Biomedical Applications of Stimulated Raman Scattering Microscopy

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Biomedical Applications of Stimulated Raman Scattering Microscopy

A dissertation presented

by

Wenlong Yang

to

The Department of Chemistry and Chemical Biology

in partial fulfillment of the requirements

for the degree of

Doctor of Philosophy

in the subject of

Chemistry

Harvard University

Cambridge, Massachusetts

April 2017
Biomedical Applications of Stimulated Raman Scattering Microscopy

Abstract

Stimulated Raman scattering (SRS) microscopy emerged as a promising tool for label-free chemical sensitivity microscopy. SRS doesn’t have non-resonant four-wave mixing background that plagued coherent anti-Stokes Raman scattering and is capable of more sensitive quantitative chemical imaging at the speed of video rate. Despite the advantages of SRS, there are still challenges preventing SRS from becoming a major tool in biology and medicine.

The thesis aims at solving these challenges and establish SRS as a major tool in biomedical research and clinical applications. The thesis first demonstrates the effort to reduce the cost of SRS with an all-fiber laser source and high-speed auto-balanced detector. Then, it shows several first of the kind applications of SRS: imaging neurotransmitters at frog neuromuscular junction, tracking disease progression in mouse models of amyotrophic lateral sclerosis (ALS), performing a drug test on ALS mice without sacrificing them, and clinical study of distribution of active ingredients of skin care product on live human in collaboration with Unilever. At last, it shows the development of a simultaneous two-color SRS setup by adding a fiber amplifier to conventional SRS setup, and efforts toward further improvement of the sensitivity of CRS by performing three-color time-resolved CARS.

In summary, the thesis shows exciting new applications of SRS. It also presents key technological developments that leads to in vivo, high-speed, cost-effective, sensitive SRS imaging, which are crucial for broader applications of label-free chemical microscopy in the future.
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Acknowledgements

First and foremost, I would like to thank my advisor, Professor Xiaoliang Sunney Xie for giving me a wonderful PhD experiences. I am so thankful that I could learn from him the characters of first-class scientists: broad and solid scientific knowledge, acute identification of good science, accurate foresight about research development, swift execution of research plans and rigorous in scientific writings. I am grateful for his trust and financial support to work on so many exciting project and explore my own research interests. His courage to pursue difficult, high-risk and important scientific problems greatly inspired me to overcome fear and tackle important problems. His care about my personal life and family are greatly appreciated.

Sunney has recruited a group of intelligent, stimulating and loving people in the lab. I am very fortunate to have the opportunity to work closely with many of them. First of all, I am very thankful for the mentorship of Dr. Christian Freudiger. He has guided me during the development of balanced detector for the fiber laser and shared with me a lot of insight into the technology of SRS. His passion in doing good science and develop useful technology greatly inspired me. I am thankful for the help from Dr. Gary Holtom. He is always ready to share his knowledge in laser, electronics, etc. Besides that, he can always tell interesting stories behind these technologies. I am grateful to his help during the development of auto-balanced detector. And whenever any instrument is not working, he is always able to make it work. I also am grateful for the discussion with James MacArthur when designing auto-balanced detector. He is a true master of electronics and always willing to share his opinions.

I want to thank Dr. Dan Fu to involved me in the project of imaging neurotransmitter in frog. He is very smart, honest and always a pleasure to work with. He has been a true friend in and out of lab. I also want to thank Prof. William Betz and Dr. Achim Klug for their selfless help on the
frog surgery, drug use and generous hosting at Denver. (I especially enjoy shooting arrows on target by blowing into a tube, when I almost considered becoming a hunter in Amazon rain forest.) Without them, we won’t be able to image neurotransmitter. I also want to thank Gary here to make the laser for the most sensitive spectral-focusing SRS. I also want to thank Prof. Jeff Lichtman to provide helpful comments on this work.

I also want to thank my wonderful collaborator Feng Tian and Prof. Kevin Eggan. I especially thank Feng for the diligent work during the three years to take care of mice and sometimes inject drugs on mice on daily basis. We had a lot of fun doing surgery and imaging on live mice and see how ALS gradually destroyed their sciatic nerves. Feng has a lot of interesting ideas. I am grateful for his gifts from China every time he went back to China. It’s always been a lot of fun to have dinner with him and his girlfriend. I would like to thank Kevin’s support and guidance on the ALS project. It’s been a great experience for me to write a paper together with him. He is a great leader and encourages his team to bring out their potentials. Every time after discussion with him, I was eager to make our paper better and had the feeling that our paper can be published in nature. It’s also been a pleasure to work with everyone else from his lab. Especially, I would like to thank Dr. Naoki Suzuki for his help on staining of dorsal raphe.

I feel grateful to have the opportunity to work with Ang Li for about two years. His knowledge impressed me greatly from the first day I met him. There are just unlimited things I can learn from him and it is not hard to learn anything from him, even though the technology looks complicated. He has deep understanding on technologies, so that he can explains them very clearly. His pursuit of perfection has greatly influenced me. From him, I learned how important it is to do things right in the beginning. Even though it may seem time consuming, eventually, it will save time and produce superior results. The paper of simultaneous-two color SRS would not look so good
without his help from experiment to paper writing. He also made substantial contribution to the *in vivo* imaging on human skin.

It’s my pleasure to have worked with Liyun (Jessica) Sang. Jessica is an honest person, a smart and curious learner, a great scientist, an inspiring entrepreneur and a strong woman. I learned a lot of biology and techniques from her. Her broad knowledge, being sensitive to new science and passion for a better health care make the exosome work possible. She designed all the cell treatment protocols and cultured the cells. Cells are so obedient in her hands. I am really grateful to have worked with this wonderful woman.

I also want to thank Dr. Fa-Ke (Frank) Lu on the help all over the years, especially the help on live mouse imaging. I am in debt to him for passing me the knowledge on career development and he is always willing to help on machine tasks. I also want to thank Yuanzhen Suo for the help in the fiber amplifier project. Yuanzhen is always willing to help. Academically, he is very smart and a fast learner. I want to thank Minbiao Ji for designing the software in tiling and wavelength scanning. I also want to thank him for hosting hot pot parties, which makes my life more colorful. It’s always an enjoyable experience to visit his home and let our children play together. I want to thank Dr. Xu Zhang for the discussions and company in the basement meeting room for 4 years.

I also want to thank Yunlong (Richard) Cao for the help on fat in muscle project and methylation of cell-free DNA project. Richard is smart and hardworking. He has a great knowledge on many fields especially food. It is always a satisfying and relaxing thing to have a conversation with him in the meeting room.

It is my pleasure to have collaborated with Lin Feng, Dane Drutis and Manoj Misra of Unilever. I want to thank them to expose me to the high-level research in industry. I am greatly
impressed about their professional attitude toward research and I am thankful for their hospitality every time I went there.

I want to thank Prof. Zeming Yao for his instructions and push on the fat in muscle project. Even though I couldn’t finish, I had the opportunity to read the literatures in this area. And it just fascinated to learn how much our food and lipid storage may affect our health. His philosophical viewpoints always make me think hard and deeper about science, faith and life. His weekend beer garden is such a wonderful event, and easily among most memorable experiences in the past 6 years. His wife, Mrs. Wang, has a heart of gold and is always welcoming and busy preparing food for everybody.

I want to thank David Lee as a great company of me sitting next to me for five years. It’s one of my favorite things to discuss research and social issue with David. He is always my source of Western culture. I and all my lab are in debt of him for organizing several outing events. BBQ in blue mountain and paint balls are among the most memorable events in the past 6 years. His dedication to friends is undoubted.

I want to thank Dr. Patricia Purcell for the care, the help on paper writing and providing suggestions on career developments. Patty is such a wonderful gift from God to our lab. She helps and cares everybody in the lab. She transformed the lab into a family.

I want to thank Tony and Hertz for the warm receptions when I first join the lab. I want to thank Tan for being such a wonderful coworker that sharing instruments and space has never been an issue. I am so thankful to be able to enjoy Tan’s skillful art work and funny introduction in the beginning of each of his group meeting talk. I want to thank Dr. Xiaohui Ni for discussions on SRS and cancer genomics. I want to thank Dr. Chongyi Chen on discussions about science and
society. I want to thank Chi-Han Chang on the help of microfluidic devices. I want to thank Lawrence Valles, Lin Song, Tracey Schaal for all the support during these years. Larry has been a great help on reimbursing all the travel costs. He is a truly responsible lab manager. I want to thank staff of department of chemistry. Especially, I want to thank Kathy Oakley diligently remind me of all important dates.

I want to thank other lab members Alec Chapman, Wenting Cai, Dr. Shasha Chong, Dr. Pin Cui, Dr. Lei Huang, Dr. Terumasa Ito, Dr. Sijia Lu, Dr. Lingjie Kong, Dr. Xiaohui Ni, Dr. Sabin Mulepati, Dr. Shufang Wang, Dr. Ziqing (Winston) Zhao, Dr. Chenghang Zhong for being together and discuss and work together occasionally. I am grateful that my experience with all these people are nothing but pleasant.

I want to thank Professors Xiaowei Zhuang and Adam Cohen for serving in my dissertation committee. Questions and criticisms from them are always honest, important and inspiring. I enjoyed the committee meetings a lot and can always learn something from them.

At last, I want to thank my wonderful wife Cuilin Qin, for love, care and support for me and my two naughty children Jason and Jessica. She has never complained about our low income during all the years. When I have any career plan, she is always supportive even though it may mean a blow to her own career development. I want to thank my family in China for giving me the freedom to pursue my own career goal and even provide financial support when I need them. I want to thank my mom to come to help my family through difficult times. She is very open-minded and loves new technology, a rare case for people who grew up during culture revolution. I am heavily influenced by her. I also want to thank my father’s support and influence. He always tries to hold the highest moral standard for himself and others. It is hard to find anyone like him in China. I am heavily influenced by him on morality.
I want to thank my wonderful friends: Yingying Lv, Jianyin Li, An Huang, Lihua Zhang, Jieling and Christopher Frye, have all been great companion these years. I want to thank Dr. Xiongjun Liu, Dr. Xin Liu and Dr. Zhengxin Liu for the friendship and discussion about science and society. Dr. Huiliang Zhang has been a mentor to me when I was in Texas A&M University. He is a true believer of Jesus Christ who won’t let the people around him influence his dedication to his faith. I also want to thank the opportunity to interact with human right lawyer, and activist Biao Teng. His passions for social justice, democracy and a better China greatly inspired me. Even though we agree on some and don’t agree some other things, our discussion has always been respectful and inspiring. I hope he feels the same. I feel sorry for the unjust treatment he received in China. I hope he can come up with real, practical, and sound solutions to the social problems in China. The job he is taking on is obviously hundreds of times harder than the research I did. I want to thank our wonderful neighbors: Lucy, Juan. I am always impressed by these Jewish friends who love their country and their culture. I am inspired by Dr. George Xue for his passion in improving education in China. I also want to thank Dr. Danny Ben-zvi for invitations to his house. It’s great experience for my son Jason and myself. I want to thank Cambridge Youth Hockey, Aggasiz Baldwin Community for providing scholarships for my son Jason so that he can pursue his interest even when we are poor. I want to thank Shirley Lin and Harvard Harmony to provide the opportunity for Jason and Jessica to learn piano for free. Shirley is simply a wonderful girl who is loving, smart and hard working. I am grateful for the city of Cambridge, and the state of Massachusetts and this country United State of America for caring and protecting the poor, the weak and sojourners, and allow people to have freedom in religion, speech and publication.

I want to thank my classmates in Department of Chemistry in my year. I enjoyed our trip to Cape Cod with James Tam, Tian-Ming Fu, Ruihua Ding, Xiabing Lou, George Hao, and Leon Liu.
It’s a great experience. I want to thank Wooyoung Hong for the company and friendship during the first year. I enjoyed discussion with Jiao Ma in Sunney’s class.

I would like to thank Judon and Linda Fambrough, who has been so nice and helpful to our family since we came to US in 2008, real lifetime friends for us, who loves me and my wife like parents. We also want to thank all the friends in Texas. It’s been great to see them graduate and successful over the years. It’s been a good time to meet Zhaokai Meng at SPIE. I am thankful for Zeyang Liao, Xuezhen, Xiaoin, Yongrui Wang for hosting my wife and daughter when they visit College Station. I also want to thank especially D’Nae Weaver who have sent us wonderful gifts during the transition from Texas to Boston. We thank the Grace Bible Church leaders connected us with our current church (Redemption hill Church). It is not easy to find a good church in Boston area, it took us a few months to be convinced about that. I would like to thank friends from our current Church: Jim and Margaret Costello, Jon Vickers, Leigh and Jon Chasteen, Micah Lee, Jeremy and Chesed Broggi, Codi and Julie Gharghouzlo, Sky and Christina Zhu, Seth and Michelle Placke, Jessey Lee and Jason Lu, Chris Kinlaw and our pastor Tanner Turley and many many other brothers and sisters. These are believers who put their faith to work. Serving with them fill me with hope and strength. I want to thank my church as a place for my soul to rest. The birth, life, death and resurrection of Jesus of Nazareth is theologically a salvation of all people, a milestone of human history, practically the ultimate demonstration of love and a role model of me. The faith in a loving and righteous God provides an everlasting meaning to my limited life and work. It gives me reason and motivation to love and forgive.
To

my parents
Citation to Previously Published Work

- Chapter 2 has been previously published with changes as the following papers:


- Chapter 3 has been previously published with changes as the following papers:


- Chapter 4 has been previously published with changes as the following papers:


- Chapter 6 has been previously published with changes as the following papers:


*: Equal-contribution authors
Chapter 1

Introduction

People often say eyes are the window to the soul. While, eyes are not only window to our own souls, but also our window to the soul of nature. We use our eyes to observe nature and try to understand the underlying principle of it. From our eyes, we get two type of information: shape and color. They happen to correspond to two major tools in scientific study: imaging and spectroscopy. As both technologies advanced greatly in the past century, now, they function as more powerful versions of our eyes to look into the soul of nature. The thesis is about a technique that combines microscopy and spectroscopy: stimulated Raman scattering (SRS) microscopy and its applications in biomedical research.

1.1 Backgrounds

One of the major tasks for scientific community is to understand human body and diseases. To do that, according to the philosophy of reductionism, we need to understand the fundamental building blocks of human body: cells. To understand cells, it is often necessary to understand how molecules in cells function and interact with each other. Considering the size of a cell (1 micron to tens of microns), among all microscopy methods, light microscopy sits at the sweet spot of scientific study of biology and medicine. Common light microscopy provides the morphological information at the resolution of hundreds of nanometers\(^1\). Super-resolution light microscopy can reach sub-ten nanometer resolution\(^2-4\).

Cells are mostly transparent under common optical microscope, where no chemical information can be obtained. The standard method to obtain chemical information in microscopy is by labeling, which is to attach dye molecules that have colors or are fluorescent to target
biomolecules in the cell\textsuperscript{5,6}. Through labeling, we can obtain the location information of the target molecules by observing the distribution of the dye. Since such observation is indirect, the specificity and effectiveness of staining may affect the imaging result of the target molecules.

Even though optical microscopy with labeling is one of the dominant tools in the biology and medical research, its application in live animals and live human are limited. Direct and label-free chemical imaging is highly desirable.

On the other hand, spectroscopy is a direct measurement of the molecular properties. Normally each molecule has its own distinct spectrum, and the spectrum of mixture of several chemicals is the linear combination of the individual spectra. Therefore, by analyzing the spectrum of a sample, one can find out the chemical composition of the sample. Sometimes, this can be done quantitatively.

Among many spectroscopic methods, Raman spectroscopy is an optical spectroscopy based on the vibrational energy levels of molecules\textsuperscript{7-9}. The advantage of Raman spectroscopy is non-invasiveness, high spatial resolution due to the use of visible or near IR light, and good specificity. But Raman is a very weak process, the disadvantage of Raman spectroscopy is the long data acquisition time, fluorescence background and low sensitivity. The low sensitivity eventually limited the chemical specificity due to the presence of backgrounds from Raman scattering of other chemicals and fluorescence.

Since both Raman and optical microscopy use visible-near IR light, one can imagine, if we could combine the Raman spectroscopy and light microscopy, we would be able to directly probe the distribution of chemicals. The idea is first realized in the Raman microscopy\textsuperscript{10}, later confocal Raman microscopy (CRM)\textsuperscript{11}. In Raman microscopy, a pump laser is focused on the sample by a
tight-focusing objective, the Raman spectrum of the sample in the light path of the pump laser was recorded. In CRM, a pin hole was used as a spacial filter to improve the spacial resolution of Raman microscopy, especially in z direction. In CRM, only the Raman spectrum of a small focal volume is collected. By scanning the sample with an XY translational stage, one can obtain the spectra of each location of the sample and map out the chemical distributions on the sample by spectra decomposition. CRM has the advantage of obtaining full Raman spectrum of the sample, with which we can find out the concentration distribution of multiple chemicals simultaneously. But just like Raman spectroscopy, CRM also has the serious problem of being slow. Because CRM need to acquire the spectra of many locations on the sample, this problem is more severe. It is not unusual to spend 1 second on each pixel to collect enough signal. The spacial resolution of CRM is on the scale of 0.5 micron. If one want to scan one single 10-micron-size cell, one need \((10/0.5) \times (10/0.5) = 400\) seconds, about 7 minutes\(^{11,12}\). The problems in Raman spectroscopy make CRM impossible to perform sensitive, high speed, high throughput and \textit{in vivo} microscopy.

Fortunately, coherent Raman scattering (CRS) is found to be a high-speed imaging method that bears the promise of label-free high speed chemical specific microscopy\(^9\).

1.2 Introduction to the coherent Raman scattering microscopy

Before we introduce the coherent Raman scattering (CRS), I would like to delve deeper into the non-coherent Raman scattering, which is commonly called spontaneous Raman scattering. Spontaneous Raman scattering is the phenomenon that input photons gains or losses energies in the scattering process due to interaction with molecules’ vibration. It was first discovered by C. V. Raman and K. S. Krishnan in lipids\(^{13-15}\) and Grigory Landsberg and Leonid Mandelstam in crystals\(^{16}\) at around the same time. The effect is explained by molecules’ electron dipole interaction with electromagnetic field (pump laser) at the level of molecular vibration\(^7-9\). The energy diagram
of spontaneous Raman is shown in Figure 1-1, where a pump laser excites the molecules to a virtual state, which is far below the electron excitation state and spontaneously go back to ground state or vibrational states.

Another molecular vibrational spectroscopy, Infrared (IR) spectroscopy use direct absorption to probe vibrational energy levels\(^\text{17}\) as shown in Figure 1-1. The difference between Raman and IR is that Raman process has two photons involved in the process and the Raman vibrational levels need to have same angular momentum as ground state. IR spectroscopy depends on single photon absorption, so its vibrational levels need to have angular momentum different from ground state by ±1. (This is because photons have angular momentum of ±1 and Raman and IR absorption transitions need to conserve the energy, momentum and angular momentum.) So, IR and Raman spectroscopy probe different sets of the vibrational levels and are complementary to each other.

![Figure 1-1 Energy diagram of spontaneous Raman scattering, IR absorption and coherent Raman scattering.](image)

Coherent Raman scattering (CRS) utilize two or three lasers to probe a specific Raman peak or band\(^\text{18}\), as shown in Figure 1-1. The two most popular CRS techniques are stimulated Raman scattering (SRS)\(^8,\text{9,19-22}\) and coherent anti-Stokes Raman scattering (CARS)\(^\text{23-25}\). In SRS, two lasers:
pump and Stokes are used to target a specific Raman peak, by which I mean the photon energy difference between pump and Stokes laser normally matches the target Raman peak of the chemical one wants to probe. Due to the Raman process, there will be increase of power in the Stokes laser and loss of power in the pump laser, which are called stimulated Raman gain and stimulated Raman loss respectively. In CARS, the three lasers are used; and they are called pump, Stokes and probe lasers. The photon energy difference between pump and Stokes laser normally matches the target Raman peak of the chemical one wants to image. The probe laser’s wavelength can be arbitrarily chosen, but are normally chosen to be shorter than pump and Stokes lasers, so that the CARS signal can be easily obtained at an anti-Stokes wavelength of probe laser by using a short pass or bandpass filter. In many cases, it is hard to have three synchronized lasers, only pump and Stokes lasers are used, where the pump laser is also used as a Stokes laser. We will mainly discuss this case in the following paragraphs, since most CARS microscopy is performed this way.

One obvious difference between CRS and spontaneous Raman is that spontaneous Raman uses one monochromatic light to probe all Raman levels, and CRS uses two lasers to target one Raman peak. The benefit of targeting one Raman peak is that CRS can excite molecules more effectively and therefore CRS has higher signal than spontaneous Raman. CRS is at least three orders of magnitude faster compare with the fastest spontaneous Raman experiment.

Another important difference between CRS and spontaneous Raman is that CRS is nonlinear optical process and spontaneous Raman is linear. In spontaneous Raman, the intensity of Raman signal depends linearly on the pump laser intensity. In SRS, the generated SRL or SRG intensity is proportional to the multiplication of pump and Stokes laser intensities. In CARS, the generated CARS laser intensity depends on the multiplication of pump, Stokes and probe intensities.
There are important differences between linear and nonlinear optical process on the effect of spacial and time scale of lasers on the resultant signal strength. First, we consider the spacial dimensions of lasers on sample. Assuming we shine excitation laser(s) into a homogeneous bulk of chemicals as shown in Figure 1-2.

![Figure 1-2 Tight vs loose focusing for linear and non-linear optical process.](image)

For linear process: spontaneous Raman scattering, the final signal doesn’t depend on how tight we focus the pump laser. If the pump laser beam size doubles, the area become four times of original size, the light intensity become quarter of previous intensity, so the total spontaneous Raman signal, which is the summation of Raman signal of all area being illuminated by pump laser, doesn’t change. The same principle applies in time domain. Spontaneous Raman are normally excited by continuous wave (CW) laser. If we use pulsed laser, keeping the average intensity the same as CW laser, each pulse has ten times higher peak intensity than the continuous wave laser, for example, then there are a tenth of time that there is light, while nine tenth of time there is no light. So, within a tenth of time there are ten times more of Raman signal, and nothing within nine tenth of the time. Total Raman signal doesn’t change either.

For nonlinear process, SRS for example, if we double focal size, the lasers’ intensities become one fourth of previous value for each laser, since the final SRS signal depends on multiply of pump
and Stokes intensities, the SRS signal for unit area become square of one fourth: sixteenth of previous value. Since the focal size become four times of previous value, the total SRS signal is one fourth of previous SRS signal. The same rules apply to time domain. For example, we compare the SRS signal when pump and Stokes are CW lasers and nanosecond lasers with same average power. When pump and Stokes are 1-nanosecond \(10^{-9}\)s lasers and there is only one pulse in one second, the peak power is \(10^{9}\) of that of CW lasers. Since the signal only happens in 1 ns, the SRS signal obtained with nanosecond laser is \((10^{9})^2 \times (10^{-9}) = 10^9\) times of that obtained with CW lasers. So, to obtain the highest SRS signal with a fixed average power, it is desirable to have tight focus, low repetition rate and short pulse lasers.

But in fact, shorter pulse duration will not always result in higher SRS signal. As pulse shortens, the spectrum of the pulse broadens\(^1\). At certain point, the spectrum bandwidth of the laser is already wider than the Raman bandwidth. Then shortening the pulse no longer improve the SRS signal. It can be understood if we think about an extreme example when the Raman peak is a Dirac delta function, then no matter how short the optical pulse is the SRS signal will not improve when the pulses are shorter. Shorter pulse will have higher nonlinear signal only when the bandwidth of nonlinear response of media is broader than the laser’s bandwidth (some coefficient may apply depending on the order of nonlinear optical process).

For nonlinear optical process, a phase matching condition is needed for efficient signal generation\(^8\). This is due to the need of conservation of momentum of light. This problem is extremely important for CARS, so let use CARS as an example to demonstrate phase-matching. Different colors of light travels in materials at different speed. For CARS, the generated CARS signal travels at different speed with pump and Stokes lasers. If the phase matching is not satisfied, as pump and Stokes lasers passes the sample, the CARS signal generated in the beginning of
sample has different phase with CARS signal generated later, they can destructively interfere and the final CARS signal will be weak. When the phase-matching condition is met, CARS signal generated along whole sample (a cylinder) added up coherently, the final CARS signal is strongest. The way to achieve such phase-matching condition is to let pump and Stokes laser go through the sample with an angle calculated from the dispersion of the media and pump, Stokes, CARS wavelengths. Because the requirement of phase-matching, it is used to be the case that pump, Stokes and probe lasers are focused on sample from different directions by a lens with centimeters of focal length, and only samples are scanned in CARS imaging experiments\textsuperscript{25}, because it is hard to maintain the correct angle if lasers are scanned across the sample by galvo mirrors as was done in the two-photon excited fluorescence microscopy\textsuperscript{26}. Scanning speed is at most a few milliseconds per pixel when the sample is scanned, while the laser scanning microscopy can reach 100 ns per pixel scanning speed with resonant scanner.

The application of CARS as a chemical microscopy method become popular after the paper of Xie lab in 1999\textsuperscript{23}. The key breakthrough of that work is to use a high numerical aperture (NA) objective instead of a low NA lens to focus the beam into the sample. The second breakthrough is that the lasers were no longer arranged in angles but propagated in the collinear geometry. The benefit of using high NA objective is that laser can be focused to a smaller spot so that the CARS signal is greatly improved as we discussed above. Secondly, the phase matching condition in CARS microscopy is satisfied due to wide angle of light of all colors in the focus. The collinear geometry allows us to use laser scanning microscope to perform CARS microscopy, even though it wasn’t until later when this was done for CARS. This really allows us to perform high speed chemical sensitive and even \textit{in vivo} microscopy\textsuperscript{27}. 
However, the notorious non-resonant four-wave mixing (FWM) background problem has been plaguing CARS microscopy\textsuperscript{9,28}. For narrow band Raman microscopy (picosecond or longer laser pulses), the non-resonant background exists even if the pump and Stokes’ photon energy difference doesn’t correspond to any Raman peak. Non-resonant FWM background reduces the image contrast, interfere with CARS signal, and twisted the spectral shape. It also introduces noises and reduce SNR, especially when detecting low concentration chemicals. Overcoming non-resonant FWM background became the No. 1 task since its application in microscopy. The effort of about 9 years to reduce FWM background resulted in SRS microscopy.

SRS belongs to coherent Raman scattering and was discovered even earlier than CARS\textsuperscript{8,20,22}, however, to my knowledge, it has never been used in microscopy before 2008\textsuperscript{19,29,30}. The energy diagram of SRS is shown in Figure 1-1. In SRS, the pump laser and Stokes laser effectively excite some molecules to the target Raman level, just like in the case of CARS; then the pump and Stokes lasers interacted with the excited molecules to produce SRS signals at the frequency of Stokes and pump lasers respectively. For the SRS signal generated at Stokes frequency, the field is $E_{SRS} \propto \chi^{(3)}_R(\Omega)E_p^2E_s$. The laser generated at Stokes wavelength has the same phase with input Stokes laser, they add up coherently. As a result, there is a gain of power in the Stokes wavelength, which is called stimulated Raman gain (SRG). At the same time, the light generated at pump frequency is $E_{SRS} \propto \chi^{(3)}_R(\Omega)E_s^2E_p$. But, it has an opposite phase and interfere destructively with pump laser, therefore, there is an attenuation in pump laser, which is called stimulated Raman loss (SRL). SRS is an important method to perform wavelength shifting and is the physical process behind Raman lasers\textsuperscript{20,22}. The phase matching condition that is required for nonlinear process like CARS are easier to be met in SRS, because the generated lights have the same wavelengths as the input lasers, so there is no need to worry about phase matching in SRS. Another advantage of SRS is that there
is no non-resonant FWM background which plagues the CARS process. Even though there are other non-resonant background in SRS e.g. cross-phase modulation, two-photon two-color absorption, and thermal lensing effects, but they are orders of magnitude smaller than the FWM background in CARS.

The measurement of light is normally a measurement of intensity. The amount of change of intensity in SRG and SRL can be calculated by adding up the generated field and input laser field, and take the square of it. For SRG, the increase of laser intensity: \( I_{SRG} \propto (E_{SRS}\!+\!E_s)^2 \!-\! E_s^2 \propto \left(\chi_R^{(3)}(\Omega)E_p^2E_s\right)^2 + 2\left(\chi_R^{(3)}(\Omega)E_pE_s^2\right)E_s \equiv 2\chi_R^{(3)}(\Omega)E_p^2E_s^2 \propto 2\chi_R^{(3)}(\Omega)I_pI_S \). The term \( \left(\chi_R^{(3)}(\Omega)E_p^2E_s\right)^2 \) is omitted because, in reality, the generated field \( \chi_R^{(3)}(\Omega)E_p^2E_s \) is very small compared to the intensity of input laser field \( E_s \). In the same way, for SRL, \( I_{SRL} \propto (E_{SRS}\!+\!E_p)^2 \!-\! E_p^2 \propto \left(-\chi_R^{(3)}(\Omega)E_s^2E_p\right)^2 + 2\left(-\chi_R^{(3)}(\Omega)E_sE_p^2\right)E_p \equiv -2\chi_R^{(3)}(\Omega)E_p^2E_s^2 \propto -2\chi_R^{(3)}(\Omega)I_pI_S \).

Even though SRS can be effective for certain material, most chemicals in the cells has moderate to weak SRS signal. Also, because the SRS signal field overlaps with the input pump or Stokes lasers in both spectral and time domain, it is impossible to separate them from input lasers. When they are measured together with input laser, we have to measure the SRG or SRL signal on a background, produced by input lasers, \( 10^6 \) bigger than amplitude of the signals.

In 2008 and 2009, the work by Xie Group and a few other labs overcome these challenges and make SRS an new tool for label-free chemical imaging. The first important technical breakthrough is the use of modulation transfer scheme. Modulation transfer is to modulate one of the input lasers, Stokes laser for example. During the SRS process in the focal volume in the sample, the generated SRS signal in pump wavelength will also be modulated due
to the modulation in Stokes laser. The transferred modulation can be picked up by a lock-in amplifier, which is widely used to extract signals at a specific frequency in the presence of big background and/or noise. The second breakthrough is the realization that the choice of modulation frequency is important. Solid state lasers have a lot of noise at low frequencies, as shown in Chapter 2 Figure 2-3. If frequency modulation is performed at low frequency, the noises in laser will also make the SRS imaging very noisy. The laser only become quite at frequencies larger than 4 MHz, so the modulation needs to be higher than 4 MHz to reach high quality imaging; 7 MHz, 10 MHz, and 20 MHz are all very common frequencies used in SRS microscopy. These breakthroughs together with the fact that SRS doesn’t have FWM background are what make SRS successful.

The advantage of SRS over CARS is obvious. SRG or SRL depends linearly on the $\chi_R^{(3)}(\Omega)$, which is proportional to concentration of chemical in the sample. Therefore, SRS signal depend linearly on the concentration of chemicals in the sample, compared with the quadratic dependence of CARS signal on chemical concentration. This make SRS spectral data processing much easier than that of CARS. There is no non-resonant FWM background in SRS. The spectrum obtained by SRS is the same as the spectrum of the chemical obtained in spontaneous Raman scattering, while the spectrum of CARS is distorted due to the interference between CARS signal and FWM background.

1.3 Challenges in SRS microscopy

Two years after the initial development of SRS microscopy, there have been great technically advancements: epi-detected SRS that can be imaged at video-rate were also developed. Applications have been demonstrated in lipid quantification, label-free histology, drug
penetration test\textsuperscript{38}, biomass measurement\textsuperscript{39}, and visualization of lipid, protein in food\textsuperscript{40}. Several multi-color SRS techniques are also developed\textsuperscript{41-43} to expand the spectroscopic capability of SRS.

Despite these successful cases, there are great challenges in SRS. We talk about these challenges by putting evaluating SRS on several aspects: spacial resolution, penetration depth, imaging speed, chemical specificity and sensitivity.

In SRS, considering the NA of water immersion objective is about 1, the spacial resolution is about $\frac{0.61\lambda}{NA} = 0.61 \times \frac{800 \text{ nm}}{1} = 488 \text{ nm}$ for pump laser alone, 610 nm for Stokes laser alone, and 381 nm for the combined beam. Even though it is desirable to have higher resolution, the current resolution is already suitable for many cell and tissue level microscopy.

The imaging speed of SRS can reach video rate. However, that is limited to single color. For multi-color SRS, the speed is normally slower due to low signal. Later in the thesis, we show some methods can be used to achieve high speed imaging with more colors.
The chemical sensitivity of SRS varies among chemicals. For common chemicals, the sensitivity is at millimolar to tens of millimolar level. For molecules like β-carotene, the sensitivity can be much higher and reach hundreds of nanomolar level. However, such molecules also tend to suffer more from photodamage. Current sensitivity is enough for lipid imaging and general protein imaging, but most molecules in a cell doesn’t reach this high concentration level. The sensitivity is estimated assuming a few hundreds of milliwatt of optical power on the sample and microsecond pixel dwell time, which is common case in SRS imaging experiments.

The chemical specificity is not great since Raman peaks correspond largely to the chemical bonds. Many chemicals share similar bonds, so it is hard to use SRS alone to confirm chemical, especially when SRS only targets one a or a few Raman peaks. Prior knowledge, or control experiments are needed to confirm chemicals. Deuterium labeling of chemicals can shift the Raman bands to new locations, so that the chemicals can be more specifically imaged\textsuperscript{44,45}. But this requires labeling and may not be easy for \textit{in vivo} imaging and may not be better than alternative fluorescence labeling in other cases. Despite these difficulties, there are still many cases where one Raman peak combined with prior chemical and morphological information is enough to identify certain chemicals.

From the above analysis, we can see that SRS is more suitable for surface imaging tasks when staining is not possible and the target chemical needs to have high concentration or strong Raman signal. The important task of researchers in this field is to find applications that is suitable for SRS especially in \textit{in vivo} animal and human studies where staining is difficult. SRS also face other difficulties including expensive laser source and the need of high-speed, multicolor imaging. At last all these challenges sum up as the need to improve sensitivity. This is where my PhD research starts.
1.4 Organization of this thesis

The thesis focuses directly on these challenges in SRS microscopy. Chapter 2 describes the development of all-fiber laser source and design of high-speed auto-balanced detector, which reduced the cost of laser source of SRS by 20 times and will make SRS accessible to more people. Chapter 3 describes the SRS imaging of neurotransmitter acetylcholine in live tissue. Acetylcholine is a small molecule and labeling may alter its function greatly, so SRS is very suitable for its visualization without labeling it. This is the first time that acetylcholine was imaged in live tissue and it gave us hope to detect neuronal activity by imaging neurotransmitter with SRS. Chapter 4 describes how SRS can be used to track the development of a deadly neurodegenerative disease amyotrophic lateral sclerosis (ALS, i.e. Lou Gehrig’s disease). SRS imaging of peripheral nerve degeneration in the form of lipid ovoids was shown to be among the earliest detectable biomarkers in a mouse model of the disease. A drug test by tracking the number of lipid ovoids on individual mouse set an example of using SRS in drug screening. Chapter 5 describes a clinical study of active ingredients deposition on human skin. This is the first time, SRS has been used in human clinical study. Chapter 6 describes a new technique for simultaneous two-color SRS by adding a simple fiber amplifier to conventional SRS setup. It is suitable for speed demanding imaging tasks, for example, we imaged white blood cells in the flowing blood stream of live mice. The principle can be applied to perform simultaneous multi-color imaging. That is badly needed for in vivo imaging. At last, time-domain CRS was performed aiming at further improvement of the sensitivity.

A significant portion of this thesis has been published in a series of papers^{46-49}. However, I will explain the electronics in more details in Chapter 2. I will describe the work in my own
perspective and logic in Chapter 4. I also added unpublished materials in Chapter 6. I hope this thesis can be a good complementary material to the published papers.
Chapter 2

Enabling economical SRS microscopy by building all-fiber laser source and high-speed auto-balanced detector

2.1 Backgrounds

Even though the application of SRS is limited at the time when I started my PhD, there is one promising application for SRS: the label-free histology. SRS microscopy can be used for label-free histology by imaging just lipid and protein these at two Raman bands. However, one hurdle for this application is that the common histology by H&E staining cost tens of dollars to do and SRS instrument cost half a million dollars. The question is whether we can reduce the cost of SRS.

The gold-standard of laser source for SRS is a solid-state picosecond OPO system. There are several advantages for such a system. First, it provides two optically synchronized pulses and one of them is tunable. Optical synchronization make pump and Stokes lasers have low timing jitter, which is necessary for high quality imaging. Secondly, it has high spectral resolution. The 6-picosecond pulses have about 0.3 nm spectral bandwidth, which corresponds to a SRS resolution below 5 cm\(^{-1}\). This is enough to resolve most Raman peaks. Thirdly, wide tuning range of OPO allows us to image most Raman peaks from 500 cm\(^{-1}\) to 3500 cm\(^{-1}\). In general, solid state OPO system are versatile and competent instrument suitable for imaging most chemicals in a research lab.

However, the SRS setup used for scientific research is not well suited for clinical applications. First, the cost of the laser system is high. Solid state OPO system cost at least 200,000 dollars. The H&E staining used in histology labs is very cheap. This has limited the Secondly, the laser system
requires good environmental control, which includes a water cooling equipment, heavy and floated optical table and sometimes a stable humidity and temperature. Thirdly, the solid-state laser faces the problem of misalignment. It may require occasionally realignment, which requires laser technician to service the laser.

Fiber laser technology has the potential to overcome these limitations as components are inexpensive and light-guiding by the fiber core avoids misalignment. Fiber lasers can be operated without an optical table. Consequently, different concepts of fiber laser sources have been proposed and implemented for CRS. From the free-space OPO system, we have learned that optical synchronization avoids timing jitter and improves long-term stability. However, previous approaches to fiber-based optically synchronized systems are less than optimal: super-continuum generation (SC) does not scale to sufficiently high power, implementation of a fiber-based OPO has proven difficult, and un-seed four-wave mixing requires low-repetition rates, which ultimately limit the imaging speed.

Here we combine the advantages of optical synchronization with straight-forward power scaling in fiber amplifiers. It turns out that the difference frequency of the two major fiber gain media, Erbium (Er) and Ytterbium (Yb), corresponds to the high-wavenumber region of Raman spectra, where most SRS imaging is performed. We have implemented an all-fiber dual-wavelength laser system based on optical synchronization of two picosecond amplifiers using a broadband SC and have optimized the pulse parameters for high-speed, sensitive SRS imaging.

A remaining challenge for using amplified fiber laser sources for sensitive SRS imaging is the presence of white laser noise at the demodulation frequency. This is fundamental as power amplification cannot decrease the shot-noise of the low power oscillator.
It is well known that balanced detection can remove common mode noises or backgrounds. Balanced detection is normally performed on balanced detector, which has two photo detectors and the difference signal of the two is the output of the balanced detector. In balanced detection, the (noisy) laser is split into two beams. One go through sample, and the other, called reference beam, is measured without going through sample, by subtracting the electric signals from the two detectors, the common mode noise that is in the laser before the split of laser beams can be removed. In some other cases, the attenuation due to sample is measured by balanced detector. Complete noise removal only happens when the amounts of noise are exactly the same on both detectors. This requirement make it difficult to cancel noises in SRS imaging, because the sample has different light transmission at different locations. So, when the laser scan through the sample, the optical intensity that go through the sample and fall on the detector are fast changing. Adjustment of power of reference beam based on the transmitted light is needed to remove noise perfectly.

The technique of auto-balanced detection can solve this problem. Auto-balanced detection is basically to achieve perfect noise cancellation by making the photocurrent or optical power of reference laser automatically follow that of the signal laser after it went through the sample. It can be done optically or electronically. Optical auto-balanced detection can be done by inserting an amplitude modulator in the light path of reference laser and modulate the reference laser according to the transmission of the signal laser. Electronic auto-balanced detection adjusts the gain of photocurrent of the reference laser to match the photocurrent of the signal laser. Researchers have applied commercially available auto-balanced detector to perform SRS imaging with fiber laser. Some researchers use optical auto-balanced detection to remove noise. However, due to the limitations in the commercially available detectors and the fiber lasers they used, previous SRS
microscopy with all fiber lasers have low signal to noise ratio and/or imaging speed compared with the SRS imaging using solid-state laser system.

We designed a new voltage-subtraction auto-balanced detector to achieve high speed imaging. To emphasize our design is ‘voltage-subtraction’ is because a lot of balanced detector and the auto-balanced detector use current subtraction to achieve balanced detection. We also designed a new filtered transimpedance amplifier to reduce the electronic noise floor so that we could measure and reduce the noise from fiber laser better. The new electronics combined with the optimized fiber laser design allows high quality SRS microscopy with much cheaper laser source\textsuperscript{46}. This is a milestone toward a broader application of SRS in and outside of research labs.

2.2 Fiber lasers for coherent Raman microscopy

The successful of fiber laser SRS thanks to the fact that Raman peaks used in SRS microscopy can be matched by fiber laser gain medium’s operating wavelength.

Many CRS microscopy are performed in the high wavenumber region of CH stretching. The advantage of using this region instead of lower wavenumber region is mainly technical. First of all, the high wavenumber CH stretching is strong in lipid and protein. Secondly, when imaging at the finger-print region (500 - 2000 cm\textsuperscript{-1}), the imaging quality is reduced due to low sensitivity at longer wavelength of PMT (for CARS) and silicon diode (for SRS). Even though the spectra of lipid and protein are less distinct at CH high wavenumber region, it is still not difficult to separate them with two-color imaging. That’s why two-color lipid protein imaging is very popular in CRS. Most label-free histology SRS imaging are done by two-color SRS in that region\textsuperscript{37,50-52}.

On the other hand, fiber lasers’ wavelengths are limited by the gain media’s absorption and emission wavelengths. Ytterbium (Yb) and erbium (Er) ion doped fibers are most commonly used
in fiber lasers. Yb fiber laser can emits from 1010 nm to 1070 nm. Er fiber laser can emits from1525 nm to 1585 nm. The photon energy difference between the two falls between 2788 - 3592 cm\(^{-1}\), which includes the whole C-H and O-H stretching high wavenumber region. But, in reality, we perform a frequency doubling on the Er fiber output to make it in the range of 763 - 792 nm\(^{68,69}\). This will allow us to use large area silicon detector with high efficiency and avoid loss of light in the microscope, since most microscopes don’t have high transmission for 1550 nm laser. After frequency doubling of Er fiber laser, we could probe Raman peaks between 2725 - 3760 cm\(^{-1}\).

The gain media’s working wavelength allow us to probe CH high wavenumber region where most SRS microscopy were conducted.

The design of optically synchronized fiber laser is shown in Figure 2-1.

![Figure 2-1 Schematic of all-fiber laser source for SRS.](image)

It starts with an Er-doped fiber oscillator at 1560nm that is mode-locked with a carbon nanotube (CNT) saturable absorber\(^{70}\) at a repetition rate of 59MHz. It has a pulse duration of 300fs, which is preferred to generate a low-noise super continuum (SC) for optical synchronization. We
used the polarization maintaining components for oscillator to achieve high stability. The output power of the oscillator is about 7mW.

The main goal of the design is to probe Raman peak between 2850 - 3050 cm\(^{-1}\), which is the CH stretching high wavenumber region. This means that we need to mainly use the Er fiber at long wavelength end of the tuning range around 1580 nm and Yb gain fiber output around 1020 - 1040 nm, even though it is possible to allow both Yb and Er laser output tunable in big range (Figure 2-2 a-b). 1580 nm is near the end of tuning range of Er fiber laser, and in order to achieve high performance, we fixed the wavelength of Er arm at 1580 nm and optimized the fiber amplifier for this wavelength.

The oscillator output is split by a 50/50 splitter into two arms to generate the pump and Stokes beams for SRS. The pump arm is amplified to 80 mW in a normal dispersion Er-doped pre-amplifier (30 dB Er fiber for about 1 m). Its output is broadened due to self-phase modulation (SPM) so that we can reach 1580 nm. We use a narrowband filter at 1580 nm to reduce the bandwidth. The power (about 2.2 mW) after filtering is restored in a low-nonlinearity Er-doped power amplifier (5 dB for about 3 meters). We obtained about 200 mW of laser centered at 1580 nm. The bandwidth is broadened to about 10 nm. We frequency double the output in a periodically poled lithium niobate (PPLN) crystal (Covesion, MSHG1550-1.0-10). We obtain a near-IR pump beam for SRS with up to 70 mW average power with bandwidth of about 1 nm, whose spectrum is shown in Figure 2-2 c and autocorrelation in Figure 2-2 d. The reason for such frequency bandwidth reduction is due to the sum frequency generation of negatively chirped pulses in the PPLN crystal\(^{68,69}\).

The Stokes arm is frequency shifted in an SC unit based on a highly nonlinear fiber (HNLF). Stable SC generation requires the use of a short piece of HNLF (5 cm). In order to achieve broad
bandwidth, it is pumped with high peak power by first pre-amplifying the 3.5 mW seed in a normal dispersion Er-doped amplifier to about 100 mW and then temporally compressing the output in 85 cm anomalous dispersion single mode fiber (SMF). By careful optimization of the splice between the SMF and HNLF, it is possible to reduce the splice loss to <1dB, enabling an all-fiber implementation. The length of SMF needs to be carefully adjusted 1 cm a time until the appearance of successful SC generation. The output covers a range from 1000 nm to > 1700 nm (detection limit of the optical spectrum analyzer). We then use an Yb-doped pre-amplifier (Yb1200, 10 cm) to increase to power of the spectral end around 1 micron to about 40 mW and a narrowband tunable filter (Agiltron, FOTF-01-6) to produce a picosecond pulse train that can be amplified in a low-nonlinearity Yb-doped power amplifier. The tunability of Stokes beam is shown in Figure 2-2 b. The autocorrelation is shown in Figure 2-2 e. We now have a Stokes beam with up to 120 mW average power that is synchronized to the master oscillator, and thus the pump beam.
Figure 2-2 Characterization of all-fiber laser source for SRS. a. Tuning range of Er fiber amplifier output. b. Tuning range of Yb fiber amplifier output, which was used as Stokes laser. c. The spectrum of Er fiber amplifier after second harmonic generation in PPLN crystal, which was used as the pump laser. d,e. Autocorrelation of pump and Stokes lasers respectively. e. Measurement of cross-correlation (inset) of pump and Stokes lasers at peak and half of peak during five minutes for timing jitter quantification.

At last, we measured the jitter between pump and Stokes laser by measuring the noise. The two beams are spatially overlapped with a free-space dichroic mirror. The rough time delay is adjusted by adding un-doped fiber before the Yb-doped power amplifier. Fine delay is obtained by adjusting a mechanical delay line while monitoring the sum-frequency generation (SFG) signal from a BBO crystal. This setup was used to measure the cross-correlation of the two pulse trains and determine the timing jitter. Figure 2-2 f shows a 5 minutes’ time-trace of the SFG signal at the half-maximum of the cross-correlation. Using the slope of the cross-correlation to convert from
amplitude to timing fluctuations, the timing jitter is estimated to be <24fs. Such approach only provides an upper limit estimate as laser intensity fluctuations can be falsely interpreted as timing jitter. However, it is sufficient to demonstrate that timing jitter is much smaller than the cross-correlation bandwidth of 4.4 ps and does not affect SRS imaging capabilities.

2.3 Laser noise: shot noise and excess noise

In this section, I will talk about one major issue of fiber laser: the noise.

![Graph showing noise characterization of solid state laser pump OPO output and fiber laser.](image)

Figure 2-3 Noise characterization of solid state laser pump OPO output and fiber laser.

Figure 2-3 shows the noise character of a fiber laser and a laser from solid state OPO, both of which are 28 mW. We could see that the laser from a solid-state laser pump OPO is noisy at low frequencies (<4MHz), and becomes quite (shot noise limited) at high frequencies (>4MHz). This explains why frequency modulation needs to be performed at high frequencies to achieve shot-noise limited imaging for SRS as I mentioned in Chapter 1 section 2. But the fiber laser has a white excess noise at high frequencies. And at the same time, it explains why fiber lasers cannot be directly used in SRS microscopy: high-frequency modulation no longer avoids excess noise. Noise in fiber laser came from shot noise of seed laser and amplification process especially the amplified spontaneous emission. The high frequency excess noise in fiber laser is also white noise. The unit, relative intensity noise, used in Figure 2-3 will be introduced later in this section.
Now I will explain in detail what is shot noise and excess noise in laser.

The intensity of light can be measured by the number of photons arrives at the detector in unit time. This intensity is not a fixed number but fluctuating, which we call noise. The origin of laser noise come from several sources. First, quantum mechanics tells us that laser is a state of light called coherent state. This state has intrinsic uncertainty for number of photons during a certain measurement time duration. For a special state of light called squeezed state lasers, this noise can be compressed or enlarged. But we won’t talk about it in this thesis, because it is not suitable for imaging due to its high cost and low effect when the loss is high. Secondly, the detector detects photons, and transfer photons to electrons in the electrical circuit. This transfer is never 100% efficient. This low efficiency caused by randomly missing of photons will introduce extra Poisson noise. We call the total noise of the previous two noise shot noise of laser. Thirdly, the light can have other noises due to the fluctuations of light source. For example, the standard wall power supply provides alternating current (AC) at 60 Hz. Light from light bulb powered by the wall power supply may have periodic fluctuation at 60 Hz. The extra noise that is added on top of the shot noise is called excess noise.

There are several differences between shot noise and excess noise. First, shot noise measured in photon number is proportional to the square root of the average photon number of laser being measured, while excess noise measured in photon number is proportional to the average photon number. So, excess noise is like a signal carried by the laser and scales with light intensity, while shot noise is a special character of the laser. Secondly, shot noise cannot be cancelled by any tools or measurement schemes, while excess noise can be cancelled by balanced detection or other noise cancellation schemes. Thirdly, shot noise is broad band white noise and excess noise very often has a specific noise spectral distribution.
The terminologies in noise can be very confusing. In previous paragraph, we talked about the noise in light as a fluctuation of detected photon numbers. These photons excite photo currents on light detectors: photodiode, in this case. What people normally measure is the electrical power of electric current, which is the square of photo current multiply the load resistor (very often 50 Ohm). Noise is often presented in a noise power (electric) spectrum chart, where the density of noise is shown. To get the real amount of noise, one have to integrate the noise density among the frequency that is interested. This also corresponds to the fact that bandwidth is inverse of integration time (or twice of it). When we integrate noise longer, we kept add noises up and they cancel each other because they are random, so we have less noise with narrower bandwidth. When we integrate shorter time, we have higher noise and wider bandwidth.

We can consider an example on excess noise and shot noise. When we double the laser intensity, the excess noise in the laser light doubles too. Photocurrent will double. So, the measured electronical noise power will become four times of the previous value (6 dB). But the shot noise of the laser only increase $\sqrt{2}$ times (3 dB), so the electronically measured shot noise power only doubles. This gives us an interesting result, when a laser is shot-noise limited, which means that shot-noise is bigger than excess noise, the electric noise power grows proportional to the laser’s average optical power. If a laser’s excess noise is higher than shot noise, the electric noise power grows quadratically with the laser’s average optical power. Assuming we have a laser source with unlimited power, we can measure an arbitrary portion of it on a detector, at low power, the shot noise will dominate. At low power, shot noise always dominate, because it reduces with power slower than excess noise does. As we put more power on the detector, the excess noise will start to dominate, which was perfectly illustrated in Figure 2 of this reference\textsuperscript{73}. 

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In order to quantify laser noise regardless of laser power, people very often use relative intensity noise (RIN) to describe laser noise\textsuperscript{73}. It is defined by the electronic noise power divided by the electric power of DC component. The DC component of electric current is proportional to the average photon numbers. The excess noise’s RIN is independent of the optical power being measured, as long as the excess noise is larger than the shot noise. But shot noise’s RIN does depend on the optical power being measured. The shot noise’s RIN is inverse proportional to the optical power of laser.

To put these in equations, assume the laser being measured has an average optical power of $P_0$ with unit of Watt, where subscript ‘o’ stands for optical. This is what we measured with optical power meter. It quantifies the amount of energy of the light (photons) in one second. Assuming the laser is monochromatic, which means it has only one wavelength (remember light is wave also?), $P_0 = N_0 \frac{h c}{\lambda}$, where $h$ is Plank constant, $c$ is speed of light, $\lambda$ is laser wavelength, and $N_0$ is the average photon number per second.

The photocurrent measured by detector is $i = \eta e N_0 = \eta P_0 / (h \frac{e}{\lambda})$, where $\eta$ is the quantum efficiency of the detector for the light at the wavelength of $\lambda$ and $e$ is the amount of charge of one electron. Within a certain time $T$, if we measure the number of photon electrons repeatedly, the average number of electrons are $\eta N_0 T$. If the laser is shot noise limited, according to Poisson distribution, the standard deviation of number of electrons will be $\sqrt{\eta N_0 T}$. The standard deviation of current will be $i_{\text{noise}} = e \sqrt{\eta N_0 T}$. Now, if we wrote the noise level in the form of noise power $P_{\text{noise}} = R (e^2 \eta N_0 / T) / 4$ (the 1/4 is due to the measurement from 50 Ohm impedance matching system and the voltage halves). Divide this value by the average electric power $P_e = R (e \eta N_0)^2 / 4$. The RIN is calculated by $RIN = \frac{1}{\eta N_0 T} = 2 \frac{BW}{\eta N_0} = 2 \frac{h c}{\eta \lambda P_0}$, with $T = \frac{2}{BW}$ for single
sided spectrum, where the spectrum is never negative. Shot noise is not frequency dependent, because it is purely random. A frequency domain derivation of shot noise can be found in literature\cite{74}.

2.4 Laser noise measurement

Now we will discuss how we obtained the data shown in Figure 2-3. It was measured with the setup shown in Figure 2-4.

![Figure 2-4 Laser noise measurement setup.](image)

The laser was attenuated by a half-wave plate followed by a polarizing beam splitter. The photodiode is a 1 by 1 cm Si photodiode (S-100CL). It was biased by 50 V power supply, it was then terminated by 50 Ohm resistor and connected to bias-T to isolate and measure DC level. High bias voltage on photodiode is important to reduce the capacitance of the diode. The 1 cm Si photodiode can be biased safely at 70 V and 100 V is also possible; the dark current may go up with high bias voltage but is not a major source of noise. The RF output from the bias T was sent to a spectrum analyzer (Signal Hound usb-sa44b) after passing through a 50-MHz low pass filter to remove the signal due to laser repetition rate at 80 MHz, which would otherwise saturate the preamplifier of spectrum analyzer.

We could tell the performance of this setup by analyze the results shown in Figure 2-3. The black line shows the electronic background of the detection electronics. It is only about 3 dB below the shot noise, which means that the electric noise and shot noise are about the same. It is not the
ideal case, if we want to claim the laser is shot noise limited. We would like the electronic noise to be about 10 dB below the shot noise level to claim that the electronics can measure the laser’s noise very well.

The source of this noise is the thermal noise of resistor ($R = 50\, Ohm$) and other noise picked up from environment. The thermal noise of a resistor is calculated by $i_{tn} = \sqrt{4kT \frac{BW}{R}}$, which corresponds to noise power density (divide bandwidth) of $P_{tn} = \left(\frac{i_{tn}}{2}\right)^2 \frac{R}{BW} = kT = 4.15 \times 10^{-21}\, W/Hz$, which corresponds to -174 dBm/Hz. The shot noise from laser has the noise power density of $P = Re^{\frac{2\eta P_0 \lambda}{2hc}} = 5.39 \times 10^{-20}\, W/Hz$, which corresponds to about -163 dBm/Hz. Even though theoretically, the optical noise is much smaller than the electrical noise from 50 Ohm resistor, the noise can be picked up during signal transmission in cable, and they may overwhelm the electronic noise. In our case, the electric noise is only a few dBs below the optical shot noise. Though the noise power density of shot noise from laser can be increased by using resistor with higher resistance, the network requires 50 Ohm resistance so we are basically stuck with 50 Ohm. Even if we get rid of the networks, we still face another difficulty which is the capacitance of photodiode. The Si photodiode has an active area of about 1 cm$^2$. With 50 V of bias, the capacitance of the photodiode is about 140 pF. At the frequency of interest (10 MHz, where we modulate our laser), this capacitance corresponds to an impedance of $Z = \frac{1}{140 \times 10^{-12} \times 2\pi \times 10^7} = 113.7\, Ohm$. So once the resistor is higher than this value, bigger resistor can no longer improve the measured noise power density of shot noise from laser.

A very common way to overcome noise is to add a preamplifier that amplifies photocurrent before sending it through noisy downstream electronics. A typical way of amplifying photocurrent is to use transimpedance amplifier, as shown in Figure 2-5. For our application, we need a high
speed operational amplifier (opamp), because we need to amplify signals at 10 MHz, if we need to amplify 100 times, we need a opamp with a gain bandwidth product larger than 1 GHz. The opamp also need to have low input noise. Fortunately, our former research scientist Dr. Gary Holtom found a very suitable opamp LMH6624, which has 1.6 GHz gain bandwidth product and 2.3 pA/√Hz; the photocurrent of 28 mW of laser has a current shot noise of 46.4 pA/√Hz. As long as a correct feedback capacitor is used (1~2 pF for R = 1-2.2 k Ohm), the circuit will be stable, if wrong feedback capacitor is used, the opamp will oscillate.

However, circuits in Figure 2-5 will not work for our application. Detectors for SRS need to take optical powers of up to 50 mW and a high gain. Circuits in Figure 2-5 will result in saturation in DC level. On the high frequency end, we use pulse lasers with 80 MHz of repetition rate. The gain of 80 MHz is about 8 times less than the gain at 10 MHz. However, the 80 MHz signal from laser is very high and can easily saturate the detector. Therefore, I designed a filtered transimpedance amplifier (shown in Figure 2-6) to avoid this problem.

![Figure 2-5 Common transimpedance amplifier design to amplify photo currents.](image-url)
Figure 2-6 The design of a filtered transimpedance amplifier that amplify signals around 10 MHz.

The idea behind the design is to model the photodiode as a current source and a 140 pF capacitor connected in parallel and build a filter with the capacitor. This model for photodiode is commonly used in circuits designs involving photodiodes\textsuperscript{75,76}. The key component is the capacitor and inductor pairs. The 140 pF capacitor is connected in parallel with 100 pF capacitor. We added an 100 pF capacitor in parallel with the 140 pF capacitor (photodiode), because the photodiode is not a perfect capacitor, their capacitance is not stable, so we added a capacitor to make the capacitance more stable. We used 1 \( \mu \)H inductor so that their resonance frequency is about 

\[
f_0 = \frac{1}{2\pi \sqrt{240 \times 10^{-12} \times 1 \times 10^6}} = 10.3 \text{ MHz}.
\]

But if we only use this pair, the DC current will go through the transimpedance amplifier and the response of the amplifier will be resonant around 10 MHz. So, we used another pair of inductor and capacitor pair to completely block the DC component from going through the amplifier and make it a band pass filter between about 9-12 MHz. The capacitor connected in series with the 1 \( \mu \)H inductor is used to block DC current from going to ground, so
that the DC current can go to the 10 Ohm resistor and can be measured if necessary. The typical spectrum analyzer measurements of the detector are shown in Figure 2-7.

![Figure 2-7](image)

**Figure 2-7** The frequency response of filtered-transimpedance amplifier with and without laser on the detector.

The spectrum of the amplifier shows two peaks and between the two peaks are the working frequencies of the circuit. For this particular one, the working frequency is between 10 and 13 MHz. The noise floor of the circuit is higher than the noise floor of spectral analyzer by at least 7 dB. The photocurrent is about 10 mA, which corresponds to about optical power of about 17.5 mW of optical power, and the shot noise is already 8 dB above the electric noise floor. Now we are able to measure shot noise with greater confidence, the next task is to reduce the excess noise in fiber laser and make it shot-noise limited for SRS imaging.

Even though I have made this design in 2013, the scheme was published much later\(^ {47} \). The detector is especially useful in cases where the measured optical power is low. In fact, the detector was used in many works of our group before it is published. Of course, it was used for the balanced
detector for all-fiber SRS\textsuperscript{46}, where the laser power is low (about 10 mW). It improved the image quality in live cell DNA imaging\textsuperscript{77}, where optical power needs to be low to make the cells go through mitosis, (because high optical power will stop mitosis.) It was used in epi and transmission detected SRS imaging of brain tumor\textsuperscript{51} and other tumors\textsuperscript{52,77}. It is also extremely helpful in the \textit{in vivo} imaging of skin care products on human skin described in Chapter 5. However, the phase of the signal for this detector depends more on the optical power on the detector, it caused some trouble for simultaneous two-color imaging described in Chapter 6.

2.5 Noise cancellation and design of voltage subtraction auto-balanced detector

Balanced detectors are routinely used to remove background and noises in optical experiments\textsuperscript{63-65}. Normally, the balanced detector has two photodiodes (detectors) and works like this. The light sources were splitted into two beams: signal beam and reference beam. Signal beam transmits through sample, bears information about the sample (attenuation or amplification) and was measured by one of the detector on the balanced detector; and the reference beam is sent directly to the other detector on the balanced detector. The common mode fluctuations in the original laser are then electronically cancelled in the balanced detector. We can imagine that only when the reference and the signal beam has exactly the same amount of noise, the noise can be cancelled perfectly. For example, if the two detectors have 10\% of difference of optical powers, the noise in the signal beam can only be cancelled by 90\%, and become 10\% of previous value. This corresponds to 20 dB of reduction in noise power.

However, high speed SRS imaging requires more than that. In SRS microscopy, the laser is scanned over a sample surface and the sample has different transmissions at different location. So, the intensity of the signal beam changes its intensity as it was scanned through the sample. To ensure perfect noise cancellation, the reference beam must also change its intensity following the
signal beam. This requires the technique called auto-balanced detection, in which the reference of the beams’ intensity or photocurrent is adjusted according to that of the signal’s.

To perform fiber laser SRS with auto-balanced detection can be achieved in several ways. A Japanese group used optical noise cancellation technique by sending both signal and reference beam to the sample so that they experience the same transmission. By delaying one of the beam by half of the period of modulation frequency, the noise cancellation was performed in lock-in amplifier by subtraction. However, the imaging speed is only the 3 ms/pixel. This may be due to lower power in their fiber laser and their use of electric synchronization, which resulted in high timing jitter and high noise. Another way to achieve auto-balanced detection is to electronically adjust the gain of reference arm or signal to match the intensity of the two arms and commercials auto-balanced detector used this method. An Italian group used commercial available auto-balanced detector to perform SRS. However, the imaging speed is 10 ms/pixel. Even though both groups are able to cancel the noise the imaging speed is about 1000 times slower than the SRS microscopy performed with solid state OPO systems.

To make all-fiber laser a source for high-speed SRS microscopy, we developed a voltage-subtraction auto-balanced detector. Our auto-balanced detector use electronics to adjust the electric gain of the photocurrent of the signal beam at high speed to guarantee the removal of noise regardless of change of signal laser intensity on the detector.

The drawbacks of the commercial auto-balanced detector are low saturation power and narrow bandwidth. The commercial auto-balanced detector is based on current subtraction of transistor pair with a feedback circuit. The beauty of this design is its simplicity. However, the product 2007 Nirvana Auto-Balanced Photoreceiver from Newport Inc. can only accept 1 mW of optical power. SRS microscopy commonly use anywhere from 10 mW up to 100 mW on the detector. The
The bandwidth is 125 kHz, which limit the pixel dwell time to longer than 16 µs. It is not possible to work in the modulation transfer scheme which is used in SRS microscopy, because that requires the noise cancellation at much higher frequency (> 4 MHz).

We found a way to solve these problems. We designed a voltage-subtraction auto-balanced detector that effectively cancelled the noise at 10 MHz with a bandwidth of about 1 MHz.

Several concepts are important to our new auto-balanced detector’s design.

First, I believed that voltage is much easier to handle than current, so it is convenient to convert photocurrent to voltage signal with high gain and low noise. Current is considered as the carrier of the signal and the voltage is the signal. Designing the circuit with voltage as signal is logically more straight-forward even though they are equivalent in many cases.

Secondly, the DC and AC component of photocurrent are can be treated separately. The frequency modulation scheme used in SRS make the two parts of the spectrum useful. The DC component provide the information about amount of light transmitted through the sample. The AC component around 10 MHz provides the SRS signal. DC and AC components can be separated by a bias T and treated separately in the so-called DC arm and AC arm in the circuit. The filtered transimpedance amplifier shown in Figure 2-6, the DC component is sent to a 10 Ohm resistor. The reason we did not use 50 Ohm is because the frequency is low and there won’t be much complicated effect due to unmatched impedance; also, the optical power of 50 mW will produce a voltage of 1.6 V on a 50 Ohm resistor, which is too high for later signal processing. The AC component around 10 MHz was amplified by the transimpedance amplifier to so that we can see the shot noise clearly. The signal due to laser repetition rate at 80 MHz was also attenuated. Even
though we can now process the AC and DC separately, the two components are proportional to each other, because they come from the same source.

Thirdly, a variable gain amplifier (VGA) can adjust the output level with voltage. This allows us to process the AC and DC signals for reference and signal beams and then feedback the result to gains of channels in VGA to achieve auto-balancing.

Fourthly, a PID feedback loop is widely used in automatic control to lock a voltage to a set point. In our case, we want to lock the noise in signal arm to the noise in the reference arm and cancel them. If this can be done for every pixel, which requires the feedback loop to be fast, then we could achieve the auto-balancing detection for high-speed imaging.

At last, the perfect match of delay is needed for perfect noise cancellation; delay adjustment can be achieved by electric phase shifter without the need of matching the light path. The perfect noise cancellation near a specific frequency can be regarded as trying to cancelling a signal with certain carrier frequency and a slowly random varying envelop. The phase of the carrier frequency and the amplitude (envelope) both needs to be matched perfectly. Intuitively, the reference and signal laser need to arrive at the detectors at the same time and the electronics on reference arm and signal arm needs to be exactly the same to perform such perfect cancellation. However, since the envelop is slowly varying, a small delay in optical path will make very little difference on the matching of amplitude (envelope), we only need to use a phase shifter to shift the carrier phase to achieve the perfect noise cancellation around a certain frequency.
Figure 2-8 The schematic of the voltage-subtraction auto-balanced detector.

Figure 2-8 shows the schematic of voltage-subtraction auto-balanced (VAB) detector. It is the realization of above design ideas.

The signal laser and reference laser both detected by 1 cm Si photodiodes. The filtered transimpedance amplifier shown in Figure 2-6 separate AC and DC components for photocurrents generated from reference and signal lasers and amplified AC currents well above electronic noise. A four channel VGA (AD8334) is used to amplify four channels. We fixed the gain for the reference laser’s AC and DC channel. Then we let the reference DC arm to be a set point for the DC arm of signal laser. The PID feedback loop is used to keep the both DC arms about the same by adjusting the gains of DC arm of signal laser. We know the DC and AC components from the same diode are proportional, by using the same gain voltage for DC on the AC arm for the signal laser, when the DC components of signal arm are locked to that of the reference arm by the PID feedback loop, the common mode noise in both AC components are the same too. Therefore, they can be perfectly cancelled in a differential amplifier. Before subtraction, the relative phase of AC
components is adjusted to match each other perfectly. This phase does not depend on laser intensity and, therefore, does not need to be adjusted during imaging experiment.

Figure 2-9 SRS microscopy of 1.1 μm polystyrene beads with -all fiber laser source to demonstrate the performance of voltage-subtraction auto-balanced detector. a. the maximum amount of noise that can be cancelled by the VAB detector around 10 MHz. b. The noise cancellation of the detector in real experiment. c-f. SRS image of 1.1 μm polystyrene beads with all-fiber laser and auto-balanced detector. c. images with full function of auto-balanced detector. d. block the reference arm shows the image without
balanced detection. e. when the auto-balancing feedback loop turned off and Stokes laser blocked. f. the auto-balancing feedback loop turned on and Stokes laser blocked.

The performance of VAB detector is shown in Figure 2-9. Figure 2-9 a shows the noise cancelling ability. We added white noise in the laser and see how much noise can be cancelled by the VAB detector. At a frequency near 10 MHz, it can cancel 45 dB of noise from the laser. The performance lowered as the frequency gets far from the center but are mostly higher than 25 dB noise cancellation between 9.5 and 10.5 MHz. Which is enough to remove all the noise from fiber laser source. Figure 2-9 b shows the noise cancellation performance for our fiber laser source. About 22 dB of excess noise is cancelled. The shot noise level is reached. Figure 2-9 c shows the SRS image of 1.1 µm polystyrene beads taken with all fiber laser source and VAB detector. The pump power on the sample is about 14 mW and the Stokes power on the sample is about 10 mW. Figure 2-9 d shows the image without balanced-detection; the noise make it hard to identify beads. The image was taken by blocking the reference laser from hitting the detector. Figure 2-9 e was taken when the Stokes laser was blocked and auto-balancing function was turned off. We blocked the Stokes laser so that we can see the results of auto-balanced detector better. Without auto-balancing function, the noise at the edges of spheres are clearly visible due to imperfect noise cancellation. Figure 2-9 f shows the image when the Stokes laser was blocked and the auto-balanced function turned on. We can see the background is flatter and even at the edges of the spheres the noise wasn’t visible. All the images were taken with pixel dwell time of 2 µs.

2.6 SRS microscopy with all-fiber laser source

As the noise cancels out, we can then take clear SRS images with all-fiber laser at the pixel dwell time of 2 µs, just like the parameters we use for solid state OPO system. An image of sebaceous gland on mouse ear in transmission mode is shown in Figure 2-10. Figure 2-10 a shows the images taken at 2850 cm⁻¹, 2950 cm⁻¹ and the overlap of the two images. The image taken at
2850 cm\(^{-1}\) (left) has high signal on lipid rich sebaceous gland. The image taken at 2950 cm\(^{-1}\) (middle) has relatively high signal not only on sebaceous gland but also on the nearby tissues and the hair in the middle of sebaceous gland. Figure 2-10 b shows a 3D image of sebaceous gland on mouse ear at 2850 cm\(^{-1}\). The imaging depth is 100 µm. The lipids layer in the skin are visible.

![Figure 2-10 SRS microscopy of mouse sebaceous gland with all-fiber laser source and auto-balanced detector. a. SRS image taken at 2850, 2950 cm\(^{-1}\) and the overlap of them. b. Three-dimensional SRS imaging at 2850 cm\(^{-1}\). The total imaging depth is 100 µm and the scale bars are 50 µm.](image)

2.7 Summary and Discussion

To achieve shot-noise limited image quality with all-fiber laser will greatly reduce the cost of light source for SRS microscopy. There are several advantages of this technique. First of all, unlike the previous current subtraction versions of auto-balanced detector, the photodiodes of signal and
reference arm can be separated from each other and the auto-balancing part of the circuit. This is the benefit of using voltage-subtraction instead of current subtraction and IC components like opamps compared with using transistor pairs. The delay between pulses doesn’t have to be exact and can be adjusted by a phase shifter. Secondly, the ratio of subtraction between reference and signal DC components can be adjusted to further reduce the noise in auto-balanced detection. Figure 2-8 shows the difference opamp between DC components of signal and reference arm are adjustable. The purpose for this design is to further reduce shot noise introduced by balance detection. Balanced detection can remove excess noise but can also add the shot noise in the reference arm to the final noise. If the reference beam and signal beam has the same optical power, then they have the same amount of shot noise, the final noise power of balanced detection will have twice the noise power of shot noise in signal beam or reference beam. To write it out in formula, the final noise power \( P_n = P_{\text{shotnoise,signal}} + P_{\text{shotnoise,reference}} = r(i_{\text{shotnoise,signal}} \times G_{\text{signal}})^2 + r(i_{\text{shotnoise,reference}} \times G_{\text{reference}})^2 \), where \( i \) represents photocurrent and \( G \) represent electric gain in VGA on that arm. We know that shot noise in photocurrent is proportional to square root of photon numbers and photon numbers are proportional to optical power so \( P_n \propto P_o,signal \times (G_{\text{signal}})^2 + P_o,reference \times (G_{\text{reference}})^2 \), where \( P_o \) represent optical power of lasers. On the other hand, the excess noise is directly proportional to optical power, in the case of perfect removal of excess noise, we have \( i_{\text{excessnoise,signal}} \times G_{\text{signal}} = i_{\text{excessnoise,reference}} \times G_{\text{reference}} \Rightarrow P_o,signal \times G_{\text{signal}} = P_o,reference \times G_{\text{reference}} \). We put this relation to the final noise expression, we can obtain \( P_n \propto G_{\text{signal}} + G_{\text{reference}} \), as long as \( P_o,signal \times G_{\text{signal}} = P_o,reference \times G_{\text{reference}} \). In principle, we can use more optical power in the reference beam so that the electric gain on the reference can be lower and the final noise power can be closer to the shot noise of signal beam. At last, this method also has limited bandwidth, and only in the center we
have 45 dB of noise suppression. The reason is dispersion in each component are not perfectly matched. So, at different frequencies, the phase or delay may be different and cause limited bandwidth. In fact, the differential amplifier does not treat two arms equivalently, so the subtraction is intrinsically imperfect. A digital implementation that carefully adjust the phase over a broad spectrum may solve this problem.

One obvious drawback of all-fiber laser is the limited wavelength, so the current design only works for CH high wavenumber SRS microscopy. For finger-print SRS microscopy, other gain medium needs to be used. Obviously, it won’t be easy to switch from CH to finger-print SRS imaging. Luckily, one of the major applications of SRS is to perform label-free histology in CH high-wavenumber region$^{37,50-52}$. Traditional H&E histology takes from half an hour to hours depending on the medical condition of the hospital. In some cases, it is desirable to have fast histology during the surgery to give precious information about the surgery. All-fiber laser SRS is well suited for this application.

To summarize, the development of the all-fiber laser source and the voltage-subtraction auto-balanced detector has greatly reduced the cost of the light source of SRS to below ten thousand dollars, a twenty times reduction of cost in laser source, without compromising in image quality and imaging speed.

This exact technology has resulted in a start-up company in Silicon Valley and is being used in a surgery room to perform label-free histology$^{51}$. We believe the lowered cost will make SRS more accessible to people working in biology, medicine and other areas.
Chapter 3

Imaging neurotransmitter in frog neuromuscular junctions by SRS

3.1 Backgrounds

How brain works is one of the biggest questions scientists face today. There are on the order of 100 billions of neurons in brain forming 1,000 trillion connections between each other. Scientists are trying to map out these connections and study brain activity\(^80\). Electron microscopy (EM)\(^81,82\), optical microscopy\(^83\) and magnetic resonance imaging (MRI)\(^84,85\) are all used to tackle this problem. EM has the highest spacial resolution, however, it is time consuming, has limited chemical specificity and works on fixed tissue only. MRI can detect brain activity \textit{in vivo} but has limited spacial resolution and chemical specificity. Optical imaging has sub-cellular resolution and high chemical specificity. Most optical imaging use labeling of specific proteins to achieve functional imaging. But not all molecules are suitable for labelling. One of the missing component in the current push for imaging brain activity and connectome is neurotransmitter.

Neurotransmitters are small molecules or peptides that transmits neural excitation at chemical synapses, which are fundamental building blocks of neural circuits. The major neurotransmitters in mammals are glutamate, dopamine, serotonin, acetylcholine and GABA, who play important roles in neural activity\(^86-91\). They mediate the transfer of signals between different neurons and between neurons and other types of cells such as muscles or glands, as shown in Figure 3-1. The property (excitatory or inhibitory) and efficacy of synaptic signal transmission depends not only on the type of neurotransmitters and receptors, but also on the ongoing activity of the synapse itself. Therefore, the complexity of the chemical synapses allows for complicated behaviors\(^92\).
Figure 3-1 Different ways neurotransmitters function in neural system and other organs. (image from https://en.wikipedia.org/wiki/Synapse)

Figure 3-2, shows the fundamental components of a chemical synapse. The presynaptic part is the nerve ending and contains vesicles that stores neurotransmitters in high concentration. The size of vesicles is about 40 nm. When neuronal excitation arrives at the synapse, the vesicles will fuse with the presynaptic membrane and release neurotransmitter to the synaptic cleft, which is a space of about 20 nm between nerve ending and the receiving part of the chemical synapse. The neurotransmitters will then attach to the receptors that open the ion channels to let the ions flow into the post synaptic membrane and excite or inhibit the excitation of the postsynaptic neuron or organs.

Figure 3-2 Components of a chemical synapse.
Studying neurotransmitter distribution and dynamics in the nervous system are critically important not only for the mapping of neural circuits, but also for revealing the pathophysiology of many neurological diseases and gaining new insights into developing therapeutic treatments. The imbalance of neurotransmitter levels at the synaptic cleft or defects in neurotransmission are associated with many psychiatric and neurological disorders such as Schizophrenia, depression, Parkinson disease, and Alzheimer disease$^{93,94}$

Despite the pivotal role that neurotransmitters play in neural circuit function, to date, it is still challenging to study them due to the lack of tools that can map their distribution and concentration at the subcellular level. In recent years, there have been intensive efforts in search of engineered fluorescent probes$^{95,96}$. However, since such labels are much bigger than the small neurotransmitter molecules, the results obtained with such labels may not necessarily reflect the behavior of the neurotransmitter themselves. To avoid this problem, label-free approaches were attempted. Maiti et.al performed the first multiphoton fluorescence imaging of serotonin in live cells$^{97}$. However, serotonin fluoresces in the UV range, which could easily be overwhelmed by tissue autofluorescence background$^{98}$. Due to the same reason, fluorescent detection of other neurotransmitters in tissue are even more challenging.

The unique vibrational signature of neurotransmitter molecules offers the opportunity to directly image neurotransmitters in tissues without the need of labeling. Surface enhanced Raman scattering (SERS) has been used to determine neurotransmitter level in cells$^{99}$. However, the application of SERS in tissue is difficult due to the requirement of colloid nanoparticles for Raman signal amplification. Confocal Raman imaging of neurotransmitters has also been attempted, but is still limited to artificial chemical samples$^{100}$. 

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Here, we demonstrated that frequency-modulation spectral-focusing (FMSF) SRS can probe an endogenous neurotransmitter for motor neurons: acetylcholine in frog neuromuscular junctions (NMJ).

We chose acetylcholine (ACh) as our target molecule because ACh is highly concentrated in neuromuscular junctions (NMJ) and acts as the chemical messenger that is released by motor neurons to activate muscles. Frog NMJ is one of the mostly studied neural structures. Publications show that the concentration of ACh at frog NMJ may reach 30 mM. This is higher than the detection limit of ACh by SRS. ACh also plays critical roles in the central nervous system by functioning as a neuromodulator.

3.2 Frequency modulation spectral-focusing SRS

Narrowband SRS imaging allows high sensitivity imaging of a particular Raman band defined by the photons’ energy difference between the pump and the Stokes lasers. Even though it does not suffer from the non-resonant background which plagues narrow band coherent anti-Stokes Raman scattering (CARS) microscopy, it is not entirely background free. Cross-phase modulation, a nonlinear optical process that is induced by the Kerr effect of the medium, also presents a background that is dependent on the optical alignment and collection efficiency of the pump beam. In addition to the purely electronic background, Raman backgrounds are ubiquitously present due to many Raman active modes of lipids, protein, and nucleic acids in the fingerprint region. In many cases of biological imaging, even though concentration of target molecule is relatively high, the backgrounds still dominate the measurement.

In order to image neurotransmitter, ACh in particular, we developed a frequency-modulation spectral-focusing SRS (FMSF-SRS) technique. It combines the advantages of the spectral focusing
method\textsuperscript{110}, which improves sensitivity, and the frequency modulation method\textsuperscript{107,111}, which provides real-time background subtraction.

First of all, the principle of spectral focusing SRS is shown in Figure 3-3 c-d. Narrow band SRS (Figure 3-3 a) use lasers with narrow spectral bandwidth (<1 nm) and long pulse durations (>2 ps). To probe different Raman peaks, the center wavelength of at least one of the lasers needs to be tuned. SRS using femtosecond lasers (Figure 3-3 b) excites a broad Raman band $\Omega_{\text{min}} - \Omega_{\text{max}}$. Therefore, the chemical specificity is poorer in femtosecond SRS. Some researchers filter the femtosecond pulses to narrow its spectra and make them into narrowband picosecond pulses. But doing this will reduce the optical powers, which results in reduced signal, low quality image or low imaging speed. Spectral focusing (SF) method was developed to solve this problem\textsuperscript{110}. In SF SRS, broad band femtosecond lasers are arranged as shown in Figure 3-3 c, where both pump and Stokes are chirped, which means that the high frequency and low frequency components are equally separated in time. The pulses are no longer femtosecond pulses even though they have broad spectrum, instead they become picosecond pulses. Because of this, at each time delay, the difference frequency between pump and Stokes lasers is the same narrow Raman peak at $\Omega$ just like in the case of picosecond narrow band SRS. SF SRS achieved narrow band SRS with femtosecond lasers without reducing its power. One feature of SF SRS is that the position of Raman peak can be adjusted without the need of tuning laser wavelengths. Figure 3-3 d shows that when the time delay between chirped pump and Stokes pulses are slightly adjusted, the lasers will excite a nearby Raman peak $\Omega'$ compared with $\Omega$ before the change of relative delay between pump and Stokes. This feature allows us to design a frequency modulation scheme that can be used to reduce imaging backgrounds.
Figure 3-3 The principle and imaging setup of FMSF-SRS. a. Pulse shape and time delay of pump and Stokes pulses in picosecond (narrow band) SRS. b. Pulse shape and time delay of pump and Stokes pulses in femtosecond (broad band) SRS. c. Pulse shape and time delay of pump and Stokes pulses in spectral-focusing SRS. d. Tuning of target Raman band in spectral-focusing SRS. e. Optical setup of FMSF-SRS. DM: dichroic mirror. EOM: electro-optical modulator. HM: half mirror. LA: lock-in amplifier. PBS: polarizing beam splitter. PD: photodiode.

The optical setup of FMSF-SRS is shown in Figure 3-3 e. Both pump and Stokes pulses are chirped by going through a 54 or 60 cm SF57 glass rods with anti-reflection coatings. Normal SF-SRS will remove either perpendicular arm or parallel arm, so that the pulses are modulated at 20 MHz, which is a quarter of laser repetition rate. The modulation is synchronized to laser repetition rate to achieve this synchronous modulation. In FMSF-SRS, neither arms are blocked, instead there is a different time delay between perpendicular arm and parallel arm, which means that pulses in parallel arm are probing a different Raman peak than pulses in perpendicular arm. Since the modulation phase of two arms are 180 degrees different, the signals of two different Raman peaks are automatically subtracted in the lock-in amplifier. When we want to perform SF-SRS without FM, we can simply block one of the arms.
3.3 Imaging ACh at frog NMJ

First, we obtained the spontaneous Raman spectrum of ACh, as shown in Figure 3-4 a. We decided to use the Raman peak at 720 cm$^{-1}$, corresponding to the symmetric stretching of the C-N bonds of the quaternary ammonium group in choline$^{112}$.

We then determined the detection linearity and sensitivity of ACh at the 720 cm$^{-1}$ Raman peak using the FM SRS setup. We performed hyperspectral scan by scanning the delay of pump beam. From Figure 3-4 c we can see that there is some background in the spectrum. Obviously by dividing each spectrum by the concentration, we cannot make them overlap with each other. But if we subtract the background of pure water before we divide the concentration, the lines overlap with each other Figure 3-4 c (bottom). This shows that ACh spectrum intensity depends linearly on the concentration of ACh. We plotted the concentration curve in Figure 3-4 b. The detection limit is about 20 mM with the integration time of 4 µs. Literature shows that the concentration of ACh in frog NMJ is about 30 mM$^{101}$, so it is very likely that SRS can probe ACh in frog NMJ. In fact, 20 mM is the sensitivity for single pixel in single image. If averaging is used and pixels are combined, the sensitivity can be much higher. As shown in Figure 3-4 c, at such concentration, the background could easily overwhelm the Raman signal from ACh. In pure solutions, only after subtraction of the background, SRS signals are linearly proportional to concentrations of ACh (Figure 2 c). However, in live tissue, the background signal is non-uniform, necessitating real-time background subtraction using FMSF-SRS.
Figure 3-4 Raman characteristic of acetylcholine. a. Spontaneous Raman of ACh. b. The concentration curve of ACh measured by spectral focusing SRS. c. Spectral focusing SRS spectra of ACh at different concentrations. d. The frequency modulation spectra of ACh at 100 mM concentration.

Frequency modulation can remove backgrounds because the backgrounds are normally broadband and bears no spectra feature. For SRS, the background produced by cross-phase modulation is such kind of background. Figure 3-4 d shows the spectra of ACh taken with parallel and perpendicular arms of 100 mM ACh by blocking the other arm. There is a shift of spectra between parallel and perpendicular arms, because the Stokes pulses, went through two arms had a slight difference of time delay relative to the pump pulses. When we allow both arms to go through the microscope for imaging, the signal are exactly the difference of the signals produced by individual arms. The signal of FMSF-SRS at certain delay of pump is identical to that obtained
through direct subtraction of two SRS signals at different wavenumbers. FMSF-SRS performs the derivative operation optically in real time instead of digitally during post-processing, offering a significant advantage of resistance to sample movement and pump power fluctuation. These advantages lead to better signal fidelity and signal to noise ratio.

We applied our imaging technique to study the frog NMJ. We used adult *Rana pipiens*, a common north American leopard frog, as our experimental model. The animal was anesthetized and sacrificed following the animal protocol approved by Harvard University Institutional Animal Care and Use Committee. The *cutaneous pectoris* muscles were dissected for imaging and electrophysiology experiments following the protocol described by Wu et al. The *cutaneous pectoris* is especially suitable for imaging because it is translucent single layer muscle that is relatively flat. The nerves on it is easily accessible for electrical excitation. Thanks to the tremendous help of Prof. William Betz and Dr. Achim Klug. We learned how to dissect the muscle and obtained the device to excite the muscle.

Figure 3-5 The location of cutaneous pectoris muscle, the microscopic structure of frog NMJ and nerve ending.

The location of *cutaneous pectoris* muscle and structure of frog NMJ is shown in Figure 3-5. The neurotransmitter ACh is stored in synaptic vesicles with an average size of ~ 40 nm. Since the spatial resolution of microscope is limited to ~300 nm, we imaged the whole synaptic vesicle pool in the focal volume instead of individual vesicles. A typical image of NMJ by FMSF-
SRS is shown in Figure 3-6. We also showed an image taken with SF-SRS without frequency modulation. We can see that frequency modulation greatly reduced background.

To locate the NMJ in the frog *cutaneous pectoris* muscle, we used alpha-Bungarotoxin-Alexa488 (α-BTX-Alexa488) staining. α-BTX binds to the ACh receptors of NMJ that are closely located with vesicles containing ACh. The two-photon fluorescence images of α-BTX-Alexa488 excited with the same lasers for SRS are shown in Figure 3-6. Indeed, they colocalized with FMSF-SRS images of ACh.

![Figure 3-6 FMSF SRS imaging of frog NMJ. a. FMSF SRS imaging of frog NMJ. b. SF SRS imaging of frog NMJ. c. Two-photon excited fluorescent imaging of ACH receptor stained by α-BTX. d. The line profile of a cross section in the image to show the effect of frequency modulation.](image)

3.4 Quantifying ACh at frog NMJ

To verify that the detected SRS signal came from ACh and to estimate its concentration, we performed hyperspectral FMSF-SRS imaging of the muscle tissue. After subtracting the signal of nearby muscle, we obtained the spectra of the NMJs in Figure 3-7. The solid blue line shows the
average SRS signal level at each delay and it matches the signal of a 13 mM ACh solution (black line). The result is based on the measurement of 14 NMJs from 5 animals.

![Graph showing average SRS signal level at each delay compared to ACh 13 mM solution.](image)

**Figure 3-7** The estimation of ACh concentration at frog NMJ by FMSF SRS hyper-spectral imaging. The spectra of the NMJs are shown and compared with ACh in water solution.

It is important to note that acetylcholine is not the only chemical that has the 720 cm\(^{-1}\) Raman peak. It is known that phosphatidylcholine that contains choline is highly present in the membrane of synaptic vesicles. It is estimated that there are about 2,524 phosphatidylcholine molecules in each synaptic vesicle\(^{114}\). Even though this number is much smaller compared with 11,000 ACh in each vesicle\(^{104}\), it is still contributing to the measured FMSF-SRS signal. Because vesicular membrane and vesicular acetylcholine are colocalized and cannot be resolved due to insufficient spatial resolution, the obtained FMSF-SRS image contains both species.

If we consider the contribution from both ACh and phosphatidylcholine, the concentration of ACh will be \(\sim 10\) mM, which is twice lower than previously published data\(^{101}\), but still on the same order of magnitude.

### 3.5 Imaging the NMJ after the release of ACh

Lastly, we performed control experiments to corroborate that when vesicle release ACh, SRS signal will decrease. It is known that strong electrical excitation will force the vesicles at NMJs to release neurotransmitters\(^{113}\). After exciting the neuromuscular junction with a 20 Hz square wave
at 4V for 10 mins, the majority of the ACh in the vesicles should be released and diffused out, but empty vesicular structure remained, which was stained with FM1-42 dye that were taken in during membrane fusion between the vesicles and the cell membrane. To ensure the efficient release of ACh, we incubated the cutaneous pectoris muscles in Ringer’s solution with 1 µM neostigmine, 25 µM of vesamicol and 300 nM hemicholinium-3 (HC-3) for 30 mins before and during excitation. Neostigmine can inhibit the function of acetylcholinesterase, while HC-3 can block the high-affinity choline uptake and vesamicol blocks the uptake of ACh by synaptic vesicles. The imaging results are shown in Figure 3-8 a-f. We observed that after excitation, α-BTX staining remains (Figure 3-8 b, e), and FM dye staining became visible (Figure 3-8 a, d). The FMSF-SRS signal of ACh had greatly decreased in NMJs (Figure 3-8 c, f).

Figure 3-8 Imaging of ACh at NMJ with and without strong excitation. a-c. Imaging of frog NMJ without electrical excitation. d-f. Imaging of frog NMJ after strong electrical excitation. a, d. Imaging of FM 1-43 dye contrast. b, e. Imaging of α-BTX contrast. c, f. Imaging of ACH with FMSF-SRS. g. Concentration of ACh measured at frog NMJs with and without electrical excitation.

We recorded the FMSF-SRS spectra of the NMJs after excitation, and compare them with that of unexcited NMJs (Figure 3-8 g). We found that the average concentration of choline group in
the NMJ has decreased from 12.9 mM to 5.7 mM. This provided a lower limit of 7.2 mM of ACh concentration in NMJ, which marks the amount of ACh that can be released from NMJ.

The control experiments further demonstrated that the FMSF-SRS signal of NMJs originated mostly from ACh in vesicles, and ACh concentration was significantly reduced when it was released from vesicles. The residue signal may have several sources, the most significant one being the phosphatidylcholine in vesicular membranes.

3.6 Discussion

This work demonstrates the first step of imaging neuronal activity by imaging neurotransmitter. We note that synaptic vesicles are characterized into three groups: the reserve pool, recycling pool and ready release pool. Only the vesicles ready release pool can be released right away. Only under long time excitation or very strong excitation the other vesicles from all three pools can be fully released. Since the reserve pool consists of 80% of synaptic vesicles. Our experiments were mainly visualizing the reserve pool. Further study of vesicle release from the ready release pool of the NMJ are currently limited by the sensitivity of FMSF-SRS. We believe that with further improvement of sensitivity of SRS, this method may open up new avenues for in vivo study of synaptic activity.
Chapter 4

Monitor amyotrophic lateral sclerosis in mouse model and drug testing

4.1 Backgrounds

Coherent Raman scattering microscopy is suitable for imaging peripheral nerves due to the high lipid content in myelin\textsuperscript{116-121}. Before SRS microscopy was invented, CARS has been used to image nerves in mouse models of multiple sclerosis (MS)\textsuperscript{122} and nerve crush\textsuperscript{123}. There are other neurodegenerative diseases that haven’t been studied by CRS before. Amyotrophic lateral sclerosis (ALS) is one of them. ALS is also called Lou Gehrig’s disease and is a progressive neurodegenerative disease\textsuperscript{124-126}. 50\% of ALS patients die within 30 months after symptom onset\textsuperscript{124-127}. There is no definitive diagnosis for ALS, which make the diagnosis time consuming\textsuperscript{125,126}. These factors make it difficult to perform drug testing and find a cure for ALS. Up to now, there is no cure for ALS. The only FDA approved drug for ALS is Riluzole that can extend the life span of ALS patients by a few months.

Improved methods for monitoring motor neuron degeneration are much needed, in part due to the high-degree of variability of disease progression in rodent models\textsuperscript{127,128} and ALS patients\textsuperscript{128-131}. End point analyses currently used to monitor disease progression in mouse models are also either laborious histological measurements or behavioral assays that can be impacted by operator-specific variance. Unbiased imaging methods could provide more precise measures of disease onset and progression as well as reduce the length and ambiguity currently inherent to trials of candidate interventions in these models.

Here, we describe the use of SRS imaging to visualize peripheral degeneration in several mouse models of ALS and human postmortem tissue\textsuperscript{47}. We found that peripheral nerve
degeneration as monitored by SRS imaging was one of the earliest detectable pathological events in ALS mouse models and that disease progression could be followed reliably over time in living animals through serial imaging47. We also demonstrate that SRS imaging could be employed to evaluate candidate therapeutics, confirming that the compound minocycline significantly slows peripheral nerve degeneration in the SOD1G93A mouse. To demonstrate the potential clinical utility of our approach, we showed that motor nerve degeneration can similarly be monitored in postmortem tissue from ALS patients.

4.2 Sciatic nerve of ALS mouse

It is known that ALS damage the peripheral nerves132-134. However, the relationship between the nerve degeneration and disease development is still unclear.

![Figure 4-1](image.png)

Figure 4-1 Three-dimensional ex vivo SRS microscopy at 2850 cm\(^{-1}\) on sciatic nerves from age matched SOD1G93A and wild type mice. a. One section of ex vivo SRS image of sciatic nerve from a 16-week old wild type mice and its 3-d reconstruction shown in b. c. One section of ex vivo SRS image of sciatic nerve from a 16-week old SOD1G93A mice and its 3-D reconstructions sectioned at various locations.
SRS provides a way to monitor the pathological change of peripheral nerves in ALS mice by imaging the lipid rich myelin. Compared with previous imaging methods: immunostaining\textsuperscript{132} or electron microscopy\textsuperscript{133}, SRS is label-free and can be performed on live animals. Even though its resolution is not as good as electron microscopes, it is enough to image myelin structures\textsuperscript{116-119,122,123}.

We started the imaging experiment by imaging sciatic nerves of late stage ALS mice and age matched littermates. Figure 4-1 shows a typical comparison of sciatic nerve between wild type (WT) mice and SOD1G93A mice. An XYZ scanning allows us to reconstruct the 3-D structure of the nerves. The structure difference between the two samples is obvious. The sciatic nerve of WT mice composed of straight tubes (myelin sheath). The sciatic nerve of SOD1G93A mice are composed of wiggled tubes and broken pieces. One prominent feature of the nerve of ALS mice is the aggregation of lipids, which we call lipid ovoids. Figure 4-1 c shows that lipid ovoids have different shapes and sizes. The cross sections, Figure 4-1 d-g, shows that some lipid ovoids are filled, and others are empty or half-filled.

To study the biological content of lipid ovoids, we stained the neurofilament and myelin basic protein (MBP) on sciatic nerves of ALS mice, as shown in Figure 4-2. The ovoid shape structure has positive staining of myelin basic protein, and inside some of the ovoid shape structures are the neurofilaments. Therefore, we believe that the lipid ovoids are broken myelin sheath with some neurofilaments enclosed. These observations are based on morphology. We could not confirm lipid ovoids with SRS and MBP very well, because MBP staining requires the removal of lipid content in the myelin.
4.3 Chemical decomposition with least square method

Other than verifying the biological content of the lipid ovoids, we also measured the change of chemical contents of the lipid ovoids and myelin, thanks to the chemical specificity of SRS. We performed hyperspectral SRS imaging of sciatic nerves from SOD1G93A and WT mice. Then we performed chemical decomposition by least square method. We know that myelin is mainly composed of lipid, protein and water\textsuperscript{135,136}. The lipids in myelin are mainly composed of cholesterol (C), galactocerebroside (GC), sphingomyelin (SPH), phosphatidylcholine (PC), phosphatidylethanolamine (PE). There spectrum measured by hyperspectral SRS are shown in Figure 4-3a. By using least square method, we calculated the chemical compositions of lipid ovoids and myelin sheaths, as shown in Figure 4-3b.
Figure 4-3 Chemical decomposition of myelin sheath and lipid ovoids in ALS mice and WT mice. a. The hyperspectral SRS measurement of Raman spectra of major chemicals in myelin. b. the chemical contents of myelin and lipid ovoids obtained by decomposition of hyperspectral SRS images by least square method.

From Figure 4-3 b, we could see that the lipid ovoids has significantly lower water content and shows a more hydrophobic nature. This justifies the name we used for these aggregated degenerated myelin as lipid ovoids. Another character is that lipid ovoids has significantly less cholesterol. It is known that cholesterol is crucial for myelin growth\(^{137}\). The reduction of myelin in lipid droplets may be a result or a cause for myelin degeneration. In this work, we focus on number of lipid ovoids. The change of chemical composition of myelin and lipid ovoids will be a good topic for future investigation.
Multivariate analysis or principle component methods are commonly used for analyzing hyperspectral analysis. However, those methods does not always provide direct chemical assignments. Here we use the least square method to decompose myelin and lipid ovoids to its main chemical contents. The least square method was credit to Carl Friedrich Gauss, and it is mathematically described as follows.

First of all, we assume there are only 7 chemicals in the myelin. We measured their spectra between 2820 cm\(^{-1}\) and 3020 cm\(^{-1}\) with hyperspectral SRS by tuning the wavelength of pump laser. We measured about 30 spectral points for each chemical, we interpolate them to a continuous function \(\text{spec}_i(\lambda)\), where \(i = 1\) to 7 for 7 chemicals. Now we consider each pixel in a hyperspectral image stack of 30 images taken at 30 wavenumbers. We took hyperspectral SRS images of nerves with 30 spectral points denoted by \((\text{pixelspec}_j, \lambda_j)\), where \(j = 1\) to 30. We can write \(\text{pixelspec}_j\) as a vector with 30 components \(\text{pixelspec}\). We can also calculate the spectral value for each chemical at these 30 wavelengths of \(\lambda_j\), we denote them as \((\text{chemspec}_i(\lambda_j), \lambda_j)\), remembering that for each sample, we may have different \((\text{chemspec}_i(\lambda_j), \lambda_j)\), because \(\lambda_j\) are a little bit different for each sample, and we use the same the interpolated spectral function \(\text{chemspec}_i(\lambda)\) for all samples.

With our assumption that the sample is only composed of 7 chemicals, we can write 30 equations \(\text{pixelspec}_j = \sum_{i=1}^{7} c_i \times \text{chemspec}_i(\lambda_j)\) for \(j = 1\) to 30 under ideal condition. Our task is to find \(c_i\), which represent the concentration of chemicals in the sample. Of course, there are measurement errors in \(\text{samplespec}_j\), so we want to find out \(c_i\) that minimize the error \(\sum_{j=1}^{30} [\text{pixelspec}_j - \sum_{i=1}^{7} c_i \times \text{spec}_i(\lambda_j)]^2\). Mathematicians found a way to calculate \(c_i\), and called the method least square method. The mathematical formula is in fact very simple, if we arrange all
these numbers in matrix form. We use a vector $\mathbf{c}$ with 7 components to represent the concentrations we are trying to calculate. We use matrix $\mathbf{SPEC}$, to represent all the $\text{spec}_i(\lambda_j)$ with $\mathbf{SPEC}_{ij} = \text{spec}_i(\lambda_j)$. Then we can calculate $\mathbf{c}$ by $\mathbf{c} = (\mathbf{SPEC}^T \cdot \mathbf{SPEC})^{-1} \cdot \mathbf{SPEC}^T \cdot \text{pixelspec}$.

We argue that least square method suit the spectral fitting in SRS better than principle components method. Least square method only considers the spectral error and assume the $\lambda_j$ are accurate. On the other hand, principle components treat independent variables $\lambda_j$ similarly as spectral errors and try to minimize the combined error of both variables. In our hyperspectral SRS imaging, the independent variables $\lambda_j$ are very accurately measured, and only the spectral error needs to be considered. So, we believe least square method is better than principle component method.

### 4.4 Quantification of lipid deposition

We need an unbiased way to quantify the lipid ovoids to use it as a biomarker for disease tracking. Researcher used Fourier space to characterize nerve in nerve crush experiment\textsuperscript{123}, where the myelin sheath degrade similarly as myelin in ALS mice. Here, we decided to quantify number of lipid ovoids or number of myelin sheaths, because lipid ovoids have similar shapes. So, we used spacial correlation to quantify them.

The general shape of lipid ovoids is solid sphere or spheroid. We therefore used three lipid ovoids, shown in Figure 4-4 a-c, as template lipid ovoids and search how many similar lipid ovoids are present in the imaged nerve section by calculating the spacial correlation. The correlation image is then marked by a certain threshold and the highlighted spots with roundness and size above set values are counted. The lipid ovoids are then counted in this way for images taken at
different imaging depths. The resultant counting is transformed according to the unit counts per 50,000 square microns per optical slice. The detailed procedure is shown in Figure 4-4 d-f.

![Image Description](image.png)

**Figure 4-4 Quantification methods of lipid ovoids.** a-c. Three templates for lipid ovoids identification. d. original SRS image as an example. e. The spacial correlation between image d and one of the templates. The areas with high correlation are counted as lipid ovoids. f. The lipid ovoid counting are performed at different depth to improve detection range.

With this method, we can automatically, consistently and unbiasedly quantify the SRS images of nerves of mouse.

The counting method heavily depends on the choice of lipid ovoid templates, and the parameters chosen for thresholds in data processing, the exact number obtained from this counting algorithm is somewhat arbitrary. But, as long as we use the same templates and same standard to process all images, the relative values can provide us the meaningful comparison between different tissues. For human samples as shown later, we used another set of lipid ovoids templates to quantify human samples. So, the lipid ovoid counts are not comparable between human and mouse. Since the absolute lipid ovoid count depends on several arbitrary values, the meaning of the value is not very meaningful, I will omit the unit of lipid ovoids counts in most part of this thesis.
The quantification is not perfect, since we only use three templates, we cannot count all lipid ovoids. There are false positives too, where the quantification algorithm may still count a few lipid ovoids even though there is none in the image. But, fortunately, the false positive counts don’t change significantly as mice increase in age. However, not all lipid ovoids counts in WT mice are false positive. We do observe lipid ovoids in a few WT mice. We assume those are due to aging or other unknown causes.

4.5 Monitoring the progression of ALS

Before we start imaging ALS mice at different ages, we performed whole sciatic nerve imaging and trying to find out the location of the appearance of lipid ovoids. A dying back hypothesis of ALS says that the nerve ending may cause the death of the motor neurons, because it is found that nerve ending degenerate early in ALS mouse models\textsuperscript{127,138-142}. If dying back were true, there should be more lipid ovoids in the distal end of the nerve than that of the proximal end. However, we cannot see any trend of dying back in our data.

Figure 4-5 shows the sciatic nerves from proximal to the distal end from a WT mouse and an age matched SOD1G93A ALS mouse.
Figure 4-5 Whole sciatic nerve SRS imaging and quantification of lipid ovoids at distal and proximal. a. The whole sciatic nerve imaging from SOD1G93A and WT mice. We quantified a few views in proximal and a few in distal end, and the results are shown in b.
We can see from Figure 4-5 that the lipid ovoids is visible along the whole nerve from the proximal (closer to spinal) to distal (closer to foot) sides. The statistics show no significant different on the number of lipid ovoids between proximal and distal side of the nerve. There is significant difference between WT health mice and the ALS mice. The whole image of Figure 4-5 is composed of hundreds of images. It takes hours to obtain those images and it will be time consuming for high throughput experiments. Luckily, since the number of lipid ovoids are similar everywhere, we can sample anyplace along the nerve as a representation of the status of the whole nerve. That’s how we quantify the progression of the disease in mice especially for live mice.

We realized early on that the lipid ovoids starts to appear very early in nerves of SOD1G93A mice. We started the experiment with ex vivo imaging to achieve the best image quality. Afterwards, we performed in vivo imaging to make sure that the lipid ovoids isn’t artifacts in the process of surgery or perfusion.

Then we further improved the surgery procedure so that the invasiveness of the surgery is so small that we can close the wound and put the mouse back to cage after imaging experiment. The incision on mice grow back in a few weeks. The mouse can perform normal activities. Then we can repeatedly image each individual mouse and track the changes of counts of lipid ovoids in their sciatic nerves. In this way, we can minimize the effect of variance between individuals.

We started imaging mice from 5 weeks of age. We image also at week 8 and week 11 as shown in Figure 4-6 a. The mouse is mounted on a rack and was imaged by an upright scanning microscope as shown in Figure 4-6 b. Figure 4-6 c shows the image of the incision covered by a glass cover slip. Microscope objective image the nerve (highlighted by a black arrow) through the cover slip. The results of in vivo survival imaging are shown in Figure 4-6 d-f.
Figure 4-6 In vivo imaging of SOD1 G93A and WT mice. a. Time point of in vivo imaging. b. In vivo SRS imaging setup. The mouse under anesthesia was mounted on a rack. The incision was exposed under the objective. The sciatic nerve is covered by a cover glass window, shown in c, in which the sciatic nerve is pointed by the black arrow. The scale bar is 1 cm. d-f. The typical SRS images of sciatic nerves of SOD1 G93A and WT mice at 5, 8, and 11 weeks of age. The scale bar is 50 µm. g. The quantification of lipid ovoids of SOD1 G93A and WT mice at 5, 8, and 11 weeks of age.
As early as 5 weeks, some mice’s sciatic nerves already have lipid ovoids. Even though we could not see lipid ovoids in every ALS mice at week 5. But on average, the statistics (Figure 4-6 g) show that the lipid ovoids counting for ALS mice is significantly higher than that of WT mice. The number of lipid ovoids grows significantly and rapidly after week 5. The increase depends linearly on age, this make lipid ovoids a good biomarker for tracking the disease progression. Interestingly, one of the ALS mice shows very mild increase of number of lipid ovoids compared with WT mice. Later, through quantitative PCR, we found that the copy number of the disease-causing gene has a much lower copy number in that mouse. We could find out an abnormal gene type by analyzing the lipid ovoid county shows that this method is very sensitive. Such kind of data will not be possible without the in vivo survival imaging, because an individual abnormality will be buried in data from typical SOD1G93A mice.

To summarize, SRS and the lipid ovoids quantification proves to be a sensitive biomarker that can be used to track the progression of ALS in SOD1G93A mouse model. Lipid ovoids revealed by SRS imaging is one of the earliest biomarkers in the ALS mouse model. A list of current biomarkers of ALS in SOD1G93A mice is shown in Figure 4-7.
4.6 Drug test with SRS

Drug testing on mouse normally involve applying certain test drug systematically to the mouse model of certain disease and certain biomarkers of that disease are monitored at different age of the animal. Normally, a group of litter-mate mice are also given placebo as a control group. (The control group is called vehicle because it has everything that carries the drug but the active ingredient of the drug.) The difference between the control and test group are compared and a conclusion is drawn to state whether the difference between the two groups are statistically significant or not. As shown in Figure 4-7, sacrifice of the mice is required for probing many of the biomarkers in ALS mice. Therefore, if researchers want to know the development of the biomarker as the mouse ages, the researchers need to sacrifice many mice at each age point. For SRS imaging, individual mouse can be imaged at different age points without being sacrificed. After multiple times of imaging experiment, we are still able to track the life span of drug test group and control group. Most importantly we can track the number of lipid ovoids on individual mice, so we don’t have to average the drug effect among different mice, which can be especially useful if the drug is effective for a specific sub-group of mice.

We chose minocycline as the subject of our drug study. There are several studies on the effect of minocycline on SOD1G93A mice and human\textsuperscript{143-148}. Most research shows that minocycline can delay the onset of ALS in SOD1G93A mice\textsuperscript{143,145,146}. Since we know minocycline is effective on SOD1G93A mice, it is an ideal candidate to test SRS imaging for ALS drug screening in mice. We did not choose the only FDA approved drug Riluzole because the effect of Riluzole is not positive on SOD1G93A mice\textsuperscript{149}. 
The experiment was performed as follows. Thirty mice were randomly separated into two groups. Mice in one group was treated by minocycline and the other group was treated by saline only and is called vehicle group. (Two vehicle treated mice died accidentally due to the technical problem of isoflurane anesthesia system during the first imaging, which caused the fact that there are two mice less in the vehicle treated group.) Minocycline was injected intraperitoneally daily at a concentration of 10 mg/kg animal body weight\textsuperscript{143}, while the vehicle group were treated with the same volume (100 µL) of saline. Figure 4-8 a shows the three age points for in vivo imaging: 5 weeks, 8 weeks and 11 weeks. Each mouse was immediately injected with Meloxicam (1mg per 1kg mouse body weight) post-operation to reduce the inflammatory effects and pain. All mice after surgery would be wound-clipped on their incision for 7-day recovery and monitored closely based on the procedure as the described long-term serial in vivo SRS imaging. The imaging parameters were the same as those used in long term in vivo imaging. The optical power used on the sample was about 120 mW of pump (815.7 nm) and 80 mW of Stokes (1064 nm). The imaging speed was 1.1 s/frame, and the image size was 512 by 512 pixels.

![Graph](image)

Figure 4-8 Minocycline drug test timeline and lipid ovoids counting for drug treated and vehicle treated mice.

The numbers of lipid ovoids for the drug test are shown in Figure 4-8 b. By analyzing the data, we found that at the age of 5 weeks, before the drug treatment, there is no significant difference of
number of lipid ovoids between two group of mice (11 ± 3 ovoids for minocycline treated versus 10 ± 2 for vehicle treated, p = 0.82), while both surpassed that of WT animals of the same age (3 ± 1 ovoids). Mice in one group were later injected with minocycline and the vehicle groups were injected with saline only. At the age of 8 weeks, which were three weeks into the treatment with minocycline or vehicle only, the minocycline treated group has significantly less lipid ovoids in their sciatic nerve, (30 ± 9 ovoids for minocycline versus 62 ± 12 ovoids for vehicle, p = 0.036), though both groups have more lipid ovoids than WT control animals (5 ± 1 ovoids). The effect minocycline became more significant at the 11-week imaging time point (64 ± 10 ovoids for minocycline versus 96 ± 7 ovoids for vehicle, p = 0.012). WT animals still has little change of number of lipid ovoids 4 ± 1 ovoids.

![Figure 4-9 Disease onset and survival for minocycline treated and vehicle treated mice.](image)

We are also able to track the disease onset and life span of the mice in our drug test experiment, thanks to the low invasiveness of surgery and imaging procedure. Figure 4-9 shows disease onset and survival results. We found that the disease onset time of minocycline treated group 107.6 ± 3.1 days is significantly (p=8×10^{-6}) later than that of the vehicle treated group (100.8 ± 3.2 days). This means that minocycline effectively delayed the onset time of ALS. We found that the lifespan of minocycline treated SOD1G93A mice is 135.4 ± 10 days, which is also longer than that of the
vehicle treated group (129.8 ± 12.4 days). However, the difference is not statistically significant, p = 0.112. We further find out that the duration between disease onset and death is 27.9 ± 10.5 days for minocycline treated group and 29.0 ± 11.6 days for vehicle treated group. This result echoes the previous results in mice and failed stage 2 clinical trial. The reason causing such difference in mouse and human may be the timing of drug use. For mice, we always treat the mice well before the disease onset. However, in clinical trial, minocycline was used to treat patients who already showed the symptom of ALS. As we discussed before, the diagnosis of ALS is time consuming. The life span for ALS patients is only about 3 years. So, after the diagnosis of ALS, ALS is already in the mode of fast progression in patients’ bodies. But the good news is that since minocycline is effective for SOD1G93A mice before the disease onset, as genomic testing become cheaper and more common, if minocycline was prescribed to SOD1G93A gene bearers before the disease onset. There is hope that this kind of target treatment may also delay the onset of ALS for human.

4.7 Next step

SRS imaging of sciatic nerve and the quantification method is very effective at distinguishing SOD1G93A and WT mice. There are at least two applications of this technique. The first one is to use SRS imaging as a tool for drug screening as we have demonstrated. It’s ability to monitor disease progression without sacrificing the mice is highly desirable for continual monitoring of single mice or the combination of SRS with other downstream analysis. Even though needle EMG doesn’t require the sacrifice of animal either. We believe that imaging provides more information with deeper image analysis, which is another direction that we could pursue. I believe that SRS is the top choice for animal based drug screening for ALS.
The second goal is to use SRS for early and more definitive diagnosis of ALS. This goal is difficult in several aspects, because we have to prove lipid ovoids is specific to ALS to achieve this goal. This will involve showing that lipid ovoids is present in peripheral nerves of all the known ALS patients and lipid ovoids are not present in the nerves of patients with those diseases.

We made some effort in this direction and the result is promising but not complete. ALS is a complicated disease, people bearing disease causing SOD1G93A gene is only 2-4% of all ALS patients. Even for the SOD1 gene alone, there are other mutations that can also cause ALS. We quantified another mouse model with mutated SOD1 genes: SOD1G37R. Figure 4-10 a shows the disease progression timeline and the SRS imaging age points.

![Figure 4-10 SRS imaging of sciatic nerve of SOD1G37R and WT mice. a. Mouse age points for imaging and disease development stages. b. Lipid quantification of SRS images.](image)

The results, as shown in Figure 4-10 b, tells us the degeneration of peripheral myelin in the form of lipid ovoids can also be detect in SOD1G37R mice. The appearance of lipid ovoids also
much earlier than other known biomarkers. This implies that lipid ovoids universally exist in SOD1 transgenic mouse models.

To confirm that lipid ovoids is universally present in ALS patients, at least we need to show that lipid ovoids are present in other ALS mouse models. The next model we examined was created by adeno-associated virus (AAV) mediated expression of the C9ORF72 hexanucleotide repeat expansion throughout the central nervous system\textsuperscript{151}. This C9ORF72 model has been reported to exhibit neuronal RNA foci, dipeptide repeat protein inclusions, TDP-43 pathology, cortical and Purkinje cell loss as well as locomotive defects by 24 weeks\textsuperscript{151} (Figure 4-11 a). By imaging ex vivo sciatic nerve samples of AAV-C9ORF72 mice carrying pathogenic (G\textsubscript{4}C\textsubscript{2})\textsubscript{66} repeats (66R) (N = 12) and their littermate controls carrying (G\textsubscript{4}C\textsubscript{2})\textsubscript{2} repeats (2R) (N = 13) between 26 weeks and 28 weeks.

Figure 4-11 SRS imaging and quantification of lipid ovoids for AA-C9ORF72 a, b and FUS mouse model c, d.

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\textsuperscript{151} Indicate the reference number for all citations mentioned in the text.
weeks, we observed lipid ovoid deposition in the majority of 66R mice. Quantification demonstrated a significantly increased number of lipid ovoids in 66R animals (29 ± 6 ovoids for 66R versus 13 ± 3 ovoids for 2R, p = 0.024; Figure 4-11).

We next examined a mutant FUS transgenic model in which either human WT or P525L mutant FUS is expressed from the mouse MAPT (tau) promoter (Figure 4-11 c). In this mouse model, It was been shown ~30% of motor neurons were lost and ~40% of tibialis anterior (TA) neuromuscular junctions were denervated at 52 weeks postnatal152. By examining sciatic nerves from hFUSP525L mice, we found significant lipid ovoid deposition was already present in these mice at 12 weeks of age (43 ± 6 ovoids for hFUSP525L versus 15 ± 2 ovoids for hFUS WT, p = 0.0008; Figure 4-11 d). Overall, these results validate SRS imaging as a powerful resource to study peripheral nerve degeneration in emerging ALS mouse models.

In both cases, ALS mouse models show significantly more lipid ovoids than control mice. However, for both AAV-C9ORF72 and FUS model, there are sub groups that has low number of lipid ovoids. This does not necessarily challenge the universality of lipid ovoids in ALS patients because these mouse models are not faithful representations of human ALS. But these observations told us that more study are needed to confirm lipid ovoids as a universal biomarker for ALS mouse models.

On the other hand, to use lipid ovoids as diagnosis of ALS we also need to make sure other neurodegeneration diseases don’t present the same phenomenon. As a small step towards this direction, we imaged the sciatic nerves of the Autoimmune encephalomyelitis (EAE) mouse model of multiple sclerosis.
Figure 4-12 SRS imaging of sciatic nerves from EAE mouse models. a. Timeline for the EAE model and SRS imaging time points. b. Typical SRS images of sciatic nerves from EAE mouse. c. lipid ovoids counting based on the SRS images.

In fact, other researchers used SRS or its predecessor CARS to study EAE model, and they didn’t see lipid ovoids. Our results shown in Figure 4-12 confirmed the previous observations. There is no significant increase of number of lipid ovoids in the sciatic nerves of EAE mice as they age.

The results in mouse model mostly support the statement that lipid ovoids is a unique biomarker in peripheral nerves of ALS mouse model. More work needs to be done to test this statement in human ALS patients. We imaged some postmortem human nerve tissue, as shown in Figure 4-13. We also found a significant increase of number of lipid ovoids. (For human nerve tissue, I chose another group of lipid ovoids templates and threshold parameters.). But I have to
admit that more human data are needed to draw any real conclusion about the feasibility of using SRS as a diagnostic tool.

Figure 4-13 SRS imaging and quantification of lipid ovoids of human postmortem nerve tissues. a. Location of dorsal, ventral root nerves. b. Lipid ovoids counting of nerves from dorsal and ventral root. c. Typical SRS images of dorsal and ventral root nerves from ALS patients and control.

To summarize, we have shown that SRS imaging peripheral nerve and the quantification of lipid ovoids are among the most sensitive method to monitor the progression of ALS in SOD1G93A transgenic mouse model. We have shown great potential to use SRS in ALS drug screening in mouse models. But there is still a long way to go to use SRS for early and accurate ALS diagnosis.
Chapter 5
Clinical study of active ingredients in skin care products

5.1 Backgrounds

SRS bears the promise of *in vivo* label-free clinical microscopic study. Most existing studies were done on live animals\(^47\), and SRS has never been used in a human clinical study before. The reason is that when it comes to the imaging or any experiment on humans, the standards are much higher compared to that of animal experiments. For example, you can use animal imaging experiment to study a very risky hypothesis. But for humans, there must be enough evidence showing that the method is already working on live animals or on postmortem human tissues before the *in vivo* clinical study can be carried out. We need to justify that the study really need live humans and cannot be replaced by other animals or *ex vivo* experiments.

On the technical side, there are strict limitations on how much optical power one can use in human clinical studies\(^153\). For human skin, the highest optical power can be used is power equivalent to about 30 mW of 800 nm ultrafast laser. Another difficulty is that on top of the limitation of power, only epi-detection mode can be used, because there is no way for light to effectively penetrate live human tissues. Current lack of a flexible imaging arm also limits the locations the imaging can be carried out. Most SRS microscopy are performed on a commercial microscope, it is not easy to put the instrument into clinical setting. Factors like the comfortability of subjects require detailed designing of the imaging stage. These factors are all preventing SRS from being used in clinical setting for live human imaging.

Existing nonlinear optical microscopic clinical study on human skin by cosmetic companies use CARS microscopy or two photon auto-fluorescence\(^154-157\). As we know, auto-fluorescence
lacks chemical information, and CARS microscopy has non-resonant background and has low contrast. SRS has the potential to overcome these problems. Here we show that by optimizing the imaging setup and collaborate with Unilever research center at Trumbull CT., we performed the first clinical study with SRS on live human. In the study, we mapped the distribution of an anti-dandruff active component zinc pyrithione (ZnPTO) on human skin.

5.2 The anti-dandruff active component: ZnPTO

Dandruff is common problem for approximately 30% of population\textsuperscript{158}. While it doesn’t cause health issues, it does affect people’s appearance and cleanness. Anti-dandruff is one of the major goal of shampoos.

It is believed that there are three major factors for dandruff\textsuperscript{159}: sebaceous secretion, Malassezia fungi and other philological factors. Malassezia fungi metabolizes triglycerides present in sebum by the expression of lipase, resulting in a lipid byproduct oleic acid (OA)\textsuperscript{159,160}. During dandruff, the levels of Malassezia increase by 1.5 to 2 times its normal level\textsuperscript{161}.

ZnPTO has been found as an effective anti-dandruff component through its cytotoxic effects\textsuperscript{162,163}. It is found that the amount of Malassezia can be effectively reduced by using shampoo with ZnPTO\textsuperscript{158}. It is believed that the amount of ZnPTO left on the scalp affect the anti-dandruff effects, however, there is no direct imaging experiment to prove this. This clinical study is designed to study the deposition of ZnPTO on human skin.

5.3 Safety concerns of Clinical SRS imaging

According to American National Standards Institute (ANSI) standard\textsuperscript{153}, the laser power used below the maximum permissible exposure (MPE) on skin is considered as safe. ANSI standard
considered thermal damages of laser pulses only. For pulsed lasers, the shortest laser pulse considered in ANSI standard is 1 nanosecond (ns) laser. Laser pulse shorter than 1 ns is treated the same as 1 ns lasers.

The MPE for pulsed lasers with repetitive pulses have two limiting MPEs, the actual MPE should be the lower limit of the two.

The first MPE is defined to prevent sub-threshold pulse cumulative thermal injury and is called single pulse MPE. For 400 nm to 1.4 μm lasers, the MPE is calculated by $0.02 \times C_a \frac{J}{cm^2}$, where $C_a$ is the wavelength correction coefficient. For laser with wavelength between 700 and 1050 nm, $C_a = 10^{2 \times (\lambda - 0.7)}$, where $\lambda$ is laser wavelength in μm. In stimulated Raman scattering (SRS) imaging experiment, for pump laser ($\lambda \sim 792$nm), $C_a = 1.528$ and for Stokes laser ($\lambda \sim 1031$nm) $C_a = 4.592$. The MPE for single pulse energy is calculated 30.5 $mJ/cm^2$ for pump laser and 91.8 $mJ/cm^2$. Since the repetition rate of our laser is 80 MHz, the single pulse MPE can be used to calculate the corresponding average power, which is $2.4 \times 10^6 mW/cm^2$ for pump laser and $7.3 \times 10^6 mW/cm^2$ for Stokes laser.

The second MPE is specified to prevent thermal injury due to heat buildup during the pulse train. The MPE is to consider the pulse laser as continuous wave (CW) laser and the power is limited by the CW laser MPE. In this case, the exposure time is considered as longer than 10 seconds, which is true for SRS imaging experiments. The MPE is calculated by $0.2 \times C_a \frac{W}{cm^2}$. For pump laser, the MPE for laser average power is 305 $mW/cm^2$ for pump laser and 918 $mW/cm^2$ for Stokes laser.

From the above calculation, we can see that for SRS imaging, we are limited by the average power MPE.
To calculate the laser power from the MPE, we need to introduce the concept of limiting aperture. The limiting apertures is the area used to measure the laser power. Since the laser spot is completely contained in circle with 3.5 mm diameter in SRS imaging experiments, the 3.5mm diameter is used to calculate the limiting aperture.

This give us the final MPE for pump lasers to be 29 mW and Stokes to be 88 mW.

Since SRS signal is proportional to the product of pump and Stokes, we want to maximize the product $SRS \propto I_{\text{pump}} \times I_{\text{Stokes}}$ with the limitations of this $\frac{I_{\text{pump}}}{29 \text{ mW}} + \frac{I_{\text{Stokes}}}{88 \text{ mW}} \leq 1$. We can obtain the optimized power for pump to be 14.5 mW and Stokes to be 44 mW.

One aspect need to be noticed is that ANSI standard does not consider multi-photon damage. This may not be a problem for nanosecond lasers, since nanosecond laser has relatively low peak power. But for femtosecond lasers, multi-photon excitations may cause damages like UV light can do. The limiting aperture is used by assuming heat can dissipate very fast on skin and did not consider the non-linear photo damage. Therefore, as shorter pulses, shorter wavelength, higher peak power and smaller imaging areas are used for imaging, the non-linear damage may need to be reassessed to guarantee the safe use of laser. With near infrared laser used in current experiment and two-picosecond pulse duration. I assume the non-linear damage is negligible.

5.4 Raman spectra and ex vivo study of ZnPTO

As how every SRS experiment starts, we took spontaneous Raman spectrum of ZnPTO first, as shown in Figure 5-1 a. The detailed spectra around 3070 cm$^{-1}$ is shown in b. We choose this region because the signal from ZnPTO is very strong and it does not overlap with lipid or protein spectra (2850 - 3000 cm$^{-1}$). We performed ex vivo hyperspectral SRS imaging of a shampoo product 1 on pig skin. The spectrum obtained from the product is shown in Figure 5-1 c. Two of
the hyperspectral imaging is shown in Figure 5-1 d at 3082 cm\(^{-1}\) (on resonance) and f at 3120 cm\(^{-1}\) (off resonance). It is in fact not easy to see where the ZnPTO particles are with only the on-resonance one image at one Raman peak Figure 5-1 d. The reduce of intensity of some part is obvious in the image at a nearby off resonance wavenumber. The ZnPTO particles become obvious if we look at Figure 5-1 e, the subtraction of images in Figure 5-1 d and f. These images are all shown in the Red Hot look up table with the same scale.

Figure 5-1 *Ex vivo* SRS microscopy of ZnPTO. a. Spontaneous Raman of ZnPTO. b. The spontaneous Raman spectrum of ZnPTO near 3070 cm\(^{-1}\). c. SRS spectrum of ZnPTO near 3070 cm\(^{-1}\). d. SRS image of Shampoo product 1 on pig skin taken at 3082 cm\(^{-1}\). f. SRS image of Shampoo product 1 on pig skin taken at 3082 cm\(^{-1}\). e. The subtraction of d and f. The images are all 220 µm wide.

5.5 *In vivo* clinical SRS of ZnPTO on human

Several efforts are made make clinical SRS possible. Some efforts are technical, some are for the comfort of human subject, and some others are for compliance with safety standards. Technically, the SRS imaging was performed in an Olympus inverted microscope IX 81 with
scanning unit of FV1000. The objective in this study was Olympus UPLSAPO 60X IR NA 1.2 objective. We need to use high NA objective because ZnPTO form small particles in the shampoo we tested. To make the human subject comfortable, we made the microscope translation stage flat so that human subject’s arm can rest on it. We made a mechanical arm to press the arm of human subjects down during the imaging to reduce its movement. For the safety, we need to limit the optical power on the subject. The biggest risk of power change is when we change wavelength, the optical power can jump to higher value. We measured the optical power of each laser at laser focus before imaging. We keep them within safety range by monitor their intensity continuously during the imaging experiment and make sure that their power stay within safety range. We have shutters outside of microscope to block the lasers once the powers exceed the safety limit power. We also have manual shutter on the microscope in case the electronic shutter fails. We made a labview controlling software, shown in Figure 5-2, to control and monitor the imaging process.
Figure 5-2 Laser and imaging controlling labview program for *in vivo* human skin microscopy

After these are implemented, we performed clinical SRS imaging on human subjects. Figure 5-3 shows one of the results we obtained on a human subject treated with shampoo product 2. Figure 5-3 a is taken at on resonance wavenumber and b at off resonance wavenumber. The images are obtained from a time lapse image stack by a z-projection of showing maximum intensity. We do this because the subject’s arm is moving all the time, it is impossible to have two frames at the same height and the small ZnPTO particles move in and out of focus. By projecting the time lapse images, we could catch all the small particles in one image. It is obvious that the on-resonance image has some particles that doesn’t exist in off-resonance image. We believe those are ZnPTO particles. Figure 5-3 c was obtained by performing z-projection by averaging all images. It shows the skin structure. These images show that ZnPTO particles stay on both the skin surface and the trenches of skin.

Figure 5-3 *In vivo* SRS microscopy of ZnPTO: a. on resonance and b. off resonance. c. The structure of skin. The size of the image is 110 µm.
Chapter 6

Simultaneous two-color SRS imaging and imaging of white blood cells \textit{in vivo}

6.1 The pursuit of high speed screening

As we have seen in the previous chapters, two-color SRS is necessary for \textit{in vivo} imaging due to the subject movement and the need of imaging at on- and off-resonance wavenumbers to suppress background and identify chemicals. In fact, not just for \textit{in vivo} imaging, many label-free histology works are also performed with two-color SRS\textsuperscript{37,50-52}. Simultaneous two-color SRS will make label-free histology with SRS at least twice faster.

Most CRS only image one narrow Raman band at a time, in fact, this is one of the reasons that CRS is so effective. When it comes to multi-color imaging, this way of excitation becomes a drawback. To perform SRS imaging at multiples wavenumbers, one needs to take images for each wavenumber sequentially, which reduces the imaging speed.

One way to speed up the multi-color SRS imaging process is by exciting a broader band of spectrum simultaneously with one picosecond laser and one femtosecond laser\textsuperscript{41,42,166-168}. The femtosecond laser is then spread out spectrally by grating and the SRL or SRG of individual narrow band is measured by a detector array and demodulated by demodulation electronics.

The advantage of such methods is that all the Raman bands within a certain range are probed at the same time; the disadvantage is that each spectrum in the Raman band has relatively low signal and the Raman band corresponding to the edge of the femtoseconds laser’s spectrum has further reduced signal. Another disadvantage of using a grating is that only one dimensional
scanning is allowed, since the other dimension is used for spreading the spectrum. Therefore, to perform XY scanning, either the sample needs to be moving in one dimensional in liquid form, e.g. in the case of flow cytometry; or the sample stage need to scan in the other dimension.

One way to avoid such limitation is to perform spectral encoded pico-femto SRS\textsuperscript{42,168}. In these methods, each narrow Raman band is modulated at a slightly different frequency. The SRS signal for each wavenumber can be read out by a fast Fourier transform. Each Fourier components corresponds to each narrow Raman band. These methods allow two-dimensional scan and high speed spectral mapping and epi-detected SRS.

However, the limitations still exist, for example, the signal for each narrow Raman band will be weak and the Raman band corresponds to the edge of the spectrum will be weak. In fact, most of such techniques are aiming to map out the chemical distribution of a few chemicals. Optical powers are wasted on bands that bears little information for that purpose.

The best way is to optimize the choice of bands for specific chemicals that needs to be imaged and image those bands simultaneously. This work represents such spirits in its simplest form: a simultaneous two-color SRS microscopy\textsuperscript{49}.

6.2 The blood cells, cell-free chemicals and exosome

One of the goals for developing high-speed imaging is to use it for biomedical analysis like high-speed histology and flow cytometry. One of possible applications is to count and/or sort circulating tumor cells (CTCs) in blood. CTCs is regarded as a valuable tool for cancer diagnose, monitor and research\textsuperscript{169-172}. CellSearch is an FDA approved CTC counting machine and CTC counting by this machine is routinely prescribed in cancer diagnosis and monitoring\textsuperscript{173-175}. There are many new ways to count and sort CTCs\textsuperscript{176-180}. Most developments focus on detecting CTCs
by immuno-staining. Even though immune-staining is a powerful tool. It also has its drawbacks. For example, there are problems of non-specific staining and lack of specific surface marker for certain cancer cells. We believe that SRS can be helpful in solving some of the problems. SRS can sensitively image and quantify lipids. It is found that prostate cancer circulating tumor cells are rich in lipid\textsuperscript{180,181}. Therefore, it is possible that high-speed SRS imaging can be helpful in identifying CTCs.

In fact, there are many components in the serum of the blood that also worth characterizing: lipoprotein, lipid, and exosome. Among then, exosomes are small (30 ~ 100 nm) extra-cellular vesicles excreted from living cells\textsuperscript{182,183}. They exist in almost all body fluids, e.g. blood, spinal fluid, urine, etc. Up to now, it is found that exosomes can contain proteins, RNAs and DNAs\textsuperscript{184-190}. It is basically a small sample of cytoplasm. Through the analysis of the content of exosomes, researchers can monitor the change in the body. Furthermore, people are using exosome to deliver drugs to brains because exosomes can penetrate the blood-brain barrier\textsuperscript{191}. There is great significance to analyze and isolate exosomes.

We developed a simple method to extract and quantify exosomes with fluorescence staining. Exosome are mostly extracted by ultracentrifuge for hours\textsuperscript{192}. It is slow and the instrument is expensive. The challenge is how to extra and analyze exosome effectively and easily.

It is only recently known that exosome also contains double-strand (ds) DNA\textsuperscript{189,190}. Sequencing DNA in the exosome may provide valuable information about disease, e.g. cancer\textsuperscript{190}. While most people focus on what DNA in exosome tell us, we focus on how we can take advantage the existence of dsDNA in exosome for extraction and quantification of exosome.
We noticed that with the existence of dsDNA in exosome, there are very good live-cell dsDNA dyes (SYBR green) that can penetrate the membrane of exosome and stain the dsDNA effectively. With that kind of dyes, we can easily stain exosomes.

The separation of exosome cannot be done with optical microscopy or flow cytometry directly because exosomes are too small. Normally exosomes are attached to beads before the extraction. We notice that PEG that is commonly used to enhance DNA attachment can absorb exosomes and form bigger particles, i.e. precipitate exosomes, that enable the flow cytometry sorting of exosomes. With some effort, we can sort exosomes from human serum. The flow cytometry sorting data is shown below.

We performed our technique on exosomes from cancer cell lines. The experiment started by incubating cancer cells SW480 and fibroblast cell line BJ in serum free (SF) media (without pheno red to reduce background in FACS sorting) for a day. We used SF media because normal serum from cows has cow exosomes, which we wanted to avoid. We used BJ cells lines as a control and we used two ways to split SW480s (1 to 2 and 1to 3) to achieve different cell density. After centrifuge at 3000 g for 15 minutes to remove cells and cell debris we filtered the supernatant of media with 0.22 μm pore filter to remove other smaller cell debris. Then we add appropriate volume of ExoQuick-TC Exosome Precipitation Solution to each sample (4ml media + 0.8ml ExoQuick-TC). After vortexing the tube, we incubate the sample in refrigerator overnight. The tubes do not need to be rotated during the incubation period. The sample was mixed with SYRB green dye with the ratio of 1:10000 and the mixture was incubated in dark for at least 30 mins. The product is ready for imaging and FACS analysis.

We used FACS JAZZ for flow cytometry sorting. We used PBS as sheath solution. The 1.5 drop enrich was used as sorting method. Gates are drawn to include events when the dye and PEG
are both added to the sample. In general, it is believed that cancer cell lines have more exosomes due to active cell division and metabolism. Our FACS sorting results are shown in Figure 6-1 and Table 6-1. The media of SW480 cells has orders of magnitude more events counting than that of BJ cells. Without the PEG, the event counts are very low. With PEG added, the signal increased drastically. We sorted the sorting results again with the same parameters for SW480 with exo (PEG). We found that only 36% of events was in the same gate the second time, which means that the sorting efficiency is at most 36% among all exosomes that contain DNA as shown in Figure 6-2. This means that some exosomes are not big enough to be sorted by the FACS machine, and they are sorted in the first time only because two or three of them were in the same droplet and their combined signal surpassed the threshold for sorting. It means that this method is still not perfect in sorting exosomes that contains DNA. Some of exosomes that did not aggregate to a certain size or doesn’t have enough DNA may still be omitted by this method. A direct comparison of our method and others are not performed.
Figure 6-1 Flowcytometry data for BJ and SW480 with and without PEG. The blue gate P3 is used for sorting of exosome.

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<td>No</td>
<td>52</td>
</tr>
<tr>
<td>BJ</td>
<td>No</td>
<td>Yes</td>
<td>93</td>
</tr>
<tr>
<td>BJ</td>
<td>Yes</td>
<td>No</td>
<td>2,906</td>
</tr>
<tr>
<td>BJ</td>
<td>Yes</td>
<td>Yes</td>
<td>814,855</td>
</tr>
<tr>
<td>SW480 (1 to 2)</td>
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<td>No</td>
<td>123</td>
</tr>
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<td>Yes</td>
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Table 6-1 The sorting events for BJ and SW480 with or without PEG. SW480 has 1:2 and 1:3 split ratio. 1:3 split result in smaller cell density.

Figure 6-2 Resorting of a sorted exosome sample with gate P3.

We also notice that after sorting, the volume become larger instead of smaller than the sample before sorting. The sorting did not enrich the exosome instead it diluted the exosome. This is because we are sorting small particles with 100 µm nozzle. The problem could be solved by using 30 µm nozzle or smaller ones. However, our machine FACSJAZZ only use 100 µm nozzle.
6.3 SRS for high speed screening overview

Even though staining has offered a lot of specificity in the screening of CTCs and exosome, SRS can provide extra information on lipid content protein content and even DNA content of each cell. However, this requires the imaging of at least two colors at high speed. As we have shown in section 6.1, there are several techniques for multi-color SRS, but there is always a balance between number of spectral points and imaging speed.

For the method use picosecond and femtosecond lasers\textsuperscript{41,167}, the advantage is that all Raman bands within a window of 100 ~ 200 cm\textsuperscript{-1} are monitored simultaneously and no motion will distort the spectra. The drawback is that not all the information is needed for imaging a few chemicals. For example, if we know in advance that a sample has only three chemicals, 3-color imaging will be enough to map out the distributions of the three chemicals\textsuperscript{77}. So, the imaging of all other colors is a waste of time. We can also say the average efficiency of imaging is low and a lot of optical power is not used efficiently. On the other hand, many multicolor SRS imaging sequentially image SRS image for each Raman band\textsuperscript{77}. The advantage of this method is that all optical power is used for producing a high-quality SRS image. The drawback is that sample movement will affect the spectral analysis.

A new method for high-speed multi-color SRS imaging is needed to maintain the advantage of both methods and avoid the drawbacks of them.

6.4 Simultaneous two-color (STC) SRS

The difficulty for simultaneous multicolor imaging lies in the light source. For example, in the spectral tailor SRS\textsuperscript{43} or multi-color SRS\textsuperscript{41}, femtosecond lasers are used. After pulse shaping, only a fraction of femtosecond laser power was used for imaging. In the early development of SRS,
our lab had the luxury of having two OPOs and a very powerful pump, so that we have three optically synchronized lasers to perform simultaneous two-color SRS imaging\textsuperscript{19}. But not every lab can afford that and it is not easily scalable to more colors.

Fiber laser has provided an easy way to perform wavelength shifting and power amplification\textsuperscript{46}. Here we will introduce a way to perform simultaneous two-color and potentially more color SRS imaging.

In SRS imaging, normally the Stokes has a wavelength between 1030 – 1064 nm and the pump laser is tunable from 750-1000nm. Our idea is to send a portion of Stokes laser into a single-mode fiber or high-nonlinear fiber to generate neighboring wavelengths of Stokes laser through the process of self-phase modulation. The desired wavelength is then obtained by filtering out other wavelengths. Then, an Ytterbium (Yb) fiber amplifier is used to amplify the desired wavelength to the level of hundreds of milliwatt. This power level (modulated) is very high compared with the individual color in pico-femto experiments. This is important achieve high-speed imaging.

The simultaneous two-color SRS (STC-SRS) imaging setup is shown in Figure 6-3 a.
Figure 6-3 STC-SRS setup, principle and wavelength tuning. a. Laser setup for the two-color simultaneous SRS. PBS: polarizing beam splitter. EOM: electro-optic modulator. DM: dichroic mirror. λ/2: half-wave plate. λ/4: quarter-wave plate. PC: polarization controller. SMF: 2-meter single-mode fiber. TF: tunable filter. WDM: wavelength-division multiplexer. Yb: 20 cm ytterbium gain fiber. b. Pulse delaying scheme of two Stokes lasers and the pump laser. τ is 1/(laser repetition rate) and τ ~ 12.5 ns. c. Spectral broadening of the 2-ps pulses at 1030 nm in the 2-meter SMF with different average power of laser with 20 MHz modulation. d. Wavelength tunability of the Yb fiber amplifier.

We started building the STC-SRS with a conventional SRS setup. We have a 2 ps pump laser, which produce about 10W of 1031nm laser. 1W of the power was used as Stokes laser in SRS imaging. The other 9 W of those laser was used in SHG to generate green laser to synchronously pump OPO. The OPO output is tunable between 690 to 960 nm and serves as pump laser in SRS microscopy. The conventional SRS laser source was highlighted in yellow part of Figure 6-3 a. In SRS, we used a modulation transfer scheme\textsuperscript{19,32}. Particularly, in this experiment, Stokes laser is synchronously modulated at 20 MHz, which means two laser pulses is on and the next two pulses is turned off. Therefore, the modulation deserts half of the power from Stokes laser into beam dump. In STC-SRS, we send the “off” pulses to fiber wavelength shifter and amplifier to generate another Stokes laser at a wavelength near the wavelength of the Stokes laser at 1031nm.

To be specific, The IR pulses (2 ps, 1031.2 nm) first went through two meters of single mode fiber (1060 XP, Thorlabs). SPM broadened the spectrum depending on pulse energy in the fiber Figure 6-3 c. Then the laser was spectrally filtered by a customized tunable filter (Agiltron) with 1 nm bandwidth. The filtered laser was then amplified in 20 cm ytterbium gain fiber (F-DF1100, Newport) pumped at 976 nm by a 450 mW single-mode laser diode. A wavelength division multiplexer (WDM) was used to combine the filtered seed laser and the 976 nm pump laser. The output could be tuned between 1020 nm and 1040 nm by adjusting input power of the SMF and the center wavelength of the tunable filter Figure 6-3 d. The average power is above 200mW for any of the color, it can reach 250 mW for middle colors. This kind of power can never be achieved.
by frequency chopping of a femtosecond laser. Since the pulses sent into fiber are already modulated at 20 MHz, the 200 mW output laser from fiber amplifier is also modulated. This power level is enough to produce high quality SRS images.

The hybrid of OPO and fiber laser has new advantages over all-fiber lasers. All-fiber laser’s wavelengths are limited by gain medium of Yb and Er to be around 1030 nm and 1560 nm respectively. Here, even though the generated wavelengths are still in the Yb gain region, the OPO output is still tunable. This method can be used in any Raman band for imaging including CH stretching high-wavenumber region and C-D stretching, fingerprint region and even low-wavenumber region.

The two Stokes lasers are organized in time as shown in Figure 6-3 b. The two-color images, as shown in Figure 6-4 a-b, are obtained from the orthogonal channels in lock-in detection, because the two Stokes’ modulations are exactly 90 degrees different. Figure 6-4 c was obtained by normalization and subtraction of b and a. Figure 6-4 d is obtained by assigning pseudo colors and overlap a and c. This kind of operation was always performed to emphasize the cell location and shape. It is more an image processing method to emphasize cell morphology than to qualitatively measure the concentration of chemicals. This is very common for lipid-protein two-color imaging because the main focus of such imaging is to separate lipid and protein content. The fact that both lipid and protein are not a single chemical can be omitted for this purpose.
Figure 6-4 STC-SRS image processing for lipid-protein two-color imaging. a. SRS image at 2850 cm\(^{-1}\). b. SRS image at 2930 cm\(^{-1}\). c. Subtraction of a from b. d. Overlap of a and c.

Figure 6-5 9 by 9 tile of processed \textit{ex vivo} STC-SRS images of a fresh mouse brain. a. Lipid–protein STC-SRS tiling of fresh 1-mm-thick mouse brain slice in transmission mode, pixel dwell time 4 µs, scale bar 250 µm. Green and blue represent lipid and protein contrasts respectively. b-d. Zoom-in images, scale bar 50 µm.
Figure 6-5 shows a 9 by 9 tile of two-color SRS images of a fresh mouse brain slice. Figure 6-5 b-d show a few enlarged fields of view. The imaging quality is very good for ex vivo SRS imaging in transmission detection mode.

We also performed epi-detected SRS imaging. This is an important capability for multi-color SRS imaging. Because live mice or human’s movement during in vivo imaging make it hard to register images taken at different colors. Many multi-color SRS method does not allow epi-detection\textsuperscript{41,167}. Therefore, it is utterly important to have a way to efficiently perform simultaneous multi-color imaging in epi-detected mode. Figure 6-6 shows the epi-detected STC-SRS imaging of mouse ear of live mouse in a, b and fresh brain slice from a mouse in c, d. We could see that the imaging quality is very good too.

![Epi-detected STC-SRS imaging](image)

Figure 6-6 Epi-detected STC-SRS imaging of mouse skin and brain slice. a, b. In vivo mouse ear skin imaging in epi-detected mode. Scale bar 50 µm. c, d. Ex vivo mouse brain slice imaging in epi-detected mode. Scale bar 50 µm. Green and blue represent lipid and protein contrasts respectively.
6.5 imaging white blood cells *in vivo*

To demonstrate the capability of STC-SRS in high speed imaging, we performed *in vivo* white blood cells (WBCs) imaging with it. As far as we know, it is the first time anyone has done this.

We performed *ex vivo* whole blood imaging as the first step. We stained the WBCs from mice by CD45 antibody conjugated to Alexa 488 dye (Biolegend Cat. # 103121). We used the CD45 staining as a verification for WBCs. The typical image of single layer blood cells is shown in Figure 6-7 a. From this image, we can see that the WBCs have less signal than that of RBCs at on-resonant wavenumber (2945 cm$^{-1}$). At off-resonant wavenumber (3026 cm$^{-1}$) the WBCs have almost no signal, while the RBC signal remains. This is because two-color two-photon absorption of hemoglobin generated a strong non-resonant background at both Raman bands $^{32}$. In any blood vessels, other than capillaries, the blood cells are not single layer. So, we imaged thick multi-layer blood smears and the average intensity of 32 WBCs and their nearby RBCs was plotted in Figure 6-7 d. The statistical analysis showed that the ratios of SRS signals of RBCs and WBCs were significantly higher at off-resonant than at on-resonant wavenumbers. A typical image of blood cells for thick blood smear was shown in Figure 6-7 e.

For *in vivo* blood imaging, we imaged the blood vessels under the belly skin of mice. We exposed the blood vessels by flipping the skin out and imaged the blood cells in transmission mode. This method has been previously used for *in vivo* blood imaging $^{193}$. Animal imaging procedures were approved by Institutional Animal Care and Use Committee (IACUC) at Harvard University. Figure 6-7 b shows the images of blood cells *in vivo*. We can see clearly that the WBCs were visible in the on-resonant images and vanished in the off-resonant images. We also divided the signal of on-resonant images by that of the off-resonant images to visualize WBCs in the image. Because the blood was flowing in relatively steady speed in one direction in the blood vessel, we
were also able to use line scan mode to image the blood cells, as shown in Figure 6-7 c. The image was obtained by scanning in horizontal direction and the vertical direction is time. Because the blood was not single-layer in the blood vessels, the WBCs also showed some signal at off-resonant wavenumber (3026 cm\(^{-1}\)) due to the off-focus RBC signal, which was obvious in the ex vivo thick mouse blood smear imaging show in Figure 6-7 e. Based on the statistics of data in Figure 6-7 d and the assumption that cells in the blood vesicle were either WBCs or RBCs, we can confirm that the cells shown in Figure 6-7 b, c were WBCs. This is the first time that multi-color SRS was used to image white blood cells in the blood stream of live animals.

Figure 6-7 STC-SRS imaging of white blood cells in flowing blood stream. a. Ex vivo mouse blood on-resonance (2945 cm\(^{-1}\)) and off-resonance (3026 cm\(^{-1}\)) imaging. Anti-CD45 antibody was used to label white blood cells. Alexa 488 dye conjugated to CD45 antibody was visualized through two-photon excited fluorescence. b. In vivo mouse blood flow imaging in time lapse mode c. In vivo mouse blood flow imaging in the line scan mode. d. Average signal level 32 WBCs and nearby RBCs at on- and off-resonance Raman
bands. e. Examples of WBC in thick mouse blood smears. Pixel dwell time was 2 µs in (a, b, c, and e).

*In vivo* imaging of blood showed that simultaneous two-color SRS can be very useful for high speed SRS imaging in live animals. Label-free WBCs detection has been achieved recently by third-harmonic microscopy\textsuperscript{194} and spectrally encoded reflection imaging\textsuperscript{195}. However, these techniques lack the chemical specificity compared with SRS.

### 6.6 Discussions

Our method could also be extended to simultaneous multi-color imaging. It was reported that circulating tumor cells (CTCs) of prostate cancer are rich in lipid\textsuperscript{181}. We expect that, by adding one more color, it may be feasible to image the CTCs with simultaneous SRS imaging at lipid (2850 cm\textsuperscript{-1}), protein (2930 cm\textsuperscript{-1}) and an off-resonance Raman frequency.

There are several technical details worth noticing in current setup. The two Stokes lasers were combined by a PBS with orthogonal polarizations. Neither of the Stokes lasers can be fully aligned with pump in polarization to produce maximum SRS signal. A grating based beam combiner can solve this problem and allows more colors to be added, but it would increase the complexity of the optical setup. We chose single mode fiber for spectrum broadening to demonstrate the simplicity of this method and reduce splicing losses of highly nonlinear fiber. We also found spectral broadening difficult for a 6-ps laser system, since the nonlinear effect was too weak. The fiber laser source was stable during the imaging period on the scale of hours, which can be seen from Figure 6-5.

One of the advantages of this method is that the power generated from the fiber amplifier is relatively high. With single stage amplification pumped by a 450 mW laser diode at 976 nm, the output power was 200–270 mW within the tuning range of ±10 nm from the seed laser. In fact, if
tunability can be sacrificed, it is possible to generate laser at longer wavelengths to image other Raman bands (e.g. 1060 nm for OH vibrations) by optimizing the length of the spectrum broadening fiber and the design of the Yb fiber amplifier. That would allow simultaneous imaging of lipid, protein and water, which will be useful for applications such as skin imaging.

In conclusion, we have developed a simultaneous two-color SRS microscopy system by adding a simple fiber amplifier to a conventional OPO based SRS imaging setup pumped by a 2-ps laser source. We imaged mouse brain in both forward- and epi-detected mode and performed \textit{in vivo} imaging of mouse skin and white blood cells in blood streams. We believe that STC-SRS will be suitable for \textit{ex vivo} and \textit{in vivo} label-free histology and other speed demanding chemical imaging applications.
Chapter 7 Time-domain CRS aiming for better sensitivity

7.1 Introduction

One major challenge limiting the application of SRS in biology and medicine is its relatively low sensitivity. Generally, the sensitivities for most biomolecules are about tens of millimolar for 2 µs pixel dwell time with current laser source without burning the sample. This is the sensitivity for single pixel. By increase data acquisition time for each pixel and combine several pixels together, one can get higher sensitivity. In a biological tissue, many chemicals are in even lower concentration than the above detection limit. Lipid tends to aggregate into lipid droplets, which make them has high concentration, so they are relatively easy to image with SRS. Water is always in high concentration in live tissues. All proteins together have high concentration. DNA has very high concentration in the nucleus. Except a few others like glucose may have high concentration, other chemicals normally have concentrations lower than millimolar in cells.

For clinical applications that involve human, the imaging speed need to be high to avoid subject movement with limited optical power on the subject\textsuperscript{153}. Therefore, it is highly desirable to improve imaging sensitivity with fixed optical power.

Here, we are looking for possible improvement of sensitivity in time-domain measurements of CRS. We try to avoid non-resonant FWM background in CARS by performing femtosecond three color time-resolved CARS\textsuperscript{196}. It is shown that surface enhanced time-resolved CARS can detect single molecule\textsuperscript{197}. Both plasmonic enhancement and CARS played a role in the high sensitivity. It is believed that there are $10^8$ of enhancement in surface enhanced Raman, which may make one doubt how big a role CARS played in the sensitivity improvement in the technique. But the fact that combination of both techniques are used to observe single molecule shows that time-
resolved CARS has very good sensitivity. Here we tried to improve the time-resolved CARS by using a three-color scheme and combining it with modulation transfer technique.

### 7.2 Three-color time-resolved CARS

We performed three-color time-resolved CARS, because we believe it can be more sensitive than the two-colored time-resolved CARS\(^{197}\). The energy diagram of two and three-color CARS is shown in Figure 7-1 a.

In two-color CARS, the input lasers are pump and Stokes. Their energy difference is the Raman peak of the target chemical. In three-color CARS, two-color CARS also exist between pump and Stokes lasers. But there is an extra laser called probe laser that can interacts with pump and Stokes to generate three-color CARS signal. Probe laser is normally chosen to have a higher photon energy (shorter wavelength), so that we can use optical filter to remove two-color CARS.

The reason three-color CARS can be more sensitive is that the three-color CARS can be explained in the time arrangement of three-color CARS, as shown in Figure 7-3 b. For two-color CARS, two pulses pump and Stokes lasers overlap in time. As how coherent Raman works, pump and Stokes pulses will pump some molecules to excited Raman state. In CARS, the molecules will go back to ground state from excited state by interacting with pump laser again. Since in two-color CARS, pump laser was used in the process twice, in order to get high signal, the pump and Stokes lasers always perfectly overlap. For that reason, the non-resonant FWM background is also highest. Because non-resonant FWM is completely instantaneous interaction, it only happens when pulses overlap. In fact, CARS on the other hand, is not necessarily instantaneous. Once the molecules are excited to excited states, they decay from that state to ground state in a few picoseconds, which is
relatively long for femtoseconds pulses. This is why three-color CARS can avoid the problem of FWM.

In three-color CARS, we have the freedom to move the probe pulse away from pump and Stokes lasers. By doing so, the three-color non-resonant FWM vanishes. That’s what makes three-color CARS more sensitive than two-color CARS.

![Energy diagram and time diagram of two-color CARS, three-color CARS, and non-resonant FWM.](image)

Beside using three-color SRS, we also modulated the pump laser to perform the modulation transfer scheme. This can remove some background. We still have two-photon excited or three-photon fluorescence background and maybe little bit of Raman anti-Stokes background. We want
to use modulation transfer to complete remove those backgrounds. The whole optical setup is shown in Figure 7-2. The 1040 nm laser is Stokes laser, the tunable output is pump laser and the SHG of 1040 nm laser is used as probe laser.

![Figure 7-2 Three-color time-resolved femtosecond CARS setup. PBS: polarizing beam splitter, SHG: second harmonic generation, EOM: electro-optical modulator, λ/2, λ/4: half and quarter-wave plates, DM: dichroic mirror.](image)

The sample we used for this test is DMSO. It has peaks at 2920 cm\(^{-1}\) and 3001 cm\(^{-1}\). We used femtosecond pulses for all three pulses. We basically excited both peaks with pump and Stokes. Probe pulses will generate two CARS signals at two wavelengths because two peaks are excited. These two CARS signals will interfere and we can see from the result that the CARS signal oscillates with delay between probe and pump/Stokes lasers, as shown in Figure 7-3 a, which is obtained on pure DMSO. This kind of oscillations can be transformed to spectra information by Fourier transform, and this is the way to get Raman peak position in three-color CARS with femtosecond pulses.
Then we tested the sensitivity of three-color CARS. Since the peak at 2920 cm\(^{-1}\) is stronger, we try to excite this peak more to achieve high sensitivity. The result is shown in Figure 7-3 b. We obtained the data with different voltage in PMT and calibrated the gains of neighboring voltages with the same sample. This allow us to have a dynamic range of eight orders of magnitude. As the data shows, clearly, CARS signal reduces quadratically with the concentration. The signal reduce too fast so that we are unable to detect lower signals, because the voltage already reached 1000 V. So even though theoretically, this method has high sensitivity, but in reality, the PMT start to get noisy and its sensitivity is similar with SRS. Figure 7-3 c shows the data of deuterated DMSO. The reason this is interesting is that for two-color CARS, it is in fact very difficult to do any imaging other than CH stretching at high wavenumber. Because PMT’s efficiency start to drop drastically for near IR wavelengths. But for three-color CARS, CARS signal is always near the probe laser, 520 nm in out case, so the PMT always has high efficiency, and you can use this setup to probe any Raman peak. We can see from Figure 7-2, the sensitivity of DMSO-d6 is similar to normal DMSO. It also has the problem of lack of signal for low concentration chemicals.

Since the sensitivity of three-color time-resolved CARS is not better than SRS and it is also so hard to get chemical information in time-resolved CARS, this technique is basically not practical for imaging.
Figure 7-3 Time-resolved three-color CARS of DMSO with femtosecond laser. a. CARS with two-Raman peaks excited. b. CARS with two Raman peaks excited but one peak is more dominant. c. CARS signal for deuterated DMSO (d6).

7.3 Epilog

This thesis presented several breakthroughs in the field of biomedical applications of SRS. We now know that SRS can be cheaper, and accessible to more researchers and users by using all-fiber laser source with high-speed auto-balancing detector. It can probe important biomolecules like neurotransmitter acetylcholine that other techniques cannot image. It can accurately monitor disease progression, and improve drug screening process in motor neuron disease. It can be used
to study the effect of skin care products on human skin. It can perform high simultaneous multi-color high speed chemical microscopy. As the push for higher sensitivity of SRS continues, we believe that these inventions and discoveries are not the end, but the beginning of broader application of SRS.
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