ウェブサイト公開用

（様式 10）
（海外特別研究員事業）
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海外特別研究員最終報告書

独立行政法人日本学術振興会 理事長 殿

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（氏名は必ず自署すること）

海外特別研究員としての派遣期間を終了しましたので、下記のとおり報告いたします。
なお、下記及び別紙記載の内容については相違ありません。

記

1. 用務地（派遣先国名）用務地： イサカ （国名： アメリカ合衆国 ）

2. 研究課題名（和文）※研究課題名は申請時のものと違わないように記載すること。
生きた動物内での光駆動タンパク質の in vivo 光化学

3. 派遣期間： 平成 31 年 4 月 1 日 ～ 令和 元 年 10 月 31 日

4. 受入機関名及び部局名
受入機関：コーネル大学応用工学物理学科

5. 所期の目的の遂行状況及び成果…書式任意 書式任意（A4 判相当 3 ページ以上、英語で記入も可）
（研究・調査実施状況及びその成果の発表・関係学会への参加状況等）
（注）「6. 研究発表」以降については様式 10－別紙 1～4 に記入の上、併せて提出すること。

The goal of this research project is to understand the photochemical processes of photoreceptor proteins in living animal cells, especially in living mouse brain. Photoreceptor proteins are widely used in essential tools in life science research; such as optogenetics and fluorescence imaging including multiphoton microscopy for deep-tissue imaging. To study the photochemistry of proteins in living animals, I apply pump-dump fluorescence spectroscopy. For photoexcitation of fluorophores in scattering tissues like the mouse brain, multiphoton excitation such as two-photon and three-photon excitation is preferable because of the long penetration depth in tissues. In my first year at the host research group, I accomplished two things: (1) mouse brain fixing in awake condition; (2) signal enhancement of multiphoton excitation.

(1) Mouse brain fixing in awake condition

To study the photochemistry of photoreceptor proteins *in vivo*, the movement of the mouse brain has to be reduced in order to obtain fluorescence signal from the same region in the brain over time. My PhD study was involved in femtosecond spectroscopy and physical chemistry of photoreceptor proteins *in vitro*; and I had no

experience of living animal handling. Therefore, acquiring knowledge and skills of treating living animals, especially mouse brain, was first required to accomplish the goal.

In the first half year at the host institute, I acquired mouse brain surgery skills including craniotomy and fluorescent dye injection to the mouse brain. To prevent the mouse brain movement in awake condition during experiments, the cranial window was fixed using dental cement and a metal scaffold. By fixing the metal scaffold to a moues holder during experiment, the mouse brain movement was successfully restricted by several-micrometer precision. The mouse movement was evaluated using laser-scanning microscopy with blood-vessel staining, observing the blood vessel movements within several micrometers over 10 min in awake condition. This mouse-brain fixing method was used for mouse brain experiments that are described below.

(2) Signal enhancement of multiphoton excitation

For *in vivo* spectroscopy deep in the mouse brain, multiphoton excitation is preferable because of the longer-penetration depth in tissues and less phototoxicity than one-photon excitation. Especially, with three-photon excitation, selective excitation to deep regions such as hippocampus, which is essential for memory formation, in the moues brain. In three-photon excitation, near-infrared laser at ~1300 and ~1700 nm is typically used to excite green and red fluorophores, respectively, because the water absorption is relatively low in those wavelengths. The longer-wavelength excitation at ~1300 nm and ~1700 nm enables longer penetration in tissues because of the low light scattering. Despite the advantage of multiphoton excitation, probability of multiphoton excitation is much lower than that of one-photon because multiphoton excitation occurs only when 2- or 3- photons arrive at the same molecule simultaneously (within ~1 fs). Since the moues has to be fixed in the moues holder during *in vivo* spectroscopy, the measurement time is limited to several hours, different from *in vitro* spectroscopy in cuvette. Moreover, the laser power is limited to prevent photo-induced damage to the proteins and tissues, derived from non-linear damage and thermal damage. Therefore, signal enhancement of multiphoton-excited fluorescence is essential to perform *in vivo* spectroscopy.

To enhance multiphoton fluorescence signals, I utilized resonance enhancement effect. In one-photon absorption, photo-excitation to an electronic excited state from the electronic ground state. In two- and three-photon excitation, intermediate states (i.e. virtual states) mediates the transition from the ground state to the excited state. A reason of low multiphoton absorption probability is derived from the very short lifetime of the intermediate states; typically ~1 fs. The intermediate state lifetime highly dependent on the energy gap between the intermediate and excited states; as it follows the uncertain principle between time

and energy ($\Delta t \Delta E \geq \hbar/2$). Therefore, when the energy of excitation laser is close to the energy gap between the ground and excited states, ΔE gets smaller; *i.e.*, Δt can be longer; which results in larger signals. The signal enhancement using larger-energy laser for a multiphoton process is called as resonance enhancement. Resonance enhancement is widely applied to resonance Raman scattering for spectroscopy and microscopy. The advantage of resonance enhancement is that large enhancement can be achieved without inducing additional molecules, different from surface plasmon resonance.

I attempted three-photon excitation with resonance enhancement, which has not been reported before. Red functional proteins are usually excited at ~600 nm with one-photon and at ~1700 nm with three-photon. Instead, to achieve resonance enhancement, excitation wavelength at ~1300 nm was selected; therefore, the energy gap between the intermediate and excited states is smaller. In the shorter-wavelength (*i.e.*, higher-energy) excitation, excitation to a higher excited state occurs, instead of the lowest-energy excited state. After excitation to a higher-energy excited state, internal conversion to the lowest-energy excited state happens quickly (typically in a femtosecond time scale), therefore fluorescence is observed mostly from the lowest-energy excited state.

I first tested using Texas Red, which is a common red fluorescent dye to label blood vessels or proteins. As a result of using shorter-wavelength excitation, ~10-fold fluorescence signal enhancement against conventional three-photon excitation at ~1700 nm was observed. Similar signal enhancement was observed in other red fluorophores, including some red fluorescent proteins. It is expected it works for other red proteins widely; therefore, this resonance enhancement method can be applied to the *in vivo* photochemistry research.

The resonance-enhanced three-photon excitation is also useful not only for spectroscopy, but also for microscopy. Texas Red is injected to the mouse brain to label the blood vessels. Upon excitation at ~1300 nm, three-photon images were obtained with high contrast and enhanced fluorescence signals at deep brain region such as CA1 hippocampus.

Moreover, resonance-enhanced excitation method gives another application in microscopy: that is multi-color three-photon fluorescence microscopy. In life science, there is a high demand for multi-color fluorescence imaging at the same time to study molecular and cellular interactions in living tissues. However, because of larger spectral separation of excitation of green and red fluorophores, multi-color three-photon fluorescence imaging has been challenging using single excitation source despite the high demand. As discussed above, with resonance-enhanced three-photon excitation, ~1300-mn excitation is used to excite red fluorophores, differently from the conventional excitation using ~1700 nm. Hence, combining the conventional and resonance-enhanced excitation methods, both of green and red fluorophores can be excited at ~1300 nm; meaning dual-color fluorescence imaging

is possible using single-laser source. Texas Red was injected to a transgenic mouse with GCaMP6s expression. GCaMP6s is a green [Ca^{2+}] indicator to monitor neural activities in real time. We demonstrated multi-color fluorescence images of Texas Red and GCaMP6s up to ~1200 μm, which is in the CA1 hippocampus region, with high contrast. Moreover, neural activity was traced up to 10 min deep in mouse brain in awake condition.

I presented the result in the postdeadline session (which is regarded as a prestigious section) of Conference on Lasers and Electro-Optics (CLEO) 2020. Also we have published a peer-reviewed conference paper and submitted two papers to high-impact peer-review journals.