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IR imaging of living cell by VSFG detected IR super-resolution microscopy

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IR spectroscopy gives highly useful information about the molecular vibrations, structures and environments around molecules. IR spectroscopy has also been applied to biological molecules and achieved a pile of significant findings. However, because of longer wavelength and larger diffraction limit of IR light, its application to minute samples such as single cell with microscopic technique is relatively undeveloped compared to conventional microscopy with visible light. Recently, we have developed a new vibrational sum-frequency generation (VSFG) detected IR super-resolution microscope. VSFG is second-order nonlinear optical effect process. If we introduced visible and IR beams simultaneously, and frequency of IR beam matched to molecular vibration, photon equal to sum-frequency of visible and IR beams be generated. We consider information IR absorption biological molecules at interface such as cell membrane and nuclear membrane has been obtained by detecting VSFG. The present work aims at the IR super-resolution imaging of living cultured cells by using this technique. We applied our VSFG detected IR super-resolution microscope to study yeast cell and cultured cells, and succeeded to observe IR images of them on IR super-resolution. In addition, we observed intensity change of emission from the cells by changing IR wavelength. Briefly, it would appear that obtained IR images reflect the IR absorption of molecules in cell interior. In our presentation, these results will be also discussed in detail.

1P-265 振動和周波発生赤外超解像顕微鏡の開発と細胞への応用

Development of vibrational sum-frequency generation IR super-resolution microscope and its application to cells

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Infrared (IR) spectroscopy is powerful method to study structure of molecule based on vibrational spectrum. However, its application to microscope did not show large progresses so much. The largest difficulty is the lower resolution of IR microscope due to the longer wavelength of IR light, and it is limited at several μm . To overcome this limitation, we developed a novel IR microscope based on vibrational sum-frequency generation (VSFG). VSFG is a non-linear optical process and we irradiate visible and IR beams on the sample. If IR beam is resonant to vibrational mode of target molecule, VSFG signal photon whose frequency is equal to sum-frequency of the two pump beams will be strongly generated. Therefore, we can detect IR absorption of the molecule by monitoring VSFG and IR spectrum can be obtained by scanning IR wavelength. Because VSFG signal has visible wavelength, IR absorption can be imaged at the resolution of visible light (several 100 nm). In this study, we applied our VSFG microscope to onion root cells. As a result, we successfully obtained IR image of the cells and measured IR absorption spectra of each cellular structure. It showed that, while the signal of aliphatic -C-H stretching vibration was stronger in cytoplasmic part, aromatic -C-H stretching vibration was strong in nucleolus. These differences may represent the different chemical composition between those structures. These results strongly show the capability of our VSFG IR super-resolution microscope to study the IR absorption of microscopic cells.

1P-266 アストロサイトにおけるカルシウムシグナルの空間的制御機構の解明

Spatial Regulation of Calcium Signals in Astrocytes

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Our nervous system consists of two major types of cells, neuron and glia. For a long time glia were only thought to be the support cells of neurons which played the main role in brain function. However one type of glia, the astrocyte has been shown to actively regulate two critical factors in brain function, namely neuronal transmission and local blood flow. These functions are enabled by intracellular calcium increase at astrocyte membrane protrusions referred as processes. In the previous study it has been shown that each astrocyte process display independent calcium dynamics. Given the fact that astrocyte calcium dynamics reflect astrocyte function, it is important to understand how each process can be a compartmentalized calcium domain. In this study, we focused on astrocyte metabotropic Glutamate Receptor (mGluR) which is mainly responsible for receiving neuronal input evoking IP₃ receptor mediated calcium release. Calcium imaging revealed that when astrocytes were stimulated with mGluR agonist, different processes have independent calcium increase initiation sites. To address the mechanism underlying these calcium dynamics, we studied the distribution and the dynamics of mGluR by single particle tracking using quantum dots. mGluR concentration was significantly higher at processes in comparison to the soma. We found that the mGluR movement is confined within the same process. This result indicates that mGluR faces a diffusion barrier blocking its entry to the soma. It is probable that this mGluR diffusion barrier is playing a key role in compartmentalization of calcium domains in astrocyte.

1P-267 神経成長因子による細胞内軸索輸送の促進機構

Augmentation of retrograde axonal transport velocity after the application of nerve growth factor

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Nerve growth factor (NGF) is essential in the survival of neuronal cells during developmental process. After NGF binds to the receptor TrkA, they are incorporated into the membrane bound vesicles by endocytosis. The TrkA-NGF vesicles are then transported to the cell body. This axonal transport is thought to be essential for further survival of the cells. To investigate the effect of NGF on the axonal transport of TrkA, NGF was applied to PC12 cells expressing TrkA labeled with a fluorescent protein (TrkA-mSEGFP). After 4nM NGF application, there were remarkable increases in the velocities of TrkA retrograde transport (before NGF application, $0.12 \pm 0.07 \mu\text{m/sec}$, after the application, $0.37 \pm 0.2 \mu\text{m/sec}$). To test if there is any difference between the transport velocity of TrkA bound to NGF and that of TrkA without NGF, we simultaneously observed the translocations of fluorescent labeled TrkA and NGF. We found that the velocities of both TrkA increase, regardless of the NGF association. This result implies that the NGF application increases the overall velocity of axonal transport in the whole cell. To confirm this idea, we observed the axonal translocations of various membrane-bound vesicles by using differential interference contrast microscope (DIC). The retrograde velocity of these vesicles increased after the application of NGF, as was observed in the case of TrkA translocation. We are now exploring the candidates of the factors that increase the velocity of retrograde axonal transport after NGF application.

1P-268 新規ローダミン骨格に基づいた近赤外蛍光イメージングプローブの開発

Evolution of Group 14 Rhodamines as a Platform for Far-red to Near-infrared Emitting Fluorescence Probes

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Far-red to near-infrared (NIR) emitting fluorescent dyes are attractive for in vivo imaging and biological applications because of low background autofluorescence from serum, proteins, and other biological macromolecules, low light scattering, and low risk of cell damage by exciting light in this wavelength region. The absorption and emission wavelengths of group 14 xanthenes and rhodamines, containing silicon, germanium, or tin at the 10 position of the xanthene chromophore, showed large bathochromic shifts compared to the original rhodamines, owing to stabilization of LUMO energy levels by $\sigma^* - \pi^*$ conjugation between group 14 atom-C (methyl) σ^* orbitals and a π^* orbital of the fluorophore. These group 14 xanthenes and rhodamines retain the advantages of the original rhodamines, including high quantum efficiency in aqueous media ($\Phi_f = 0.30 \sim 0.45$), tolerance to photobleaching, and high water solubility. To confirm the validity of group 14 rhodamines for developing NIR fluorescence probes, we developed several kinds of novel fluorescence probes emitting in the far-red to NIR region, i.e., pH-sensors for acidic environment, a Zn^{2+} sensor, and a probe for reactive oxygen species.

1P-269 線形アンチセンスプローブを用いた生細胞の内在性 mRNA のリアルタイム追跡

Real time monitoring of endogenous mRNA in living cells using linear antisense probe

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In eukaryotic cells mRNA plays a key role in gene regulations. However, the function of mRNA is not fully understood because direct detection of endogenous mRNAs in living cells has been difficult. We developed a novel method for the observation of endogenous mRNA in living cells using fluorescently labeled linear antisense 2'-O-methyl RNA oligonucleotides.

Here we applied linear antisense probes to the real time monitoring of endogenous mRNA, which will be useful in understanding the function of mRNA as well as the intracellular localization. The kinetics for the hybridization reaction of antisense probe examined both in vitro (solutions) and in vivo (living cell) were compared with those of Molecular Beacon (MB), the conventional probe for endogenous mRNAs. The MB that has antisense sequences to *c-fos* mRNA was prepared and subjected to the hybridization with mRNA. Although the spectral change owing to the hybridization with the target *c-fos* mRNA was big enough, the hybridization reaction of MB preceded very slowly in vitro and in vivo. The time constants of MB were almost 70 times and 80 times larger than those of linear antisense probe obtained in vitro and in vivo, respectively. Next, the induction of *c-fos* mRNA was investigated in real time using linear antisense probes. As a result, the elevation of *c-fos* mRNA expressed in the cytoplasm was observed within one hour after stimulation. In conclusions, we showed the linear

antisense probes are advantageous in monitoring of mRNAs due to their predominant kinetics in hybridizing with target mRNAs.

the tolerance against the restriction enzyme digestion by measuring the level of gene transcription.

1P-270 ストレス環境における生きた細胞の内在性 mRNA のリアルタイムイメージング
Real-time imaging of endogenous mRNAs in living cells during stress

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In response to stress, eukaryotic cells reprogram mRNA metabolism to repair stress-induced damage and adapt to changed conditions, which is associated with the assembly of stress granule (SG) and processing body (PB) in the cytoplasm. During stress, cytoplasmic mRNAs aggregate to these RNA granules, where they are sorted and remodeled for reinitiation of translation, degradation or storage. Till now, stress-induced assembly of SG and PB is mainly studied indirectly by using protein makers, and the actual behavior of endogenous mRNAs in living cells during stress has not been detected directly yet and remained uncertain.

In this study, we used a linear antisense probe to visualize endogenous cytoplasmic mRNAs in living mammalian cells in order to investigate the dynamics of mRNAs under stress in cytoplasm and obtain insight of the role and function of SG and PB. Poly(A)⁺ mRNAs were chosen as the target. A Cy3 labeled poly(U)₂₂ 2'-O-methyl RNA probe was used to detect poly(A)⁺ mRNAs. The probes combined with streptavidin were microinjected into the cytoplasm of COS-7 cells, followed by the induction of stress with 0.5 mM arsenite.

As a result, a remarkable enhance in both size and number of RNA granules was observed after arsenite stress, but such increase was not found in normal cells. The granules were rapidly induced within 20 min in response to the stress. A large amount of small granules were first aggregated, and then gathered to bigger ones. The detail of the assembly and the dynamics of mRNAs is under study.

1P-273 編むことで構成された3次元マイクロハイドロゲル構造
Three-dimensional Hydrogel Microstructures Fabricated by Knitting

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We propose a method to knit hydrogel fibers for fabricating 3D hydrogel microstructures. Hydrogel microfibers were generated with a coaxial laminar flow microfluidic device. We manually handled these microfibers in a liquid, and succeeded to knit them to form a complex woven structure.

Hydrogel-based micro-scale structures have recently garner attentions for their potential applications in biochemical science and biotechnology. Usually, hydrogel microstructures have been constructed using micro-molding techniques or microfluidic devices. However, building complex three-dimensional hydrogel structure is technically difficult and a challenging issue. On the other hand, handling of fibers is well established in fabric industries, and knitting of fibers can make various shapes in one to three dimensions such as wires, sheets and cylinders. Our concept is to use hydrogel fibers instead of hydrogel beads or molded hydrogel structures, and to knit them for constructing three-dimensional microstructures, as knitted products in our daily life.

We successfully knitted three alginate-based hydrogel microfibers to form braid structures. This demonstration indicates that various kinds of micro-scale hydrogel structures could be fabricated by our knitting approach.

1P-274 生物物理学のための細胞サイズ半透膜チャンバー
A cell-sized semipermeable chamber for biophysics

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Microchambers for biophysical research have actively developed in recent years. Since the chambers have uniform shape and size, they allow us to observe biophysical phenomena quantitatively and accurately, e.g., a single molecular analysis of a biomacromolecule, a network analysis of cells or tissue, etc. However, most microchambers are closed system, so that buffer and medium solutions cannot be exchanged easily. Thus, it has been difficult to observe environmental responses of a single biomacromolecule and to realize a long-term observation of a cell/tissue culture by addition of nutrient. Our group has given an eye to this point, and has developed a semipermeable microsphere with a diameter of several tens micrometers as a semipermeable microchamber. Now, we are developing a cell-sized semipermeable chamber with a diameter of several micrometers. The chambers are placed in a microfluidic channel, so that this microchamber system can achieve not only exchange of the solution inside the chambers but also construction of an interaction network between the chambers connected by the microfluidic channel. In this presentation, we will show the progress of our research and discuss the possibility of the cell-sized semipermeable chamber in biophysics and DNA/RNA nanobiotechnology.

1P-275 蛍光性 BODIPY ケージド化合物を用いた生体分子の時空間光制御
Spatial and temporal control of biomolecules by green light irradiation with use of fluorescent BODIPY-caged compounds

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Cellular events and biological activities are controlled by spatial and temporal distribution of biomolecules in cell system. Therefore, spatiotemporal control of biomolecular activities is one of the most important technologies used in current biological researches. Caged compound is one of the most powerful tools for the spatiotemporal control of biomolecules in cell system, which can be activated by irradiation of light. However, since present caged compounds require UV light for its activation, cell damage and poor permeability of the irradiation light into the tissues are inevitable problem in the photo-control system. In addition, users could not know the distribution of caged compounds, since they are invisible in microscope.

In this meeting, we will describe the development of novel caged compounds which are fluorescently visible, and efficiently activatable with green light. We have developed caged histamine and caged glutamate based on novel photo-reaction of 4-phenoxy BODIPY derivatives. Since these caged compounds fluoresce, we can see if the caged compounds are present where we would like to activate them. In addition, because the light of the range of wavelength is much harmless and far more permeable into biological tissues, developed caged compounds can be applied to UV-vulnerable samples, or organs and tissues. In the future, BODIPY-caged compounds would offer dual uncaging system, in which two different biomolecules are activated with different wavelength of light.

1P-276 Rc-PYP と相互作用タンパク質を用いた酵素活性の光制御系の作製

1P-271 キネシン-カルモジュリン融合蛋白質を用いた分子シャトルの開発
Kinesin Calmodulin Fusion Protein as a Molecular Shuttle

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Recently attention is focused on the application of molecular shuttles based on the motor protein kinesin and microtubule to the drug delivery system (DDS) and lab-on-a-chip. Previously biotin-avidin and antigen-antibody system have been used to attach target cargoes on kinesin. Although the systems are highly specific and tight, these are flawed as irreversible binding. In this study, we employed reversible cargo loading system using calmodulin (CaM) and M13 peptide for the molecular shuttle. We have designed kinesin K560 chimera protein fused with CaM at the C-terminal tail region of kinesin (K560-CaM). K560-CaM was expressed by *E. coli* expression system and purified. The ATPase activity and the microtubules gliding activity of K560-CaM were almost in the normal range of the kinesin wild type. The Ca²⁺ dependent reversible binding of K560-CaM and M13 peptide was monitored with HPLC using size-exclusion column (SEC-HPLC). Using rotary-shadowing electron microscopy, K560-CaM was observed as tetrameric configuration in the absence of Ca²⁺. In the presence of Ca²⁺, K560-CaM was observed as dimeric and tetrameric configuration. The Ca²⁺-dependent configurational change of K560-CaM was also monitored by SEC-HPLC and analytical ultracentrifugation. Finally, we successfully observed that K560-CaM transports M13 peptide coupled with quantum dot along the microtubule in the presence of Ca²⁺ using TIRF-Microscopy.

1P-272 プログラム死する大腸菌の開発
The development of *E.coli* programmed death system

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We developed a genetic system for an inducible programmed death of *E.coli* cells, with an aim for application to the genome engineering. We introduced, into *E.coli* cells, an expression vector of a restriction enzyme, SacI, of which expression level is strictly controlled by pET and pLysS system. Once expressed, SacI can digest the genomic DNA at ~150 positions, leading a fatal damage to the *E.coli* cell growth. When the SacI expression was induced, the cell growth which was monitored by O. D. of the culture medium actually stopped. However, unexpectedly, the cell growth again started after 5 hours. Then, we measured the population of viable cells from colony forming unit by plating the culture on an agar plate. After induction of SacI, the number of viable cells in 1ml medium decreased from 10⁸ to 10⁴ but not extinct. We carried out the same experiment on a ΔrecA strain. The percentage of survival cells decreased in RecA gene deficient strain, suggesting that the RecA-dependent recombination of genomic DNA conferred the tolerance against the SacI induction. However, extinction was still not achieved. We are analyzing what kinds of genetic alternations conferred