Form 3

Synopsis of Thesis

Title: Development of hyperspectral coherent Raman scattering microscopy system using high-speed tunable picosecond laser and its application for intracellular lipid imaging

（高速波長走査ピコ秒レーザーを用いたハイパースペクトルコヒーレントラマン散乱顕微鏡システムの開発と細胞内脂質イメージングへの応用）

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I developed a hyperspectral coherent anti-Stokes Raman scattering (CARS) and stimulated Raman scattering (SRS) microscopy systems to visualize biomolecular dynamics without any staining. In order to realize the system, I constructed a high-speed tunable picosecond mode-locked laser that was be able to easily synchronized with another picosecond mode-locked laser to provide the laser sources for CARS/SRS microscopy. By applying the developed microscopy system, movies were obtained with acquisition rates of 10 ms/image, faster than video rates (~33 ms/image). A wide range of wavelength tuning over 1000 cm^{-1} (equal to 85 nm) was achieved. During the wavelength change, the synchronization was recovered within 20 ms. It led to the realization of fast switching imaging of the treated cells with deuterated lipid. For that purpose, the wavelength was alternately change between 833 nm and 888 nm which are associated to the Raman shift of 2100 cm^{-1} and 2850 cm^{-1}. By using this method, I succeeded in imaging of the uptaken lipid by the cells.

Coherent Raman scattering (CRS), which includes CARS and SRS, refers to the nonlinear Raman process. CRS microscopy supersedes conventional Raman microscopy which lacks of fast imaging capability due to the extremely small cross section. Every molecule has molecular vibrations which highly depend on its structure and Raman spectroscopy is a method for observation of the molecular vibration. Therefore, CRS microscopy is a powerful tool for label-free biological imaging with high selectivity since it directly senses the molecular vibrations. Furthermore, the fast imaging capability is advantageous for the observation of biomolecular dynamics intracellularly. CRS microscopy usually employs two laser sources with slightly different frequencies to excite a specific molecular vibration. Hence, laser sources with high peak power and narrow spectral bandwidth are required to enable the occurrence of nonlinear Raman process and to distinguish the molecular vibration.

I constructed a new laser system to realize hyperspectral coherent Raman (CRS) microscopy system. The new laser system referred to a picosecond mode-locked Ti:sapphire laser which was equipped with an acousto optic tunable filter (AOTF) for wavelength tuning, a piezoelectric actuator driving the end mirror, galvano motors driving parallel plates pair, and a pulse synchronization system. The AOTF enabled the rapid wavelength tuning because it applied an electronically control system to change the wavelength. The piezoelectric actuator driving the end mirror and the galvano motors driving parallel plates pair are installed to compensate for the repetition frequency change of the laser during the wavelength scanning. The synchronization utilized two types of timing jitter detection system: an electronic phase detector and a balanced cross-correlator for the coarse and fine feedback control. Thus, during a wide range tuning, the pulse synchronization system recovered the synchronization automatically within 20 ms/wavelength change.

I then developed a fast spectral CARS microscopy system by applying the high-speed tunable laser into multi-focus CARS microscopy. The use of microlens array scanner allowed me to perform rapid acquisition imaging at rates of 100 frames per second, faster than video rates (~33 frames per second). The spectral imaging capability was represented by a series of CARS images due to the scanning of AOTF laser from 875.0 to 905.0 nm which corresponded to the Raman shift of 2663.21 cm^{-1} to 3049.41 cm^{-1}. For a 100 ms exposure time, the imaging process requires 120 ms/image to cover the wavelength change, laser resynchronization after the wavelength change, and image acquisition. A switching imaging was also demonstrated to show the capability of the spectral CARS microscopy for rapid detection of different molecules of which the Raman shift separated far. In

this case, I observed a mixture of deuterated and non-deuterated stearic acid that have Raman peak at 2100 cm^{-1} and 2850 cm^{-1}, respectively. For this purpose, the tunable laser was alternately set to 833 nm and 888 nm.

I continued the development of our system into spectral SRS microscopy to overcome the inevitable non-resonant background in CARS microscopy. By the observation of adipocytes (fat storing cells) that was treated by adding deuterated stearic acid into the medium, I showed that the non-resonant background did not appear in the SRS image of the sample at 2300 cm^{-1} (off resonant) while it did in the CARS image from the same sample region. I extended the observation spectra by tuning the wavelength of AOTF laser over extremely wide region from 2100 cm^{-1} to 3200 cm^{-1}. I also demonstrated a switching imaging of the same sample and alternately showed the images of lipid droplets in adipocytes which contained deuterated lipid and non-deuterated lipid. CRS microscopy is suitable for lipids observation because lipid has unique features in Raman spectra. Lipid mainly consists of CH bond of which vibrational frequency inhabits specific region in Raman spectrum with significantly strong signal level. Furthermore, I can take advantages of CD bond to enhance the detection of lipids. As the isomorphic structures of correlated CH bond, CD bond does not change the biochemical dynamics of lipid, however it can be clearly distinguished from the CH bond in the Raman spectra. CD bond dramatically downshifts the vibrational frequency of related CH bond to Raman silent region, where there should be no signal from biological specimens will interfere.

The results from CARS microscopy revealed that naturally the deuterated structure did not appear in cells. The appearance of significant signal at 2100 cm^{-1} indicated that the exogenous lipid was uptaken by the cells. Further study revealed that lipid with different structures may follow separated metabolic pathways. In this case, the long chain fatty acid seemed to be preferably accumulated than medium chain fatty acid. The observation of intracellular lipid dynamics in lipid droplets is possibly useful for studying and recognizing lipid related disease. Lipid gets larger attention lately for biomedical study since its function is more than just as energy source.

The accumulation of exogenous lipid by adipocytes might relate to its function as the lipid storing cells. However, the accumulation of lipid by fibroblasts likely described the abnormality in lipid storage due to certain diseases. In this case, the fibroblasts were obtained from a patient with indication of triglyceride deposit cardiomyovasculopathy (TGCV). TGCV is a new phenotype of heart disease which is indicated by a massive accumulation of triglyceride (TG) in the coronary arterial wall and cardiomyocytes, though its concentration in blood plasma is found at normal level. As it was observed in the observation of adipocytes, there was a dissimilarity of metabolic pathways of different lipids which might suggest the possibility of drug development for TGCV and other lipid-related diseases.

様式7

論文審査の結果の要旨及び担当者

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論文審査の結果の要旨

本論文は，コヒーレントラマン散乱顕微鏡システムの開発と細胞内脂質イメージングへの応用に関する研究をまとめたものである。ここで取り上げたコヒーレントラマン散乱法は、高感度が特長のcoherent anti-Stokes Raman scattering(CARS)と高コントラストが特長のstimulated Raman scattering(SRS)である。これらを相補的に利用することで、目的とする分子の精緻な動態が計測できる。細胞内の分子観察に応用するため、CARSとSRSを光学顕微鏡に適用し、コヒーレントラマン散乱顕微鏡を構築した。本研究では顕微鏡に高速分光画像取得能を持たせるため、高速波長走査機能をそなえたピコ秒レーザーシステムを開発した。また本レーザーを導入したラマン顕微鏡システムの性能を評価するため、細胞内の脂質同定ならびにスペクトル分解画像の高速取得を試み、開発システムの有用性を示した。具体的には次のような成果を得た。

（1）新規レーザーシステムは2台のピコ秒モードロックチタンサファイアレーザーをベースとし、一方のレーザー共振器内に、音響波長可変フィルター、ガルバノモーターに取り付けたガラス平行平板、ピエゾアクチュエーターに取り付けたエンドミラーを制御することで高速波長走査と2レーザーの時間的同期の両立を可能にした。

（2）このレーザーシステムをCARS顕微鏡とSRS顕微鏡の光源として共用し、互いの長所を生かす高速スペクトルイメージング可能なコヒーレントラマン顕微鏡システムを開発した。

（3）上記のラマン顕微鏡によって、細胞内脂肪滴のラマン分光イメージングをビデオレート以上（100 fps）で取得し、また700 cm-1以上の波長スイッチングを20 ms で２レーザーを同期させながら行えることを示した。

このように、独自に開発したラマン顕微鏡システムによって、従来法では実現不可能であった生きた細胞の無染色高速分光イメージングを可能としている。本法は細胞生理を損なうことなく細胞・組織のあるがままの挙動を捉えることができる非侵襲計測法であり、生物学および医学へ大きく貢献するものと期待される。したがって得られた成果の工学的意義は大きく、また学術的にも高いレベルの内容を有しているので、博士（工学）の学位論文として価値のあるものと認める。