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Advances in Raman Imaging of Lipid Macromolecules

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ABSTRACT OF THE THESIS

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Biomolecular imaging is one of the rapidly developing areas of research in biomedical sciences. Since the discovery of Raman scattering by Raman and Krishnan and especially in the last few decades, significant progress has been made in developing Raman scattering-based imaging tools and their applications to studying biomolecules. Raman spectroscopy with enhancement and advances especially applied on lipid imaging is reviewed and discussed in the present study. Different Raman modalities and their performances are evaluated and compared in different aspects. Experimental performance of Raman imaging on lipid study is achieved with justified outcome. Raman spectroscopy could be justified as a powerful technique for biological imaging and lipid study.

Introduction

Lipid is a chemical substance that is an important component in living cells and tissues. Besides proteins and carbohydrates, lipid is another major constituent of cells in plants and animals. Lipids can be easily stored in living beings, and therefore they become a powerful source of energy. Typical types of lipids include fatty acids, steroids, waxes, neutral fats and compound lipids such as lipoproteins and glycolipids. Since lipids play an essential role in living organisms and dynamic metabolism, it is important to study and analyze lipids in different scales in cells and living organisms. Imaging is an important and fundamental part in the process of lipid studies. It can provide information beneath the membranes of cells and tissues directly without making too much effort or damaging the tissues.

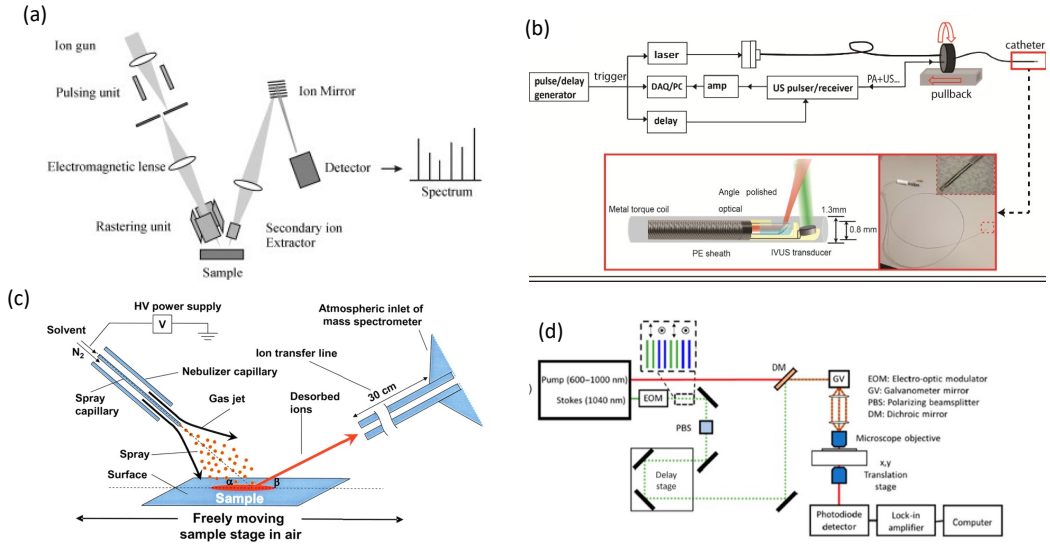

Figure 1. A summary of current imaging platforms for lipid metabolism. (a) Setup of TOF-SIM imaging system(Saleem & Galla, 2010) (b) Scheme of an intravascular photoacoustic imaging system(Wu et al., 2017) (c) Setup of Desorption electrospray ionization (DESI) imaging system(Takats, Wiseman, Gologan, & Cooks, 2004) (d) Setup of stimulated Raman spectroscopy system(Fung & Shi, 2020)

When studying living tissues and medical applications, imaging is a direct and intuitive method

that people may use to look into those organs and structures that cannot be seen by human eyes. Qualified imaging techniques and outcomes help a lot on visualizing small and deep organs and molecules that people cannot visualize directly or clearly. In lipid study, different bioimaging tools were performed and tested. Traditional techniques for lipid imaging are generally based on fluorescence microscopy via exogenous chemical probes(Donaldson & de Aguiar, 2018). Besides conventional fluorescent imaging(Goutayer et al., 2010), mass spectrometry imaging(MSI) (Gode & Volmer, 2013) was a popular group of technique that was used in lipid imaging. More specifically, MALDI-MSI(Caprioli, Farmer, & Gile, 1997), SIMS(McQuaw, Sostarecz, Zheng, Ewing, & Winograd, 2006) and TOF-based(Borner et al., 2006; Touboul, Brunelle, Halgand, De La Porte, & Laprevote, 2005) mass spectrometric techniques were performed on lipid imaging in small scales. But MSI technology is still advancing and several aspects of MSI still need improvement(Gode & Volmer, 2013). Also, MSI instruments are intractable so that limited analytical information can be obtained from a few specialized and fully equipped laboratories. Intravascular photoacoustic (IVPA) is another attempt for in vivo lipid imaging. When IVPA is combined with ultrasound (US) imaging, it was possible to assess lipid deposition with fast real-time imaging. But there were still remaining challenges in IVPA/US lipid imaging, such as difficulties in catheter design, and the low photoacoustic SNR in the targeted region close to the transducer(Wu et al., 2017). Basic setup of imaging system used for lipid study are shown in Figure 1. The different mechanisms of imaging tools lead to different outcome and emphasized advantages when being used on biological samples.

Since different types of imaging techniques still have inevitable limitations, an advanced optical spectroscopy that is high-resolution, non-invasive, easily to be approached to trace biological processes of lipid is desired. In recent years, Raman scattering imaging showed its powerful performance in lipid imaging while overcoming several challenging aspects in conventional imaging techniques.

Raman scattering or Raman imaging is a type of optical spectroscopy that can visualize cells and living tissues by revealing vibrational states and transitions. Raman scattering is basically an inelastic scattering of visible monochromatic light. During the scattering process, a quantum of energy that is involved in a photon can induce vibration in molecular bonds(Min, Freudiger, Lu, & Xie, 2011). By

modulating a given incident light with a specific wavelength, those molecular bonds will vibrate at certain specific frequencies. Each type of molecular bonds has a typical frequency of vibration. The intensity of vibration can be recognized and recorded in the spectrum, so that the certain molecular bonds in the sample can be indicated. While the incident light is not absorbed by the sample, the photons are scattered and induce an energy difference between the incident and scattered light(Min et al., 2011). The energy difference can be indicated by the change of wavelength from between the two lights and so called the Raman shift. The generation of Raman shift is directly reflected by the energy required for molecular bond vibrations. During the imaging process, a higher intensity or peak along the spectrum at certain frequency can indicate the existence and concentration of certain molecular bond.

Several categories of different types of Raman scattering include spontaneous Raman scattering, coherent Raman scattering, resonant Raman scattering, surface-enhanced Raman scattering, tip-enhanced Raman scatterings(Fung & Shi, 2020) and some other types of Raman imaging. From those, coherent Raman scattering is a group of modalities that was discovered being widely used on tissue and organ imaging.

Compared to other optical imaging techniques, Raman scattering has weaker signals of imaging(Fung & Shi, 2020). That is, achieving quick and high-quality Raman imaging is important and might be challenging. For normal Raman scattering, or spontaneous RS, obtaining high-resolution images with high signal-to-noise ratio (SNR) requires longer acquisition time(Yue & Cheng, 2016) than other techniques, so that usage of RS is limited and challenging for imaging in larger pieces of organs or living tissues. In order to visualize dynamic activities in living tissues in a fast time scale, different enhancements and improvements are investigated and tested on Raman spectroscopy.

Raman spectroscopy is a powerful tool that showed viability of lipid imaging in examinations. In recent years, lipid metabolism and dynamic activities of fatty acids were successfully visualized under several modalities of Raman spectroscopy(Fung & Shi, 2020). In these approaches, researchers are finding ways to perform quick and qualified Raman imaging without interfering or damaging internal structures and functions of lipid molecules. Since external fluorescent labeling might be toxic or intrusive to the tissues

and molecules, some conventional optical techniques with the usage of toxic labels and dyes are not suitable for lipid imaging. Therefore, noninvasive labeling and label-free Raman techniques are desired to be developed and enhanced for lipid imaging. Without external perturbation, existence, cellular structures and chemical composition of lipid molecules within tissues and organs can be visualized intuitively. While dynamic activities such as lipid uptake and its intracellular fate are also of interest to be visualized in organs, high-speed and continuous Raman imaging with high resolution or SNR is desired to be achieved ideally. Different types of biological species involving specific chemical bonds have their Raman signal peaks at specific wavenumbers on the spectrum. According to studies, lipids are imaged at the specific Raman band around the wavenumber of 1440 cm^{-1}. Moreover, the Raman band at 1440 cm^{-1} to 1450 cm^{-1} are usually used to discriminate lipid and protein since these two species have close wavenumber bands for Raman imaging(Russo et al., 2020).

Each type of biological species is supposed to have its specific Raman profile according to the chemical components and stretching bonds in the species. This profile can be viewed as a biological fingerprint that can be used to discriminate and quantify the interested molecules or substances. Figure 2 shows an example of Raman profile of fatty acids, a typical type of lipids, measured with 532 nm excitation(Czamara et al., 2015). Raman spectra of different kinds of fatty acids are shown and distinctive peaks of Raman signal are identifiable at specific wavenumbers across the profile. These peaks indicate the high concentration of certain chemical bonds in the structure of fatty acids, so that the fingerprints of fatty acids can be used in further lipid imaging and analysis.

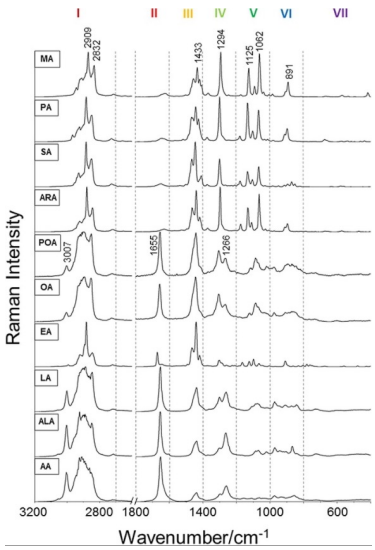

Figure 2. Raman profile of 10 subtypes of fatty acids measured with 532 nm excitation. Raman spectra are shown in high wavenumber range and distinctive peaks are identifiable(Czamara et al., 2015).

In recent years, researches have demonstrated the performance of different Raman modalities in lipid imaging. Successful cases that were reported included both labeled(Noothalapati Venkata & Shigeto, 2012) and label-free(Folick, Min, & Wang, 2011) Raman techniques but each of them may still has such challenges to be overcome. Therefore, it is still on the way to find a most suitable and enhanced Raman imaging technique for imaging of lipid macromolecules, and specific improvements and enhancements are continuously being developed in studies.

Different Raman modalities for lipid imaging

Spontaneous Raman Spectroscopy

Spontaneous Raman spectroscopy is the most traditional inelastic, non-resonant vibrational Raman scattering of photons. While an incident visible monochromatic light is given, the light cannot be absorbed by the tissue, so that the photons will start scattering and generate an energy difference from incident photon. The energy difference is then exhibited as the wavelength difference, which is later called as Raman shift.

During the inelastic scattering of incident light, vibration in molecular bonds is induced by a quantum of energy from a photon. The incident light is given a certain tunable wavelength, so that internal molecular bonds within an analyte or tissue sample will vibrate, and different molecular bonds will vibrate at certain different frequencies(Fung & Shi, 2020). During spontaneous Raman scattering, it generates specific Raman spectra according to the intrinsic molecular vibrations in small molecules, so that reflect a vibrational signature or fingerprint of internal chemical bonds. That is, spontaneous Raman spectroscopy enables identification of particular molecules without applying labeling or dying on tissue samples(Puppels et al., 1990). While Raman spectra can exhibit specific peaks according to molecular structures and conformations, spontaneous Raman spectroscopy becomes a useful technique in biomedical analysis and applications such as histological analysis, diagnosis of diseases and physiological analysis in molecular level(Ogawa et al., 2009). Spontaneous Raman spectroscopy was then considered as a selective technique for label-free detection in specific living tissues and organs(Minamikawa et al., 2013).

However, compared with other types of Raman imaging, spontaneous Raman scattering is not a very precise tool for in vivo tissue imaging. Since Raman scattering has lower energy than other optical imaging techniques, the sensitivity and resolution are limited, and the processing time of measurement could be much longer than desired. At cellular level, spontaneous Raman scattering could hardly produce high-contrast or high-quality tissue imaging(Ogawa et al., 2009). In order to overcome these challenges, some improved or hybrid methods such as a Raman confocal microscope incorporated with slit-scanning apparatus(Harada et al., 2008) were developed to enhance the speed of imaging(Hamada et al., 2008). Also, applying resonance Raman spectra instead of non-resonance spectra in biomedical imaging can induce much stronger Raman signal intensities at thousands of magnitude(Spiro, 1985). That is, in order to obtain higher sensitivity, resolution and selectivity of spontaneous Raman scattering, enhancements are always necessary in the devices or probing techniques for biomedical imaging.

For lipid imaging, people always use spontaneous Raman spectroscopy combined with other techniques in order to obtain high-quality imaging within shorter acquisition time. Spontaneous Raman

scattering itself showed ability to recognize vibrations of C-H stretching bonds in lipid molecules(Choe, Lademann, & Darvin, 2014) by displaying distinguish peaks of stretching bonds on Raman spectrum. However, due to the limited resolution, imaging speed and insufficient ability of mapping of lipid distribution(Klossek et al., 2017), spontaneous Raman spectroscopy did not always become the first choice for lipid imaging.

Coherent anti-Stokes Raman Scattering (CARS)

Coherent anti-Stokes Raman Scattering (CARS) is a kind of nonlinear parametric generation spectroscopy that was widely used in chemical analyses in gas and condensed phases(Maeda, Kamisuki, Kataoka, & Adachi, 1985). During the imaging process of CARS spectroscopy, there is energy exchange between the incident and resulting light fields while the molecules in tissue samples remain in the ground state throughout the process. The vibrational state probing of CARS is similar to that of spontaneous Raman scattering. In CARS spectroscopy, there are two laser beams, pump beam and Stokes beam, that have spatial overlapping so that can generate CARS signal by the phase-matching conditions(Zumbusch, Holtom, & Xie, 1999). When the energy or frequency difference between two incident laser beams matches the vibrational frequency of molecules in tissues of interest, the CARS signal will be significantly enhanced and displayed as a peak on the Raman spectrum.

Comparing to spontaneous Raman scattering, CARS, as a third-order nonlinear Raman scattering process, has remarkable enhancements on signal strength, autoluminescent background reduction and imaging acquisition time(Rinia, Burger, Bonn, & Muller, 2008). The nonlinear dependence of CARS signals on laser beams makes signal generation more efficient for pulsed lasers with high peak power and short duration(Maeda et al., 1985). By using lenses with high numerical aperture in CARS, the beams can be focused tightly to efficiently reduce background noise signal and photodamage to sample tissues, and can also improve lateral resolution of imaging(Sheppard, Gu, Kawata, & Kawata, 1994). However, there is

limited improvement in sensitivity of CARS because of the existence of non-resonant background signal, so that it is still a challenge to achieve high-resolution three-dimensional sectioning imaging for CARS(Zumbusch et al., 1999).

Label-free CARS spectroscopy is one of the popular Raman techniques that were used for lipid imaging, especially for quantitative measurements. Successful CARS imaging of lipid domains in single bilayers with clear vibrational contrast without labeling the lipid molecules was reported before last decade(L. Li & Cheng, 2008). Such technique could also be extended in phase segregation study in dynamic living cell membranes(L. Li & Cheng, 2008). Multiplex CARS conjugated with selected spectral-analysis tools was used in observation of thermodynamic state(Muller & Schins, 2002) and measurements of lipid phase transition with transition behavior information(Rinia, Bonn, Muller, & Vartiainen, 2007), and showed the ability of quantitative imaging of CARS spectroscopy. Advanced multiplex label-free CARS microscopy was also used in imaging of lipid composition and packing of lipid droplets that are dynamic and multi-functional(Rinia et al., 2008), and provided crucial information to further studying biological functions of lipid molecules in living organs. Therefore, the ability of quantitative imaging of CARS was re-emphasized.

CARS spectroscopy was also reported with the ability to monitor lipid storage in *Caenorhabditis elegans* (C. elegans) model in vivo while overcoming limitations of fluorescence microscopy(Hellerer et al., 2007). Such measurement was performed on single-cell level and was helpful for studying fundamental mechanisms of intracellular dynamic activities in lipid metabolism. That is, comparing to spontaneous Raman spectroscopy, CARS spectroscopy has greater potential for dynamic imaging in further lipid analysis. However, in molecular or small-scale biological imaging such as lipid imaging, CARS still has demonstrated limitations or challenges that are needed to be overcome. First of all, non-resonant background is very likely display in the outcomes or CARS imaging, so that the clarity and quality of images are inevitably influenced. Meanwhile, autofluorescence interference is another major limitation that will affect the imaging quality(Folick et al., 2011). Also, CARS imaging could hardly achieve direct

quantification in applications, so that other techniques might be utilized or conjugated with CARS in order to accomplish the quantifications. Therefore, further developed or enhanced Raman imaging modalities beyond CARS might be more suitable for such molecular imaging.

Stimulated Raman Scattering (SRS)

Stimulated Raman scattering (SRS) is a newly developed imaging platform that was enhanced and applied in the past decade. As a nonlinear imaging process, the basic principle of SRS imaging is that, when two laser beams, pump beam and Stokes beam, coincident on the sample, their energy difference matches vibrational frequency within the spectrum(Freudiger et al., 2008), the molecular vibration is then stimulated after the nonlinear interactions(Folick et al., 2011). When energy transfer occurs between two laser beams, there generate intensity loss of pump beam and intensity gain of Stokes beam, which are called stimulated Raman loss (SRL)(Jones & Stoicheff, 1964) and stimulated Raman gain (SRG) respectively. If there is no energy transfer, molecular vibration will not be stimulated so that there will be no intensity gain or loss. This property makes SRS an ideal technique to avoid non-resonant background(Min et al., 2011). Based on existing discovery, SRS has advantages involving high sensitivity(Ozeki, Dake, Kajiyama, Fukui, & Itoh, 2009), high chemical selectivity, high imaging speed(Nandakumar, Kovalev, & Volkmer, 2009) and relatively higher resolution comparing to other types of Raman-based microscopy. Currently SRS is under exploring and modified by researchers in order to make it a more compatible and convenient method that can be popularized in biological studies and clinical applications.

As an enhanced Raman imaging technique, SRS had marked performance and enabled various practical applications on label-free dynamic lipid imaging. During last decades, SRS imaging was successfully used to observe lipid regulatory mechanisms(Le, Duren, Slipchenko, Hu, & Cheng, 2010; M. C. Wang, Min, Freudiger, Ruvkun, & Xie, 2011) and lipid storage phenotype in vivo in Caenorhabditis elegans as a typical genetic model. When label-free SRS was performed in lipid analysis, image artifacts were avoided, and direct and reliable quantification were shown(Min et al., 2011). Also, when hyperspectral

SRS imaging was conjugated with multivariate analysis such as fingerprint vibration technique, this certain platform allowed quantitative mappings of lipid distribution in vivo(P. Wang et al., 2014). Meanwhile, since this platform could also differentiate saturated and unsaturated lipid molecules in cells, cellular degree of fat saturation could also be detected by this SRS imaging platform(P. Wang et al., 2014).

Since label-free SRS imaging allows high-resolution video-rate cellular imaging, it could not only allow digital quantification of lipid droplets at single-cell level(Cao, Zhou, Chen, Streets, & Huang, 2016), but also help trace intracellular dynamic motion of lipid droplets(Dou, Zhang, Jung, Cheng, & Umulis, 2012). That is, SRS imaging gave a good start of systematic cellular imaging, and therefore morphological information of cells in different chemical environments were able to be obtained without using fluorescent labeling. Beyond this point, label-free SRS imaging was then performed to quantify spatial-temporal dynamics of lipid droplets and analyze the relationship between lipid dynamics and droplet sizes as well as cellular lipid metabolism in vivo(C. Zhang, Li, Lan, & Cheng, 2017). These newly approached application of label-free SRS imaging on lipid study was superior and could hardly achieved by conventional optical imaging techniques or other types of Raman scattering imaging, so that SRS imaging became a powerful and distinguished technique for lipid analysis.

Surface-enhanced Raman Scattering (SERS)

Surface-enhanced Raman Scattering (SERS) is an inelastic, surface-sensitive type of Raman spectroscopy. The development of SERS could efficiently overcome the challenge of low sensitivity and imaging resolution that were showed in spontaneous Raman scattering. The basis of SERS is a plasmonic effect in which the incident electromagnetic field of molecules that absorbed on to a noble metal surface is increased drastically, so that the Raman signal intensities will also be much higher than that of traditional Raman scattering as well as other techniques like fluorescence(Faulds, Barbagallo, Keer, Smith, & Graham, 2004). The enhancement of signal intensity for SERS comparing to normal Raman scattering can be reached to 10^6-fold. The enhancement factor, $EF_{SERS} = 10^6$ can be explained as the product of an electromagnetic enhancement mechanism and a chemical enhancement mechanism(Stiles, Dieringer, Shah, & Van Duyne,

2008). In SERS, the noble-metal substrate plays a very important role. Such SERS substrates are generally categorized by solution-based substrates, solid-supported substrates, tip-enhanced Raman spectroscopy (TERS) substrates and single-molecule substrates(Bruzas, Lum, Gorunmez, & Sagle, 2018).

Not until last decade, SERS was limited in utilization because its imaging could hardly be reproduced due to existence of unclear substrates fabricated by electrochemical roughening(Stiles et al., 2008). After putting a great amount of effort for the enhancement of SERS, advanced ancillary techniques such as nanoparticle fabrication(Nie & Emory, 1997), surface preparation and modifications(Dick, McFarland, Haynes, & Van Duyne, 2002; Jensen, Malinsky, Haynes, & Van Duyne, 2000) were developed and improved that made SERS a more efficient ultrasensitive analytical imaging technique.

In lipid study, while lipid molecules have dynamic three-dimensional structures, SERS enables direct and label-free detection of lipid molecules through their Raman fingerprints. In order to analyze SERS imaging on lipid molecules, different types of biomimetic lipid membrane bilayers were developed. A popular one among those is hybrid bilayer membrane that is a metal surface decorated with alkane thiols under a lipid monolayer(Masango et al., 2016). Continuous development of substrates and lipid bilayers enable field enhancements and improved detection. Improved hybrid lipid bilayers that were fabricated by nanoparticles capped with gold citrate in solution phase was successfully used with to monitor time-dependent lipid oxidation(Driver et al., 2014). Another hybrid bilayer membrane fabricated in nanoparticle-on-mirror substrates enabled observations of long-time lipid dynamics and transitions(Taylor et al., 2014). Such developments showed that SERS has great ability and possibility on lipid detection in different levels of scale.

The reason that SERS is not the first choice in small-scale or single-cell level of imaging is that it still has some disadvantages that may limit its performance. When analyzing cells and their characteristics, SERS signal at single-cell level is strongly influenced by the physiological condition and cellular metabolic activity of the sample cells(Weiss et al., 2019). For example, differences in factors including labeling status and storage conditions of cell species may influence the signals in SERS spectra. These properties can lead to complicated interpretation of data and lack of distinction of signals between different interested

organisms.

Tip-enhanced Raman scattering (TERS)

Tip-enhanced Raman scattering (TERS) is another enhanced Raman modality that can provide molecular biological information at nanoscale and is aiming at improving the relatively weak signal and resolution of Raman scattering techniques(Bailo & Deckert, 2008) without labeling on the molecules of interest. Generally, TERS is developed from SERS and combines SERS with atomic force microscopy (AFM)(Gross, Mohn, Moll, Liljeroth, & Meyer, 2009) or scanning tunneling microscopy (STM) in order to further enhance spatial resolution of imaging(Schmid, Opilik, Blum, & Zenobi, 2013). Improvement of TERS is based on the intrinsic plasmonic molecular structure(Treffer et al., 2012), more specifically, bringing a fine metal tip within a few nanometers of a molecular film appeared to give strong enhancement of Raman scattered light from the sample(Stockle, Suh, Deckert, & Zenobi, 2000). TERS allows acquisition of vibrational spectra with significantly improved spatial resolution and sensitivity beyond diffraction limit(Nakata, Nomoto, Toyota, & Fujinami, 2013).

In the TERS imaging process, a metallic probing tip is illuminated by a focused laser beam, then the electromagnetic field at the tip apex is intensified so that generates a confined light source in Raman scattering(Schmid et al., 2013). Silver nanoparticles are commonly used to deposit on a scanning probing tip, so that it can improve Raman scattering in the region that is close to the probing tip because of the localized surface metallic plasmon resonances of silver itself(Nakata et al., 2013).

Label-free TERS is another Raman scattering technique that has remarkable achievements in lipid analysis and imaging in the past decade. In 2011, nanoscale reproducible TERS spectra from small number of molecules of a lipid layer was successfully represented by probing a silver tip on a golden surface(Opilik, Bauer, Schmid, Stadler, & Zenobi, 2011). In this model of segregated lipid domains, high-resolution TERS images with complete spectral information were obtained for nanoscale molecules in lipid layers(Opilik et al., 2011). Since cellular environment of lipid membranes is complex and highly dynamic, TERS application enabled qualified nanoscale imaging of molecular substances by overcoming such limitations

of sensitivity and resolution in previous Raman scattering applications. In 2013, another research reported label-free TERS imaging of lipid bilayers in water with coated probing laser tip(Nakata et al., 2013). In this approach, alumina- and silver-coated tungsten tips were developed since tips that made of such metallic material can be robust in water. Two kinds of lipid bilayers were investigated, and spectral changes of bilayers were observed and presented in terms of fluidity of liquid crystalline phase(Nakata et al., 2013). That is, TERS imaging was shown as having the potential of imaging and characterizing dynamic lipid membranes or other biomembranes(Bohme et al., 2010; Bohme et al., 2009) that are diffusive and inhomogeneous in molecular environment while maintaining the sensitivity and resolution of imaging.

Even though recent studies have explored TERS for biological and molecular imaging, practical applications of TERS still remain challenging. Technically, required TERS-active tips with sufficient local field enhancement and defined plasmon resonance are difficult to be reproducibly fabricated(Berweger & Raschke, 2010). Besides the choices of appropriate tips, limited enhancement values for local electric field that is lower than expectation is another problem influencing the imaging quality. Moreover, physical properties and limitations such as short acquisition time and tip scattering efficiency of TERS result in limited SNR and challenges in obtaining high pixel density and resolution while imaging the area of closely packed species(Jiang et al., 2017).

Different tags developed for lipid imaging

Optical techniques such as Raman spectroscopy are valuable in cell biology since they allow people to monitor intracellular activities with non-intrusive feature(Y. Li, Wang, Mu, Ma, & Guo, 2017). Even though label-free Raman spectroscopy has been applied in biological imaging for several decades, sometimes it is still limited in genetic specificity for intracellular imaging of organs or metabolites(J. Zhang et al., 2018). In order to further improve the ability of Raman scattering imaging techniques, different tags were developed and applied in imaging of small biomolecules such as lipid molecules. The Raman tags apply small modifications on the targeted molecules, while avoiding alteration to their functionality in

cellular metabolism and reduce cytotoxicity(Matanfack, Ruger, Stiebing, Schmitt, & Popp, 2020). These tags were used to enhance selectivity and molecular specificity while label-free Raman techniques have limited quality of outcomes. Therefore, appropriate Raman tags or labels can be used in imaging and detection of specific targets in complex tissues and biological systems. Generally, Raman tags are categorized into three major groups: surface enhanced Raman scattering (SERS) probes, bioorthogonal Raman probes and resonance Raman probes. Graphical illustrations of the three types of Raman tags are shown in Figure 3. Several tags where commonly chosen in lipid imaging and analysis because of their specific molecular characteristics in previous studies.

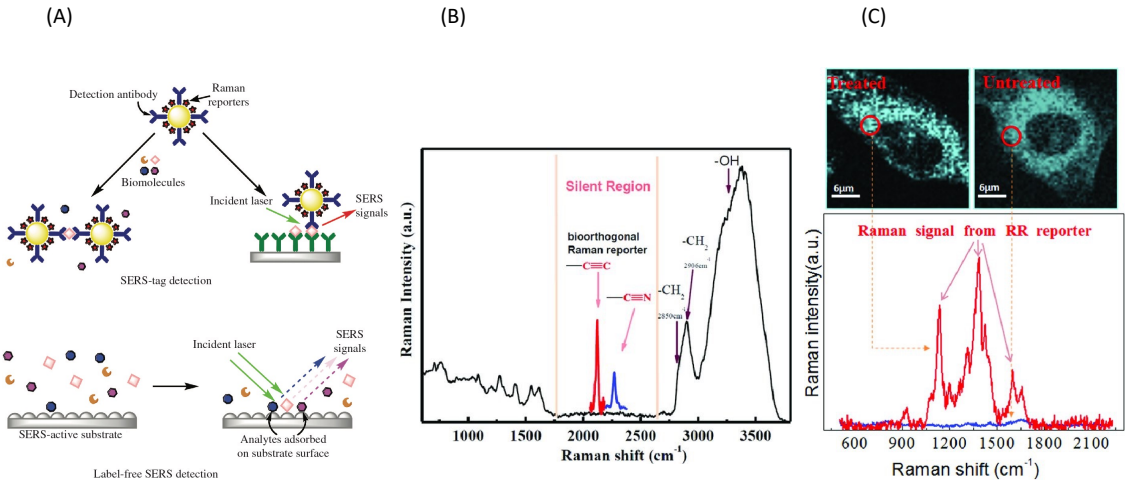

Figure 3. Graphical illustration of developed three types of Raman tags. (A) Difference between label-free SERS and detection of SERS tag(Nguyen, A. H., et al. 2017). (B) Principle of the bioorthogonal Raman probe (Li, Y., et al. 2017). Application of bioorthogonal Raman tags generate spectral peaks in silent region of spectra. (C) Principles of the Resonance Raman probe. Raman spectra of cytoplasm from treated and untreated HeLa cell are shown and different outcomes of two types of cells are indicated(Li, Y., et al. 2017)

SERS tags are ideal methods for detection of multiple targeting since they have high sensitivity and narrow spectral width of Raman peaks(Y. Li et al., 2017). The basis of SERS probing is that the proximity

of intrinsic strong Raman scattering molecules and metal nanoparticles produces a strong Raman signal(Y. Q. Wang, Yan, & Chen, 2013). While SERS tags can mark specific molecules of interest, they allow further mapping of targeted molecules in tissue samples. With the development of relevant nano-manufacturing technologies, SERS-based probes could be designed based on immune-labeling(Rohr, Cotton, Fan, & Tarcha, 1989). SERS probes are constructed by these parts: nano-structured substrates, Raman reporter, targeting molecule and protection shell(Y. Li et al., 2017). These constituents help made SERS probes a technique with great biocompatibility and biostability and reduced nonspecific binding of tags. However, SERS tags still have limitations such as the design and construction of probing components are highly specific to each application, so that the sensitivity of probing cannot be reproduced perfectly. Also, SERS tags can hardly detect or identify intracellular gradient of biomolecule distribution or mapping in a single-cell level since the size of nanoparticles in SERS substrates is much bigger than that of common molecules that we are interested in(Y. Li et al., 2017).

Bioorthogonal Raman probes are based on characteristic Raman signals of chemical bonds in cell components or molecules. Generally, natural biomolecules do not possess vibrational Raman signal in the cell's Raman-silent region, more specifically, the range from 1800 cm^{-1} to 2800 cm^{-1} in Raman spectrum. The bioorthogonal tags consist the chemical bonds that possess Raman signal in the silent region, so that they can be used as labels to mark the desired biomolecules by showing specific peaks within this region. Generally, this new class bioorthogonal Raman tags involve two typical probing methods that are stable isotope labeling, which is based on exchanges of single atoms, and addition of molecular function groups with a large Raman scattering cross section(Matanfack et al., 2020). Both of them consist specific vibrations in the silent region of Raman spectrum and such region does not overlap with other possible cellular Raman contributions with high multiplexing capabilities(Wei et al., 2017). Frequently applied stable isotope labeling in biomolecular Raman imaging includes hydrogen (^{1}H) with deuterium (^{2}H) and ^{12}C with ^{13}C. For functional group tags, common examples are characteristic chemical bonds including alkyne, nitrile and C-D bond. From previous research, it is shown that bioorthogonal tags are popular and have been widely used

in visualizing small biological molecules including lipid in living systems.

Raman spectroscopy conjugated with applications of stable isotopes as probing tags are widespread in biological, medical and pharmaceutical fields and have gained importance in recent years in cellular and molecular studies(Lorenz, Wichmann, Stockel, Rosch, & Popp, 2017). Isotopes are basically variants of same chemical elements that have different number of neutrons. Comparing to radioactive isotopes, stable isotopes do not undergo radioactive decay so that they are more secure and ensure molecular stability during biological processes in a longer time period(Abraham, 2014). With these biological and chemical characteristics, stable isotopes can be a proper Raman tag to track dynamic biological activities in living systems that may suffer from potential perturbations. Stable-isotope labeling in Raman spectroscopy refers to labeling interested molecules such as proteins, DNA as well as lipids with chosen stable isotope. While biomolecules naturally consist major biogenic elements like hydrogen, carbon, oxygen and nitrites, selected stable heavy isotopes of such elements can be used as biological tracers to probe the isotope incorporation into cellular system(Y. Wang, Huang, Cui, & Wagner, 2016). Using heavy isotopes as Raman tags leads to changes in the vibrational frequency of Raman peaks while maintaining unchanged Raman signal intensities(Matanfack et al., 2020). So that biological molecules of interest can be traced by monitoring activities of stable isotopes and induced changes in Raman peaks in within spectrum. These stable-isotope Raman tags, especially ^{13}C, had various applications in lipid imaging and analysis, more detailed labeling examples will be discussed in the following section.

Functional groups are another type of widely used bioorthogonal Raman tags in molecular imaging. People always utilize functional groups that exhibit triple bonds, for example, alkyne and nitrile, for molecular labeling in Raman spectroscopy. Labeling target biomolecules with such functional groups generates corresponding vibrations within silent region while not affecting molecular or cellular metabolic characteristics. Also, minute variations of chemical substituents close to such functional groups can generate opportunities for multiplexing in order to simultaneously study dynamic biomolecules with attractive sensitivity and specificity in Raman spectroscopy(Matanfack et al., 2020). Within these functional

groups as Raman probing tags, alkyne tags showed desirable characteristics in visualizing small dynamic cellular biomolecules and is also a popular technique in lipid imaging and analysis.

Alkyne tags, usually conjugated with stimulated Raman scattering, is a popular group of Raman tag strategy that has been used in visualization of small biomolecules or intracellular imaging of metabolites in living tissues and system. Alkynes, or C≡C bonds, are nonlinear vibrational tags that possess desirable spectroscopic and chemical characteristics. General features of alkynes include small in size, exogeneous and biorthogonal or inertness of reaction with endogenous molecules(Wei et al., 2014). Alkyne tags are small enough and suitable for labeling of small, single cellular molecule. In Raman spectrum, the internal stretching motion of alkyne bonds displays significant change in polarizability, therefore induce sharp Raman peaks in a cell-silent spectral region(Lin-Vien, 1991). Alkyne possesses a unique Raman band that does not overlap with that of those endogenous molecules in cells and tissues(Yamakoshi et al., 2012), so that the Raman signal of alkyne could be distinguished from presented mixture of scatterings from different molecules during imaging. In previous studies, relative Raman intensities of alkyne tags were successfully measured while using carbon tetrachloride as an internal standard molecule. But alkyne tags in Raman scattering still have limitations such as quantitative data of Raman intensities of alkyne-tagged molecules are not easy to obtain since the absolute Raman signal intensities of alkynes could hardly be measured directly(Larkin, Gustafson, & Asher, 1991). And also, a certain type of tagged molecule may have fluctuant intensities depending on different methods and instrumentation of measurements(Rea, 1960).

Alkyne-tag Raman imaging (ATRI) was considered as a potential technique that is suitable for lipid imaging and analysis. Studies denoted that ATRI can be used in imaging of several different species of nonaromatic, mobile noncovalent-bond-forming small molecules including lipid relevant candidate molecules in drug delivery(Yamakoshi et al., 2012). Besides morphological structure and distribution of mobile molecules, ATRI could help obtain information such as cellular concentration of molecules and allow multicolor imaging of molecules(Yamakoshi et al., 2012).

Another type of developed Raman tag is resonance Raman probes (RR probes) that are based on

resonance Raman mechanism. The chosen excitation wavelength should have energy that is close to that of Raman reporter molecules' electronic transition(Y. Li et al., 2017). Because of such energy overlapping, scattering and signal intensities of Raman reporter will be significantly enhanced, and distinctive signal peaks will be displayed in Raman spectrum. Since previously reported RR probes were challenged in several factors including biocompatibility and inherent accompanying fluorescence, practical RR probing applied in cell imaging was not developed until several years ago in last decade. In 2015, a study reported developed RR probe as a new generation of Raman molecular probes and presented application of RR probes in living cell imaging and immuno-labeling. This novel technique could successfully provide unparalleled high detection sensitivity because of such resonance Raman enhancement(Y. Li et al., 2015). The reported RR probe was designed to achieve RR enhancement by excitation in visible spectral range that is far away from cellular absorption of biomolecules and therefore friendlier to biological samples. And the certain approach allowed both mapping of biomolecules and fast identification of labeled cellular domains under confocal or spontaneous Raman scattering(Y. Li et al., 2015). One year later, a BBQ-650 RR probe that worked based on excitation in red spectral range was developed. It was found that cellular molecules do not absorb in this spectral range, so that phototoxicity, or laser-induced cell damage, was minimized and the Raman signal intensities was significantly enhanced(Kuzmin et al., 2016).

Comparing with previous Raman tags, newly developed resonance Raman tags in visible or red spectral range had limited reported applications in lipid imaging. Previously before these developments, RR probing in UV spectral range was applied in lipid study in 2009. Quan's group presented UV resonance Raman spectroscopy with 229 nm excitation in study of tryptophan-containing antimicrobial peptides with a broad-spectrum activity against bacteria in lipid vesicles(Quan & Ianoul, 2009). In this research, UV resonance Raman spectra of peptides were shown and dominated by tryptophan bands. However, this research only gave outcomes that focused on localization and molecular interactions between peptides and surrounding lipid membrane, and the systematic information and dynamic monitoring of molecules were still limited. For resonance Raman tags, molecular Raman imaging in visible or red spectral range could be

a novel field to discover in further studies, since it seems have significant potential in biological imaging of living systems.

At the present time, bioorthogonal Raman tags are most popular in molecular and cellular biological imaging in different scales or imaging levels. For lipid imaging, stable-isotope labeling and functional group tags are being widely utilized in previous and current studies in the recent decades. These labels can help better target biomolecules of interest without damaging samples or induce cytotoxicity while producing enhancement in biological selectivity, specificity and sensitivity. These labeled Raman spectroscopies are another group of powerful imaging technique besides label-free approaches in lipid imaging, and people can choose proper methods or techniques in Raman imaging according to specific desired characteristics in different biological applications.

In vivo labeling for lipid and membrane imaging

Labeling is a conventional approach in small scale cellular imaging since it can help target and distinguish molecules of interest from the complex cellular environment and it is the major advantage of labeled optical imaging comparing with label-free imaging approaches. Ideal labeling techniques should be noninvasive, nontoxicity to cells and tissues, and should not alter molecular characteristics and dynamics of cellular substances. In all of the Raman imaging techniques incorporated with molecular labeling that were applied on lipid imaging, stable-isotope labeling Raman spectroscopy is always popular for spatiotemporal, functional or dynamic detections in living cells and tissues.

Raman spectroscopy incorporated with stable-isotope labeling was used in multiple aspects in lipid imaging. In 2012, a study by Matthaus et al. performed noninvasive imaging of intracellular lipid metabolism in macrophages by this Raman technique. Fatty acids and cholesterol in the form of lipid droplets were labeled with deuterium, and the deuterated derivatives of saturated and non-saturated fatty acids and cholesterol in a well-established model of human cells were successfully visualized. Therefore,

the uptake and storage dynamics of lipid droplets were informed and so that visualize macrophage foam cell formation directly at single lipid species level(Matthaus et al., 2012). Raman images of such labeled cells are shown in Figure 4. Another type of stable isotope Raman label, ^{13}C labeling, was also performed for lipid imaging in the model of single fission yeast cells. In a study by Venkata et al. in 2012, the in vivo time-lapse Raman imaging was coupled with ^{13}C labeling in the culture of *Schizosaccharomyces probe* cells as a designed model(Noothalapati Venkata & Shigeto, 2012). They cultured labeled ^{13}C-glucose in the cellular medium and visualized its dynamic in vivo process which could reflect dynamic activities of proteins and lipids according to their characteristic Raman bands(Noothalapati Venkata & Shigeto, 2012) (Figure 5). That is, while tracing the labeled molecules in vivo, certain dynamics and metabolism of corresponding intracellular components could be clearly visualized and analyzed.

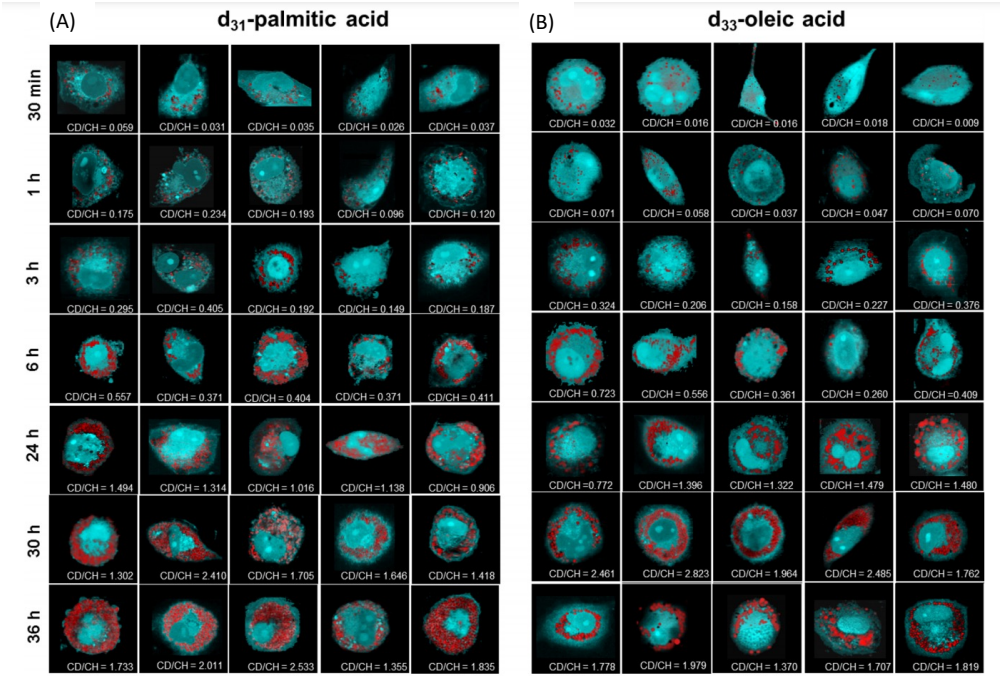

Figure 4. THP-1 macrophages incubated with serum albumin-complexed labeling substances. (A) Macrophages incubated with d31-palmitic for the times indicated. (B) Macrophages incubated with d33-oleic acid for the times indicated. (Matthaus, C., et al. 2012)

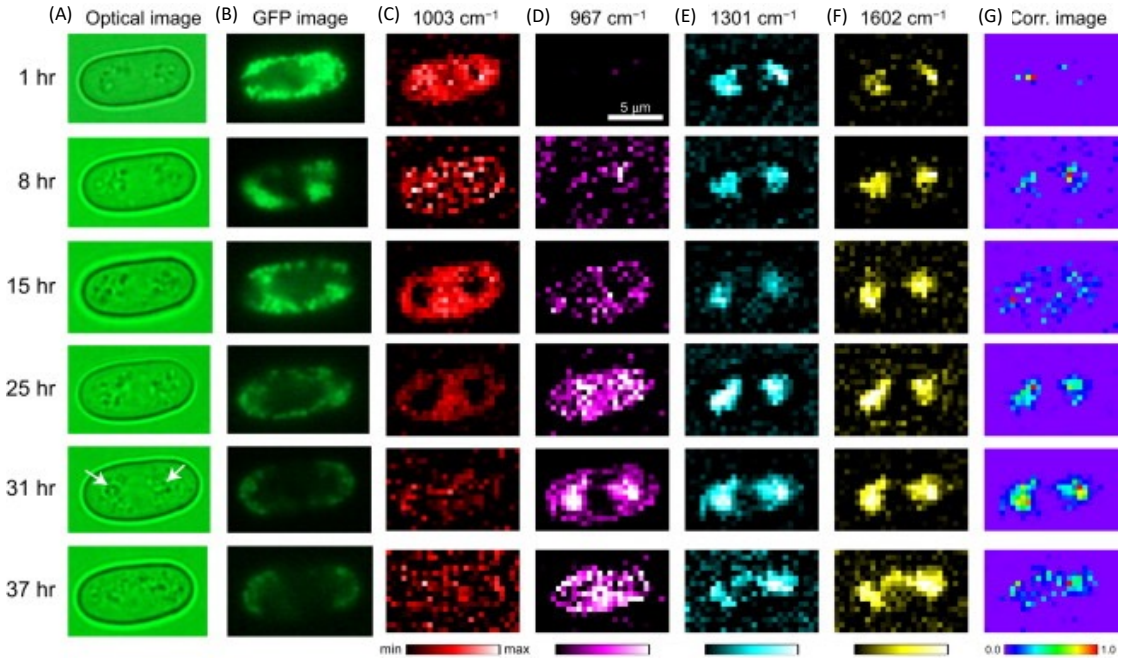

Figure 5. Raman Imaging of a Single Living S. pombe Cell Grown in ^{13}C-Glucose-Containing Medium(Noothalapati Venkata, H. N. and S. Shigeto 2012). (A) Optical images of target cells. Black dots pointed by arrows are lipid droplets. (B) Fluorescence images of mitochondria in cells. Raman images are constructed at wavenumbers of (C) 1003 cm^{-1}, (D) 967 cm^{-1} , (E) 1301 cm^{-1} , (F) 1602 cm^{-1}. (G) Correlation between images (D) and (E).

Labeling with function groups is another popular method of labeled Raman spectroscopy that was applied in imaging of lipid molecules. In a previous study, alkyne-tagged fatty acids in single cell were monitored using Raman spectroscopy and therefore provide direct graphical information of intracellular lipid distribution and uptake dynamics. The ability of Raman spectroscopy with alkyne labeling was highlighted since it helped understand fine details in lipid uptake, metabolism and distribution(Jamieson et al., 2018). Alkyne tagging is a key step in the study since it can help target and localize the fatty acids, or biomolecules of interest with very high specificity. The utilization of alkyne tag is based on its unique Raman signal in the silent region within the spectrum, more specifically, approximately between the wavenumbers of 1800 and 2000 cm^{-1}. (Yamakoshi et al., 2012) Meanwhile, alkyne is not a natural functional group or inherently exist in native biological systems, then it became an appropriate precursor to imaging specific biomolecules and became a typical Raman tag for in vivo labeling of lipid molecules.

Laboratory application of SRS for lipid study

Insulin metabolism is a crucial part or process in lipid activities including synthesis and separations. In the current study, we performed laboratory experiments for studying insulin metabolism and lipid analysis using stimulated Raman spectroscopy (SRS). Insulin signaling pathway plays an important role in nutrient transportation, metabolic activities and maintenance of hemostasis in living beings. During the metabolic signaling, insulin binds to the receptor tyrosine kinase on the surface of cell, and then activate the receptor and initiate the signaling. What happen next is the auto-phosphorylation of insulin receptor, and then phosphorylation of insulin receptor substrates (IRS). The phosphorylation activates the PI3 kinase/Akt signaling pathway, while the IRS proteins bind and active downstream targets. Then mediation activities of PI3 kinase initiate signaling cascades which closely link to a series of intracellular metabolic activities including up-regulation of glucose transport, glycogen synthesis and lipogenesis, as well as down-regulation of gluconeogenesis and lipolysis. The experiments of imaging are focused on investigation of this certain PI3K/Akt insulin signaling pathway and how modification of related gene and enzymes influence the metabolic activities of lipid molecules. We choose *Drosophila* as a biological genetic model because the gene expression of *Drosophila* is easy to monitor and suitable for modification and mutation on genes. We focused on imaging four typical types of interested biological species or genes under SRS and for following comparisons. The four species or genetic types are marked as: *Ctr* (control animal), *InR del* (insulin receptor deletion), *InR dn* (dominant negative insulin receptor) and *Pten* (tumor suppressor gene).

A schematic illustration in Figure 6 of the insulin signaling pathway can provide a direct idea of the downstream activities. I highlight the important genes that are going to be modified in the following experiments in red color.

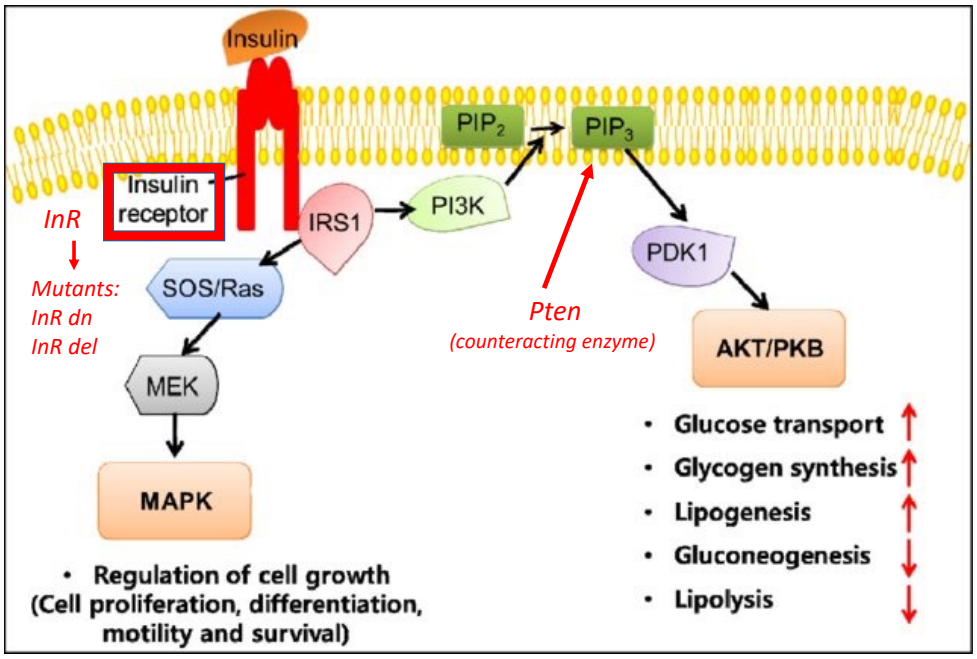

Figure 6. Schematic illustration of insulin signaling pathway. The downstream binding of substances and enzymes are shown for the PI3K/Akt pathway. Corresponding metabolic activities under regulation are shown (Jung, U. J., & Choi, M. S. (2014)). Genes or enzymes that are mutated or knocked down in the experimental design are marked as red.

Then experimental procedures were designed based on the background knowledge of lipid metabolism that mentioned earlier. Two gene expression types were driven from fat body of *Drosophila*. One is *Pten*, which is a lipid phosphatase, or an enzyme counteracting activity of PI3 kinase. The other is *InR DN*, the dominant negative form of insulin receptor. Sine these gene expressions are supposed to down-regulate the signaling pathway, I hypothesized that when these gene types are knockdown, the insulin signaling should be promoted and the metabolic activities of lipid controlled by this pathway should be increased. (Hypothesis 1)

Another driven gene expression type I considered was *InR del*, a catalytic active form of insulin receptor. The second hypothesis was while the *InR del* gene type is overexpressed, the signaling pathway is hyperactivated so that the metabolic activities of lipid will be downregulated or blocked, and lipid synthesis or lipogenesis process will be decreased. (Hypothesis 2)

These two hypotheses regarding lipid metabolism are about to verified by following SRS imaging

processing and analysis.

Both label-free and labeled SRS imaging were performed on lipid molecules. The four types of species were visualized using tuned lasers of CH_2 and CDL stretching bonds, or lipid channels. CH_2 stretching bond was visualized by label-free SRS at wavenumber of 2850 cm^{-1}. Meanwhile, CDL indicates CD labeled lipids, that are the lipid molecules labeled by deuterium, or heavy water, and the corresponding wavenumber of imaging is 2135 cm^{-1}. SRS images of four species obtained by tuned CH_2 and CDL lasers are shown in the first two rows of Figure 7. The third row in the figure marked as CDL/CH_2 involves ratiometric pictures indicating the percentage of turned over CH_2 bonds.

After raw SRS images from two lipid channels were obtained, the images were processed in FIJI imaging processing software. The raw pictures from two lipid channels are colored. And then the images of each genetic type were calculated as dividing the signal intensity of CDL channel by that of CH_2 channel to obtain ratiometric images. The calculated images were colored and tuned with appropriate contrast to have an intuitive visualization of the status and distribution of molecules that were turned over. In Figure 7, the third row marked as CDL/CH_2 are ratiometric pictures indicating the percentage of turned over lipid molecules. Considering the biological basis of metabolism, we can have the idea that green molecules from CH_2 lipid channel indicate the old lipid molecules, while purple ones from CDL channel indicate the newly synthesized lipids. The ratiometric images marked as CDL/CH_2 in the third row then became the ratio between newly formed lipid molecules and the old ones. Quantifications and cellular distribution of lipid molecules under each circumstance could be visualized.

The result shows that the sample groups with knockdown *Pten* and *InR dn* have more synthesized lipid droplets than that of the wild type samples. While the two counteracting enzymes are blocked, the insulin signaling pathway is promoted so that the lipogenesis, which is upregulated by insulin signaling pathway, is also increased. The result from imaging processing is consistent with the hypothesized consequence after the mutations, so that my hypothesis 1 is verified as true.

For the sample group of overexpressed *InR del* genetic type, the ratiometric image showed that the newly synthesized lipid droplets are less than that of wild type samples. It indicates that the insulin signaling pathway with this genetic mutation is downregulating or blocking lipid synthesis, so that the hypothesis 2 is also true.

From dynamic visualization of CDL/CH_2 turned over percentage, we can have an idea on the process and distribution of CH bonds labeling by deuterium in lipid molecules. This information would be helpful for following investigations of insulin metabolic pathway and processing activities.

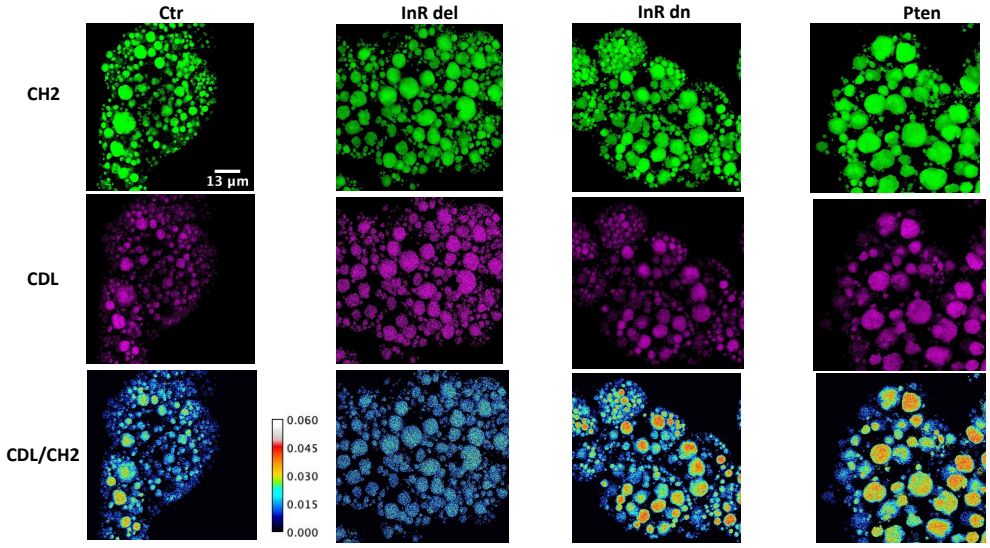

Figure 7. Experimental images of lipid droplets processed by stimulated Raman spectroscopy to study insulin metabolic pathway. SRS images were constructed for 4 groups of samples: *Ctr*, *InR del*, *InR dn* and *Pten*. Old molecules indicated by CH_2 channel are shown in green (row 1) and newly formed molecules indicated by CDL channel are shown in purple (row 2). Ratiometric images processed are also displayed in row 3.

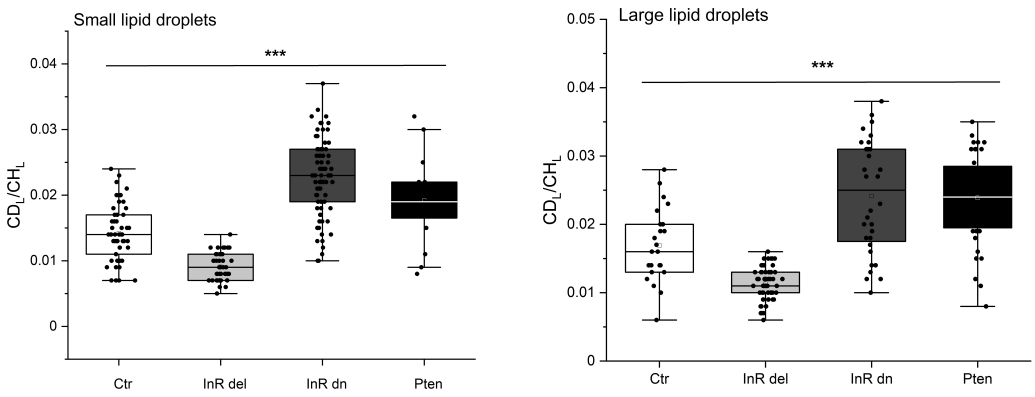

Figure 8. Statistical data from ratiometric images is calculated and displayed for each of the four sample groups. Approximate values of ratios between new and old lipid molecules and the distribution of data points are shown.

Following the SRS imaging process on insulin receptors to study the insulin metabolic pathway in lipid study, data analysis was also performed. The ratiometric data points of lipid droplets for each of four species were marked and compared. As shown in Figure 8, for small lipid droplets, the ratiometric values between new and old molecules for Ctr, InR del, InR dn and Pten are around 0.014, 0.008, 0.023 and 0.019, respectively. The values for large lipid droplets of the four species are around 0.013, 0.011, 0.025 and 0.024, respectively. We can see that for small lipid droplets, mutant groups of *InR dn* and *Pten* have more newly formed molecules than the wild type while *InR del* mutant has the least. For large lipid droplets, the results are consistent with that of the small molecules. Large and small droplets in these four species show the same trend of synthesizing activities in this analysis. The statistical results could further verify the conclusion we got from previous imaging analysis.

Lipid subtype detection with Raman spectroscopy

While the relationship between function of insulin signaling pathway and lipid metabolism could be successfully investigated by SRS imaging processing and analysis, I thought about and generated a further interested question. Is it possible for us to get a more detailed information about which specific subtype of lipid is regulated or modified by mutations of genes in insulin metabolic pathway? Or how should we detect the dominant lipid subtype from an SRS image with mixture of lipid subtypes?

Lipids are basically classified into saponifiable and nonsaponifiable lipids according to their characteristics of whether they can be disintegrated through hydrolysis. Saponifiable lipids are always composed by several ester groups and typical examples are sphingolipids, phospholipids, waxes and triglycerides. Nonsaponifiable includes cholesterol, prostaglandins and some other types of lipids. These lipid subtypes always have unique biological characteristics and properties in living systems, so that lipid subtype detection is an important aspect of lipid imaging and analysis.

Why Raman Imaging for lipid subtype detection?

As a newly developed and enhanced biological imaging technique, Raman microscopy was always compared with some other conventional imaging tools throughout the developments and applications.

Among hyperspectral imaging techniques, matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry imaging and confocal Raman microspectroscopic imaging were found to be compared together. Both of the hyperspectral imaging techniques enable label-free imaging and visualization of spatial distribution of molecular substances(Lasch & Noda, 2017). They can provide detailed molecular information while probing various types of biomedical species and samples. Besides the similarities, there are also some important differences that people may consider when choosing appropriate methods. Mass spectroscopy (MS) is destructive to biological samples during imaging process, so that MALDI-TOF MS seems not that friendly to cells and tissues while it requires the application of more

complex matrix(Lasch & Noda, 2017). At this point, vibrational spectroscopic tools such as Raman imaging show their characteristics of nondestructive and friendly to biological tissue and samples. However, vibrational spectra may exhibit broad or overlapping bands that need extra effort on separation and make the following analysis more difficult. As same issue may also happen to MS techniques, MS imaging shows its advantage of enabling coupling with separation techniques and chemical methods, so that the outcome of MS techniques often display high-resolution signals to interested molecular substances(Lasch & Noda, 2017). For the same reason, some other types of MS techniques such as LC-MS share the same disadvantages on molecular imaging including lipid analysis.

Secondary ion mass spectroscopy (SIMS) is another type of MS technology. During biological imaging, while MALDI can provide higher upper mass range, SIMS focuses more on obtaining high spatial resolution(Boxer, Kraft, & Weber, 2009; Lanni et al., 2014). A previous study mentioned that SIMS has relatively high selectivity, but the sampling matrix effect may result in inaccurate imaging outcome(Gallagher et al., 2004). In this way, Raman imaging may show clearer and better contribution images when analyzing complex biological samples.

Nuclear magnetic resonance (NMR) is a multifaceted biological imaging technique. NMR spectroscopy is good at providing information on local environment including type, number and symmetry of chemical bonds in interested biological sample and also sensitive to local radial distribution(Kosinski et al., 1988). Comparing with Raman imaging technique, NMR is weak in providing spatial and dynamic information, so that not that good for detecting lipid subtypes and their distributions in molecular species.

Positron-emission tomography (PET) is a type of nuclear medicine imaging method that uses radioactive tracers. PET is ideal for functional imaging including metabolic process and physiological activities in a larger scale. Since accurate radioactive tracers should be carefully chosen and its property that might be harmful to cells and tissues, PET should not be a good choice for small-scale molecular imaging or lipid analysis.

As a small-scale molecular imaging application, lipid subtype detection requires high resolution, selectivity and outstanding ability of in vivo or in situ dynamic visualization in living cells and tissues. Then Raman spectroscopy started to show its ability in this field of study.

In recent decades, Raman spectroscopy became a popular imaging method that was being used in lipid analysis. Enlightened by such developments, people also started trying to perform Raman microscopy in detection of lipid subtype specifically. In a recent study, the researchers showed their achievement in determination of protein content, chemical profiles and dynamics of lipid particles in *C.elegans* as a biological model of living system. The study revealed the diversity and different subtypes of lipid particles in intestine and skin-like epidermis and indicated that the lipid particles in adult intestine were diverse while those in epidermis, mostly contained triglyceride content, were less heterogenous and dynamic(Chen et al., 2020). Since *C.elegans* was a typical biological model to perform Raman imaging of lipid synthesis and lipolysis dynamics of lipid subtypes quantitatively, such outcome obtained from a same model as before was representative and showed persistency and comprehensive application of Raman imaging in lipid analysis. Moreover, while another approach applying stimulated Raman scattering microscopy conjugated with bioorthogonal molecular labeling, metabolic dynamics and concentration of specific fatty acids in lipid droplets could be visualized and monitored(X. S. Li et al., 2019). Here in this study, it was shown that different kinds of fatty acids that were labeled by specifically chosen Raman tags were able to display typical vibrational Raman peaks at specific wavenumbers on the Raman spectrum(X. S. Li et al., 2019). That is, with the help of appropriate Raman labels, several lipid subtypes could be targeted individually with Raman labeling so that they could be distinguished by the visualized Raman peaks throughout the spectrum.

The detection of lipid subtype using Raman technologies is still a newly developed or premature approach, and it was just being explored in the very recent years. However, while the outstanding characteristics and performance of Raman spectroscopy were investigated and justified in many different

aspects, and its various modalities also allow for conjugation with other ancillary imaging techniques, Raman spectroscopy should have huge potential to become an emerging technique in lipid subtype detection in future studies. In the meantime, for the purpose of practically detect the interested lipid subtypes in samples, an imaging clustering platform is needed to be developed specifically to solve this problem.

Popular platforms that are designed for vibrational spectroscopic imaging analysis are investigated. Multivariate curve resolution–alternating least squares (MCR-ALS) analysis platform is a powerful technique that showed successful pixel-by-pixel analysis of biological substances. It enables extracting the spectrum of a single pixel and analyze the dominant chemical compound in the specific pixel by comparing the spectrum with reference standard spectrum of pure compound. The pixel-by-pixel analysis can finally reach out to an outcome with distribution of substances across the entire sample and provide information about dominant substance in the specific sample under investigation.

According to investigation, this MCR-ALS technique should be a potential powerful platform for lipid subtype detection according to its features and properties. But this platform has unresolved limitations before applying on lipid samples. The processing time is slow since it takes about hours for analyzing a single piece of sample. Some major steps such as comparison between acquired spectrum and reference spectrum of pure substance still need to be done manually during the analysis. Meanwhile, since the platform only accept txt files with arrays of data set, supplemental platform to convert raw images to acceptable data sets might need to be developed. These features limit the practical application of MCR-ALS on lipid subtype detection. More importantly for lipid subtype detection, after investigating the spectra of several lipid subtypes, it is found that some of the subtypes have Raman spectra with similar structure and peaks, and that may reduce the accuracy of analysis under interference of background noise and mixing status of compounds in tissue samples. And since the vibrational spectra of native in situ compounds might not be identical to extracted pure component spectra, the error could be further enlarged during analysis.

Therefore, in my hypothesis, some future enhancements can be developed for MCR-ALS platform before it can be applied for lipid subtype detection. Multi-task processing that allows simultaneous analysis

for different pixels could be investigated to reduce the long processing time of pixel-by-pixel analysis. The comparison between acquired spectra and reference spectra needs to be further monitored manually to improve the accuracy of analysis. And the reference spectra of lipid subtypes needed to be distinguished and marked with details in the library data set in order to obtain the precise result when there is high similarity between subtypes of interest.

Since lipid subtype detection is a newly developed field of lipid analysis, limited approaches were done for the detection and the outcomes were not that substantial. There still need efforts to find and develop an optimal technique or platform for the purpose of lipid subtype detection.

Concluding remarks and future perspective

From various research studies in recent decades, Raman spectroscopy and its proper modalities were considered as a powerful imaging technique in biological and molecular analysis, including lipid analysis, at small scale in different aspects. With properties of high resolution, sensitivity and selectivity, as well as label-free and nondestructive to biological samples, Raman scattering should soon become a prominent tool and replace the position of conventional imaging techniques such as mass spectroscopy for lipid imaging. However, as a newly developed vibrational imaging technique in molecular imaging applications, Raman scattering still has unsolved limitations and challenges in small-scale biological imaging and lipid analysis.

For lipid imaging itself, Raman spectroscopy had been justified to overcome pertinacious challenges in conventional techniques, more specifically, the mass spectroscopy in different aspects. Successful cases were shown in studies that Raman imaging can be used to trace metabolic activities and molecular movements of lipid substances. Multiple types of information including dynamic tracing and quantification of molecules were able to be visualized in more and more attempts. In the near future, Raman modalities are expected to be enhanced with higher intensity of signals, wider range of imaging scale and

various type of sample-friendly labeling substances to further improve the quality and preciseness of biological imaging.

For future respective and further investigation of lipid subtype detection, current platforms that is the potential optimal method for such detection purpose are evaluated. Lipid subtype detection with imaging techniques is a brand-new field under investigation and conventional approaches consist inevitable challenges and the outcomes were less than satisfactory. It is necessary and important to construct a new or enhanced platform especially for the desired detection.

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