CARS images, indicating that there are few oscillators in the nuclei. The uneven background in CARS images is a common issue, most likely due to the chromatic aberrations of the lenses inside the microscope. Before taking CARS images, we adjusted the pump and Stokes laser beams so that their pulses were temporally and spatially overlapped and tightly focused at the center of the FOV. When the incident laser beams moved away from the center of the lenses during laser-scanning procedure, the dispersive lenses would fail to focus the two colors to exact the same convergence point and weaken the phase match. This could explain why CARS images always look brighter in the middle and darker at the edges. However, this phenomenon is consistent in every image, which does not affect the subsequent image processing and diagnosis.
In the image-stitching algorithm, we overlapped part of the adjoining CARS images so as to reduce the effect of the uneven illumination.

### 5.2. Photodamage

Photodamage may result from a number of mechanisms, which are closely related with the excitation conditions and the material properties of the sample. Yan Fu et al. has studied the photodamage to the myelin sheath in spinal tissues under CARS microscopy[51]. They measured the photodamage rate by altering different parameters including laser power, Raman shift, excitation wavelengths, and repetition rate. It took about one minute to induce blebbing at average power of 9.24 mW (CARS image can be acquired in just a few seconds). They concluded that keeping average powers below 10 mW and pulse energies below 1 nJ on the focus point was generally safe for imaging live cells under fast scanning conditions. However, because myelin sheath is very rich in lipid, they can generate plenty of CARS photons with low laser powers. In our experiment, we took CARS images on solid tumor tissues which possessed a low lipid level relative to normal tissues. As a consequence, a higher excitation power was required so as to obtain enough image contrast. In our previous work, we used 200 mW (2.63 nJ, peak power at 463 W) for pump beam and 100 mW (1.32 nJ, peak power at 165 W) for Stokes beam to image breast cancer[52, 53] and lung cancer[37, 54-56]. The average power at the focal plane was 75 mW and 35 mW for the pump and Stokes beams, respectively. This power combination was higher than that typically used for CARS imaging, but it did not cause any photodamage. We used roughly the same power in our thyroid and
parathyroid applications, and we did not observe photodamage here either. The
tolerance to laser power in human body is considered to be higher than that in
thawed tissues, therefore less photodamage is expected for clinical applications.

5.3. Segmentation algorithm

MATLAB provides a function called `graythresh` that computes a global threshold
using Otsu’s method[57] to minimize the intraclass variance of the black and white
pixels. However, Otsu’s method failed to separate adjacent cells on our images as
shown in Figure 5.1 (B), which led to under-segmentation issue. MATLAB also has a
watershed function that identifies different watershed regions and labels them.
Direct calling of `watershed` function on our images would still cause over-
segmentation (Figure 5.1 (C)), as the watershed transform treats every little dark
spot as a regional minimum and thus creates intensive catchment basins. Hence,
pre-processing, which normally involves identifying and selecting the catchment
basins that contain objects, is a must for successful segmentation. To implement this
using MATLAB built-in functions, we first separated the objects from the
background by using the same binarization method that was contained in our
proposed algorithm. Instead of applying the GVF to detect the seed points, we then
used `imextendedmax` and `imcomplement` functions to locate regional minimum. The
resulting image after applying watershed function is shown in Figure 5.1 (D) and the
corresponding codes can be found in the supporting materials. Notice that although
the cell nuclei are mostly segmented out, the boundaries are coarse and not as
smooth as the result that we got. What’s more, we would be unable to detect the centers of the nuclei without the use of GVF.

Figure 5.1 - (A) Original H&E image of a benign thyroid tumor tissue (cropped). (B) Nuclei segmentation using Otsu’s method. (C) Direct calling of watershed function in MATLAB. (D) Watershed regions obtained by watershed function after preprocessing. (E) Our proposed algorithm result.

Our proposed nuclei detection and segmentation algorithm resolved the under-detection issue by using the nuclei’s shape information to refine the segmentation results (Figure 5.1 (E)). We first classified all the nuclei into well-segmented and under-segmented nuclei by measuring the Gaussian probability density function (PDF) values. In the PDF model, nine features were empirically selected from the
training data set to predict the possibility of a given nucleus belonging to the well-
segmented group[39]. The under-segmented candidates would then go through a
splitting method to find the final splitting line. Two points located in the quarter and
three-quarter positions of the major axis of a given under-segmented nucleus would
be selected as the centers of the overlapped nuclei. One disadvantage of our CARS
microscopy system is that it cannot reveal the nuclear structure in detail. The nuclei
of papillary carcinoma are typically irregular, along with nuclear grooves and
pseudoinclusions[2]. These features could hardly be seen under our CARS
microscopy system, because the size of a nucleus is close to our current resolution
limit, and the nuclei seldom contain chemical bonds that are readable for CARS.

5.4. Summary and future work

In this project, we acquired the first sets of CARS images of normal thyroid tissue,
benign thyroid tumor, papillary thyroid carcinoma, and parathyroid adenoma. We
developed algorithms to differentiate different cellular structures based on CARS
images. This proposed technique could be very useful especially when it is
incorporated with a fiber optic endoscope[58, 59], so that we could help surgeons to
identify cancerous thyroid tissue when FNA fails, reserve good parathyroid glands
during thyroidectomy, and find parathyroid adenoma during parathyroidectomy.
The current approach is of great value even without the incorporation of an
endoscope probe, as it could be readily used to examine surgically excised samples
without the need of frozen section analysis. The estimated time to completely
analyze each sample is under 5 minutes, which is much shorter than the typical 20
minutes delay for pathological evaluation[48]. Therefore, our approach can significantly accelerate the surgical procedure and thus reduce surgical costs and patient suffering.

Our next step would be taking CARS images on the other two types of thyroid carcinoma, i.e., medullary carcinoma and follicular carcinoma. Regional node metastases are common for thyroid carcinoma. We want to see how thyroid cancerous cells spread into the neighboring lymphatic channels. Also, we want to acquire some normal parathyroid tissues to see the difference between the normal parathyroid and parathyroid adenoma. The current study serves as a proof-of-concept for future in vivo applications. In order to do real-time point-of-care optical molecular imaging and diagnosis, we need to combine the CARS technique with a microendoscope probe[60, 61]. Further improvement of the image processing algorithm will be another strategy to facilitate the integration of the CARS imaging system with current surgical process such as computerized decision making systems. Also, we will extend this technology for other disease applications, such as neurodegeneration and brain tumors. Finally, we have a plan to combine the diagnostic imaging technique with the ultrafast femtosecond athermal laser to do image-guided microsurgery[62].
References


