spatial distribution by binning mass spectrum. This bundle of mass spectrum corresponds to maximum likelihood estimation and entropy image calculated from bundled spectrum shows more distinct pattern than normal entropy image.

3PS029 シャペロン GroEL フットボール型複合体を介したタンパク質フォールディングの直接観察

Direct observation of protein folding mediated by the football-shaped GroEL-GroES complex

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The chaperonin GroEL is an essential molecular chaperone that mediates protein folding together with its cofactor GroES in *Escherichia coli*. It has been commonly believed that GroES binds alternatively to each ring of GroEL, which is composed of two identical rings stacked back to back, during the functional cycle. In other words, an asymmetric GroEL-GroES complex (the bullet-shaped complex) is formed throughout the cycle, whereas a symmetric GroEL-(GroES)₂ complex (the football-shaped complex) is not formed. We have recently shown that the football-shaped complex coexists with the bullet-shaped complex during the functional cycle. However, how protein folding proceeds in the football-shaped complex remains poorly understood. Here, we used green fluorescent protein (GFP) as a substrate to visualize the folding in the football-shaped complex by single-molecule fluorescence techniques. First, we directly counted the number of refolded GFP molecules in the football-shaped complex, and showed that GFP can fold in both rings of the complex. Next, we performed the real-time imaging of GFP folding in the football-shaped complex. Remarkably, the folding was a sequential two-step reaction, and the kinetics was in excellent agreement with that in the bullet-shaped complex. These results demonstrate that the same reactions take place in both cavities of the football-shaped complex in the same manner to facilitate protein folding. These findings shed new light on the functional cycle including the football-shaped complex.

3PS030 等方性と偏光変調性の 2 つの全反射型蛍光顕微鏡を組み合わせる Combining of isotropic TIRFM and polarization-modulation TIRFM

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Two different types of total internal reflection fluorescence microscopy, TIRFM, have been independently developed in our research group. The first one is an isotropic TIRFM, *iTIRFM* (Adachi *et al.*, *Cell* 2007), in which the hollow-cone light generated by a diffractive diffuser is parallelized and focused to the back focal plane of the objective. Single fluorophores at the sample plane are almost uniformly excited even when their orientation are randomly distributed, because the incident light comes from all directions and so the resultant evanescent field contains all (*X*, *Y* and *Z*) polarization-components. The second one is a polarization-modulation TIRFM, *pmTIRFM* (Nishizaka *et al.*, *Nat. Struct. Mol. Biol.*, 2004; Masaike *et al.*, *Nat. Struct. Mol. Biol.*, 2008), which enables us to detect orientation of single fluorophores in *XY*-plane by analyzing a phase of the oscillation of their intensity.

We here combined these two techniques on the same Nikon microscope equipped with a commercial 'perfect-focus system.' Two different illuminations are switchable in a second by translating two filter cubes, which are located at the different points on the optical axis, with electric motors. This experimental setup allows to characterize single fluorophores quantitatively in terms of intensity, orientation and anisotropy, which uncover the change in local environment of multiple catalytic sites in a single protein molecule, such as F₁-ATPase, by directly visualizing repetitive binding and hydrolysis of 2'-Cy3-EDA-ATP.

3PS031 炎症時におけるヒト単球サイトカイン分泌のライブセルイメージング Live-cell secretion imaging assay of the inflammatory cytokine from human monocytes

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Inflammatory cytokines play a key role in the initiation of inflammation. However, their secretion dynamics especially at the single cell resolution still

remain unclear. For example, IL-1 β is a leaderless protein, lacking the signal peptide that typically directs secreted proteins through the classical endoplasmic reticulum-Golgi secretory pathway. Therefore the mechanisms of cellular release into the extracellular environment remain the subject of much investigation. To overcome this, we developed live-cell secretion imaging assay by total internal reflection fluorescence illumination with micro-fabricated well array chip. Here we tried to monitor the release dynamics of LPS activated human monocyte before and after additional ATP stimulation. Forty wells were observed every 1 minute for 4 hours. While little signals of IL-1 β could be observed before ATP stimulation, the fraction of the IL-1 β secreting cells largely increased after ATP stimulation. Moreover they showed strong IL-1 β signal and increased in their membrane permeability indicated with SYTOX dead cell staining reagent. This change seems to represent a "Terminal release", which is usually associated with cell death called "pyroptosis". This work thus succeeded in imaging of IL-1 β secretion from living cells at a high temporal resolution, which will enable us to investigate acute-phase inflammatory reactions of monocytes at the single-cell level for the first time.

3PS032 大腸菌異物排出タンパク質複合体コンポーネントの細胞内動態解析 Intracellular dynamics of the xenobiotic efflux proteins in *Escherichia coli*

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The rising occurrence of multidrug-resistant bacteria is a serious worldwide issue. One of the major causes of the multidrug resistance is the elevated expression of an RND-type xenobiotic efflux transporter complex, which consists of three components, the inner membrane transporter, the membrane fusion protein, and the outer membrane channel. Among the five inner membrane transporters of *Escherichia coli*, only AcrB, which forms a complex with the membrane fusion protein AcrA and the outer membrane channel TolC, is constitutively expressed. Interestingly, the other inducible transporters are also coupled to TolC. This raises an interesting question: does a newly synthesized inner membrane transporter replace the pre-existing transporter in complex with TolC or form a ternary complex *de novo*? In this study, we aim at visualization of the construction processes of the efflux protein complex. First, we constructed a strain expressing a chromosome-encoded fusion protein AcrB-GFP. We observed with total internal reflection fluorescence microscopy (TIRFM), most fluorescent foci of AcrB-GFP did not show lateral movement, whereas they were moving incessantly in the absence of TolC. Second, a residue in the extramembrane loop of TolC was replaced by Cys, and the resulting mutant was stained with the thiol-reactive fluorescent reagent to demonstrate co-localization of AcrB-GFP with labeled TolC. These results suggest that AcrB is fixed when it forms a complex with TolC, which penetrate through the peptidoglycan layer.

3PS033 1 分子蛍光イメージングを用いた転写活性化による Arp4 の動態変化の定量解析

Quantitative analysis of changes in molecular dynamics of Arp4 upon transcriptional activation using single-molecule fluorescence imaging

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Actin-related protein 4 (Arp4) is a component of a chromatin-remodeling complex. Chromatin-remodeling is thought to occur with direct binding of Arp4 to histones, indicating that Arp4 is involved in transcription control. Consequently, molecular dynamics of Arp4 is expected to change in correlation with transcriptional activity. However, the details of relationship between molecular dynamics of Arp4 and transcriptional activity remain unclear. In this study, we performed quantification of molecular dynamics of Arp4 both before and after stimulation with the phorbol ester PMA, as a transcriptional activator. We carried out single-molecule fluorescence imaging of the nucleus of CHO cells expressing TagRFP-T fusion proteins of Arp4 at a low level. The residence time of individual Arp4 molecules, the time from appearance to disappearance of the fluorescence spot, was measured in the images. We found that a residence time after stimulation tends to become shorter than before. It means that transcriptional activation causes dissociation of Arp4-containing chromatin-remodeling complexes from the target sites. In yeast, the number of the

chromatin-remodeling complexes is estimated to be much less than that of the target sites. Upon transcriptional activation, Arp4 needs to cover the increased target sites with the limited number of the molecules. Our result may reflect this molecular mechanism. We will also discuss how Arp4 is involved in transcription with regard to RNA polymerase II.

3PS034 ATP 結合部位変異体 Arp4 β のイメージング解析

Imaging analysis of Arp4 β mutants in ATP-binding site

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Actin related proteins (Arps) are evolutionarily and structurally similar with actin, and they have ATP binding activity like actin. Actin and Arp4 β are critical components of ATP-dependent chromatin remodeling complex, which regulates the dynamic modification of chromatin architecture. Arp4 β found to play a key role as a regulator of the activity. However, the detail of the regulatory mechanism remains unclear.

To clarify whether ATP binding to Arp4 β handles the regulation, we planned to analyze dynamics of the Arp4 β mutant which have the defect of ATP binding activity. We constructed GFP fusion protein of Arp4 β (GFP-Arp4 β) and GFP-Arp4 β -G173D by single nucleotide substitution to deprive ATP binding activity. We established CHO cell lines expressing GFP-Arp4 β or GFP-Arp4 β -G173D at a low level.

When we observed the cells with fluorescence microscopy, wild type Arp4 β were localized mainly in the nucleus. In contrast, Arp4 β -G173D was scarcely localized in the nucleus, and most of them were localized in the cytoplasm. To check the effect of nuclear export or actin polymerization to this cytoplasmic localization of the mutant protein, we observed the mutant under nuclear export inhibitor (LeptomycinB), or actin polymerization inhibitor (LatrunculinB) existence. However, the localization of the mutant did not change. It is suggested that ATP binding activity may regulate its localization in the nucleus.

3PS035 遺伝的にコードされたカルシウムインジケータを用いた細胞内カルシウム分布の動態解析

Dynamics of intracellular Ca²⁺ distribution measured by genetically encoded Ca²⁺ indicators

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Ca²⁺ mediates the signal transduction pathways in cells as a second messenger. By stimulus, the distribution of intracellular Ca²⁺ changes dramatically. After Ca²⁺ is released to the cytoplasm from the endoplasmic reticulum, calcium influx occurs via calcium ion channel on the cell membrane to the cytoplasm. Ca²⁺ is thought to diffuse into nucleus from the cytosol through nuclear pores. Free calcium in the nucleus regulates important functions such as transcription of some genes. There could be some mechanism for precise regulation of Ca²⁺ concentration in the nucleus. Recently genetically encoded Ca²⁺ indicators have been developed. The fusion proteins of the indicator enable us to analyze the local Ca²⁺ concentration. To analyze the Ca²⁺ distribution after stimulation, we constructed fusion proteins of membrane protein or nuclear protein to Ca²⁺ indicator GCaMP3 and established the HeLa cells expressing these Ca²⁺ indicators. Upon stimulation with Histamine, We observed the propagation of Ca²⁺ over the cytoplasm and into the nucleus. We will discuss the difference of Ca²⁺ distribution pattern between in the cytoplasm and the nucleus.

3PS036 大腸菌二成分制御系センサーおよび応答調節因子の蛍光イメージング

Fluorescence imaging of histidine kinases and response regulators of *Escherichia coli*

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Escherichia coli has many two-component regulatory systems (TCS) that respond to specific environmental signals. Each TCS consists of a sensor histidine kinase (HK) and a response regulator (RR), together forming a signal transduction pathway via histidyl-aspartyl phospho-relay. We previously constructed GFP fusions to each HK and classified their localization patterns into six groups. In this study, to explore the physiological significance of

localization of HKs, we constructed a series of plasmids encoding mCherry fusions to each RR. Unexpectedly, expression of mCherry fusions to some RRs resulted in occurrence of elongated cells. This appears to be due to overexpression because cell sizes were normal when the expression levels of the relevant RR-mCherry fusions were tightly controlled. Out of 24 RRs tested, mCherry fusions to AtoC that is involved in acetoacetate metabolism and TorR that is involved in regulation of anaerobic respiration showed (polar and/or lateral) dotted patterns of localization. These RR-mCherry fusions appeared to co-localize with the GFP fusions of the cognate HKs (AtoS and TorS, respectively). We next examined the effect of the cognate HKs (AtoS and TorS) on the localization of these RR-mCherry fusions. Localization of AtoC-mCherry was lost when the *atoS* gene was deleted, whereas TorR-mCherry retained its localization pattern in the *torS* deletion background. These results suggest that AtoC (and possibly TorR also) localize to a pole or lateral membrane regions by interacting with its cognate HK.

3PS037 ラマン分光顕微鏡を用いて細胞の分化状態を識別する

Raman microscopy distinguishes the status of differentiating cell

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We demonstrate that Raman microscopy distinguishes the status of cell differentiation without any labeling. Raman scattering arises from molecular vibration of sample molecules. When a molecule is exposed to photons, a small fraction of photons are scattered with a different frequency from that of incident photons. The shift of the frequency is equal to the molecular vibration frequency. Spectra of Raman scattering are considered as “molecular fingerprints”, because one can reveal molecular species, structures, and surrounding environment by the shift. We expect that the Raman scattering spectrum of a cell can be considered as “cellular fingerprint”, and that we can distinguish, in particular, the status of cell differentiation without any labeling. In order to prove it, we observed the transition of the status of cells during differentiation by Raman microscopy. We built a confocal slit-scanning microscope with excitation wavelength of 532 nm, and continuously obtained Raman images of ES cells from the beginning of differentiation. In analyzing the spectra of nuclei of the ES cells, we found a significant difference between before and after differentiation. Furthermore, to analyze the spectra quantitatively, we employed principal component analysis. The plot of principal component scores showed the translation of the scores in the principal component hyperspace with progression of the differentiation. We believe this result verifies the applicability of Raman microscopy to monitoring of the transition of cellular status.

3PS038 VSFG 検出赤外超解像顕微鏡を用いた毛髪サンプルの分子構造解析

Molecular structure analysis of the human hair samples by using VSFG detected IR super-resolution microscope

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We have developed vibrational sum-frequency generation (VSFG) detected IR super-resolution microscope with a sub-micrometer spatial resolution until now, and have reported the applications to various biological samples such as onion root cell, A549 cultured cell, bio-cellulose, etc. In this study, we applied an IR super-resolution microscope to human hairs. Human hair is a complex biological material consisting of distinct morphological components and is well-known to compose of the medulla, cortex, and cuticle regions from inside to outside. At the cortex area occupying 90% of human hair, keratin protein, which is primary component of human hair, forms a line in the shape of fiber along the longitudinal direction of human hair, and controls physical properties such as flexibility, strength, softness and curliness. To understand the relationship between physical properties and the internal nanostructure, we attempted IR imaging at IR super-resolution in the 6-9 μ m mid-IR region.

As a result, we succeeded in obtaining VSFG image in the amide III band at 1250 cm⁻¹ with sub-micrometer spatial resolution, and clearly observed strong VSFG signals only from the cortex area. VSFG image seems to be contributed to the IR mapping of the distribution of α -helix structure at the cortex. This is supported by very weak emission from the cuticle and medulla, those do not have an α -helix structure. In the presentation, the internal nanostructure of human hairs will be discussed in detail.