国際研究集会

〔 目　　次 〕

1．From photon to mind-advanced non-linear imaging and fluorescence-based biosensors（多光子イメージングの最前線）
（代表者：岡部繁男　2008 年 4 月 18 日－4 月 19 日）..... 460

From photon to mind-advanced non-linear imaging and fluorescence-based biosensors
（多光子イメージングの最前線）

2008 年 4 月 18 日－4 月 19 日

代表・世話人：岡部繁男（東京大学）
所内対応者：鍋倉淳一（生体恒常機能発達機構）

（１）Rapid developmental desynchronization of neocortical network activity

Portera-Cailliau C (University of California, Los Angels)

（２）Targeted whole-cell recordings from unlabeled neurons *in vivo*

Kitamura K (University of Tokyo)

（３）3D imaging modes for in vivo 2-photon microscopy

Helmchen F (University of Zurich)

（４）Microglial surveillance of synapses in the normal and damaged brain

Wake H, Nabekura J (National Institute for Physiologiccal Sciences)

（５）Imaging glia-synapse interactions

Okabe S (University of Tokyo)

（６）The promise of giant micro-photonics

Taira T (National Instiute for Molecular Science)

（７）Prospects of pin-point photoregulation of biological processed using transfected photoactivated adenylyl cyclase (PAC), the microbial blue-light sensor flavoprotein with intrinsic effector function

Watanabe M (School of Advanced Sciences, Graduate University for Acvanced Studied [SOKENDAI])

（８）Direct measurement of protein dynamics in living cells using a rationally designed photoconvertible fluorescent protein

Nagai T (Hokkaido University)

（９）Design, Synthesis and Biological Application of Chemical Probes Which Convert Biological Signals to Output

Kikuchi K (Osaka University)

（10）Visualization of biomolecules in live cells using split-reporter reconstitution analysis

Ozawa T (University of Tokyo)

（11）Imaging of intracellular hydrogen peroxide signaling with genetically encoded fluorescent indicator HyPer

Belousov V (Institute of Bioorganic Chemistry)

（12）Stroking the Synapse: Insight into Ischemic Damage and Recovery from In Vivo Two-Photon Imaging of Individual Synapses and Circuits

Murphy T (University of British Columbia)

（13）Two-photon imaging, uncaging and photoactivation of dendritic spines

Kasai H (University of Tokyo)

Poster Session

（Ｐ１）Morphological changes in spines in behaviorally activated neurons

Takuma Kitanishi, Yuji Ikegaya, Norio Matsuki, Maki K. Yamada (Grad Sch. Pharm. Sci., Univ. Tokyo)

（Ｐ２）Stress caused by mother-separation affects dendritic spine development in layer V pyramidal neuron of mouse motor cortex

Yusuke Takatsuru, Junichi Nabekura (National Institute for Physiological Sciences and JST, CREST)

（Ｐ３）In vivo imaging of intercellular messengers released from neurons

Hiroshi Sekiya, Daisuke Ino, Yohei Okubo, Shigeyuki Namiki,
Hirokazu Sakamoto,Sho Iinuma, Kenzo Hirose, Masamitsu Iino
(Graduate School of Medicine, The University of Tokyo)

（Ｐ４）In vivo time-lapse imaging of migration of cortical interneurons during postnatal development

Hiroyuki Inada[1], Hiroaki Wake[1], Tomomi, Nemoto[1], Yuchio Yanagawa[2], Junichi Nabekura[1]
([1]National Institute for Physiological Sciences,
[2]Department of Genetic and Behavioral Neuroscience, Gunma University)

（Ｐ５）Visualization of hippocampal polysynaptic computation

Rie Kimura (Laboratory of Chemical Pharmacology, Graduate School of
Pharmaceutical Sciences, The University of Tokyo)
Norio Matsuki (Laboratory of Chemical Pharmacology, Graduate School of
Pharmaceutical Sciences, The University of Tokyo)
Yuji Ikegaya (Laboratory of Chemical Pharmacology, Graduate School of Pharmaceutical Sciences,
The University of Tokyo and Precursory Research for Embryonic Science and Technology ,
Japan Science and Technology Agency)

（Ｐ６）Simultaneous monitoring of three 2nd messengers using a prism-based TIRFM system

Hideo Mogami, Eisuke Adachi, Yuko Suzuki, Tetumei Urano
(Department of Physiology, Hamamatsu University School of Medicine)

（Ｐ７）Imaging glucose uptake into single, living mammalian cells

Katsuya Yamada (Hirosaki University)

（Ｐ８）Development of Ratiometric Fluorescent Phosphatase Probes with Novel Switch Mechanism

Shuji Watanabe, Shin Mizukami and Kazuya Kikuchi (Graduate School of Engineering, Osaka University)

（Ｐ９）Characterization of newly synthesized fluorescent probes targeting hexahistidine based on molecular recognition

Atsushi Murata (Department of Life Science and Medical Bio-Science)
Su-In Yoon (Laboratory of Neural Disorders, Waseda Olympus Bioscience Research Institute)
Satoshi Arai (Department of Life Science and Medical Bio-Science)
Ritsuko Fujii (Laboratory of Neural Disorders, Waseda Olympus Bioscience Research Institute
and Advanced Research Center for Biological Science, Waseda University, Japan)
Xiaoyu Wu (Department of Chemistry, National University of Singapore, Singapore)
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Shinji Takeoka (Department of Life Science and Medical Bio-Science)
Miwako Ozaki (Laboratory of Neural Disorders, Waseda Olympus Bioscience Research Institute
and Institute for Biomedical Science, Consolidated Research Institute for Advanced Science and Medical Care
and Advanced Research Center for Biological Science, Waseda University, Japan)

（Ｐ10）Monitoring of IP3 dynamics during Ca^{2+} oscillations with FRET-based IP_3 biosensors

Akihiko Tanimura[1], Takao Morita[1], Akiko Shitara[1], Akihiro Nezu[1], Takeharu Nagai[2], Yosuke Tojyo[1]
([1]Department of Pharmacology, School of Dentistry, Health Sciences University of Hokkaido,
[2]Nanosystems Physiology, Research Institute for Electronic Science, Hokkaido University)

（P 11）In vivo two-photon calcium imaging of somatosensory cortex in a mouse model of inflammatory pain

Kei Eto (Division of Homeostatic Development, Department of Developmental Physiology,
National Institute for Physiological science and Laboratory of Pathophysioly,
Gradated School of Pharmaceutical Science, Kyushu University)
Hiroaki Wake (Division of Homeostatic Development, Department of Developmental Physiology,
National Institute for Physiological science and JST, CREST)
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National Institute for Physiological science)
Mami Noda (Laboratory of Pathophysioly, Gradated School of Pharmaceutical Science, Kyushu University)
Junichi Nabekura (Division of Homeostatic Development, Department of Developmental Physiology,
National Institute for Physiological science and JST, CREST)

（P 12）*In vivo* two-photon Ca^{2+} imaging analysis of orientation selectivity by using transgenic mice

Kazuhiro Sohya[1], Katsuro Kameyama[1], Teppei Ebina[2], Yuchio Yanagawa[3], Tadaharu Tsumoto[1]
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【概要】

革新的非線形光学系である多光子励起法は近年生物イメージング法として国内外で，急速な広がりを示している。光学系技術，光感受性化学物質の作成，および生物系への応用は急速に発展している。そのため，平成 20 年 4 月 18-19 日の 2 日間，岡崎カンファレンスセンターにて上記名の第 1 回生理研国際研究集会が開催された (http://www.nips.ac.jp/hsdev/iws2008/index.html)。国外 4 名と国内 9 名の最先端研究者による講演，12 題のポスター発表，および 126 名の国内外からの参加者があった。1) レーザー光学，2) 光感受性物質合成，および 3) 多光子励起顕微鏡の生体脳・組織応用技術についての各セッションで討論を行った。最新のマイクロチップレーザー光学技術紹介に加えて, 光感受性物質のセッションでは，光活性化アデニールサイクラーゼ，新たな蛍光蛋白，酵素活性観察プローブや蛍光分子イメージング解析について紹介があった。生物系への応用について，シナプスサイズ調節とその意義，各種グリア細胞動態，高速カルシウムシグナル取り込み法，および発達や病態時のシナプス動態が紹介された。また，集会終了後に生理学研究所において，頭蓋骨観察窓の形成術のデモンストレーションを行い 20 名以上の参加者があった。参加者が最先端レーザー顕微鏡技術・蛍光プローブ・応用技術の三位一体の最先端技術について，有意義な討論や共同研究の立ち上げを強力に推進するきっかけの場を提供して国際研究集会を終了した。

Recent advance in the non-linear imaging provides a various new finding not only in the Neuroscience, but in the wide range of biology. However, the necessary techniques for imaging are still in progress.

The first NIPS international workshop was held on April 18-19, 2009 at the Okazaki Conference Center. This meeting provided the presentations regarding the recent advances in 1) laser apparatus, 2) fluorescent probe, 3) various applications of multiphoton excitation microscopy on living animals and tissues, e.g. brain, presented by four foreign and nine internal outstanding speakers. In addition, 12 presentations were given on poster by young researchers (126 participants in total). Main topics were 1) the cloning of photosensitive enzyme and the developments of photo-probe sensing enzyme activity and new fluorescent proteins. 2) advance applications to image the spine plasticity in size, astrocyte and microglia motility, neuronal Ca^{2+} transient with a high speed acquisition system and neuronal circuits remodeling in development and neuronal damage. In addition, the surgical operation to make a cranial window for imaging was demonstrated at NIPS.

For this purpose, outstanding scientists in each field will present their recent advances in their science and technique employed.

（1）Rapid developmental desynchronization of neocortical network activity

Peyman Golshani[1], J. Tiago Goncalves[1], Ricardo Mostany[1], Stelios M. Smirnakis[2], Carlos Portera-Cailliau[1,3]
([1]Department of Neurology, David Geffen School of Medicine at UCLA,
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[3]Department of Neurobiology, David Geffen School of Medicine at UCLA.)

We use *in vivo* 2-photon calcium imaging (OGB-1-AM) and *in vivo* patch-clamp recordings of Layer 2/3 neurons in mouse barrel cortex from postnatal day (P) 4 to adult stages. Recordings are done in awake and in lightly anesthetized mice. We can monitor the activity of dozens-hundreds of neurons simultaneously at acquisition rates of 4-16 Hz. We find that synchronous activity across large ensembles of cortical neurons persists well into the second postnatal week and that a rapid switch occurs around P11-P13, after which network activity is largely de-synchronized. This abrupt change in network behavior is not modulated by sensory experience.

（2）Targeted whole-cell recordings from unlabeled neurons *in vivo*

Kazuo Kitamura (Department of Neurophysiology, Graduate School Medicine, University of Tokyo)

We have developed a new approach for making targeted whole-cell patch-clamp recordings from single neurons *in vivo* visualized using two-photon microscopy. The improved recording properties have also made it possible to make whole-cell recordings from awake animals with considerably high success rate. The method can also be used for targeted *in vivo* single cell electroporation of plasmid DNA into identified cell types, leading to stable transgene expression. This approach thus allows recording, labeling and genetic manipulation of single neurons in the intact native mammalian brain without the need to pre-label neuronal populations. Therefore, this technique will promise a wide range of applications in neuroscience research.

（3）3D imaging modes for in vivo 2-photon microscopy

Fritjof Helmchen (Brain Research Institute, University of Zurich, Switzerland)

We introduced a novel 3D laser scanning technology for two-photon microscopy that for the first time permits fast fluorescence measurements from several hundred cells distributed in 3D space. The basic idea is to perform a 3D linescan that passes through as many cell bodies as possible. We found that more than 90% of cell somata can be sampled by the scan line within volumes of 250 microns side length (containing on the order of 400 cells). We applied this method to reveal spatiotemporal activity patterns in neuronal and astrocytic networks in layer 2/3 of rat somatosensory cortex in vivo. Activity was monitored in response to local electrical or sensory stimulation and was visualized using the 3D visualization software Amira. In addition, we have devised several other laser scanning modes, which permit new views on single neurons, especially the activity pattern that occur in their dendritic trees. These modes include an “arbitrary plane imaging” mode, which is especially well suited for imaging neuronal activity in the cerebellar cortex, and a “3D ribbon scanning” technology, which enables simultaneous measurements from multiple neocortical pyramidal cell dendrites.

（4）Microglial surveillance of synapses in the normal and damaged brain

Hiroaki Wake, Junichi Nabekura
(Division of Homeostatic Development, National Institutes of Physiological Sciences.
Core Research for Evolutional Science and Technology, Japan Science and Technology Agency)

Using dual two-photon imaging of neurons and microglia *in vivo* we show that resting microglial processes make brief (～5 min) and direct contacts with neuronal synapses about once per hour, or less frequently when neuronal activity is decreased. In contrast, the contact duration between microglia and synapse following transient cerebral ischemia were markedly prolonged (～1 hour). Such prolonged microglial contact frequently resulted in disappearance of presynaptic boutons in the ischemic area. Our results directly demonstrate that resting microglia *in vivo* are monitors of the functional and pathophysiological status of synapses and its activity, and may determine the subsequent fate of ischemic synapses.

（5）Imaging glia-synapse interactions

Shigeo Okabe (Department of Cellular Neurobiology, Graduate School of Medicine, University of Tokyo)

We visualized astroglia and pyramidal neurons in hippocampal slice cultures by combining adenovirus - mediated, Cre - dependent expression of GFP with electroporation of rhodamine - dextran. Two-photon time-lapse imaging of immature dendritic protrusions and astroglial processes revealed longer lifetime of dendritic protrusions having experienced astroglial contacts than those without contacts. Dendritic protrusions with astroglial contacts also showed higher tendency to form spines. Inhibition of astroglial motility and interference of ephrin-Eph signaling affected normal stabilization and maturation of spines. These findings suggest an involvement of direct astroglia-filopodia contacts in subsequent maturation of dendritic spines.

（6）The promise of giant micro-photonics

Takunori Taira (Laser Research Center, Institute for Molecular Science)

We have demonstrated the high power cw operation (power of 414 W and power density of 0.19 MW/cm^3) in edge-pumped ceramic Yb:YAG microchip lasers, the high brightness (brightness of 0.14 PW/cm^2-sr and brightness - temperature of ～2x10^{20}K) giant pulse generation in passively Q-switched Nd:YAG microchip lasers, and the widely wavelength coverage (from UV to THz wave range) in QPM and BPM nonlinear frequency conversions). Few- to hundred-micrometer-sized ceramic grains or chips should open the door to high-average power giant pulse micro - solid-state lasers and their fruitful applications with micro - domain controlled nonlinear devices, so to speak “Giant Micro Photonics” The demonstrated widely tunable microchip lasers owing to its high brightness-temperature nature and functional wavelength conversion devices will extend the frontier of photonics.

（7）Prospects of pin-point photoregulation of biological processes using transfected photoactivated adenylyl cyclase (PAC), the microbial blue-light sensor flavoprotein with intrinsic effector function

Masakatsu Watanabe (School of Advanced Sciences and Hayama Center for Advanced Studies,
Graduate University for Advanced Studies [SOKENDAI] and
National Institute for Basic Biology, Okazaki, Japan)
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The purified flavoprotein (ca. 400 kDa), with noncovalently bound FAD, seemed to be a heterotetramer of α- and β-subunits. Predicted amino acid sequences of each of the subunits were similar to each other and contained two FAD-binding domains each followed by an adenylyl cyclase catalytic domain. As thus expected, the flavoprotein showed an adenylyl cyclase activity, which was near hudred times elevated by blue-light irradiation. Thus, PAC can directly transduce a light signal into a change in the intracellular cyclic AMP level without any other partner proteins in contrast to the GPCR systems wherein sets of three different proteins are involved to transduce the extracellular signals into intracellular output signals such as cyclic nucleotide levels.

Thus it was proposed that expression of PAC in heterologous cells would allow scientists to photo-manipulate intracellular cAMP levels with exquisite spatiotemporal control. Recent demonstrations of successful realizations as well as future prospects of this idea will be shown in the talk.

（8）Direct measurement of protein dynamics in living cells using a rationally designed photoconvertible fluorescent protein

Takeharu Nagai, Tomoki Matsuda (Research Institute for Electronic Science, Hokkaido University)
Atsushi Miyawaki (Brain Science Institute, RIKEN)

I introduce a new member of photoconvertible fluorescent protein, Phamret, which consists of CFP and PA-GFP. The photoconversion mechanism of Phamret is based on photoactivation-mediated Förster resonance energy transfer (FRET) from CFP to PA-GFP. Before UV stimulation, PA-GFP in Phamret does not work as an acceptor for FRET, then cyan fluorescence is detected by excitation at 458 nm. After UV stimulation, PA-GFP is irreversibly activated to function as a FRET acceptor, thereby Phamret emits green fluorescence by excitation at 458 nm. Thus, Phamret can be monitored by single excitation-dual emission mode allowing mobility analyses over a broad range of kinetics. HeLa cells expressing Phamret with several targeting sequences showed the clear localization to the correct destination of subcellular organelles, and were successfully highlighted in specific region by UV stimulation. In addition, we have also devised a new microscopic method for accurate measurement of diffusion coefficient of biomolecules from the fluorescence decay after photostimulation. By combining these two technologies, we are now able to measure diffusion kinetics ranging from less than 0.1 μm^2/sec up to approximately 100 μm^2/sec, and have found significant changes in free protein movement during cell cycle progression.

（9）Design, Synthesis and Biological Application of Chemical Probes Which Convert Biological Signals to Chemical Output

Kazuya Kikuchi (Graduate School of Engineering, Osaka University)

We propose a novel design strategy for ^{19}F-MRI probes to detect protease activities and to demonstrate its practical applicability. The design principle is based on the paramagnetic relaxation effect from Gd^{3+} to ^{19}F. A peptide was synthesized, Gd-DOTA-DEVD-Tfb, attached to a Gd^{3+} complex at the N-terminus and a ^{19}F-containing group at the C-terminus. The ^{19}F-NMR transverse relaxation time (T_2) of the compound was largely shortened by the paramagnetic effect of intramolecular Gd^{3+}. The peptide was designed to have a sequence cleaved by an apoptotic protease, caspase-3. When the peptide was incubated with caspase-3, the peptide was cleaved and subsequently the Gd^{3+} complex and the ^{19}F-containing group were separated from each other. T_2, after cleavage, was extended to cancel the intramolecular paramagnetic interaction. T_2 is a parameter that can be used to generate contrasts in MR images. Using this probe as a positive contrast agent, the probe could detect caspase-3 activity spatially from a phantom image using ^{19}F MRI.

（10）Visualization of biomolecules in live cells using split-reporter reconstitution analysis

Takeaki OZAWA (Department of Chemistry, School of Science, The University of Tokyo and JST)

The most common reporters are firefly luciferase, renilla luciferase, green fluorescent protein (GFP) and its variants with various spectral properties. Herein, novel design of split GFP and split luciferase will be described; the principle is based on reconstitution of the split-reporter fragments when they are brought together into close proximity. To demonstrate the usefulness of the split-GFP reporters, we have used the reporters for developing a genetic method to identify mitochondrial proteins and their localization, and imaging dynamics of endogenous mRNA in single living cells. We have used a split-luciferase reporter for noninvasive imaging of protein interactions in living subjects. We have developed another design of reporter proteins; a cyclic luciferase by protein splicing to monitor protease activities in living mice. These reporter proteins are generally applicable for visualization of complex cellular processes in living cells and animals.

（11）Imaging of intracellular hydrogen peroxide signaling with genetically encoded fluorescent indicator HyPer

Belousov V (Institute of Bioorganic Chemistry)

Using HyPer we investigated the formation of H_2O_2 levels during phagocytosis in realtime. Upon phagocytosis of opsonized zymosan (OPZ) particles RAW 264.7 cells transiently elevated H_2O_2 that was started to rise immediately following particle ingestion and peaked after about 2 minutes. Within the following 10-16 minutes H_2O_2 concentration decreased to the basal level without further changes. Increased H_2O_2 levels were delocalized throughout cytoplasm. The use of a reversible probe for intracellular H_2O_2 detection, allows us to re-evaluate the existing view on the function of ROS in phagocytoting cells.

（12）Stroking the Synapse: Insight into Ischemic Damage and Recovery from In Vivo Two-Photon Imaging of Individual Synapses and Circuits

Timothy H. Murphy (Univ. of British Columbia, Vancouver Canada)

In vivo two-photon calcium imaging was used in adult mice within reorganized forelimb and hindlimb somatosensory functional maps to determine how the response properties of resident neurons and glia are altered during recovery from ischemic damage over 2-8 weeks. Single cell imaging reveals that the limb-selectivity of individual neurons was altered by ischemia, such that neurons normally selective for a single contralateral limb processed information from multiple limbs. Altered limb-selectivity was most prominent in border regions between stroke-altered forelimb and hindlimb macroscopic representations, and peaked one month after the targeted insult. Two months after stroke, individual neurons near the center of reorganized functional areas became more selective for a preferred limb. These data indicate that in adult animals seemingly hardwired cortical neurons first adopt wider functional roles as they develop strategies to compensate for loss of specific sensory modalities following forms of brain damage such as stroke.

（13）Two-photon imaging, uncaging and photoactivation of dendritic spines

Haruo Kasai (Graduate School of Medicine, The University of Tokyo)

We have recently succeeded in applying two-photon uncaging methodology to adult neocortex in vivo. We found caged-compounds to rapidly diffuse into the brain, and uncaging of glutamate at the dendrites of whole-cell clamped neurons yield the map of glutamate sensitivity akin to those in slice preparations, indicating that the structure-functions relationship is preserved in the brain in vivo. We also succeeded in inducing long-term enlargement of spines by repetitive uncaging in 0 Mg solution. The enlargement occurs rapidly in vivo as in vitro. Thus, structural plasticity of dendritic spines can be utilized to further clarify brain function.