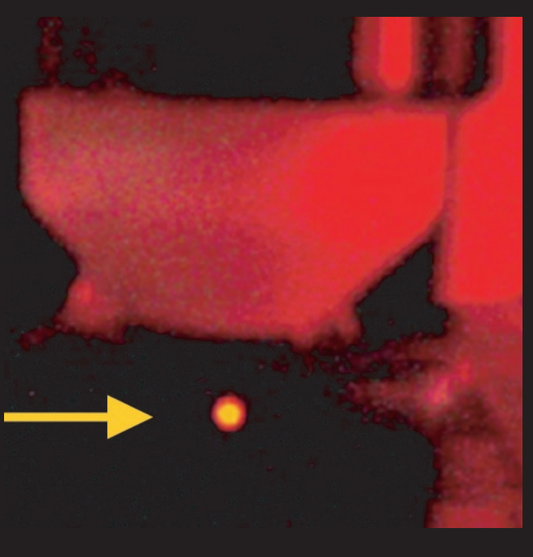
NIPS - JST　国際ワークショップ

- From photon to mind -

Advanced Nonlinear Imaging
and
Fluorescence-based Biosensors

２００８年4月１８日（金）−１９日（土）

参加費：無料



多光子励起顕微鏡の個体・生物応用
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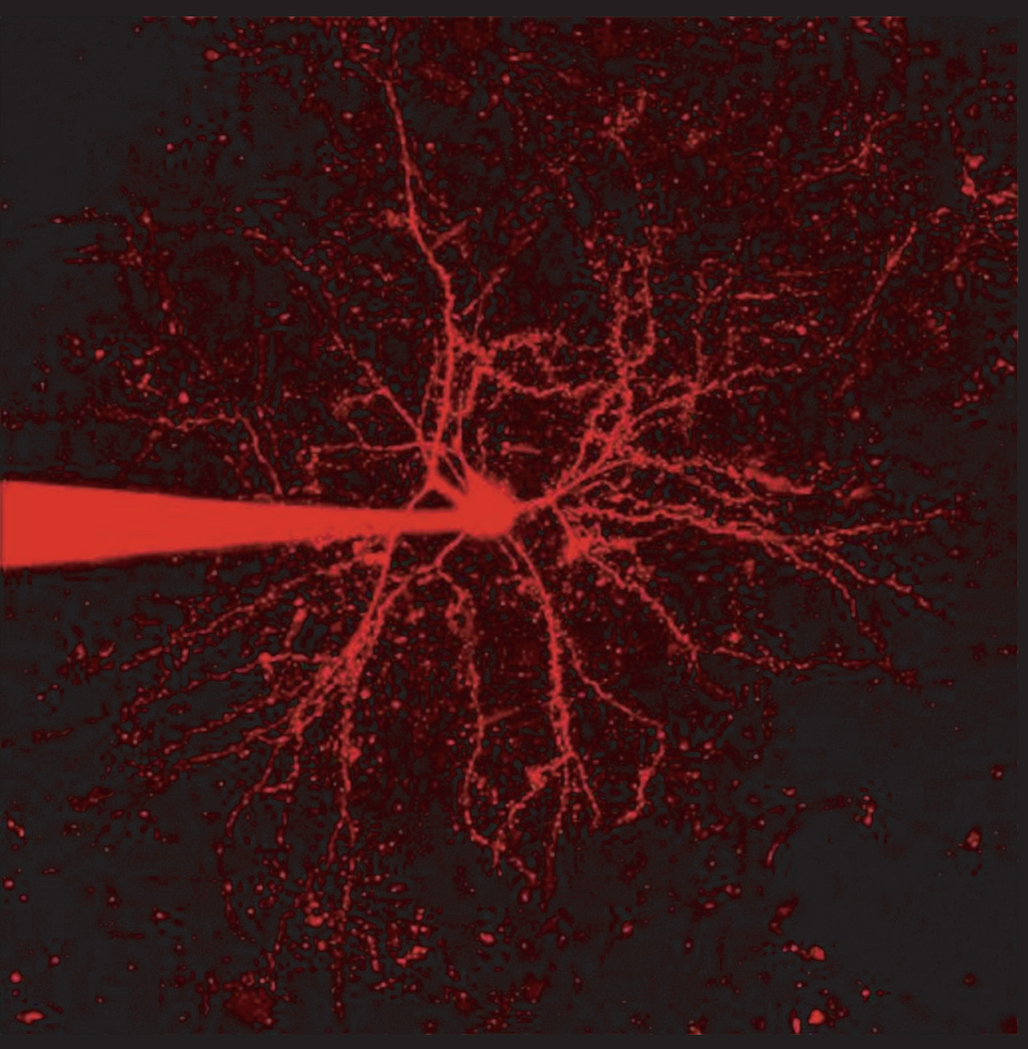
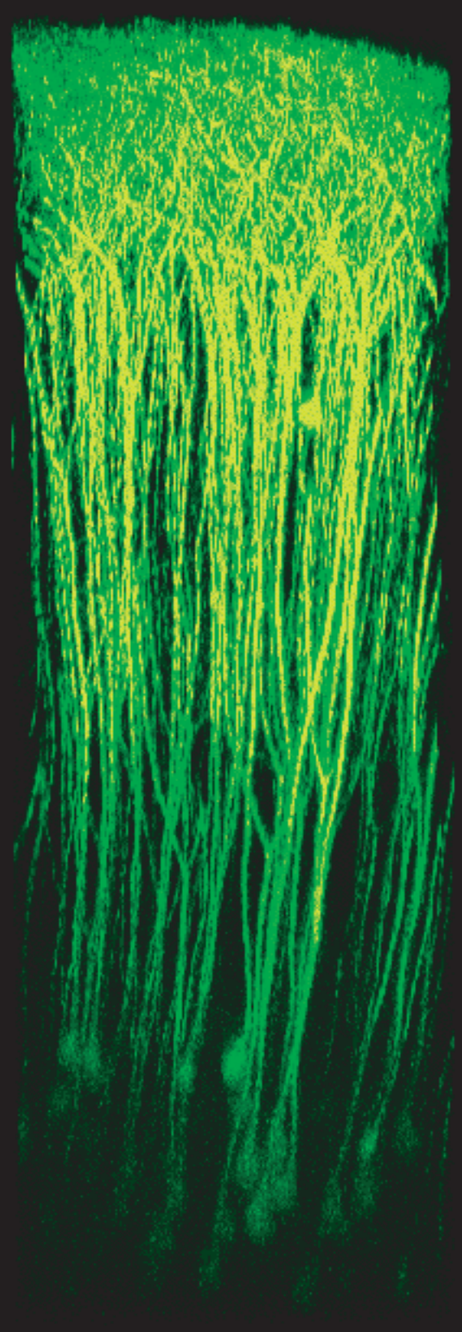
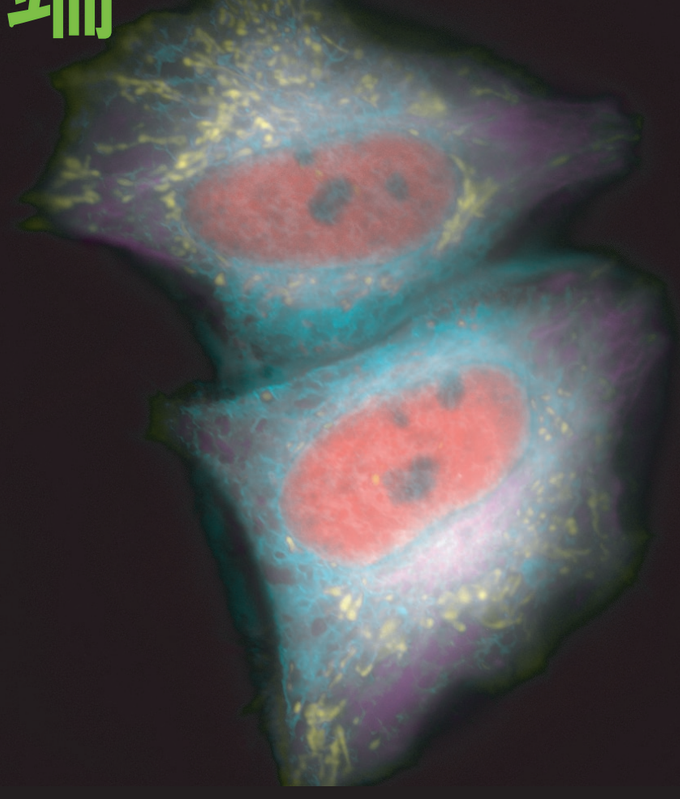
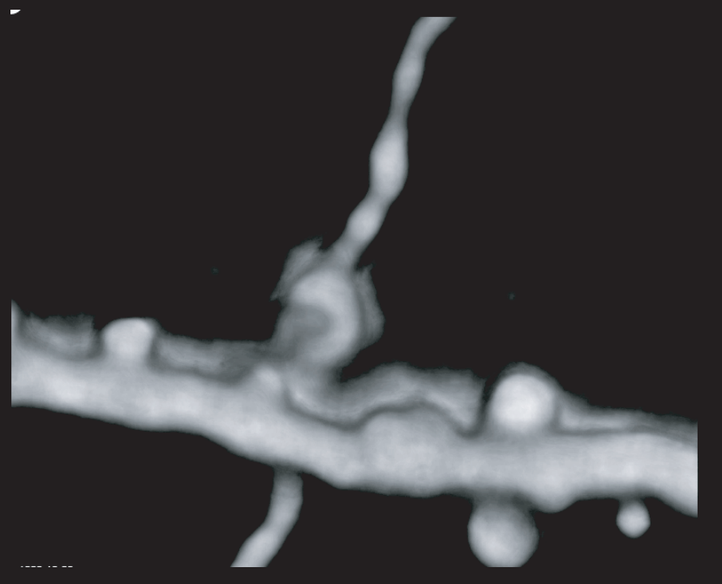








主催：生理学研究所、科学技術振興機構

NIPS－JST International Workshop

-From photon to mind -

Advanced Nonlinear Imaging

&

Fluorescence-based Biosensors

April 18th-19th, 2008
Okazaki Conference Center
(7-min walk from Higashi-Okazaki Station of Meitetsu Railroad)
Admission fee: free
Reception (including Snack at 19th): 5,000yen
Registration: March 1st to April 4th
http://www.nips.ac.jp/hsdev/iws2008

Program

April 18th (Friday)

12:00－13:00 Registration
13:00-13:05 Opening Remark (Okabe S)

JST Workshop

Advances in Non-linear Imaging I (Chaired by Matsui K)

13:05-13:50 Portera-Cailliau C (University of California, Los Angels)
Rapid developmental desynchronization of neocortical network acitivity

13:50-14:20 Kitamura K (University of Tokyo)
Targeted whole-cell recordings from unlabeled neurons in vivo

14:20–15:05 **Helmchen F** (University of Zurich)
3D imaging modes for in vivo 2-photon microscopy

15:05–16:00 Group Photo (in front of the Okazaki Conference Center)
Coffee Break & Poster Session

NIPS Workshop

Advances in Non-linear Imaging II (Chaired by Kitamura K)

16:00–16:30 **Wake H、Nabekura J** (National Institute for Physiological Sciences)
Microglial surveillance of synapses in the normal and damaged brain

16:30–17:10 **Okabe S** (University of Tokyo)
Imaging glia-synapse interactions

Recent Advances of Laser & Photoactivated Probe (Chaired by Nemoto T)

17:10–17:50 **Taira T** (National Institute for Molecular Science)
The promise of giant micro-photonics

17:50–18:30 **Watanabe M** (School of Advanced Sciences, Graduate University for Advanced Studies [SOKENDAI])
Prospects of pin-point photoregulation of biological processes using transfected photoactivated adenylyl cyclase (PAC), the microbial blue-light sensor flavoprotein with intrinsic effector function

Reception 18:30－20:30

April 19th (Saturday)

Fluorescence-Based Biosensor (Chaired by Watanabe M)

8:50 - 9:30 **Nagai T** (Hokkaido University)
Direct measurement of protein dynamics in living cells using a rationally designed photoconvertible fluorescent protein

9:30 - 10:10 **Kikuchi K** (Osaka University)
Design, Synthesis and Biological Application of Chemical Probes Which Convert Biological Signals to Output

10:10－10:50 **Ozawa T** (University of Tokyo)
Visualization of biomolecules in live cells using split-reporter reconstitution analysis

10:50-11:35 **Belousov V** (Institute of Bioorganic Chemistry)
Imaging of intracellular hydrogen peroxide signaling with genetically encoded fluorescent indicator HyPer

11:35-13:00 Poster Session & Snack

Advances in Nonlinear Imaging III (Chaired by Nabekura J)

13:00-13:45 **Murphy T** (University of British Columbia)
Stroking the Synapse: Insight into Ischemic Damage and Recovery from In Vivo Two-Photon Imaging of Individual Synapses and Circuits

13:45-14:25 **Kasai H** (University of Tokyo)
Two-photon imaging, uncaging and photoactivation of dendritic spines

14:25－14:30 Closing Remark (Nabekura, J)

15:00-17:00
Demonstration: Procedure to make Cranial Window
(Portera-Cailliau C)
at NIPS, 5th Floor

Poster session

P-1 **Morphological changes in spines in behaviorally activated neurons**
Takuma Kitanishi, Yuji Ikegaya, Norio Matsuki, Maki K. Yamada

P-2 **Stress caused by mother-separation affects dendritic spine development in layer V pyramidal neuron of mouse motor cortex**
Yusuke Takatsuru, Junichi Nabekura

P-3 ***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

P-4 ***In vivo* time-lapse imaging of migration of cortical interneurons during postnatal development**
Hiroyuki Inada, Hiroaki Wake, Tomomi, Nemoto, Yuchio Yanagawa, Junichi Nabekura

P-5 **Visualization of hippocampal polysynaptic computation**
Rie Kimura, Norio Matsuki, Yuji Ikegaya

P-6 **Simultaneous monitoring of three 2nd messengers using a prism-based TIRFM system**
Hideo Mogami, Eisuke Adachi, Yuko Suzuki, Tetumei Urano

P-7 **Imaging glucose uptake into single, living mammalian cells**
Katsuya Yamada

P-8 **Development of Ratiometric Fluorescent Phosphatase Probes with Novel Switch Mechanism**
Shuji Watanabe, Shin Mizukami and Kazuya Kikuchi

P-9 **Characterization of newly synthesized fluorescent probes targeting hexahistidine based on molecular recognition**
Atsushi Murata, Su-In Yoon, Satoshi Arai, Ritsuko Fujii, Xiaoyu Wu, Yixin Lu, Shinji Takeoka, Miwako Ozaki

P-10 **Monitoring of IP_3 dynamics during Ca^{2+} oscillations with FRET-based IP_3 biosensors**

Akihiko Tanimura, Takao Morita, Akiko Shitara, Akihiro Nezu, Takeharu Nagai & Yosuke Tojyo

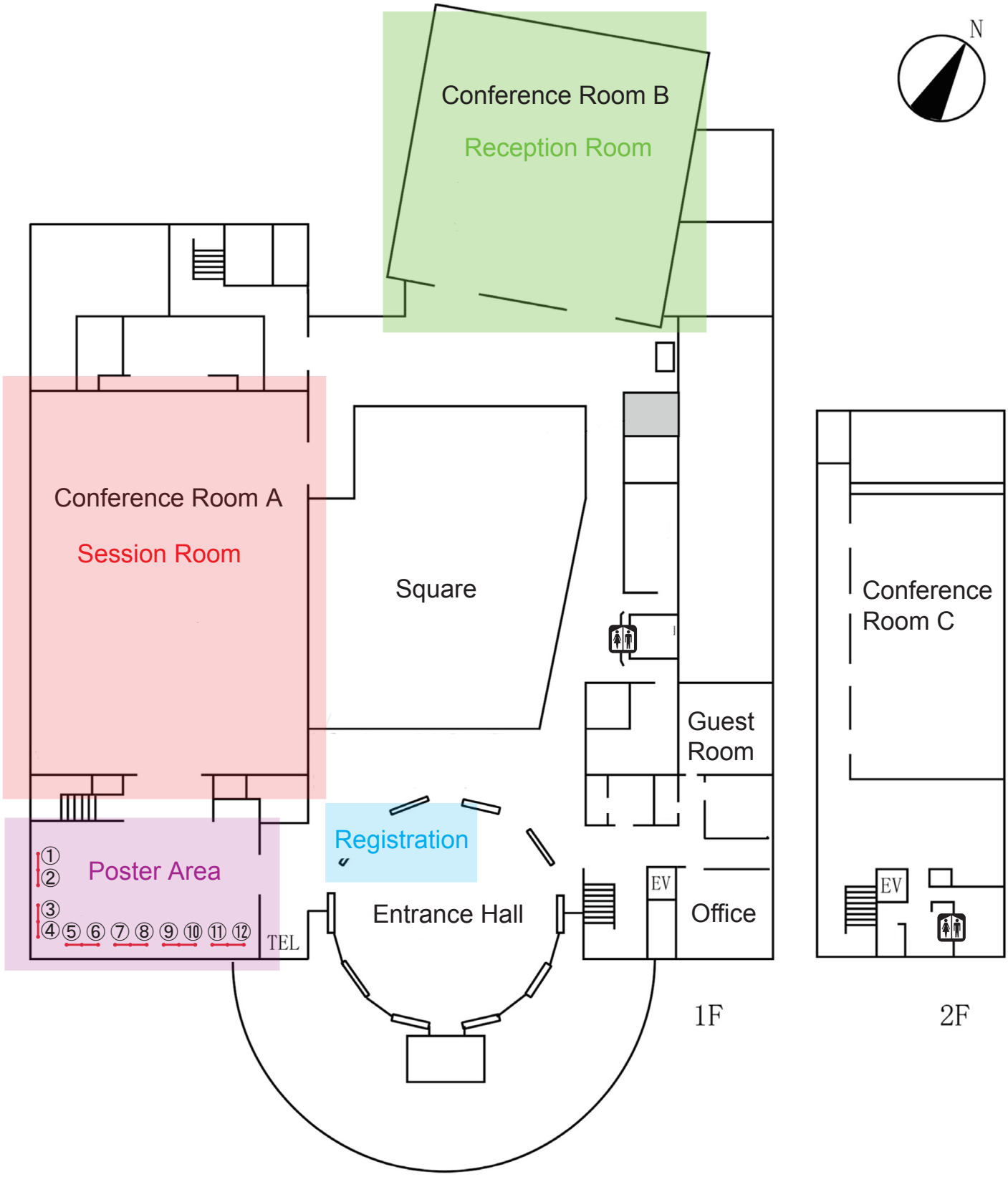
P-11 ***In vivo* two-photon calcium imaging of somatosensory cortex in a mouse model of inflammatory pain**

Kei Eto, Hiroaki Wake, Hitoshi Ishibashi, Mami Noda, Junichi Nabekura

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

Kazuhiro Sohya, Katsuro Kameyama, Teppei Ebina, Yuchio Yanagawa, Tadaharu Tsumoto

Okazaki Conference Center Information Map



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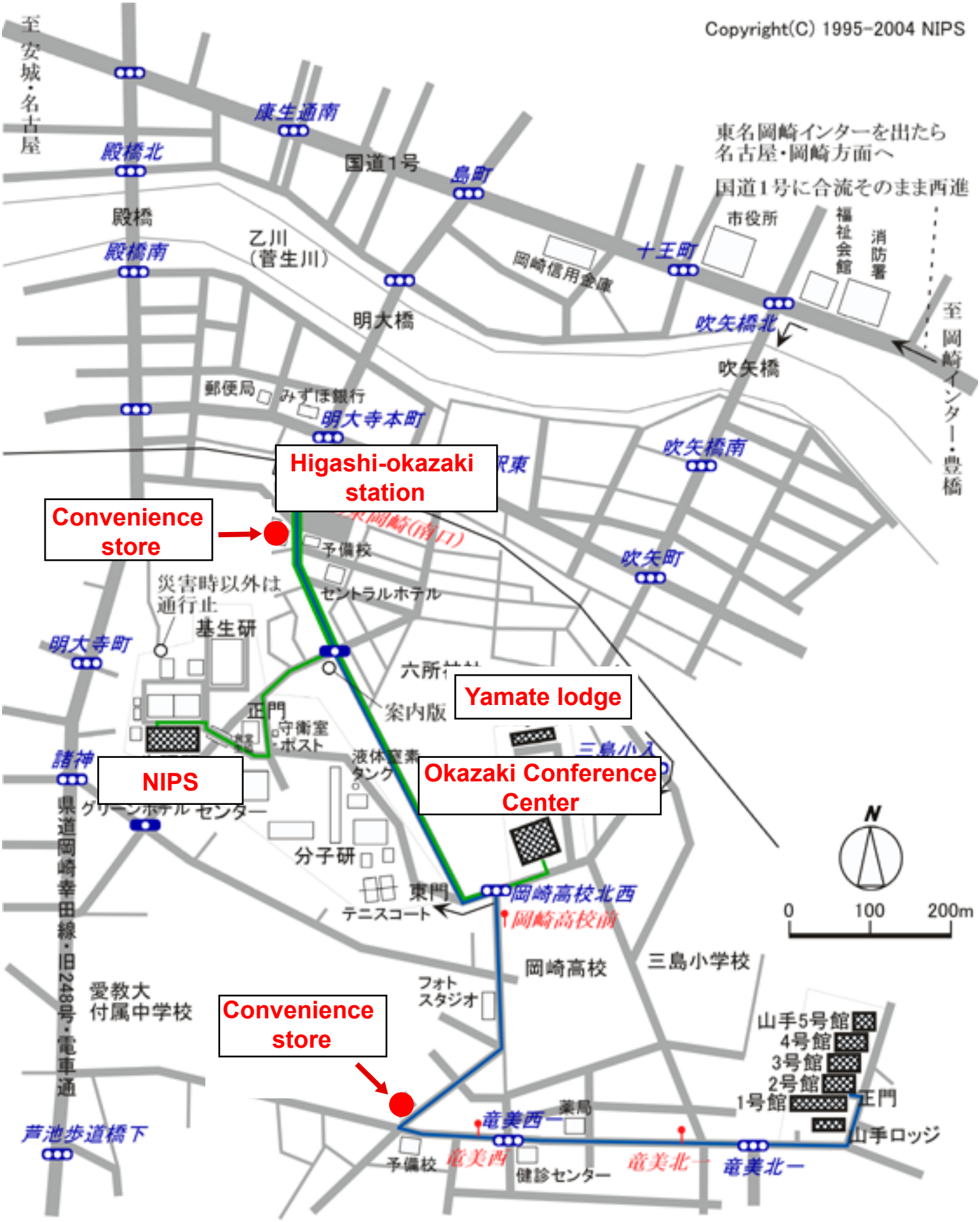
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Rapid developmental desynchronization of neocortical network activity

Peyman Golshani[1], J. Tiago Goncalves[1], Ricardo Mostany[1], Stelios M. Smirnakis[3], and Carlos Portera-Cailliau [1,2].

[1]Department of Neurology, David Geffen School of Medicine at UCLA. [2]Department of Neurobiology, David Geffen School of Medicine at UCLA. [3] Department of Neurology, Baylor College of Medicine

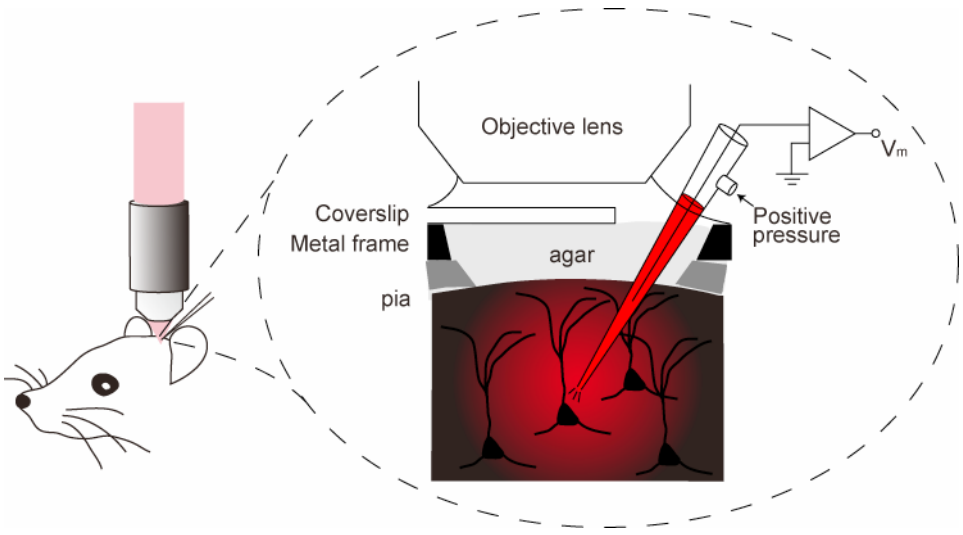
Propagation of synchronized network activity through immature neocortical networks is thought to play an essential role in the activity-dependent refinement of neocortical maps. Several *in vitro* and *in vivo* studies have demonstrated the existence of widespread synchronous firing of neocortical neurons during the first postnatal week (e.g., spindle bursts and early network oscillations). Eventually, slow (0.2 - 0.5 Hz) oscillations with sparse firing of action potentials predominate in adult neocortex, but when or how fast the switch occurs is not known. 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.

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. A patch electrode is used to perfuse the extracellular space surrounding the neuron of interest with a fluorescent dye, thus allowing the neuron to be visualized as a negative image ('shadow') and identified on the basis of its somatodendritic structure. The same electrode is then placed on the neuron under visual control to allow gigaseal formation ('shadowpatching'). We demonstrate the reliability and versatility of shadowpatching by performing whole-cell recordings from visually identified neurons in neocortex and cerebellum of rats and mice. 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.



3D imaging modes for in vivo 2-photon microscopy

Fritjof Helmchen
Brain Research Institute, University of Zurich, Switzerland

Spatiotemporal activity patterns in three-dimensionally organized cellular networks are fundamental to the function of the nervous system. Recently, new methods for *in vivo* staining of cell populations with anatomical and functional indicators have been developed. Combined with two-photon microscopy they enable studies of network dynamics in the neocortex. In particular, bulk-loading with calcium indicator dye now permits indirect measurements of neural activity based on spike-evoked calcium transients, even with single-spike resolution. In addition, calcium signals in the surrounding astrocytic network and the neuropil can be measured simultaneously. We are developing imaging techniques for functional 3D imaging of neuronal activity with sufficiently high temporal resolution to resolve spiking activity (>= 10 Hz). 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. In this talk, I will give an overview of the new imaging modes, present recent advances in 3D image processing for analysis of the functional data, and show preliminary data from imaging experiments in rat barrel cortex.

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

As well as in development, the remodeling of neuronal network has been proposed during the recovery after various neuronal damages. Indeed, time lapse imaging reveals characteristic remodeling of peripheral neuron network with neuronal injury. Time lapse imaging by using in vivo two photon microscopy combined with transgenic mice expressing various fluorescent proteins in specific brain cells leads to observe fine structures of labeled cells in an in vivo animal. Resting microglial processes in the intact brain are dynamic, extending and retracting their processes, with occasional pauses, as if actively surveying the neuronal environment. 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. Our results demonstrate the functional relation of the migroglia-synapse connections in the pathological state and indicate the possibility of the future investigation of the various therapy of the injured brain targeted to the microglia.

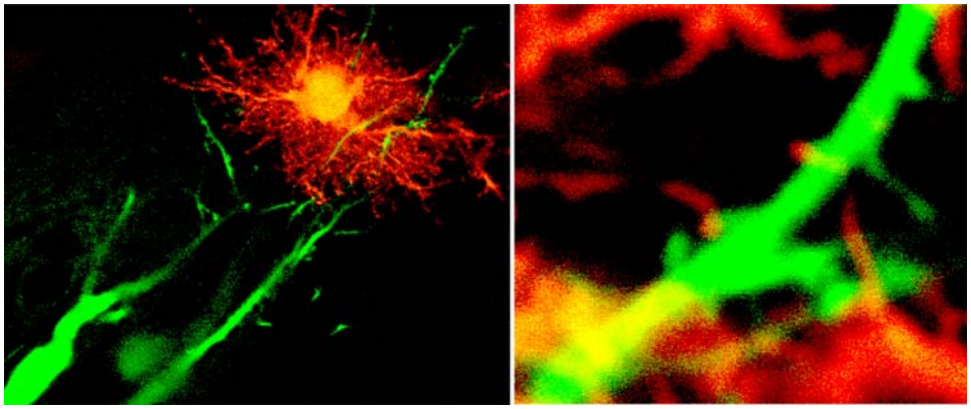
Imaging glia-synapse interactions

Shigeo Okabe

Department of Cellular Neurobiology, Graduate School of Medicine, University of Tokyo

Several lines of evidences indicate roles of astroglia in synaptogenesis, possibly mediated by either cell adhesion or diffusible factors. However, structural evidences supporting this claim are virtually lacking, mainly due to technical limitations in simultaneous imaging of neuronal and astroglial structures. 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.

Interaction between immature apical dendrites of CA1 pyramidal neurons (green) and an astrocyte (red)　(Left: lower magnification view, Right; higher magnification view).



The promise of giant micro-photonics

Takunori Taira

Laser Research Center, Institute for Molecular Science (IMS), Myodaiji, Okazaki 444-8585, Japan

Progress of the micro domain structure and boundary engineering brings the new optical function in the conventional materials; their compact solid-state optical devices are reliable, efficient and multi-functional light sources. The new generation of micro and/or microchip solid-state [1], ceramic and single-crystal, lasers can provide excellent spatial mode quality and narrow linewidths with enough power. High-brightness nature of these lasers has allowed efficient wavelength extension by nonlinear frequency conversion. On the other hand, the quasi phase matching (QPM) is an attractive technique for compensating phase velocity dispersion in frequency conversion. Inasmuch as the pool of mature nonlinear optical materials is limited and development of new materials takes time and cost, QPM is a useful method for extending the range of birefingent phase matching (BPM) based available nonlinear optical materials.

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 (Fig. 1) [2], 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 (Fig. 2), and the widely wavelength coverage (from UV to THz wave range) in QPM and BPM nonlinear frequency conversions [3, 4] (Fig. 3). 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.

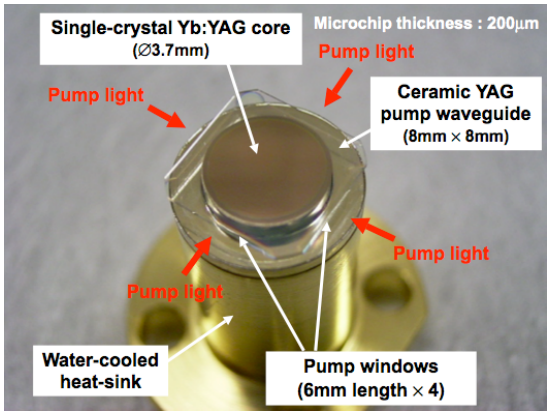


Fig. 1 Edge pumped Yb:YAG ceramic microchip laser

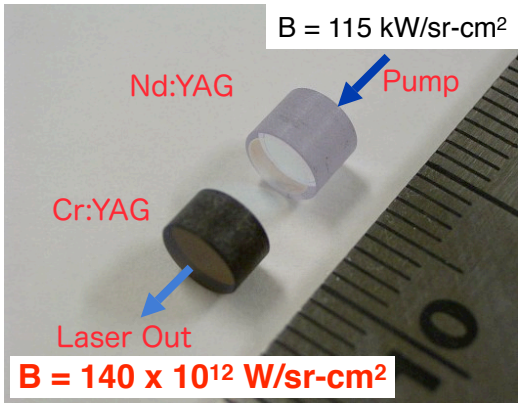


Fig. 2 Passively Q-switched Nd:YAG microchip laser

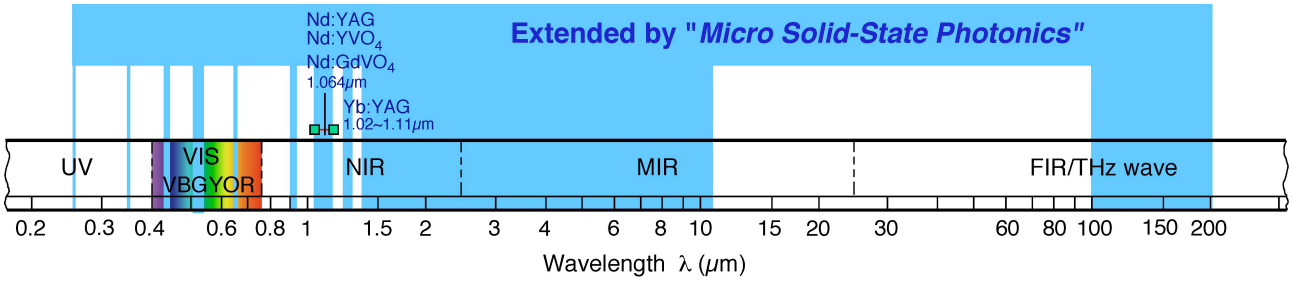


Fig. 3 High brightness-temperature microchip lasers allow us the wavelength extension from THz wave until UV region with the efficient nonlinear wavelength conversion.

References

1. T. Taira, A. Mukai, Y. Nozawa, and T. Kobayashi, "Single-mode oscillation of —" Opt. Lett., **16**, 1955 (1991).
2. T. Taira, "RE^{3+}-ion-doped YAG ceramic lasers" IEEE J. Sel. Top. Quantum Electron., **13**, 798 (2007). ***Invited***
3. T. Taira, Y. Matsuoka, H. Sakai, A. Sone, and H. Kan, "Passively Q-switched—" CLEO, CWF6, in Long Beach, USA (2006).
4. S. Hayashi, T. Taira, and *et al.*, "Compact terahertz-wave parametric —" CLEO, CTuGG2, in Long Beach, USA (2006).

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[a, b, c] and Mineo Iseki[b]

[a]School of Advanced Sciences and [b]Hayama Center for Advanced Studies, Graduate University for Advanced Studies [SOKENDAI], Hayama, Kanagawa 240-0193, Japan

and

[c]National Institute for Basic Biology, Okazaki, Aichi 444-8585, Japan

In 2002, we reported the discovery and characterization of a unique blue-light sensor protein with an intrinsic effector function (photoactivated adenylyl cyclase; PAC)[1] from *Euglena gracilis*, a unicellular flagellate alga which shows blue-light-responsive photomovement responses to locate itself at appropriate light environments. The action spectra for these responses indicate the involvement of flavoproteins as the light sensors mediating them. The paraflagellar body (PFB), a swelling near the base of the flagellum showing a flavin-type fluorescence has been considered as a light-sensing organelle for the photomovements. To identify the light sensor molecules in the PFB, we isolated PFBs and purified the flavoproteins therein using the flavin-type fluorescence as the marker. 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.[1]

Thus it was proposed that expression of PAC in heterologous cells would allow scientists to photo-manipulate intracellular cAMP levels with exquisite spatiotemporal control.[1] Recent demonstrations of successful realizations[2, 3] as well as future prospects of this idea will be shown in the talk.

References

[1]Iseki M, Matsunaga S, Murakami A, Ohno K, Shiga K, Yoshida K, Sugai M, Takahashi T, Hori T, Watanabe M. (2002). A blue-light-activated adenylyl cyclase mediates photoavoidance in *Euglena gracilis*. Nature *415*, 1047-1051,

[2]Schroeder-Lang S., Schwaerzel M., Seifert R., Struenker T., Kateriya S., Looser J., Watanabe M., Kaupp U.B., Hegemann P., Nagel G. (2007). Fast manipulation of cellular cAMP level by light *in vivo*. Nat. Meth. *4*, 39-42.

[3]Nagahama T., Suzuki T., Yoshikawa S., Iseki, M. Functional transplant of photoactivated adenylyl cyclase (PAC) into *Aplysia* sensory neurons. (2007). Neurosci. Res. *59*, 81-88.

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

Takeharu Nagai[1], Tomoki Matsuda[1] and Atsushi Miyawaki[2]
[1]Research Institute for Electronic Science, Hokkaido University
[2]Brain Science Institute, RIKEN

Application of fluorescent proteins for cell biological studies is expanding from conventional usage such as fluorescent tag for protein tracking or reporter for gene activity. Recently, several fluorescent proteins including PA-GFP, Kaede and Kindling, which change in the emission spectra upon UV stimulation, have been reported. In this symposium, 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 (Figure 1). 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.

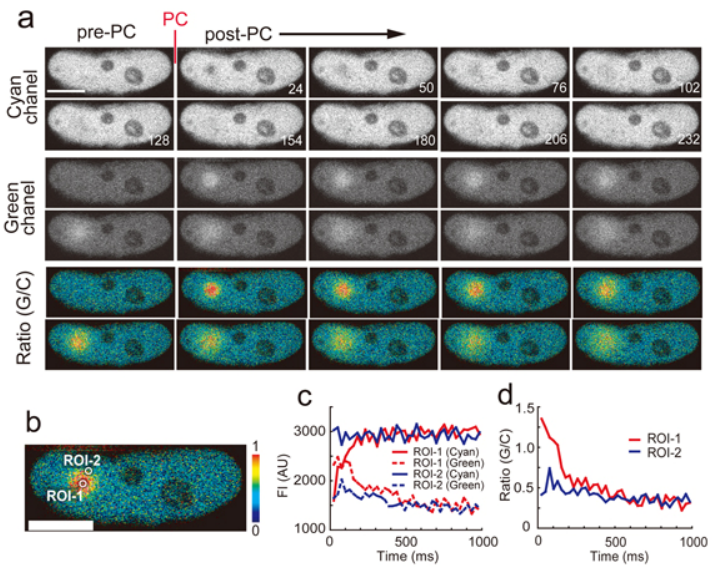


Figure 1. Visualization of rapid protein dynamics using Phamret (**a**) A series of confocal images of donor CFP (upper), acceptor PA-GFP (middle), and pseudocolored emission ratio showing diffusion of photoconverted PP2Cγ-Phamret. Images were taken every 26 msec. (**b**) A magnified view of the first image just after photoconversion. The white circles (diameter 1.10 mm) represent the regions of interest (ROI) for intensity calculation. ROI-1 was set on the photoconverted (PC) circular region (diameter 1.38 mm). ROI-2 was placed outside of the photoconverted region. Distance between the center of two ROIs is 2.1 mm. (**c**) Time course of cyan (solid line) and green (dashed line) fluorescence intensity of Phamret in ROI-1 (red) and ROI-2 (blue). (**d**) Time course of green to cyan emission ratio in ROI-1 (red) and ROI-2 (blue). Scale bar, 10 mm.

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

Kazuya Kikuchi
Graduate School of Engineering, Osaka University
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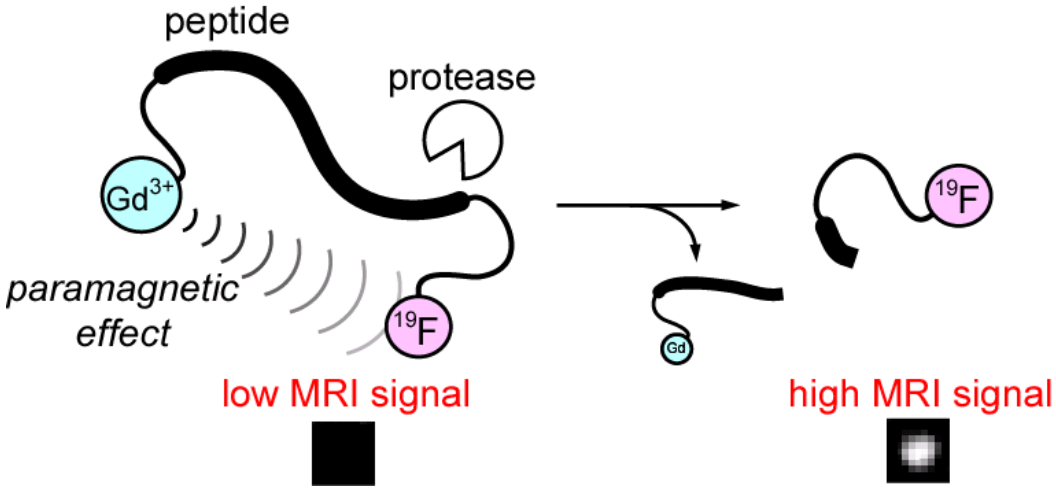
One of the great challenges in the post-genome era is to clarify the biological significance of intracellular molecules directly in living cells. If we can visualize a molecule in action, it is possible to acquire biological information, which is unavailable if we deal with cell homogenates. One possible approach is to design and synthesize chemical probes that can convert biological information to chemical output. For this purpose, fluorescent sensor molecules based on modulation of coordination space for intracellular messengers have been developed and successfully applied to living cells.

Real-time imaging of enzyme activities *in vivo* offers valuable information in understanding living systems and in the possibility to develop medicine to treat various forms of diseases. Magnetic resonance imaging (MRI) is an imaging modality adequate for *in vivo* studies. Therefore, many scientists are interested in the development of MRI probes capable of detecting enzyme activities *in vivo*. However, in the case of ^{1}H-MRI probes, interference from the background signals intrinsic to ^{1}H becomes problematic. Because such a background signal is hardly detectable, ^{19}F-MRI probes are promising for *in vivo* imaging. Despite this potential, few principles exist for designing ^{19}F-MRI probes to detect enzyme activities. 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.



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

Takeaki OZAWA

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A current focus of biological research is to quantify and image cellular processes in living subjects. To detect such cellular processes, genetically-encoded reporters have been extensively used. 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.

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

Belousov V
Institute of Bioorganic Chemistry

Reactive oxygen species (ROS) have long been implicated in a great number of pathologies such as cardiovascular and neurodegenerative disorders, cancer, inflammatory and immune diseases. Aside of that, many researchers have for a while being considering intracellular ROS as being essential in regulating the cell physiology e.g., embryogenesis, innate immunity, cell growth, etc. However, a truly new era in ROS research started a couple of years ago when H_2O_2 was positively identified as a messenger molecule acting by selective modification of thiol groups in protein tyrosine phosphatases, thereby transducing the regulatory signal from the cell surface receptors to MAP kinases. Since that discovery, a new "intracellular H_2O_2 signaling" field of science had been born.

Several approaches have been developed for estimating ROS production *in vitro*. Among them dichlorofluorescein (DCF) derivatives are widely used to detect intracellular ROS because they fluoresce after oxidation by ROS. However, DCF derivatives are sensitive to multiple types of ROS, including several reactive oxygen and nitrogen species. Also, these dyes cannot be targeted to specific intracellular compartments. An even more serious disadvantage of DCF derivatives is that they can produce ROS upon exposure to light. Thus, visualization of ROS with DCF can result in artifactual ROS generation and signal amplification.

To overcome the disadvantages of existing methods for detecting ROS, we designed a genetically encoded biosensor for hydrogen peroxide (H_2O_2). This sensor consists of circularly permuted yellow fluorescent protein (cpYFP) inserted into OxyR-RD. The indicator named HyPer demonstrates submicromolar affinity to H_2O_2, and at the same time it is insensitive to other oxidants.

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.

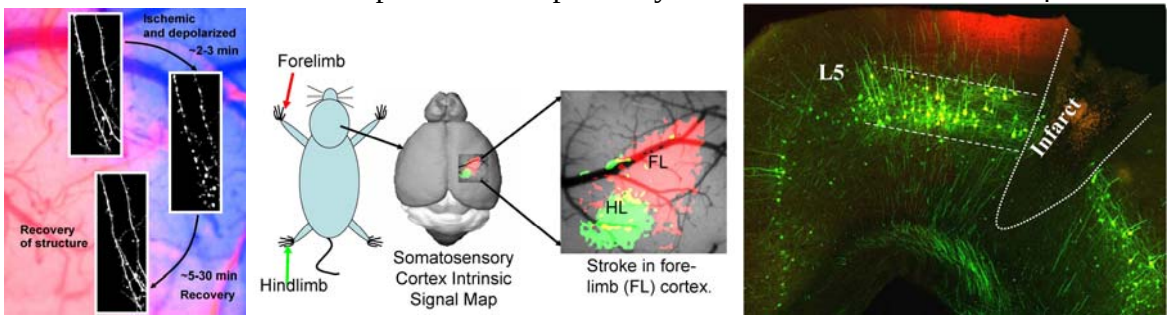
Stroking the Synapse: Insight into Ischemic Damage and Recovery from In Vivo Two-Photon Imaging of Individual Synapses and Circuits. Timothy H. Murphy Univ. of British Columbia, Vancouver Canada thmurphy@interchange.ubc.ca

Transgenic mice with fluorescently labeled neurons provide a new tool to assess mechanisms of stroke damage and recovery. Brain synaptic wiring is a target of ischemia and is degraded within minutes of stroke onset by a wave of ischemic depolarization. Prompt reperfusion within the first hour after stroke onset can reverse most structural damage. However, without reperfusion the brain must cope with lost sensory and motor modalities.

Over weeks to months after stroke, macroscopic imaging studies suggest that a key component of stroke recovery is the transfer of function from damaged brain areas to surviving peri-infarct regions. Although new areas of activation have been reported, how sensory information flows in and out these regions on a millisecond time scale, and the structural basis are not understood. Using *in vivo* voltage sensitive dye imaging combined with analysis of dendritic spine plasticity and tract tracing, we define new principles by which reorganized cortical networks operate. Several weeks after stroke in the adult mouse forelimb sensorimotor cortex, forelimb-evoked responses re-emerged in adjacent peri-infarct motor and hindlimb areas, and appeared to recruit medial-posterior cortical domains. Functional re-mapping of the forelimb representation was driven, not by an increase in peri-infarct motor and hindlimb responsiveness per se, but rather an increase in the amount of time these areas spent processing forelimb related sensations. Structurally, reorganized areas selectively-exhibited high levels dendritic spine turnover and were the recipients of both new inputs from medial-posterior cortical areas, and made functionally enhanced striatal outputs.

Although voltage sensitive dye imaging indicated new patterns of sensory activation, it was not clear how single neurons are altered in relation to cortical maps. It is conceivable that individual surviving neurons adopt new roles at the expense of their usual function. *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. Our findings indicate that the re-mapping of a sensory representation during recovery from stroke is associated with profound changes, not only in fine synaptic structure or long-range input and output connections, but changes in the fundamental manner in which recovered areas process sensory information.

Reversible structure. Maps and stroke plasticity. New connections in the peri-infarct.



Zhang et al. 2005 J. Neurosci.; Zhang and Murphy PloS Biol. 2007; Brown et al. 2007 J. Neurosci.; Murphy et al. 2008 J. Neurosci.

Two-photon imaging, uncaging and photoactivation of dendritic spines.

Haruo Kasai Graduate School of Medicine, The University of Tokyo

We have developed an optical method to stimulate single spines using two-photon uncaging of caged-glutamate, and revealed that there is a strong structure-function relationship of dendritic spines (1), and suggested that structures of spines represent their functional states (2,4). In fact, we found the rapid and long-term spine enlargement was associated with long-term potentiation at the level of single spines (3). Using two-photon photoactivation of PAGFP-actin and uncaging, we have clarified the dynamics of actin fibers underling long-term enlargement of spines (5). We have also found that cytosolic actin can prevent washout phenomenon of spine enlargement and LTP in whole-cell clamped cells (6). Using this technique in whole-cell clamped neurons, we have clarified that enlargement of spines can be induced by low-frequency pairing of pre- and post-synaptic spikes (spike-timing protocol). Interestingly, the enlargement induced by the spike-timing protocol was associated with a gradual protein-synthesis dependent enlargement, and dependent on the secretion of BDNF (6).

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.

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Morphological changes in spines in behaviorally activated neurons

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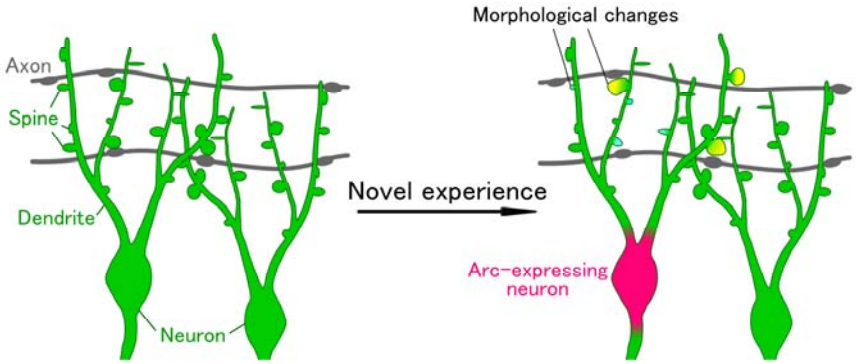
Morphological plasticity of dendritic spines underlies the fine-tuning of network connectivity. The relationship between spine plasticity and experience-dependent neuronal activities, however, is largely unknown. We simultaneously imaged spine morphology and Arc (an immediate-early gene that serves as a putative cellular marker of past neuronal activity) expression using Thy1-mGFP transgenic mice after exploration in novel environment for either 15 or 60 min. After the exploration, Arc was expressed in 25% of hippocampal CA1 neurons. We found that, after 60 min of exploration, Arc-positive cells possessed fewer small spines and more large spines compared with Arc-negative cells, although these parameters showed no difference after 15 min of exploration. These findings suggest a coordinated balance in spine morphological changes on Arc-positive cells. The 60-min mice showed less exploring activities than the 15-min mice when re-exposed to the same environment. This suggests familiarization of the 60-min mice to the environment accompanied with the changes in spine sizes. These results provide the first evidence of morphological changes in spines selective in behaviorally activated hippocampal neurons.



Exploration in novel environment resulted in changes in spine sizes

in a small portion of hippocampal neurons.

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

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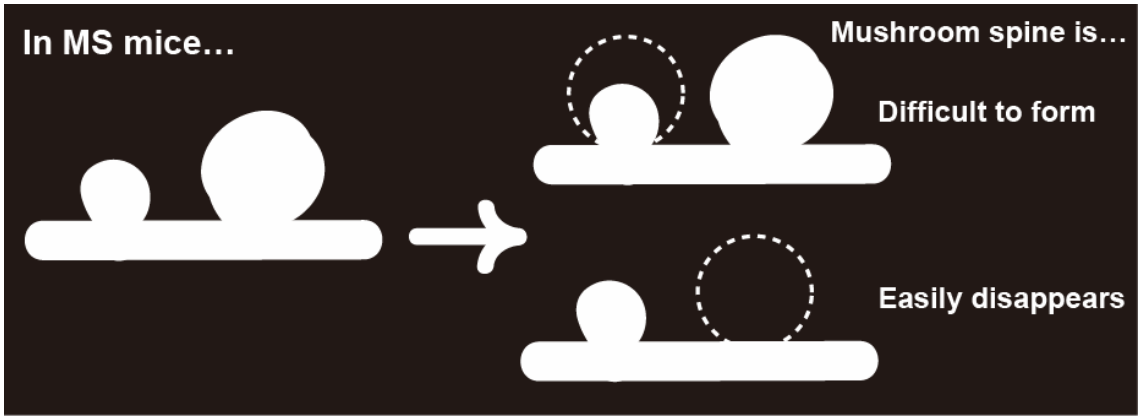
Abstract

Mother-separation (MS) is an important model of stress-induced effects on neuronal development with implications for humans. It has previously been shown that MS cause not only the changes in the serum concentration of stress-induced hormones but also causes significant structural changes of neurons in the somatosensory and/or prefrontal cortex. In this study, we investigated the effects of MS on the structure of dendritic spines in motor cortex in vitro by using Thy1-EYFP (H-line) mice which express YFP protein in layer V pyramidal neuron both in vitro and in vivo. We found that the number of mushroom spines were significantly decreased in MS mice compared with those in control mice especially at postnatal 8 weeks and the effects were prolonged even at postnatal 12 weeks. Similar effects were seen not only in frontal cortex but also, interestingly, in motor cortex. The result of in vivo imaging by using two-photon laser microscopy suggested that the survival rate of mushroom spines were significantly decreased in MS mice. We conclude that MS-induced stress cause significant effects on stability of spine globally in cortex even after grown up.



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

In brain functions such as synaptic transmission, neural plasticity and neurovascular control, neurons and supporting cells cooperate communicating each other using intercellular messengers. Various roles of the intercellular messengers are beginning to be clarified. For example, glutamate, which is released from the presynaptic terminals, not only activates the postsynaptic neurons but also activates adjacent glial cells and possibly contractile cells called pericytes in capillaries. Nitric oxide (NO), on the other hand, is released from neurons and is closely linked to the synaptic plasticity and vasodilation. Although the release mechanisms of these intercellular messengers and their effects on the receptors have been well characterized, their spatiotemporal dynamics are still poorly understood. Recently fluorescent probes of several intercellular messengers became available. Fluorescence imaging techniques are powerful methods for the analysis of spatiotemporal dynamics of signaling molecules, but they have mainly been applied to brain slices and isolated cells. We have now applied the fluorescence imaging technique of signaling molecules to the brain of living animals, employing sensory stimulus to evoke physiological neural activity. We have detected glutamate and NO signals in sensory cortex, using fluorescent probes of these molecules. We wish to extend our study to clarify the physiological spatiotemporal dynamics of glutamate and NO in the regulation of synaptic transmission, activity of glial cells and control of cerebral blood flow.

In vivo time-lapse imaging of the migration of cortical interneurons during postnatal development

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A majority of GABAergic interneurons in the rodent cortex originate from the ganglionic eminences (GEs). During perinatal days, they migrate tangentially through the marginal zone (MZ) and intermediate zone/subventricular zone (IZ/SVZ) towards the cortex. After reaching the cortex, they migrate into the cortical plate and incorporate into their appropriate laminar position. To investigate the dynamics of the migrating interneurons in intact immature animal, we attempted to develop *in vivo* time-lapse imaging with two-photon laser-scanning microscopy employed on immature transgenic mice expressing Venus in GABAergic interneurons (VGAT-Venus). To examine the correlation between Venus-positive neurons and GABAergic interneurons, we employed double-labeling immunofluorescent staining against Venus fluorescence and GABA. In result, GABA staining was confirmed in almost all Venus positive cells (more than 90%) in neocortex. To establish the time-lapse imaging of immature mouse neocortex neurons, we designed for the specialized experimental instruments. For example, we developed four-direction restraint bars (front, back, left, and right side of the head) to alleviate heartbeat and breathing and to maintain the body temperature which are critical for immature mice. Furthermore, we adopted thin skull method to improve the resolution, signal and contrast of imaging. In combination with these new methods with two-photon microscopy, we could observe the multidirectional tangential migration of GABAergic interneurons in the MZ and descending radial migration toward the CP (cortical plate) *in vivo* even at postnatal day 0-2. This approach will be a powerful tool to understand neuronal dynamics in developing intact brain.

Visualization of hippocampal polysynaptic computation

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Complex information processing in the brain embodies high-order nonlinear computations. This processing system arises from polysynaptic feedforward or feedback microcircuits consisting of excitatory and inhibitory neurons. Thus, it cannot be assessed by isolating responses of single neurons or by averaging multineuronal responses in space and time. With this respect, functional multineuron calcium imaging (fMCI) is a promising tool. This is a large-scale recording technique that simultaneously monitors the spatiotemporal patterns of spikes emitted by hundreds of neurons with single-cell resolution. Using fMCI, we attempted to understand the manner in which information is processed in the hippocampal networks. Information that the hippocampus receives from the cortex is relayed through the dentate gyrus (DG), CA3, and then CA1; and sent back to the cortex. We placed two stimulation electrodes (Stim A and Stim B) in the DG granule cell layer of cultured hippocampal networks and monitored activity evoked in CA1 pyramidal cells, i.e., a major output region of hippocampal circuits. In this experimental design, we regarded the hippocampal polysynaptic network as a logical operator that converts DG inputs to CA1 outputs. We found that hippocampal networks involve diverse logical operator-like units. Repetitive application of a combination of Stim A and Stim B induced a persistent change in firing patterns of CA1 neurons. These results suggest that hippocampal polysynaptic networks function as a complex parallel processing system that undergoes plastic remodeling.

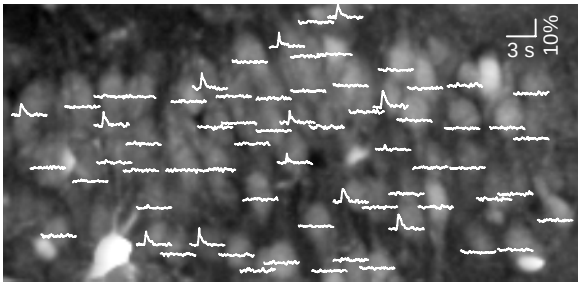

Fig 1 Spatial distribution of spiking CA1 neurons in response to a single-pulse DG stimulus.

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

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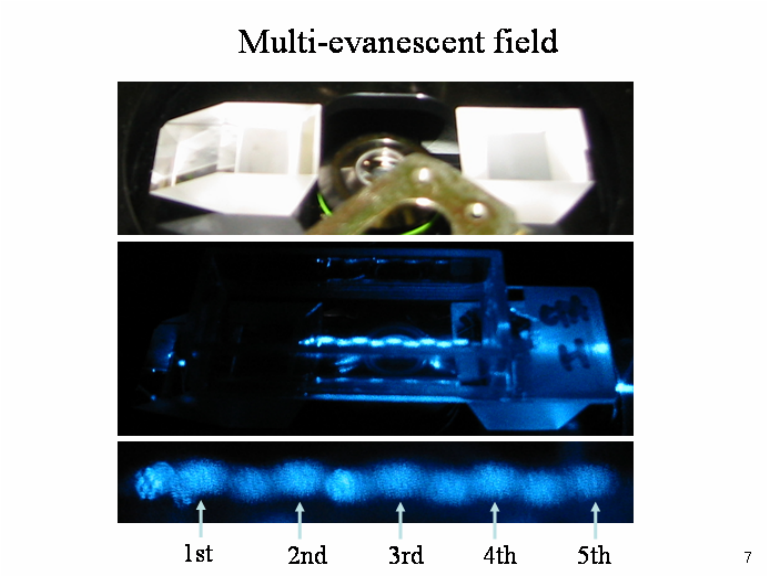
Fluorescence imaging is a powerful method to visualize and analyze spatiotemporal dynamics of molecules in a living cell. Here we have developed a prism-based total internal reflection fluorescence microscopy (TIRFM) system to simultaneously visualize and analyze three 2nd messengers, Ca2+, cAMP and diacylglycerol (DAG), that involve many cellular functions. Employed were three fluorescent indicators: 1) fura-2 AM for Ca2+, 2) Epac1-camp, a CFP-YFP FRET-based cAMP indicator, for cAMP and 3) C1-mRFP, a tandem DAG binding domain of PKC gamma, for DAG. These indicators were transfected into Cos 7 cells. DAG signal was taken by TIRFM, and Ca2+ and cAMP signals were taken by epifluorescence microscopy. When three 2nd messengers were evoked in Cos7 cell stimulated with 100uM ATP, each signal was successfully dissected without significant overlap of emission wave length among three signals. Unmixing of overlapping signals was also discussed in this study.



Imaging glucose uptake into single, living mammalian cells

Katsuya Yamada
Hirosaki University

Glucose transport activity in mammalian cells has been monitored by radiolabeled tracers such as [^{14}C] 2-deoxy-D-glucose. Although these tracers are quite effective in glucose utilization studies, they cannot measure glucose uptake in single, living cells. We have developed a fluorescent D-glucose derivative, 2-[*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-2-deoxy-D-glucose (2-NBDG), that allows a more sensitive measurement of glucose uptake in single, living cells. 2-NBDG was initially developed for the rapid non-culture count of viable microbial cells and successfully applied to *Escherichia coli* (*E. coli*) by Hideaki Matsuoka[1]. Then we demonstrated in living mammalian cells that the uptake of 2-NBDG takes place through glucose transporters (GLUTs) in a concentration-, time-, and temperature-dependent manner[2]. Kinetic analysis using fluorometry further revealed that apparent K_m values for the uptake were similar to those reported by D-glucose and the nonmetabolizable glucose analogue, 3-*O*-methyl-D-glucose, found in pancreatic islets.

For the last several years, 2-NBDG has been used for monitoring glucose uptake into a variety of mammalian cells including astrocytes and neurons. By simply superfusing 2-NBDG over cells by a flow-through system, simultaneous monitoring of differing glucose uptake into heterogeneous cells is possible. However, care should be taken in that the fluorescence intensity is an arbitrary measure. Thus, quantification requires stability of the system as well as accurate procedures. Procedures for combining this method with Ca^{2+} imaging by fura-2 and subsequent immunocytochemical identification of cells are presented. Technical limiation of the method also is discussed.

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Development of Ratiometric Fluorescent Phosphatase Probes with Novel Switch Mechanism

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Dephosphorylation is an essential biochemical reaction and plays important roles in the regulation of various metabolic and signal transduction pathways in living organisms. Fluorescent probes are attractive tools to provide spatiotemporal information about phosphatase activities in a living cell. Many phosphorylated biomolecules are alkyl phosphate monoesters, not aryl phosphate monoesters. There has been, however, no report about a small molecule fluorescent probe based on alkyl phosphate monoester structure, because conventional switch mechanism for fluorescent phosphatase probes utilizes the dephosphorylation of phosphate monoesters of phenolate.

In this research, highly specific fluorescent probes based on alkyl monoester structure were developed by using a novel switch mechanism. For the design of the novel switch mechanism, two chemical characteristics of coumarin derivatives were utilized. The first characteristic is that the excitation spectrum of 7-hydroxycoumarin is affected by the pH of the buffer solution. Deprotonation of the 7-hydroxyl group affects the fluorescence properties of 7-hydroxycoumarin. The second characteristic is that the p*K*a value of 7-hydroxy-8-phosphorylmethyl coumarin is considerably higher than that of 7-hydroxy-8-methylcoumarin. These characteristics suggest that there is a strong interaction between the phosphonate group and the 7-hydroxy group. It is, therefore, considered that the 7-hydroxy group is deprotonated and the excitation spectrum is red-shifted, when the anionic group is removed from 7-hydroxycoumarin by an enzymatic reaction (Figure 1). Based on these ideas, a novel fluorescent probe, PPHC, was synthesized consisting of 7-hydroxy coumarin with phosphate group. It was demonstrated that PPHC was efficiently dephosphorylated by acid phosphatase (ACP) and the excitation spectrum of PPHC was changed ratiometrically as the enzymatic reaction proceeded (Figure 2). Interestingly, PPHC was dephosphorylated only by ACP among six phosphatases. PPHC showed completely different enzyme specificity from known probes based on aryl phosphate monoester structure.

In conclusion, a novel switch mechanism for fluorescent probes detecting enzyme activities was developed. It is based on a pK_a change of the 7-hydroxyl group of 7-hydroxycoumarin derivatives induced by adjacent anionic groups. This mechanism was applied to the fluorescent phosphatase probes, which are the first small molecule based probes for alkyl phosphate monoesters. These probes have high specificity to ACP and spectral change of the probes is ratiometric by an enzymatic reaction of phosphatase.

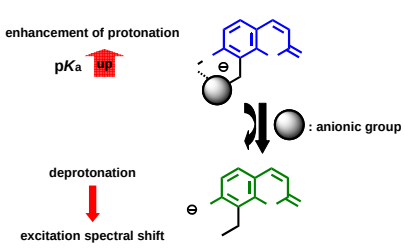

Figure 1. Detection methods of enzyme activity

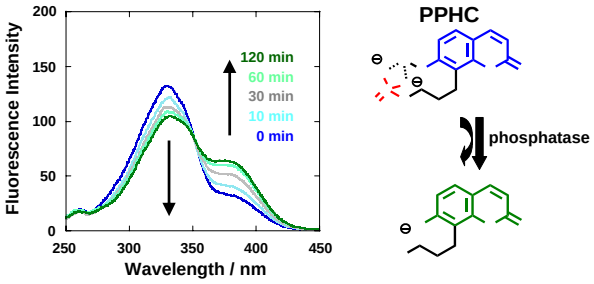

Figure 2. Excitation spectra of PPHC before and after addition of acid phosphatase (ACP) in 100 mM HEPES buffer solution (pH 7.4). λ_{em} = 470 nm.

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

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The fluorescent imaging technology is useful as the tool for examining spatial-temporal localization of proteins and their functions in living cells. Small molecular fluorescent probes especially have the advantage of suppressing the movement obstruction of the target protein to the minimum and being easy to be labeled, compared with the method of introducing a fluorescent protein by a genetic engineering technique. On the other hand, the problem has been left in respect of the selectivity to the tag and the stability of the probe after introduction. We are focusing on hexahistidine (His-tag) that is a popular tag peptide in biology's field. The His-tag is multi-used because of being handy from which it is tagged not to affect protein function relatively. A fluorescent probe targeting His-tag should be convenient for biologists and spread widely in the future.

In order to synthesize small molecular fluorescent probes by the recognition of His-tag and to evaluate the recognition, a detailed examination of zinc type probe with low toxicity was performed as first step. Concretely, the pyridylsulfonamide residue which recognizes the imidazole ring of histidine via coordination bonds through the zinc ion was built into the fluorescent frame, and two or more low molecular fluorescent probes that changed the uniting position and the number of bond of recognition units. Furthermore another new type's probe was characterized in living cells. By using these probes, we examined the property of these fluorescent probes in living cells. Then plasmids that express membrane proteins and His-tag were constructed and transfected into the NIH3T3 cells and injected to primary cultured neurons to examine the tag-selectivity of the probes in cultured cells. From the characterization of small compound probes, some problems will be posed from biology's field and the more refined ways will be discussed.

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

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Monitoring temporal changes in intracellular concentrations of inositol 1,4,5-trisphosphate ($[IP_3]_i$) is of vital importance to the ultimate understanding of intracellular Ca^{2+} signals. We have reported a ratiometric fluorescent biosensor, LIBRA, which consists of the IP_3-binding domain of the rat type3 IP_3 receptor fused between the FRET pair CFP and YFP, and preceded by a membrane-targeting signal. Here we describe improved variants of LIBRA and the utility of this biosensor by examining the IP_3 responses during oscillatory changes in $[Ca^{2+}]_i$ in single living cells.

Initially, we replaced the FRET accepter EYFP to the more bright and pH-stable mutant Venus. The resulting variant LIBRAv was less pH-sensitive than original LIBRA, and gave 1.5-fold increase in emission ratio between zero and saturating IP_3 (Rmax).

We next engineered IP_3-binding domain of LIBRAv. Mutation of a critical amino acid of IP_3-binding domain (K508A) attenuated its response to IP_3, whereas the mutation R440Q gave ~2-fold increase in the affinity for IP_3. The K508A mutant can be ideal as the negative control for IP_3 measurements. We also constructed another high affinity LIBRA using IP_3-binding domain of type 2 IP_3R with R440Q mutation, which exhibits ~5-fold increase in affinity for IP_3. To further improve the dynamic ranges of these biosensors, we constructed LIBRA variants with different linkers and circularly permutated fluorescent proteins.

The simultaneous monitoring of IP_3 and Ca^{2+} using LIBRA variants and fura-2 indicated a monophasic increase in LIBRA ratio during oscillatory changes in Ca^{2+} concentrations in COS7 cells. On the other hand, oscillatory changes in LIBRA ratio were observed during Ca^{2+} oscillations in HSY-EA1 cells. These results suggest that mechanisms for Ca^{2+} oscillation are varied in different cell types.

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

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Inflammatory diseases are frequently accompanied by severe pain. The processing of pain sensation in the somatosensory cortex was demonstrated by using functional magnetic resonance imaging. However, little is known about this processing at the resolution of the individual cell in the somatosensory cortex. To address this issue, we applied *in vivo* two-photon functional calcium imaging to inflammatory pain model made by injection of Complete Freund's adjuvand (CFA) in the mouse hindpaw. Sensory-evoked calcium transients and the response rate of those calcium transients increased under the pathological conditions. These results suggest that the activities of neurons in the somatosensory cortex increased under the inflammatory pain conditions.

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

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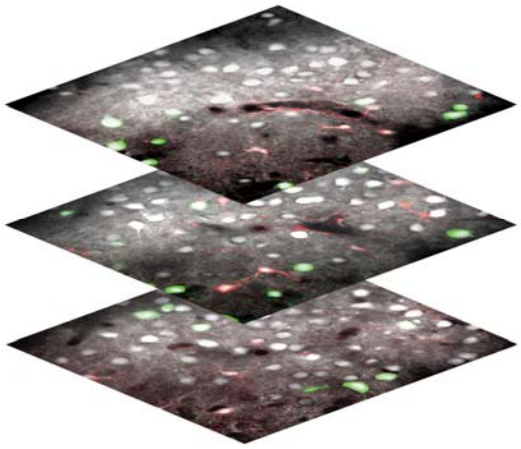
～ The method of in vivo two-photon functional Ca^{2+} imaging became a powerful tool for analysis of neuronal circuits in the physiological condition, because we can almost simultaneously measure the activity of neuronal populations at the single cell resolution with two-photon microscopy. ～

Recent studies suggest that inhibitory neurons play an important role in formation of selective responses of cortical neurons, and response properties of inhibitory neurons may be different from those of excitatory neurons.

To address this issue, we applied *in vivo* two-photon functional Ca^{2+} imaging to GAD67-GFP knock-in mice, in which GABAergic neurons express enhanced green fluorescent protein (EGFP). Astroglia were stained by an astrocyte specific dye (sulforhodamine 101). Thus, the three types of cells, GABAergic neurons, excitatory neurons and astrocytes, in layer Ⅱ/Ⅲ of the visual cortex were differentially identified by using different wavelengths of excitation light and a dichroic mirror for emitted fluorescence. And then their responses to moving visual stimuli at different orientations were measured with changes in a Ca^{2+}-sensitive dye.

In this presentation, I would like to discuss the application of two-photon functional Ca^{2+} imaging to transgenic mice and show the results that almost all GABAergic neurons have orientation-insensitive responses, while most of excitatory neurons have orientation-selective responses.



***In vivo* two-photon functional Ca^{2+} imaging of mouse visual cortex**