places with the large optical distance, but without TMRE fluorescence, These results suggest that highly polarized mitochondrion is apt to have high density. The mechanism that relates mitochondrial density with their polarization will be discussed.

2P-261 シグナル伝達活性化におけるT細胞受容体分子会合の1分子解析 Single molecule analysis of microclustering of signaling molecules on T cell activation

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We have developed a microscopic system and technologies that allow immunological responses and signaling processes to be visualized at the single-molecule level. Although the immunological synapse has been thought to mediate antigen recognition and activation of T cells, the sites and molecular mechanisms for initiating and sustaining TCR signals remain elusive. We have clearly visualized the initial process of signaling activation in T cells, from the moment of signaling activation. We reported that T cell activation is initiated and sustained in TCR (T cell receptor)-containing microclusters generated at the initial contact areas and that the activation signals precede immunological synapse formation. Microclusters containing TCR, ZAP-70 (kinase) and SLP-76 (adaptor) are continuously generated in the periphery. Microclusters containing TCR, ZAP-70 and SLP-76 migrate toward the center of the contact cell surfaces, but SLP-76 disappeared completely and ZAP-70 was mostly lost before the center.

We calculated the number of TCR molecules from the fluorescence intensity of single EGFP-CD3z molecules. Newly generated microclusters at the periphery contained about 50 molecules of CD3zeta. The number of molecules in microclusters increased linearly with time at the early phase and the periphery. At the later phase and central region, the number of molecules in microclusters are saturated and largely fluctuated. We discuss these features of microclustering with respect to molecular interactions and mechanisms.

2P-262 分子内閉環・開環を制御原理とする新規酵素活性検出蛍光プローブの設計

Novel design strategy for various fluorescence probes to detect activity of protease based on unique intramolecular spirocyclization

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Proteases play essential roles in cancer and immune disease, as well as in the generation of amino acids for protein synthesis. Sensitive and accurate protease detection systems become a crucial tool for the early diagnosis of diseases. To detect activity of protease, rhodamine110 derivatives have been widely used as fluorescent probes. However, since these substrates have two reactive sites, two enzymatic reactions are necessary to become strongly fluorescent, decreasing the rate of fluorescence manifestation and limiting the linear range of assays.

Recently, we reported that a series of tetramethylrhodamine derivatives bearing a hydroxymethyl or mercaptomethyl group instead of original carboxy group showed unique intramolecular spirocyclization. Based on these findings, we synthesized a series of new hydroxymethyl-rhodamine110 (RhoHM) derivatives. RhoHM was strongly fluorescent in aqueous solution at pH7.4, while a compound that one of the amino groups of RhoHM is acetylated, was colorless and non-fluorescent. This result suggests that we can regulate fluorescence by controlling intramolecular spirocyclization at one reactive site. Next, we developed fluorescent probes for leucine aminopeptidase and γ -glutamyltranspeptidase based on this approach. Our probes could detect the activity of these proteases sensitively, quickly and quantitatively. We will present their properties in detail in this symposium.

2P-263 がん選択的な PDT の実現を目指した activatable 光増感剤の開発

Development of an Activatable Photosensitizing Probe Aimed for Tumor Specific PDT

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Photodynamic therapy (PDT) is a noninvasive treatment modality for cancerous pathologies by combinational use of visible light with a photosensitizing chemical called "photosensitizer" which oxidizes biomolecules via generation of reactive oxygen species (ROS). Despite its promising results to date, the selectivity of PDT leaves much to be desired as it often gives rise to clinical side effects such as prolonged photosensitivity. To overcome this problem, a new class of "activatable" photosensitizing probe capable of stressing the target tumor selectively is required.

Here, we developed a novel photosensitizing probe based on a rational molecular design which generates singlet oxygen ($^1\text{O}_2$) only in the presence of β -galactosidase, a commonly used reporter enzyme. In the inactive form, the

photosensitizer is colorless and does not absorb the irradiated visible light. On the other hand, the active form, which is the product of reaction of the activatable photosensitizing probe with β -galactosidase, generates $^1\text{O}_2$ in high yield upon irradiation of light. By controlling the absorbance, we have achieved an unprecedented contrast of the photosensitivity in the result of a specific enzymatic reaction. By combination with the pre-targeting strategy of β -galactosidase against tumor which we have previously reported, the novel activatable photosensitizing probe may afford highly tumor specific PDT.

2P-264 ミューラー行列イメージング法の構築2

Construction of Mueller matrix imaging system

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We are developing Mueller Matrix microscope imaging system. Any polarization states of light can be described by Stokes vector. We need four analyzers to measure the Stokes vector that has 4 components. The emission light S from a sample for the incident light S_0 is written as $S=MS_0$, where M is a 4x4 matrix and called Mueller Matrix. The Mueller Matrix of the sample includes all information about polarization responses for any incident lights. To measure the accurate Mueller Matrix we should use some analyzers and polarizers that can make polarized light to distance from each other on the Poincare sphere. To accurate measure, we use ferroelectric liquid crystals (FLC), which are high-speed polarization switching devices and have a high stability fluctuation of temperature. We first measured the individual Mueller matrix of optical elements. On the base of these Mueller Matrices we measured the Mueller matrix of the optical system constructed from these optical elements. In addition we compared measurements with simulation results, which was calculated by using ideal optical elements. The acquirement of 16 images for each pair of four kinds of polarizer and analyzer and conversion into the Mueller Matrix image were implemented as a plug-in of ImageJ, which is public domain image processing software. To acquire images from cooled CCD, we rewrote MicroManager, which is the image acquisition package from ImageJ, and used. This method enables quantification of local anisotropy from fluorescent images and measurements of circular dichroism and birefringence from transmission images.

2P-265 有機シリカ粒子技術を用いた新規なマルチモーダルイメージングプローブの開発

Development of novel multimodal imaging probe using organosilica particle technology

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Novel multimodal imaging probes have been successfully synthesized by using organosilica particles technology. The organosilica multimodal imaging probes could show both a signal for magnetic resonance imaging for macro-imaging and a fluorescent signal for fluorescent microscopic observation and in fluorescent vivo imaging. Nanoparticles for magnetic resonance imaging such as iron oxide were encapsulated with organosilica layer. Fluorescent signal of organosilica multimodal imaging probes were synthesized via one pot synthesis by using various interactions between internal residue in organosilica layer and fluorescent dye or fluorescent dye conjugate. The resulting organosilica multimodal imaging probes were well dispersed in solution and have unique surface and internal properties. The organosilica multimodal imaging probes have functional residues on the surface that could be modified with biomolecules. We demonstrate the advantages of the organosilica multimodal imaging probes, and their usefulness for bioimaging, drug delivery system and nanomedicine.

2P-266 新規遠心顕微鏡を用いた重力感受に関わるアミロプラスト動態の解析

Analysis of amyloplast dynamics involved in gravity sensing using a novel centrifuge microscope

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Plants sense gravity and change their growth direction, known as gravitropism. Sedimentation of amyloplasts (AMs) in endodermal cells seems to be involved in gravity sensing in Arabidopsis shoots. Recent studies, however, suggested that AMs show dynamic and complicated movements toward gravity after 90°-reorientation of the specimen probably due to interaction with vacuole or cytoskeleton. Therefore, it remains unclear what kind of AM movement is required for gravity sensing. To address this issue, we analyzed gravitropism and AM dynamics under hypergravity condition where sedimentation by gravity is more dominant than other movements. Segments of Arabidopsis inflorescence stem showed a gravitropism in response to hypergravity (10g) that was applied perpendicularly to the growth axis for 30 sec in a conventional centrifuge. Real-time imaging of AMs during the 10g stimulation was performed using a recently developed centrifuge microscope. In this system, all optical devices (e.g. objective

lens, light source and CCD camera) are mounted on a motor, enabling bright-field imaging with a temporal resolution of 30 frames/sec during rotation. Almost all AMs started to move toward 10g and some reached the one side of endodermal cell within 30 sec. These results suggest that redistribution of AMs toward gravity resulting from the directional movement is involved in gravity sensing of Arabidopsis inflorescence stems. To further test this idea, we analyzed the well-known shoot gravitropic mutant, *sgr2*, which has non-sedimentable AMs and shows little gravitropism at 1g. Both redistribution of AMs toward hypergravity and an obvious gravitropism were induced by 30g for 5 min, strongly supporting the above idea.

2P-267 蛍光タンパク質の2量体界面への変異が FRET インジケータのダイナミックレンジに及ぼす影響

The effect on the dynamic range of FRET indicators by mutation in fluorescent protein dimerization interface

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Genetically encoded fluorescent protein (FP)-based FRET indicators have become indispensable tools in molecular and cellular biology as well as other biology-related disciplines. Due to the *Aequorea victoria* FP's tendency to dimerize at high concentration, an A206K repulsive mutation has been introduced to monomerize the FP dimers by adding a charged residue at a dimerization interface. Because of the popularity and wide usage of the mutation among GFP-based FRET probes, we have analyzed the effect of the charge introduction toward the dynamic range of the indicators. A genetically encoded Ca²⁺ indicator Cameleon utilizes calmodulin and M13 peptide sandwiched by color variants of the fluorescent protein. Upon Ca²⁺ binding calmodulin and M13 undergo structural changes that alter both the distance between the chromophores and relative orientation of the dipole moments, which in turn affects the FRET signal of the indicator. We have constructed Cameleon indicators with the dimerization interface mutations and compared the dynamic range of the mutants with that of the non-mutant indicator. Indeed the dynamic range of the indicator was reduced as more charges were introduced to the dimerization interface. Spectroscopic analysis revealed that the Ca²⁺-saturated samples, but not the Ca²⁺-free sample, had the dramatic reduction in FRET signal. Moreover, the sticky mutations (S208F, V224L) which enhance the dimerization of FPs also reduced the dynamic range of the indicator. The reduction was due to the FRET signal increase in the Ca²⁺-free sample. These results suggest that neither repulsive nor sticky mutations should be introduced to the dimerization interface as they both reduce the dynamic range of the indicator, by reduced FRET signal upon Ca²⁺ binding, and by increased basal FRET signal, respectively.

2P-268 FRET 指示薬における遷移双極子モーメント、FRET 効率、及びダイナミックレンジの相互関係

Correlation among dipole orientation, FRET efficiency and dynamic range of FRET-based indicators

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Förster resonance energy transfer (FRET) is a physical phenomena in which an excited state energy of donor migrates into an acceptor non-radiatively. The FRET efficiency depends on both distance between donor and acceptor, and the relative orientation of the chromophore's transition dipoles. In the case of small chromophores, the orientation factor can be neglected due to their free rotation. Thus, FRET efficiency can be considered as a function of the distance between donor and acceptor. This is reason why many scientists have used FRET as a "spectroscopic ruler" to measure intermolecular distance. In contrast, the chromophore of fluorescent protein (FP) is fixed in the β -barrel structure with a slower rotation than the excited-state lifetime during which FRET occurs. Therefore, FP-based FRET should be strongly influenced by the orientation factor. Cameleon, YC3.60, is a FRET-based Ca²⁺ indicator with the biggest dynamic range (> 500 %) among the FP-based FRET indicators reported so far. The high dynamic range is achieved by high FRET efficiency in Ca²⁺-saturated state, which is thought to be owing to the appropriate orientation of the transition dipoles, making YC3.60 a preferable model FRET indicator to investigate precise relationship among the orientation factors, the FRET efficiency and the dynamic range. Here, we report visualization of these three factors of regularly arrayed biotinylated-YC3.60 in the shape of two dimensions on the crystal of streptavidin by using a newly developed fluorescence polarization microscopy.

2P-269 植物細胞における長時間生理機能イメージングを目指した赤方化指示薬開発

Long time physiological imaging of plant cells with reduced autofluorescence by using a Ca²⁺ indicator composed of a red-shifted FRET pair.

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Förster resonance energy transfer (FRET) is a physical phenomena in which an excited energy of donor molecule migrates non-radiatively into an acceptor molecule. So far, fluorescent protein-based FRET has been widely applied to develop functional indicators. In most studies, combination of cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP) has been used as the FRET donor and acceptor, respectively. In spite of the many successful application, it remains a fundamental drawback in terms of the excitation wavelength since the FRET pair uses a relatively photo-toxic violet light (~430 nm) for specific excitation of CFP. To overcome this, we chose a green fluorescent protein (GFP) as the FRET donor, which can be excited by blue light such as a conventional 488 nm argon ion laser that is physiologically much gentle to live cells than violet light. Among various longer wavelength fluorescent proteins, mKO gave the highest FRET signal in combination with GFP. Thus, we replaced CFP and YFP in a Ca²⁺ indicator, cameleon YC3.60, with GFP and mKO, respectively, to obtain orange cameleon (OC3.60). When expressed in mammalian cells, OC3.60 reported Ca²⁺ transient and oscillation as shown by other Ca²⁺ indicator. Furthermore, we succeeded long term Ca²⁺ imaging with reduced autofluorescence signal in guard cells of plant, Arabidopsis thaliana that stably expresses OC3.60, demonstrating superiority of the FRET pair in bioimaging in plant.

2P-270 単一細胞エレクトロポレーションによって培養小脳神経細胞に導入された siRNA の効果の長期リアルタイムモニタリング

Long-term and real-time monitoring of effects of small interfering RNA introduced by single-cell electroporation in cultured cerebellar neurons

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RNA interference (RNAi) is a powerful means to elucidate gene function. Recently, we developed the method for introducing small interfering RNA (siRNA) by single-cell electroporation using micropipettes in neuronal cell cultures. In contrast to widely used transfection methods, our method allows the introduction of siRNA into single and identified cells. This feature is advantageous especially in investigations of the nervous system because the nervous system is composed of various types of cells. To explore the availability of this method in detail, we introduced siRNA against green fluorescent protein (GFP) into GFP-expressing Purkinje and Golgi cells in cerebellar cell cultures by single-cell electroporation. The temporal changes in the intensity of GFP fluorescence in the same electroporated cells were monitored in real-time over 10 days after electroporation by confocal microscopic imaging. Several parameters, including tip diameter and resistance of micropipettes, concentrations of siRNA and a fluorescent dye marker, voltage and time of pulses, were optimized to maximize both the efficacy of RNAi and the viability of the electroporated cells. Under the optimal conditions, transfer of GFP siRNA markedly reduced GFP fluorescence in the electroporated cells by 4 days after electroporation at the latest, whereas that of negative control siRNA had no effects. GFP siRNA was more efficient in Purkinje cells than in Golgi cells. Sufficient reduction in GFP fluorescence continued until at least 7 days after electroporation. After that, GFP fluorescence began to be recovered in some electroporated cells, although the recovery levels varied among cells. Thus, it is suggested that the most effective gene-silencing by this method is achieved at least between 4 and 7 days after electroporation.

2P-271 投影型X線顕微鏡によるケジラミのマイクロCT

Micro-CT of *Pthirus pubis* by projection X-ray microscopy

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The projection X-ray microscope utilized a very small X-ray source from a thin (0.1-3 μ m) target metal film that is excited by the focused electron beam of a scanning electron microscope (SEM). If an object is placed just below the target metal film, the diverging X-rays enlarge the shadow of the object. Because no X-ray optics such as zone-plate is used, focal depth is infinite in principle. Taking this advantage, we have applied projection X-ray microscope for three-dimensional (3-D) structure analysis of a louse (*Pthirus pubis*, 1 mm in length) by means of cone-beam computed tomography (CT).

In the present study, a 3 μ m thick copper film was used with an electron beam accelerating voltage of 18 keV. Thus, the characteristic X-ray of copper K α (0.15 nm) was the main X-ray. The projection images of a louse were recorded in every 3 degree for whole direction (360 degree) with a stepping motor controlled sample rotator. The 3-D image was reconstructed from cone-beam projections using filtered back-projection algorithm. Because an entire structure can be inspected using a single specimen without drying, coating, or making sections, the X-ray micro-CT technique method presented here is a powerful tool for the study of biological specimens. Three dimensional, inside and outside, structure of *Pthirus pubis* will be shown.