Conventional biochemical assays are performed via an averaging procedure with mixture of a large number of target cells. However, protein expression profiles of each individual cell fluctuate even if the cells are treated in the same culture dish. Thus the data that are obtained by an averaging procedure lose information regarding the heterogeneity of individual cells; therefore we needed to develop a quantitative assay of protein expression at the single-cell level. Fluorescence correlation spectroscopy (FCS) can detect molecular concentration in living cell under single-molecule sensitivity. However, there are difficulties in quantifying the precise total number of molecules due to differences of cell size, morphological shape and/or localization of target molecules. Thus, for quantitative assay of total expressed protein in a single cell, it is necessary to isolate single cells, extract cellular components and detect extremely small amounts of molecules from the individual cells. We developed a new system combining a microfabricated lab-on-chip device and FCS that features a simple protocol to isolate single-cells and detect small amounts of specific fluorescent molecules extracted from a single cell. The relationship between the number of foreign DNA molecules incorporated and the number of EGFP molecules expressed in the single cell was confirmed by the combination system. Results indicate that numbers of transfected DNA molecules were rather equal for each cell; however, the expression rate of protein varied in each cell.

1P324 単一細胞における遺伝子発現定量測定系を用いたマクロファージ様細胞 LPS 活性化の動的解析
Kinetic analysis of LPS-activation in macrophage-like cells based on a quantification system for gene expression level in a single cell

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Macrophages are activated by LPS to start expressing various kinds of cytokines. To address how the concerted expression of cytokines is organized during activation, we tried establishing simultaneous quantification of multiple gene expression levels in individual cells. Regarding conventional single cell sorting techniques, you often have to utilize general purpose labwares, such as FACS into 96-well plates, which would cause a considerable loss of tiny amount of materials. Here, we applied a chemically structured slide glass, AmpliGrid, to FACSARIA, which allows us to deposit a cell to the precise position and to execute amplification in the 1 μ L volume *in situ*, meaning that we can avoid a loss of both cells and any polynucleotides. Meanwhile, there are no methods to satisfy both microquantitativeness and multiplexing among current methods such as microarray and qPCR. To solve the problem, the BioMark system was adopted. The system are based on a microfluidics technique which enable to control $\sim$ nL of fluid and produce qPCR data with 48 samples x 48 target genes at the same time. We quantified the expression level of a series of genes in each single cell of LPS-stimulated J774.1, which is one of the mouse macrophage-like cell lines. The targeted genes were selected on the basis of microarray data with a mass of the cells. From the results, we found that a couple of genes sharply but rarely increased at the early phase of activation whereas the others seemed to increase gradually somewhat in synchronization with other cells.

1P325 小さい生体分子複合体のためのクライオ電子顕微鏡法の挑戦
Challenges of electron cryomicroscopy for small biomolecular complexes

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Electron cryomicroscopy (cEM) is one of the powerful techniques to observe protein structure without staining. However, because the method gives us very low contrast images, to date, cEM has been used to analyze protein complexes above 100kDa, for example virus and ribosome. Unfortunately, we cannot easily observe a typical size of proteins (approximately 30 kDa, less than 100 kDa). This is why the cEM usage is limited. In the present studies, we challenged observation of small biomolecular complexes and their three-dimensional reconstruction to expand cEM possibilities.

We here observed both porcine pepsin (34.6kDa) in gastric juice and porcine pepsinogen (39.6kDa) of pepsin zymogen, as test samples, to elucidate how to grow mature after discarding the N-terminal fragment (approximately 44 a.a., 5 kDa). As a result, we succeeded in observing both proteins directly and reconstructing three-dimensional structures with about 2 nm resolutions. When we compare both 3D reconstructions, we could certainly observe that pepsin has a cleft near an active site. Comparing them with crystal structure, loop regions of crystal structures were different in structure. Interestingly, the loop near the active site had low density, probably because of flexibility. The flexibility will be expected from crystal structure and the loop would be fixed

after binding substrates.

We will show both resolution limit of three-dimensional structures and the optimal methods for analyzing small bio-molecular complex.

1P326 赤色シフトした蛍光 ATP プローブを用いた生細胞内 ATP およびカルシウムイオンの同時イメージング
Red-shifted genetically-encoded fluorescent ATP indicator enabled simultaneous imaging of ATP and calcium ion inside single living cells

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Genetically-encoded Förster resonance energy transfer (FRET) probes have become indispensable tools for monitoring biological parameters, such as dynamics and/or distribution of ions, signaling molecules, and enzymatic activities, inside living cells. However, because excitation and emission wavelengths of CFP and YFP, which are the most commonly used FRET-pair, significantly overlap those of most of inorganic fluorescent probes, genetically-encoded FRET probes are rarely used in combination with another genetically-encoded probe or inorganic probe. Here, we report a red-shifted genetically-encoded FRET probe for ATP (GO-ATeam) that has GFP and OFP as a donor and an acceptor of FRET, respectively, instead of CFP and YFP in original ATP probe (ATeam [1]). Because there is only ignorable cross-talk between GO-ATeam and an inorganic Ca^{2+} indicator, Fura-2, we could simultaneously image ATP and Ca^{2+} concentrations of the same single living cells by loading Fura-2 into the cells expressing GO-ATeam. When Ca^{2+} spark was induced in the cells by histamine, mitochondrial ATP also elevated following Ca^{2+} . Intracellular Ca^{2+} typically returned to a basal level within 10 minutes after histamine stimulation. In contrast, most of cells retained high level of mitochondrial ATP for a much longer time. This result strongly supports the idea that intracellular Ca^{2+} promotes long-term activation of energy metabolism in mitochondria [2]. [1] Imamura et al. (2009) PNAS, 106, p15651. [2] Jouaville et al. (1999) PNAS, 96, p13807.

1P327 A genetically-encoded autoluminescent Ca^{2+} indicators with expanded dynamic range

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Bioluminescent proteins such as luciferase are a powerful tool for monitoring biological processes including gene expression in living organisms since bioluminescent signals can be acquired without an external light source. However, bioluminescence signals are too dim to be measured in real time. To overcome this drawback, Renilla reniformis luciferase was conducted on random mutagenesis to improve the intensity, yielding RLuc8-m1. In addition, the luminescence intensity was further increased to 1,000-fold by an efficient bioluminescence resonance energy transfer (BRET) between RLuc8-m1 and a yellow fluorescent protein, Venus, in VRL10.3-m1, enabling observation of subcellular structure at the single-cell level in real time at more than 1 Hz. We then applied the VRL10.3-m1 to design Ca^{2+} indicators based on reconstitution of split- VRL10.3-m1 fused with the Ca^{2+} binding domain, calmodulin (CaM) and M13. The split-VRL-10.3-m1 did not emit light due to splitting the VRL10.3-m1 domain. But Ca^{2+} binding induced reversible conformational change of the CaM-M13 between an extended and compact form, which, in turn, induced the reconstitution of the split- VRL10.3-m1 resulting in bright emission peaking at 530 nm. By optimizing the split position and linker length, we achieved expansion of the dynamic range in the Ca^{2+} indicator up to 400%, by which we succeeded long-term real time Ca^{2+} imaging in several types of cells.

1P328 マウス腫瘍細胞における微小管伸長端ダイナミクスの蛍光イメージング解析
Analysis of microtubule growing ends dynamics by fluorescence imaging of EB1-GFP in mice tumor cells

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Microtubules (MT) are highly dynamic polymers of tubulin that are essential for various fundamental processes including chromosome segregation and cell motility. EB1 is a member of MT plus-end-tracking proteins (+TIPs) and act as an exquisite marker of growing microtubule ends. In this study, we investigated the intracellular MT dynamics using EB1-GFP-expressing human KPL-4 breast cancer cells in dish (*in vitro*), tumor tissue section (*ex vivo*) and mice (*in vivo*). Using a spinning disk confocal microscope (CSU22,