20-21 MAY 2017 | SINGAPORE

MECHANOBIOLOGY INSTITUTE, NATIONAL UNIVERSITY OF SINGAPORE
NUS CENTRE FOR BIOIMAGING SCIENCES
BIOIMAGING SOCIETY OF JAPAN

JOINT SYMPOSIUM ON BIOIMAGING BETWEEN SINGAPORE AND JAPAN

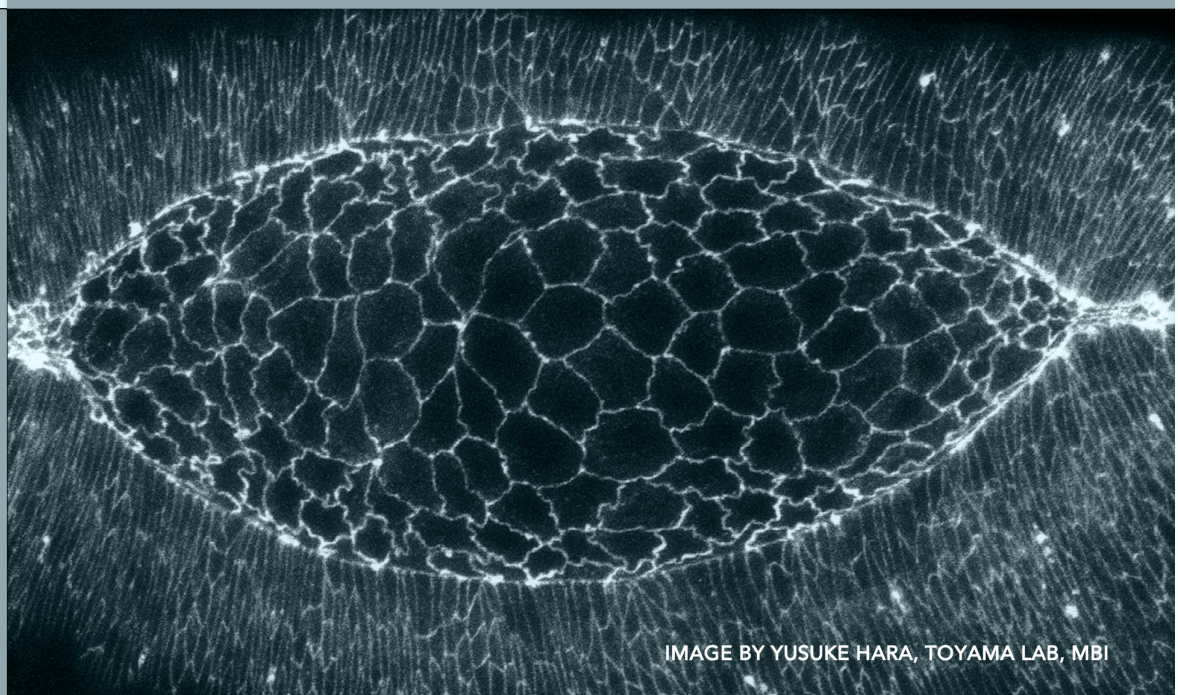

IMAGE BY YUSUKE HARA, TOYAMA LAB, MBI

JOINT SYMPOSIUM ON BIOIMAGING

BETWEEN

SINGAPORE AND JAPAN

5th International Symposium on Bioimaging

MAY 20-21, 2017

Venue:

NATIONAL UNIVERSITY OF SINGAPORE

Organizers

Paul MATSUDAIRA, National University of Singapore
Kazuo SUZUKI, Bio-imaging Society of Japan

Bioimaging Society, Japan,
Cooperation in Singapore: WABIOS

Dear All,

Welcome to Singapore! It is our pleasure to host the Joint Symposium on BioImaging, the 5th Annual Meeting of the Bioimaging Society of Japan, and the Women in Science Symposium. The MechanoBiology Institute and the Centre for BioImaging Sciences were established on the NUS campus only eight years ago but in this short period both research centers have developed novel light and electron microscopy methods and applied them to problems in structural biology, cell and developmental biology, and biophysics. The opportunity to present our research to our colleagues from Japan will open new collaborations.

Many thanks go to Prof. Thorsten Wohland, (DBS/CBIS), Prof. Linda Kenney (MBI), Ms Latha.K.S (MBI), and Ms Nurzanna (CBIS) for their hard work in planning and organizing this symposium. Finally, I would like to express my sincere gratitude to JEOL for sponsoring the conference dinner. Please take time to enjoy our wonderful campus and city before returning to Japan.

Sincerely,

Paul Matsudaira

Joint Symposium on Bioimaging between Singapore & Japan
National University of Singapore
May 20-21, Singapore

Saturday, May 20, 2017
Venue: University Hall Auditorium, 2nd Storey, Lee Kong Chian Wing

Time	**Speaker**
0830-0900am	**Registration** University Hall Auditorium
0900-0910am	**Opening remarks** **Paul MATSUDAIRA** Centre for Bioimaging Sciences & Department of Biological Sciences, National University of Singapore
Symposium 1	
0910-0935am	**Min WU** National University of Singapore ***Information Content of Intracellular Patterns***
0935-1000am	**Lu GAN** Centre for Bioimaging Sciences & Department of Biological Sciences, National University of Singapore ***Exploration of Eukaryotic Nuclei at Molecular Resolution***
1000-1025am	**Pakorn (Tony) KANCHANAWONG** Department of Biomedical Engineering & Mechanobiology Institute, National University of Singapore ***Nanoscale Architecture of Cadherin-based Cell Adhesions***
1025-1050am	**Yusuke TOYAMA** Mechanobiology Institute, Department of Biological Sciences, National University of Singapore & Temasek Lifesciences Laboratory, Singapore ***Tension-Dependent Vinculin Dynamics at Adherens Junction During Cell Boundary Oscillation***
1050-1120am	Coffee Break
1120-1220am	**Plenary Lecture 1** **Shin'ichi ISHIWATA** Department of Physics, Faculty of Science &Engineering, Waseda University, Tokyo, Japan ***Bio-motile Functions Studied by Microscopic Imaging of Physical Parameters***
1220-0210pm	Lunch at University Hall, CBIS visit (starting at 13.10)
WOMEN IN SCIENCE SYMPOSIUM **Organizers: Linda J Kenney & Naoko Iida -Tanaka, Kahoko Hashimoto**	
0210-0215pm	**Opening Address: Organizer, Japan** **Naoko Iida-TANAKA**, Otsuma Women's University, Japan **Kahoko HASHIMOTO**, Chiba Institute of Technology, Japan
0215-0230pm	**Reina IKAGA** Department of Nutritional Science, National Institute of Health and Nutrition, Japan ***The Role of Aquaporin-8 on Mitochondria***
0230-0255pm	**Tomoko MASAIKE** Department of Applied Biological Science, Faculty of Science & Technology, Tokyo University of Science, Japan ***Imaging Movements and Reactions of Biomolecules***

0255-0320pm	**Linda J KENNEY** Mechanobiology Institute, National University of Singapore ***Super-resolution Imaging of Salmonella Virulence Regulation and Secretion***
0320-0345pm	**Carol TANG** National Neuroscience Institute, Duke-NUS Medical School, National Cancer Centre, Singapore ***Targeting Brain Tumors: Improving Lives through Precision Medicine***
0345-0350pm	**Closing Remarks: Organizer, Singapore** **Linda J KENNEY** Mechnobiology Institute, National University of Singapore
0350-0400pm	**Presentation by Sponsor - JEOL Ltd.**
0400-0600pm	**Coffee break & Poster presentations**
0630-0830pm	Dinner at Kent Ridge Guild House

Sunday, May 21, 2017
Venue: Mechanobiology Institute, Level 5, T-Lab, NUS

Time	**Speaker**
Symposium 2: Organizer: Takeharu NAGAI	
0900-0925am	**Hiroki R UEDA** Systems Pharmacology, Graduate School of Medicine, University of Tokyo, Laboratory of Synthetic Biology, Quantitative Biology Centre, RIKEN, Japan ***Whole-body and Whole-organ Clearing and Imaging with Single-cell Resolution Toward Organism-Level Systems Biology in Mammals***
0925-0950am	**Satoshi NISHIMURA** Center for Molecular Medicine, Jichi Medical University, Tochigi, Japan ***Minor Changes but Major Future Impacts, can be Revealed by Extensive and High-Through-Put In Vivo Visualization Using 4K/8K CMOS Sensors***
0950-1015am	**Katsumasa FUJITA** Department of Applied Physics, Osaka University, Japan ***Raman Microscopy for Molecular Imaging of Living Cells***
1015-1040am	**Takeharu NAGAI** The Institute of Scientific and Industrial Research, Osaka University, Japan ***Development of Techniques for Imaging Physiological Functions Toward Visualization of Minority Cells***
1040-1110am	Coffee Break
1110-1210am	**Plenary Lecture 2** **Michael SHEETZ** Mechanobiology Institute, National University of Singapore ***Integrin Clusters: A Robust Way to Organize Adhesions for Cell Mechanics***
1210-1220pm	**Closing Remarks** **Kazuo SUZUKI** Asia International Institute of Infectious Diseases Control, Teikyo University Department of Immunology, National Institute of Infectious Diseases, Japan
1220-0200pm	Lunch at MBI, Level 10; MBI visit starting at 0100 pm

SPEAKER ABSTRACTS

Information Content of Intracellular Patterns

Wu MIN
National University of Singapore

Periodic wave patterns are widely observed in oscillatory or excitable chemical systems and in multicellular systems such as cardiac tissue and slime moulds. More recently, waves of cortical activity, linked to actin dynamics in many cases, have been documented in a variety of single-cell systems, including various immune cell types (1, 2). The appearance of order in these patterns raises the intriguing possibility that molecular networks favouring their generation are evolved due to specific functional advantages they confer. One particular appealing hypothesis is that wave propagation in space generates location-dependent phase information that could be harnessed to encode spatial information. However, little is known about whether or how they are employed by biological systems, especially higher eukaryotes. We will discuss our recent results characterizing cortical waves of active Cdc42, which suggest that mitotic cortical waves might potentially integrate positional and cell size information in modulating final sites of cell division.

1. Wu M, Wu X, De Camilli P. Calcium oscillations-coupled conversion of actin travelling waves to standing oscillations. Proc Natl Acad Sci U S A. 2013 Jan 22;110(4):1339-44.

2. Xiong D, Xiao S, Guo S, Lin Q, Nakatsu F, Wu M. Frequency and amplitude control of cortical oscillations by phosphoinositide waves. Nat Chem Biol. 2016 Mar;12(3):159-66.

Exploration of Eukaryotic Nuclei At Molecular Resolution

Lu GAN
Centre for BioImaging Sciences and Department of Biological Sciences, National University of Singapore

The nucleus is the information warehouse of the eukaryotic cell. Essential functions like transcription, replication, and chromosome segregation happen in the nucleus. Many fundamental questions remain unanswered because we lack a molecular understanding of the nucleus. To address this shortfall, my group has used electron cryotomography (cryo-ET) to obtain 3-D nanometer-level atlases of plankton and yeast nuclei. We found that the chromosomes *in vivo* do not form highly ordered 30-nm fibers and that they do not condense very much either. In contrast, we found that the chromatin readily forms compact 30-nm fibers when released from the nucleus. These observations suggest that while one of the lowest energy states of natural chromatin is the 30-nm fiber, the crowded and metabolically active interior of nuclei keeps chromatin in a more liquid-like state. Such disordered chromatin would enable the high levels of transcription detected in yeast and might explain how other eukaryotes remodel their chromatin in response to transcriptional upregulation.

Nanoscale Architecture of Cadherin-based Cell Adhesions

Pakorn (Tony) KANCHANAWONG
Department of Biomedical Engineering and Mechanobiology Institute
National University of Singapore

Cadherin-mediated cell adhesions are supramolecular complexes that play essential roles in ligating and mechanically integrating neighboring cells, supporting dynamic coupling between cell-cell adhesions and the contractile actin cytoskeletons. Despite well-documented functions in major aspects of tissue morphogenesis and multicellularity, the ultrastructural organization within cadherin-based adhesions remains unknown, thus obscuring insights into the underlying molecular mechanisms. We mapped the nanoscale organization of key cell-cell junction proteins within cadherin-based adhesions formed on planarized biomimetic cadherin substrate. The enhanced optical accessibility of the planar substrate together with interference-based nanoscopy methods enabled high precision (~10-nm) axial (z) position measurement using common fluorescent proteins. We observed a surprisingly well-organized molecular architecture that stratified along the z-axis, with the cadherin-catenin layer and the actin compartment separated by ~30 nm, interposed by a vinculin-containing interface zone. Our results indicated that vinculin can undergo a conformational activation to span between the cadherin-catenin layer and the actin compartment. The nanoscale positioning of vinculin is determined by alpha-catenin, while vinculin conformational state is controlled by contractility and Abl kinase phosphorylation on the residue Y822 of vinculin. Vinculin activation, in turn, modulates the positioning of VASP and zyxin, inducing VASP-mediated actin polymerization, that likely results in a positive feedback loop that regulates junction strengthening. In conclusion, our measurements reveal a modular nanoscale architecture of cadherin-based adhesions, suggesting a control principle whereby vinculin serves as a molecular clutch that integrates mechanical and biochemical signals to differentially engage the cadherin-catenin complexes to the actomyosin contraction machinery under different contexts such as developmental processes or diseases states.

Tension-Dependent Vinculin Dynamics At Adherens Junction During Cell Boundary Oscillation

Yusuke HARA[1], Murat SHAGIROV[1], Issac LIM[1], Yusuke TOYAMA[1,2,3]
1) Mechanobiology Institute, Singapore, 2) Department of Biological Sciences, National University of Singapore, 3) Temasek Lifesciences Laboratory, Singapore

Throughout development, cell boundaries undergo contraction and elongation to change tissue morphology. In comparison to cell boundary contraction, which is mainly driven by Myosin II dependent contraction, the mechanisms of cell boundary elongation are still elusive. We used amnioserosa cells, which exhibit cell oscillation during *Drosophila* dorsal closure, as a model system to uncover the mechanics underling cell boundary elongation, and to elucidate how the junctional tension evolves during cell oscillation. Here we show that the cell boundary elongation is predominantly driven by non-autonomous active process, which is associated with the transient flow of Myosin II in the neighboring cells pulling the vertices. Moreover, measurements of the junctional tensions at the specific phases of boundary oscillation by laser ablation, showed that the elongating boundaries exhibited lower tension in compare to the contracting boundaries. We extended our individual tension measurements to non-invasively estimate a tension map across the tissue. We further found that the vinculin, which is known to interact with adherens junction component a-catenin in tension dependent manner, accumulates to or dissociates from oscillating boundary, and the dynamics correlated with the estimated junctional tension. We propose that the level of vinculin at the cell boundary could be used to approximate junctional tension

Bio-Motile Functions
Studied by Microscopic Imaging of Physical Parameters

Shin'ichi ISHIWATA
Department of Physics, Faculty of Science and Engineering, Waseda University, Tokyo, Japan

The functions of bio-motile systems depend on physical parameters such as force and temperature. Here I will present the optical-microscopic imaging showing the dynamic properties of bio-motile systems (motor proteins such as myosin and kinesin, cytoskeletons such as actin filaments and microtubules, a meiotic spindle, muscle and nerve cells etc.), where the effects of mechanical perturbation and heat pulse are focused. First, I will introduce single-molecular studies: that is, unbinding force of myosin II, V and VI with an actin filament [1,2], that of kinesin with a microtubule [3,4], the polymerization dynamics of individual actin filaments [5], distortion of protomers in an actin filament by external force [6], supercoiling and rotational movement of sliding actin filaments [7,8], helical and robust processive movement of myosin V [9,10], and the force-generation of a microtubule depolymerizer named MCAK [11]. Next, I will report the viscoelastic properties of spindles self-organized in a *Xenopus* egg extract [12,13], and how the external mechanical impulse regulates the size and shape of spindles [11,14], and the timing of chromosome segregation within a HeLa cell at metaphase [15]. Then, I will introduce how environmental temperature and local heat pulse regulate the protein and cellular functions [16,17] such as the activities of molecular motors in vitro [18] and in a cell [19], the contractile properties of striated muscle [20-22], Ca-burst in a HeLa cell [23-26] and a nerve cell [27]. Finally, I will introduce spontaneous formation of a ring-shaped contractile actin bundle [28] and rotational cytoplasmic flow [29] within an artificial cell system composed of protein solutions in a cell-sized water-in-oil droplet surrounded by a monolayer phospholipid membrane.

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The Role of Aquaporin-8 on Mitochondria

Reina IKAGA[1,2]
1)Department of Nutritional Science, National Institutes of Biomedical Innovation, Health and Nutrition,
2)Department of Food Science, Otsuma Women's University

Aquaporins (AQPs) are a family of membrane transport proteinswhich play a role in water transport across biological membranes.At least thirteen AQPs (AQP0-12) have been identified in mammals.Most ofthem are localized in the plasma membrane and are involved in water transport into and/or out of cells. AQP8 is also expressed in various tissue and cells, including liver, testis, and pancreas.It is interesting that AQP8 appears to have functions on theplasma membrane and/or on the mitochondrial inner membrane. The major function of mitochondria is the production of ATP by oxidative phosphorylation. Through this process, a large amount of water is also formed. However, the mechanisms of mitochondrial water handling remain largely unknown.

We found that, in mouse adipose tissues and 3T3-L1 preadipocytes, the mitochondrial type AQP8 is dominantly expressed. We therefore created an AQP8 knock-down cell line in which we have investigated the role of AQP8 onmitochondrial function.The rate of oxygen consumption was decreased and the cellular ATP level wassignificantly reduced in the AQP8-knockdowncells.In the AQP8-knockdown cells, marked swelling of the mitochondria was observed by electron microscopy(Figure). These observations lead us to the hypothesis that the reduced AQP8 expression disrupts normal mitochondrial water flux, and lowers electron transport activity and ATP synthesis through inhibition of the water-generating processes. We also found that knockdown of AQP8 had multiple effects on the cellular metabolism including reduction of fat accumulation and changes in glycolysis and lipolysis. Thus, mitochondrial AQP8 functionally supports mitochondrial respiration probably via water excretionand cellular metabolism through maintenance of mitochondrial function.

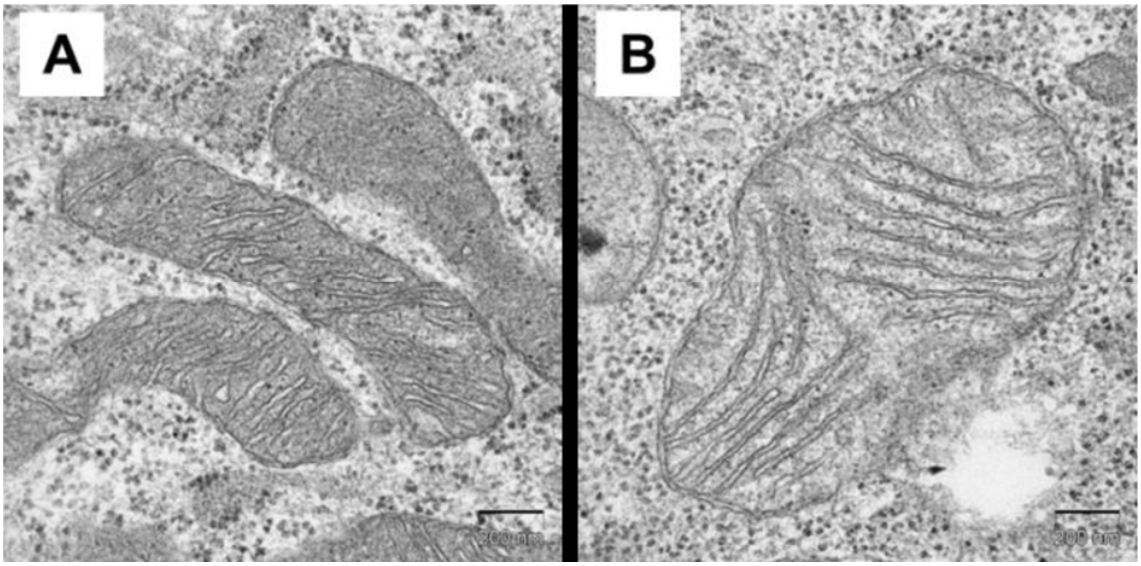

Figure.
Electronmicrographsofmitochondria.Typicaltransmissionelectronmicroscopyimagesofmitochondriainacontrolcell(A)an dinanAQP8-knockdown cell (B).Scalebaris 200nm.

Imaging Movements and Reactions of Biomolecules

Tomoko MASAIKE
Department of Applied Biological Science,
Faculty of Science and Technology, Tokyo University of Science

The aim of our bio imaging is to clarify how unitary steps of motions and chemical reactions lead to biological functions.

Our main target is F1-ATPase, the world's smallest rotary molecular motor. Its rotational behaviors have been studied in detail by the single-molecule observation method. Nevertheless, how chemical steps in ATP hydrolysis reaction drive rotation of the central shaft still remains unclear. Our aim for this target is to describe the relationship between ATP hydrolysis reaction and rotational motion by conformational mapping of the catalytic subunit. We monitored domain motions in several local regions of the catalytic subunit by detecting orientation of single-molecule fluorescent probes under TIRF microscopy, while simultaneously observing rotation of the bead attached to central shaft by dark-field microscopy.

We recently detected changes in orientation of a fluorophore attached to helices that are close to the catalytic site of the ATPase reaction. In addition, manipulation of the central shaft through magnetic beads under magnetic field seems to induce conformational changes of the catalytic subunit. These results suggest how rotation of the central shaft enhances conformational changes of the catalytic subunit. Taken together, they suggest concerted intra-subunit conformational changes of the secondary structures in the catalytic subunit. We try to discuss how conformational changes of each domain are relayed to the C-terminal helix in order to assist rotation of the central shaft.

Further studies include trials for visualization of chemical reactions catalyzed by single molecules. There has been studies that utilized fluorescent analogs of substrate ATP for observation of binding and release from single F1-ATPase molecules. Nevertheless, these analogs could not report timing of Pi release from theenzyme. Although we have not succeeded in detection of single inorganic phosphate (Pi) molecules so far, we are developing a system to detect Pi released from a single-molecule F1-ATPase.

Finally, our target is expanding to higher levels of biological hierarchy such as organelles. They require other techniques of microscopy such as 3-D tracking microscopy and subsequent interpretation of multiple molecules that work cooperatively. Therefore, future studies should be directed at single-molecule studies within an assembly of molecules.

We hope that our observation studies will open novel strategies of bio imaging and contribute to understanding of biological systems.

Super-resolution Imaging of *Salmonella* Virulence Regulation and Secretion

Yunfeng GAO[1], Christopher SPAHN[2], Yong Hwee FOO[1], Andrew LIEW[1],
Mike HEILEMANN[2], and Linda J. KENNEY[1,3,4]
1)Mechanobiology Institute, National University of Singapore, Singapore; 2)Goethe University, Frankfurt, Germany; 3)Jesse Brown VAMC; 4)University of Illinois-Chicago, Chicago, IL

In bacteria, one paradigm for signal transduction is the two-component regulatory system, consisting of a sensor kinase and a response regulator. In *Salmonella*, the EnvZ/OmpR system responds to osmotic stress and also positively regulates the *Salmonella* Pathogenicity Island 2 (SPI-2)-encoded type III secretion system required for its survival in the macrophage vacuole. EnvZ/OmpR acidifies the *Salmonella* cytoplasm in response to the acidified vacuole, driving virulence factor secretion into the host cytosol.

Acidification is required for the secretion of virulence factors, blocking acidification results in a neutralized cytoplasm that is defective for SPI-2 secretion. Using super-resolution microscopy, we visualized the emergence of *Salmonella*-secreted effectors into the host cytoplasm and followed the resulting endosomal tubulation. Our results suggest a mechanical role in needle elongation of the type three secretory apparatus and subsequent gating of effector secretion. Comparison of our laboratory strain with a recently emerging invasive strain of *S.* Typhimurium provides evidence for its invasive properties. Our work challenges existing views that bacteria regulate their pH to maintain neutrality, and provides a new model for *Salmonella* virulence factor secretion and infection. Supported by the Research Center of Excellence in Mechanobiology from the Ministry of Education, Singapore, VA 5I01BX000372 and NIH AI123640 to LJK.

Targeting Brain Tumors: Improving Lives through Precision Medicine

Carol TANG[1,2,3], Christopher Beng Ti ANG[1,2,4,5]
1) National Neuroscience Institute, 2) Duke-NUS Medical School, 3)National Cancer Centre, 4)Singapore Institute for Clinical Sciences, A*STAR, 5)Department of Physiology, NUS

In mid-2016, the National Neuroscience Institute was awarded the NMRC Translational and Clinical Research (TCR) Flagship Program grant for neuro-oncology. This 5-year endeavour seeks to address significant questions of unmet medical need, particularly in the most devastating form of adult malignant gliomas. The multi-disciplinary team comprises scientific and clinical partners from several institutes and industry.

The theme of *Patient Stratification towards Precision Medicine* serves to define specific biomarkers which can stratify patients with malignant glioma into therapeutic classes so that specific chemotherapeutics can be administered. The theme of *Deciphering Molecular Heterogeneity* focuses on elucidating the molecular profile of grade IV glioblastoma multiform (GBM) at the time of recurrence and progression. This is the stage of disease where at present, management protocols are most arbitrary and require refinement. The knowledge obtained by creation of a genomic database for recurrent GBM will be both novel and of great clinical significance. The third theme of *Novel Imaging Technologies and Predictive Biomarker Profiles* aims to develop new platforms such as imaging probes which are not only specific to malignant glioma but are also closely correlated with the molecular stratification of the tumor1,2. In addition, we also describe the development of a "fluid biopsy" exosome-based microfluidic biomarker platform which allows for clinical monitoring of the patient at diagnosis and along the course of the disease3-5. In this theme, we also address the issue of optimization of surgical resection of malignant gliomas. In this disease, the interface between tumor tissue and normal brain is indistinct, making aggressive resection hazardous. As such, we describe the clinical development and trial of a Raman spectroscopic probe fashioned for use in neurosurgery that has in addition, the capability to distinguish molecular subtype profiles. We capitalize on the fact that our clinical center has an intraoperative MR imaging facility which allows the surgeon to ascertain extent of surgical resection during surgery. This modality will be integrated into the clinical effort to determine the utility of the Raman probe in maximizing tumor resection. In summary, this TCR proposal addresses the key considerations in the management of a patient with malignant glioma from time of diagnosis onwards. It is *composed of efforts which will refine diagnosis, classification of disease, surgical resection technique and selection of chemotherapeutic protocol*.

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Whole-body and Whole-organ Clearing and Imaging with Single-cell Resolution Toward Organism-Level Systems Biology in Mammals

Hiroki R. Ueda
Systems Pharmacology, Graduate School of Medicine, University of Tokyo
Laboratory for Synthetic Biology, Quantitative Biology Centre, RIKEN

Organism-level systems biology aims to identify and analyse cellular circuits in organisms. To this end, one of the most powerful methods is optical imaging in combination with fluorescent labelling. However, the long-standing obstacle has been tissue opacity. Recently, the solutions to this problem have started to emerge by whole-body/organ clearing techniques that employ new tissue-clearing chemistry. In this talk, I introduce these advancements and discuss how to combine new clearing techniques with efficient production of genome-engineered animals, rapid volume imaging and efficient image informatics in order to obtain quantitative organ-wide single-cell-resolution data. These technologies start to bring us closer to system-level understanding of physiology and diseases of complex mammalian systems.

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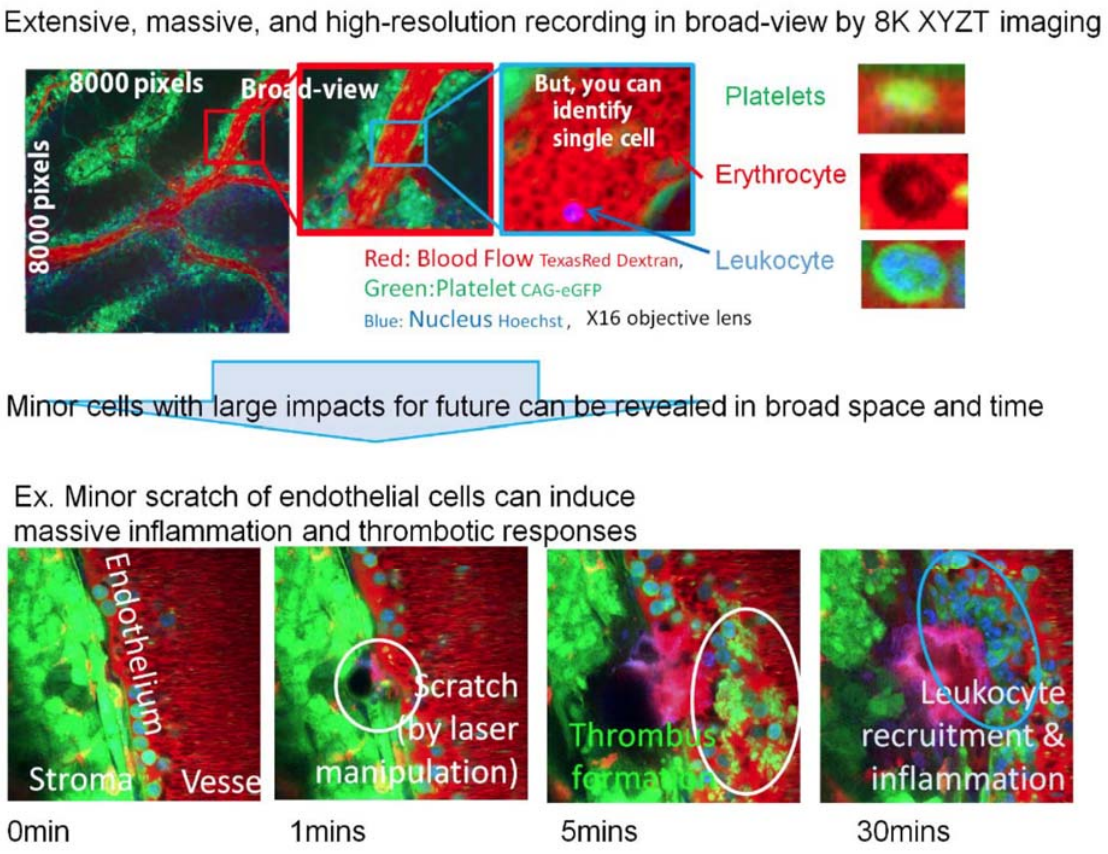
Minor Changes but Major Future Impacts, Can Be Revealed by Extensive and High-Through-Put In Vivo Visualization Using 4K/8K CMOS Sensors

Satoshi NISHIMURA
Center for Molecular Medicine, Jichi Medical University, Tochigi, JAPAN

To capture rare/minor changes but with biologically important importance for future, we developed multiscale imaging system in space and time dimensions which was optimized for living animals. Especially, recently advanced technologies including 1:CMOS sensors with large pixel numbers (4 to 8K), 2: highthroughput-image handling methods, and 3: low-power high-NA lens were integrated into one optical system. Animals are hold in customized chamber during observation, and controlled by robot-like mechanical devices which can adujust animal positions in synchronization with CMOS shutters and XYZT scanning. Large pixel numbers enhanced imaging fields and evaluated information per time. Recording time was also increased to cover from milli-second to hours to identify key points during long pathological changes. “Singularity points” can be identified form long-time recordings by reverse engineering techniques.

We identified tiny scratch on endothelium can induce massive platelet aggregations. Thrombus formation and inflammatory changes followed in later phase, which is one of the main components of cardiovascular events. Neutrophils were accumulated to repair leak of blood into lymphatic stromal space after damage, and macrophage recruitment followed. Platelet activation in millisecond order, and chronic inflammatory reactions in hours are simultaneously evaluated by single imaging modality. We covered micro to macro scale in space and time dimension, and many massive pathological changes can be attributed to small triggers. Singularity in life can be visualized, and will be the therapeutic target with minimum invasive ness.



Raman Microscopy for Molecular Imaging of Living Cells

Katsumasa FUJITA
Department of Applied Physics, Osaka University

Raman spectroscopy has been used for material analysis in the various research field. The capability of detection molecular vibrations enables to detect molecular species, conditions, and their environment in a sample to understand the function and characteristic of target materials. The recent development of techniques for highly sensitive Raman detection has expanded the application of Raman spectroscopy to the biomedical field. In particular, Raman microscopy that can obtain spatial distributions of biological molecules base on their vibrations provides various methodologies to analyze complicated biological phenomena. In our research, we have developed a Raman microscope that can rapidly image molecular distribution in a live cell [1,2]and studied the application to the analysis of molecular dynamics during cellular events, such as apoptosis, cell division and differentiation [3-7]. We measured Raman spectra during those cellular events and found that Raman spectra can represent the cell state during the activities, indicating that Raman microscopy can be used to investigate cell status without labeling. Since Raman microscopy can provide comprehensive information of intracellular molecules, it can be a tool to characterize cells and find functional singularities that may exhibit significant contribution to the cellular event.

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Development of Techniques For Imaging Physiological Functions Toward Visualization of Minority Cells

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If we carefully observe the cell population that at first glance looks uniform and homogeneous, we may find small number of heterogeneous cells with a different nature. Moreover, this minority cells would sometimes significantly alter the behavior of the whole cell population. In this symposium, I would like to discuss 1) possible mechanism by which variety output could be produced even in the cells with identical biomolecular reaction networks, and 2) development of techniques for imaging physiological function at wide range of spatiotemporal scale, which is indispensable for identification of minority cells.

Integrin Clusters: A Robust Way to Organize Adhesions for Cell Mechanics

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Mechanobiology Institute, National University of Singapore, Singapore

Recent studies at the nanometer scale have revealed that relatively uniform clusters of adhesion proteins (50-100 nm) constitute the modular units of cell adhesion sites in both cell-matrix and cell-cell adhesions. Super resolution microscopy and membrane protein diffusion studies both suggest that even large focal adhesions are formed of 100nm clusters that are loosely aggregated. Clusters of 20-50 adhesion molecules (integrins or cadherins) can support large forces through avidity binding interactions but can also be disassembled or endocytosed rapidly. Assembly of the clusters of integrins is force-independent and involves gathering integrins at ligand binding sites where they are stabilized by cytoplasmic adhesion proteins that crosslink the integrin cytoplasmic tails plus connect the clusters to the cell cytoskeleton (see clusters of paxillin in Fig. 1). Cooperative-signaling events can occur in a single cluster without cascading to other clusters. Thus, the clusters appear to be very important elements in many cellular processes and can be considered as a critical functional module.

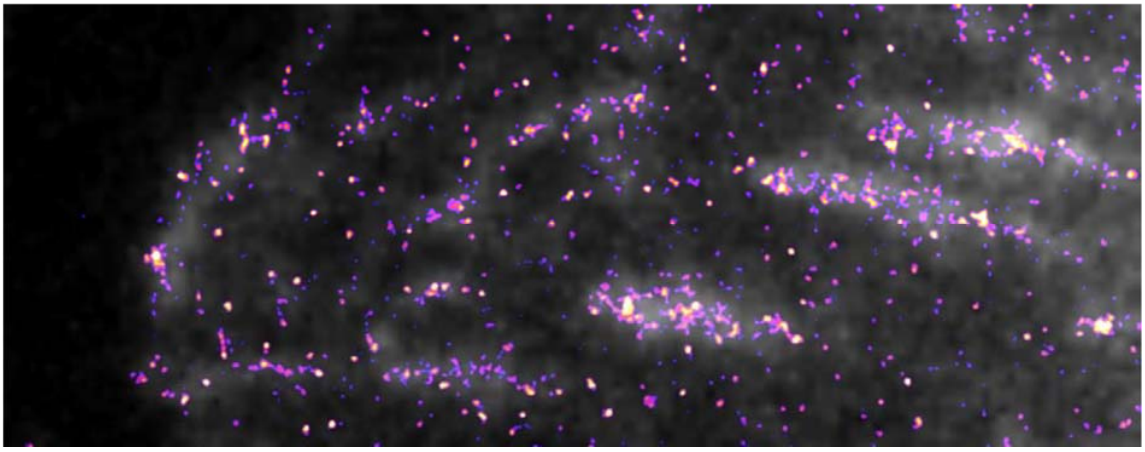

Figure 1 PALM super-resolution image of paxillin clusters (50-100 nm in diameter) in purple overlaid on a standard epi-fluorescence image of the same paxillin adhesions (diffuse white) in a spread fibroblast. Bar is 1000 nm.

POSTER ABSTRACTS

Quantitative cell biology of plant stomata

Takumi HIGAKI

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Manual evaluation of cellular structures is still a popular approach in cell biological studies. However, such approaches are laborious and are prone to error, especially when large quantities of image data need to be analyzed. We have introduced an image analysis method that overcomes these limitations by quantification and clustering of cytoskeletal structures. In our framework, cytoskeletal orientation, bundling and density are quantified by measurement of newly-developed, robust metric parameters from images. Thereafter, the microscopic images are classified without supervision by clustering based on the metric patterns. Clustering allows us to collectively investigate the large number of cytoskeletal structure images without laborious inspection. Application of this framework to images of GFP-labeled actin cytoskeletons in plant guard cells determined that microfilaments are radially oriented and transiently bundled in the process of diurnal stomatal opening. The measurements also revealed that the artificial bundling of actin cytoskeletons suppressed the diurnal patterns of stomatal opening, suggesting that changes in the level of actin bundling are crucial for promoting stomatal opening. In addition, in order to comprehensively grasp cell biological events in plant stomatal movement, we have acquired microscopic images of guard cells with various organelles markers (Figure 1). We visualized the average organelle distributions in guard cells using probabilistic mapping (Figure 2). Subtractive images of open and closed stomata showed distribution changes in some intracellular structures including endoplasmic reticulum (ER). Time-lapse imaging showed that similar ER distribution changes occurred during stomatal opening induced by laser oblation of neighboring epidermal cells, indicating that our image analysis approach has identified a novel ER relocation in stomatal opening.

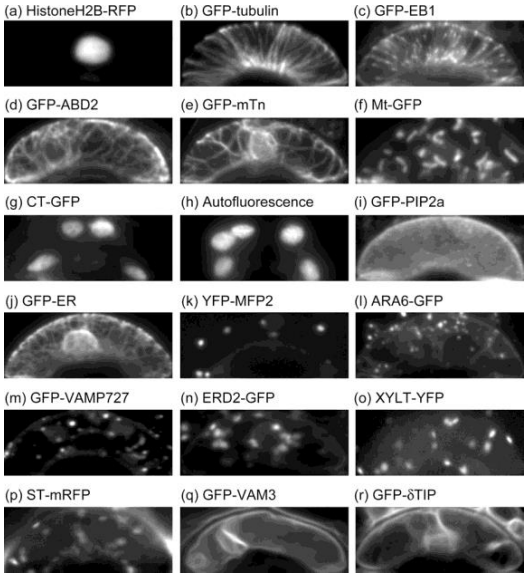

Figure 1. Representative images of guard cells.

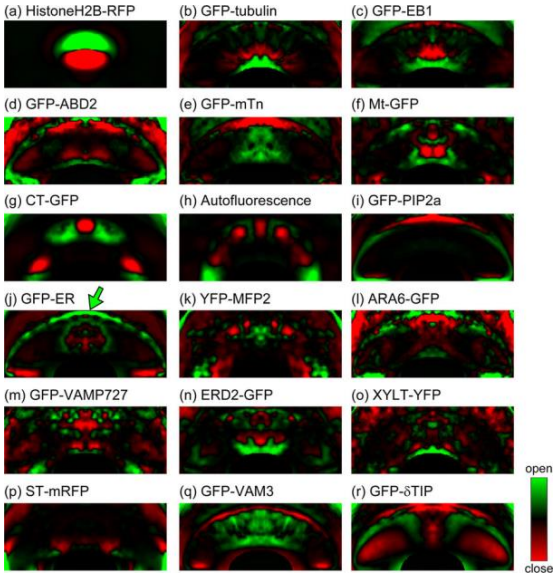

Figure 2. Probability map images of guard cells.

Development of Bioluminescent Low Affinity Ca^{2+} Indicators Applicable to Analysis of Ca^{2+} Dynamics in Endoplasmic Reticulum.

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Calcium ion (Ca^{2+}) is a crucial intracellular signaling molecule concerned with many biological processes. Ca^{2+} release from major intracellular Ca^{2+} store endoplasmic reticulum (ER) which is one of the key factors for this regulation through the controlling of cytosolic Ca^{2+} level. To investigate ER Ca^{2+} dynamics (having sub milimolar (mM) range of Ca^{2+}), a low affinity Ca^{2+} indicator is able to facilitate the Ca^{2+} imaging. Over the last couple of decade's variety of the fluorescence protein (FP) based Genetically Encoded Ca^{2+} Indicators (GECIs) have been developed for live cell imaging. These Ca^{2+} indicators are the most promising tools for investigating subcellular Ca^{2+} dynamics in cellular organelles. To monitor the Ca^{2+} dynamics in ER, we have successfully developed a bioluminescent low affinity intensiometric genetically encoded Ca^{2+} indicators named CeNL(Ca^{2+}). It does not require any excitation light; therefore, it can be overcome the potential disadvantages (phototoxicity, photobleching, and autofluorescence from the specimen) in fluorescence imaging. By using the CeNL(Ca^{2+}) and previously developed high affinity green color GECI GeNL(Ca^{2+}), we have successfully achieved the simultaneous dual color Ca^{2+} imaging in ER and nucleus in mammalian cells. We are expecting that our low affinity indicator may investigate neuronal cells and skeletal muscle Ca^{2+} imaging.

Development of a novel long-circulating ultrasound contrast agent

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Tadamitsu SHIMA[1)], Daiki OMATA[1)], Kazuo MARUYAMA[1)]

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INTRODUCTION

Ultrasound (US) imaging is comparatively inexpensive, minimally invasive and provide real-time visualization. It is important to understand the vascular structure of tumor tissue. However, it is difficult to take the US imaging of blood flow in small vessel of tumor tissue. To solve this, microbubbles as US contrast agents have been developed. In Japan, the contrast agent Sonazoid is approved. It is applied for diagnostics of liver cancer as negative staining by being uptaken by kupffer cells in the liver, but not in tumor. However, because the blood clearance of Sonazoid is fast due to the liver accumulation, it would not be suitable for other tumors and disorders. In this study, we tried to develop novel microbubbles for long circulation to observe dynamics of blood flow.

METHODS

Preparation of Lipid Bubbles (LB) Bubbles were prepared from liposomes of distearoyl phosphocholine (DSPC), distearoyl phosphatidylglycerol (DSPG) and 1,2 – distearoyl – sn – glycerol – 3 – phosphoethanolamine – N - [methoxy (polyethylene glycol) - 2000] (DSPE-MPEG(2k)), which were homogenized together with C_3F_8 gas. Then, the bubbles were freeze-dried and rehydrated before use.

Assessment of stability of LB in blood Mice (ddY, female, 6 weeks), anesthetized with isoflurane, were intravenously administrated LB or Sonazoid (1×10^7 particles / 20 μL). Then the stability in blood was assessed by comparing intensity of US imaging in kidney. The intensity of US imaging was analyzed by ImageJ software. Then, the time until half of maximum intensity ($T_{1/2}$) was calculated.

RESULTS AND DISCUSSION

$T_{1/2}$ increased with increasing DSPG content. The longest $T_{1/2}$ was achieved with LB containing 60 mol% DSPG, $T_{1/2}$ of LB containing 90% DSPG was shorter compared to 60% DSPG containing LB. In addition, we compared $T_{1/2}$ on kidney between 60% DSPG containing LB and Sonazoid. $T_{1/2}$ of LB containing 60% DSPG was longer than that of Sonazoid. From these results, it was thought that 60% DSPG containing LB could escape from reticuloendothelial system such as liver.

CONCLUSION

We succeeded to develop the long circulating microbubble by adding DSPG to the shell of LB. And there was optimal rate of DSPG content of 60 mol%. We expect that our long circulating LB could be a useful US diagnostic agent for various types of tumor where Sonazoid is not applicable.

ACKNOWLEDGMENTS

This research was partially supported by the MEXT-Supported Program for the Strategic Research Foundation at Private Universities and the Strategic Research Program for Brain Sciences from Japan Agency for Medical Research and Development.

Preparation of acoustic nanodroplets using a microfluidity device and evaluation of their physical properties

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Lisa MUNAKATA [1)], Hitoshi URUGA [1)], Mutsumi SUGII [1)], Daiki OMATA [1)], Kazuo MARUYAMA [1)]
[1)]Teikyo University, Faculty of Pharma-Sciences, Laboratory of Drug Delivery System, Tokyo, Japan

INTRODUCTION

Nanodroplets containing liquid perfluoropentane (PFP) that can be turned to gas by ultrasound irradiation have been proposed as new technology for diagnosis and treatment of cancer. However, the optimum production method has not been established, because of non-uniform size and difficulties to scale up. Therefore, to make uniform nanodroplets with a method that allows large scale manufacturing is most important for clinical application. In order to solve these problems, we focused on a mixing technology using a microfluidics device that should give uniform formulations and is easy to scale up. In this study, we compared the particle size of nanodroplets produced using a microfluidics device with nanodroplets produced by conventional ethanol injection method. We also evaluated the phase change of nanodroplets from liquid to gas and *in vivo* kinetics.

METHODS

Lipid and PFP were dissolved in ethanol, and mixed with water with a microfluidics device or by injection into water while stirring in a test tube. Ethanol was removed using ultrafiltration. The nanodroplet sizes were measured with dynamic light scattering. The phase change from liquid droplets to gas bubbles was observed with an ultrasound imaging device. Furthermore, the DiR as fluorescent probe was added to the suspension of nanodroplets and it was intravenously injected in tumor-bearing mice. The *in vivo* kinetics was evaluated by observation at 1, 3, 6, 12, 24 and 48 hours after administration of the nanodroplets by In Vivo Imaging System (IVIS).

RESULTS AND DISCUSSION

Nanodroplets had an average size of about 200 nm and a uniform size distribution. The prepared nanodroplets were reproducible with microfluidics device. Observation with ultrasound imaging showed echo signal enhancement which occurred by ultrasound irradiation. This shows that the nanodroplets had become gas bubbles. Enhancement of echo signal was not seen with the control formulation not containing PFP. This suggests that the microfluidics device is suitable tool for making the phase change nanodroplets. IVIS imaging showed that the nanodroplets were accumulated in tumor tissue. Therefore, we expect that nanodroplets could be a new ultrasound imaging agent.

Visualization of insulin secretion from rat pancreatic INS-1 cells by using a fluorescent membrane probe.

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Conventional fluorescence observation of insulin secretion in living cells has been achieved by labeling insulin itself or insulin granules with fluorescent proteins, in which genetic engineering was needed. In this study, we proposed a simple method by using a fluorescent membrane probe, FM1-43, and aimed to develop the method suitable for simultaneous observation with other parameters such as cellular, endoplasmic reticulum and mitochondrial Ca^{2+} concentrations. FM1-43 is a fluorescent dye reversibly binding to the cell membrane. The number of fluorescent vesicles in cytosol is expected to increase gradually during insulin secretion, because a part of lipid bilayer in insulin granules constantly returns back into the cytosol after transitionally fusing to plasma membrane and being stained by FM1-43.

After stimulation with glucose, the intracellular fluorescent intensity of FM1-43 in rat pancreatic INS-1 cells gradually increased as expected (Fig.1). The rate of intensity increments which is expected to correspond to the insulin secretion during the time, regularly oscillated and the oscillation frequency was in good agreement with the one of real insulin concentration in the extracellular fluid (Fig.2). Looking at the area under the curve of the increments rate, the number of fluorescent vesicles in the cells greatly increased at around 5 and 25 minutes after glucose stimulation, also in good agreement with the pattern of insulin concentration changes in the extracellular fluid. By using this method, it is expected that the secretory rhythm of individual cells can be captured, and simultaneous observation of insulin secretion and other intracellular parameters will be possible.

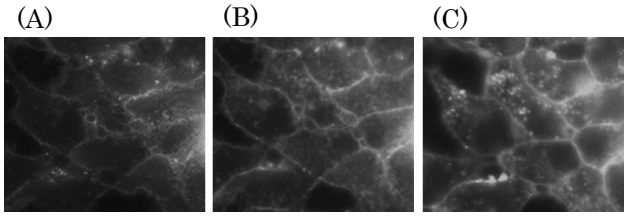

Fig.1. Fluorescent images of INS-1 cells labeled by FM1-43. The images were obtained before (A) and after 19 (B) and 39 (C) minutes of stimulation with glucose.

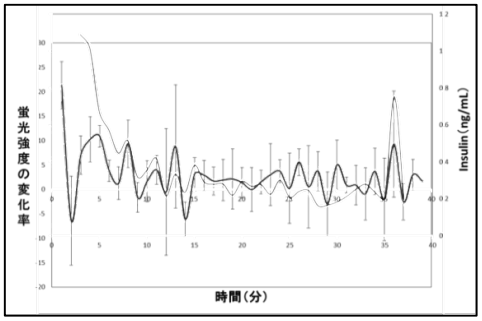

Fig.2. Time course of the increments rate of the intracellular fluorescent intensity (black line) and the amount of insulin excreted in the extracellular fluid (gray line).

Knockdown of perilipin 4 affects on lipid droplet formation and mitochondrial morphology in 3T3-L1 adipocytes.

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Adipocytes have endocrine functions and secrete hormones and cytokines to regulate metabolic states of whole body. Adipocyte enlargement resulted from lipid droplet accumulation could be a switch to signal pathway for secretion of cytokines, increasing inflammation around adipocytes and then causing lifestyle-related diseases. The switching mechanism, however, is still unclear. Perilipins are a family of proteins which localizes on the lipid droplet surface and play an important role in lipid droplet formation and maturation. Perilipins may also be involved in communication with surrounding organella. We observed that mitochondria formed a network around lipid droplets during differentiation and maturation of 3T3-L1 to adipocytes. In this study, we focused on perilipin 4, as a clue to clarify the relationship between lipid droplets, and created the cell lines (shPLIN4) from 3T3-L1 cells, in which the expression of perilipin 4 mRNA was knocked down to ca. 40%.

In shPLIN4, the lipid droplets were smaller but much more than those in the control cells (shCont) and mitochondria were shorter and thinner (Fig.1A and C), indicating that the knockdown of perilipin4 affected both of lipid droplets formation and mitochondrial energy states. The mRNA expression of enzymes in glycolysis and electron transfer system of shPLIN4 was lower than those of shCont, so the ATP synthesis could be decreased. However, the expression level of AMPK in shPLIN4 was also decreased. An possible explanation of these results is that knockdown of perilipin 4 reduced the expression level of AMPK, possibly lowering the whole energy production. Treatment with AICAR, an AMPK activator, recovered the mRNA levels of the enzymes and the mitochondrial morphology in shPLIN4 (Fig 1D).

On the other hand, the expression of MCP-1, an inflammatory cytokine, in shPLIN4 was much more increased compared to shCont, and AICAR treatment did not recover it. It is most likely that the perilipin 4 knockdown affects the inflammatory states in 3T3-L1 adipocytes through a different pathway from the AMPK signaling.

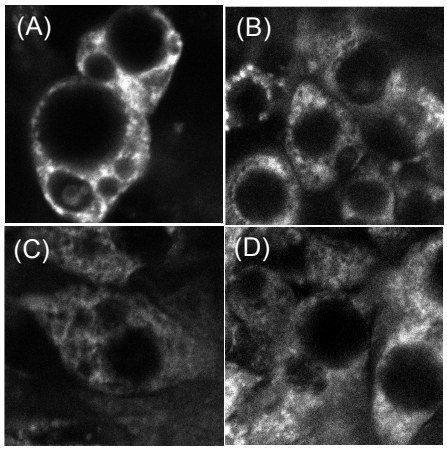

Fig.1. Fluorescent images of mitochondria labeled with MitoTracker Deep Red in shPLIN4 (C and D) and shCont (A and B). The cells in panel B and D were treated with 1mM AICAR over night.

Structure of Protein A and its derivative in solution studied by small-angle x-ray scattering

T Masaji Shinjo[1)], Kaoru Ichimura[2)], Masaki Kojima[7)] and Hiroshi Kihara[3-6)]

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Protein A (PA) is a 56 kDa surface protein with five homologous Ig-binding domains found in the cell wall of S. aureus. Each domain folds into a three-helix bundle, and is able to bind to IgGs. PA releases Igs in acidic pH, suggesting its pH-dep. conformational change. We, then, start investigating the structure of PA and its derivative, C5, in both of neutral and acidic pHs by SAXS. Results show: PA and C5 form globule conformations, and their Rg values are 3.73 ± 0.02 nm within experimental errors both at neutral and acidic pHs. However, Kratky plots and P(r) suggest the conformation of PA at acidic pH is different from the other cases. Experiments were done at PF BL 6A with the approval No. of 2013G101. Authors are grateful to the sample supply from KANEKA CORP.

At the symposium, we will show results of PA and C5 with Fc fragment Ig.

Analysis for antigen phagocytotic reaction regulated by cellular signal transduction

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1) Chiba Institute of Technology, Department of Life and Environmental Sciences, Faculty of Engineering, 2) Graduate School of Engineering, 3) Department of Life Science, Faculty of Advanced Engineering, 4) Chiba University, Graduate School of Medicine

In immune system, antigen presenting cells act as an important role both for the host defense and for immunological tolerance. Macrophage performs transrational effect on innate immunity and adaptive immunity. Like dendritic cell, macrophage is also called the professional antigen presenting cells, however, it is indicated that antigen processing potency of macrophage is predominant. In this study, we analyzed the antigen phagocytotic reaction, which activates cellular signal transduction. By using fluorescent dye labeled ovalbumin, macrophage cell line: RAW264.7 was analyzed for antigen phagocytotic manner with confocal imaging study and western blotting. When macrophage showed endocytosis of antigen proteins, such as ovalbumin, the signal of NF-κB was activated to induce cellular transcription. NF-κB activation was also observed when antigen signal had been disappeared, recognized by the fluorescent intensity of ovalbumin. We propose that antigen presenting cells, especially macrophage, may be activated when endocytosis is initiated and also when antigen degradation is initiated. To understand whether different types of antigen, or different size of antigen may occur the different patterns of cellular signal activation, we have compared the ovalbumin phagocytosis with BODIPY conjugated *E.coli* phagocytosis. Understanding of the mechanism of phagocytotic time dependent pattern would contribute the therapeutic timing of allergy and inflammation.

Fig.1

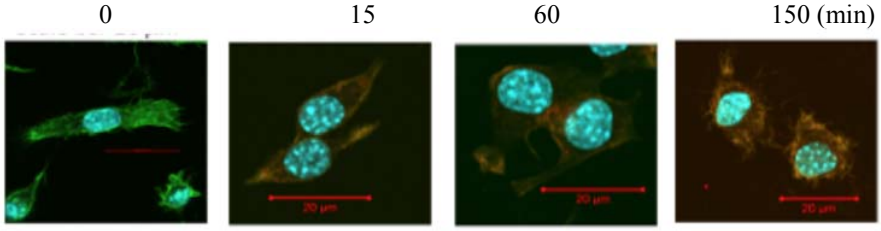
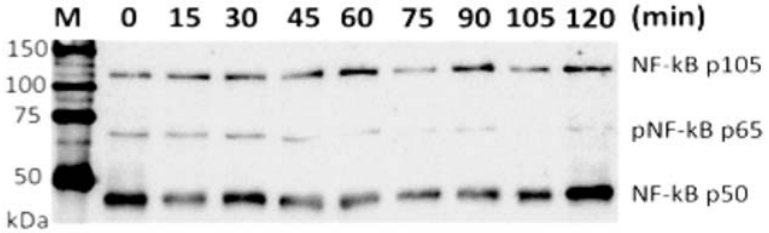


Fig.2



Macrophage cell line: RAW264.7 was treated with fluorescent dye labeled ovalbumin; Alexa 594 ovalbumin, the fluorescent intensity of antigen was recognized from 15 min to 120 min. The signal was disappeared at 150 min (Fig.1). Phospho-NF-κB band was also recognized at the early phase of phagocytosis (Fig.2).

Single-molecule observation of actin polymerization using linear zero-mode waveguides

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Takashi Tanii[2)], & Takashi Funatsu[1)]
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Actin is a ubiquitous cytoskeletal protein, which is essential for the structure and function of eukaryotic cells. Actin polymerization occurs through three phases called nucleation, elongation, and steady-state phases. A previous study showed that the ends of actin filaments grow and shorten more rapidly than expected from measured rate constants of monomer association and dissociation [1]. There are several possible polymerization mechanisms to explain this interesting result, but detailed polymerization mechanism is not known. In this study, we aimed to investigate the oligomeric state of actin incorporated into the filament ends using single-molecule fluorescence imaging in the actin polymerization process. Actin polymerization occurs when the concentration of G-actin monomer in solution exceeds 100 nM (critical concentration). Therefore, single-molecule fluorescence imaging at such a high concentration is required. However, single-molecule fluorescence imaging of actin polymerization is impossible under total internal reflection fluorescence microscopy (TIRFM). It is because the signal-to-noise ratio (SNR) cannot exceed 3 at concentrations above 100 nM due to the size of the excitation region. In contrast, SNR can exceed 3 by using linear zero-mode waveguides (ZMWs) that suppress background light by narrowing the excitation region. Hence, we observed actin polymerization using single-molecule fluorescence imaging with linear ZMWs (Fig. 1). As a result, we found that the monomer associated with filament ends in the elongation phase, but small oligomers (dimer to trimer) were also added to the filaments in the steady-state phase. Furthermore, no cooperative binding of actin at the ends of the filament was observed.

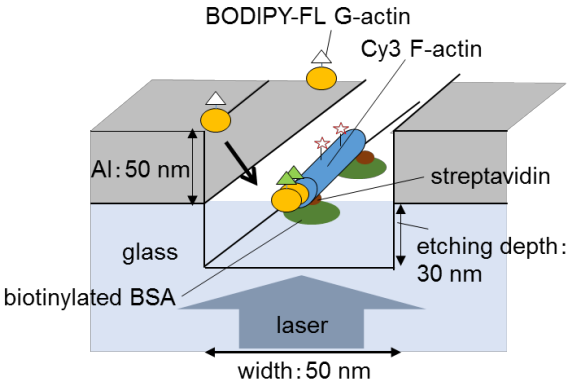

Fig. 1 Experimental setting with linear zero-mode waveguides

Reference

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Development of a method of heating a single cell using gold nanoparticles

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Temperature is a fundamental physical quantity, which has significant effects on cellular functions. Recent study has shown that the intracellular temperature distribution is heterogeneous. For example, mitochondrial chemical stimulation brought local heat generation; and there was an intracellular temperature gradient near this organelle. These results suggested that local temperature changes might regulate various biochemical reactions in a cell. Thus, understanding the relationship between intracellular temperature and cell functions may contribute to obtaining better insights into cell functions. However, the mechanism and the significance of intracellular heat generation are still unknown. Therefore, it is desired to examine cell response to intracellular local heating. In this study, we have developed a method to control the intracellular local temperature for investigation of temperature-dependent cell functions.

Here, we report a simple technique to heat a single cell using gold nanoparticles (GNPs), which efficiently convert light to heat. While heating the cell using GNPs, we measured temperature in a cell by using a molecular thermometer, fluorescent nanogel thermometer (FNT). At low temperature, the fluorescence intensity of FNT is weak while at high temperature, the intensity is strong. We irradiated GNPs incorporated into COS7 cells with a visible laser and observed temperature rise in cells by FNT. By adjusting the amount of GNPs and the laser power, we could quantitatively control the intracellular temperature rise. Furthermore, we succeeded in inducing a cell response by this heating method; we observed stress granules (SG) formation upon heating, which is responsible for the suppression of translation during stress. Through the heat generation by GNPs, SG formation was induced. These results show that GNPs have a potential for the means to heat the intracellular local region and this method is useful for the comprehension of cell responses to local temperature change in the cells. Our method will contribute greatly to investigate intracellular thermogenesis-based thermal biology.

Microfabrication techniques for observing neuronal transmission pathway

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Neuronal circuits in vivo and in vitro consist of huge number of cells and form complexed neurotransmission pathways. Therefore, it is difficult to understand how neuronal communication works at network level. If we are able to reconstruct simple neuronal circuits and regulate the transmission pathway, the mechanism of how the neuronal circuit stores memories and processes information at the neuronal circuit level will probably become clear. To this end, we developed a microfabrication technique to create artificial neuronal circuits composed of individual neurons using agarose and an infrared laser (Agarose-microfabrication technique). Using this technique, we successfully created simple neuronal circuits with controlled synaptogenesis. By combining electrophysiological technique, we further demonstrated that the artificial neuronal circuit showed original firing pattern depending on the circuit structure. The electrophysiological technique allows us to detect the frequency of neuronal activity in detail. On the other hand, bioimaging technique enables us to observe the transmission pathway of the neuronal circuit. We developed another microfabrication technique to observe the neuronal transmission pathway using bioimaging technique. To study the neuronal transmission pathway, a 2.5-dimensional (2.5D) microfabrication technique using an ultraviolet curing resin was developed. The structure formed by 2.5D microfabrication technique is steric, which allows us to cocultivate neurons and glias that are required to keep the structure of neuronal circuit for a long time. As a result, the 2.5D technique allowed us to co-cultivate neurons and glias for a long time while the designed structure is maintained. Furthermore, a combination of the 2.5D technique and the agarose microfabrication technique allowed us to guide the synaptogenesis. In summary, the new microfabrication technique with bioimaging shows possibilities for observing principles of neuronal networks such as neuronal plasticity at network level.

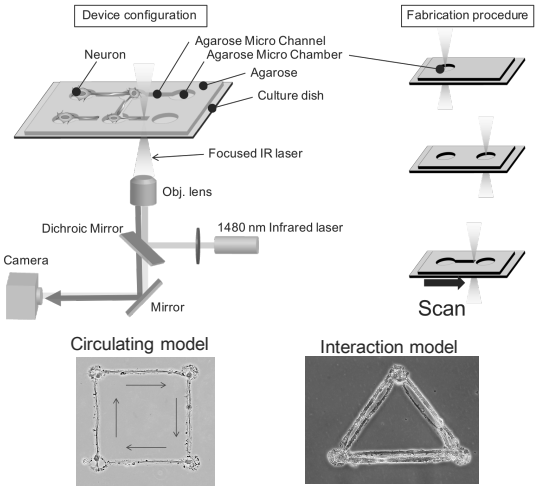

Figure 1. Agarose microfabrication technique

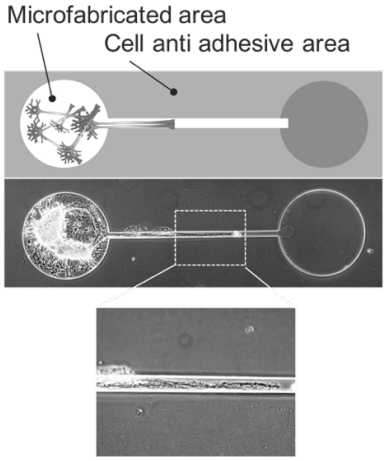

Figure 2. Combination technique of 2.5D and agarose microfabrication technique

RGB-color fluorescent genetically-encoded ATP indicators for energy metabolism research

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Adenosine triphosphate (ATP) is used for various cellular activities (for example, cell motility, exocytosis and development). For understanding of the dynamics of ATP at cellular level, fluorescent genetically-encoded indicators would be a powerful tool for optically detecting intracellular ATP. Recent years, we engineered red, green, and blue (RGB-color) intensiometric indicators using single fluorescent proteins where the fluorescence intensity at a single wavelength is enhanced upon sensing ATP. The expanded color usability of these indicators enabled monitoring the ATP dynamics in different organelles at same cell at the same time. Here, we imaged the dynamics of cytoplasmic and mitochondrial ATP of cells derived from a wide variety of species, including mammalian cells, plants, and worms. Furthermore, coupling with additional fluorescent indicators, we visualized the dynamic interplay of ATP, cAMP, and Ca^{2+} in the brown adipocyte during an uncoupling event. We do believe that the toolset of indicators will facilitate future research into energy metabolism.

Concomitant monitoring of local heating and heat-triggered cargo release from thermosensitive liposomes by fluorescence imaging

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Control of cargo release from nanoparticles is an important technology for maximizing the benefits of the nanoparticles for biological and biomedical applications such as bioimaging and drug delivery systems. Heat stimulus is a potent tool to trigger the release of cargo from the nanoparticles, for which a light energy is a practical source to generate local heating at a target region. Herein, we attempt to apply the fluorescence imaging techniques for concomitant monitoring of the heat-triggered cargo release from lipid-based nanoparticles (liposomes) and local heating by photothermal conversion of water with a 980 nm near-infrared (NIR) laser irradiation. Liposome formulation consisting of 1,2-dipalmitory-*sn*-glycero-3-phosphocholine (DPPC) with an anionic lipid and PEG-lipid as stabilizers encapsulating hypertonic solution with lipid membrane shows that a gel to liquid-crystalline phase transition at around 40 ^{o}C effectively triggers the release of cargo from liposomes at temperature above 40 ^{o}C with NIR irradiation. Our proof of concept has been demonstrated in a cancer cell with microscopic monitoring the actual "intracellular temperature" using a fluorescent thermosensor. An intracellular thermometry revealed that it was not until the intracellular temperature reached around 40 ^{o}C by NIR irradiation that the release of the cargo started gradually, showing the good agreement with the result from the extracellular *in vitro* study. This targeted release of cargo from thermosensitive liposomes based on a photothermal effect using NIR laser offers a potent nanoscale platform for the on-demand release of drugs in intracellular space with local heating. The intracellular thermometry facilitates the quantitative monitoring and control of the heating at the cellular level. Furthermore an extended concept for macroscopic temperature monitoring using the thermosensitive liposomes was examined using NIR laser-induced heating of water in a hydrogel matrix. The temperature distribution in hydrogel by photothermal conversion can now be traced by fluorescence in real time, with the use of thermosensitive liposomes that release the fluorescence cargo at different threshold temperatures. These liposome platforms, equipped with temperature-sensing capability, extend the concept of temperature monitoring for heat-triggered drug release, as well as thermotherapy. Thus fluorescence imaging techniques are powerful tool for concomitant real-time monitoring for precise control of local temperature and drug release triggered by heat stimulus at target region.

Evaluation of cell-to-cell conduction on one-dimensional line of cardiac muscle cells network for in vitro predictive cardiotoxicity measurement beyond cell-based drug discovery electrophysiology

Kenji Matsuura[1), 2)], Moe Iwamura[3)], Naoki Takahashi[3)], Yuki Yamanaka[3)], Natuki Seki[4)], Masao Odaka[1), 2)], Akihiro Hattori[1), 2)], Hideyuki Terazono[1), 2)] and Kenji Yasuda[1-4)]

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In recent years, technologies to evaluate the effect of drugs to human cardiac muscle cells have been promoted vigorously. As the one of interested questions in this field is whether the in vitro assay can predict the clinical issues such as the "*Torsade de pointes* (TdP)", which is the potentially deadly cardiac arrhythmia cause by the asynchronous beating behavior of neighboring cells. Hence, we have proposed an on-chip cell-to-cell conduction screening assay of cardiomyocytes to overcome the limitations of the conventional cell-based *in vitro* assays. In the assay, we set a thousand of cardiomyocytes one-dimensionally on the rectangular shaped agarose microchambers on the multi electrode array chip, and measured the propagation of signal in cardiac muscle cell network and measured the increase and fluctuation of their propagations and durations field potentials (FPD)(Fig. 1). The result showed that the evaluation of delay and fluctuation cell-to-cell conduction have a potential to predict risks of arrhythmia more precisely beyond the conventional *in vitro* predictive cardiotoxicity assays.

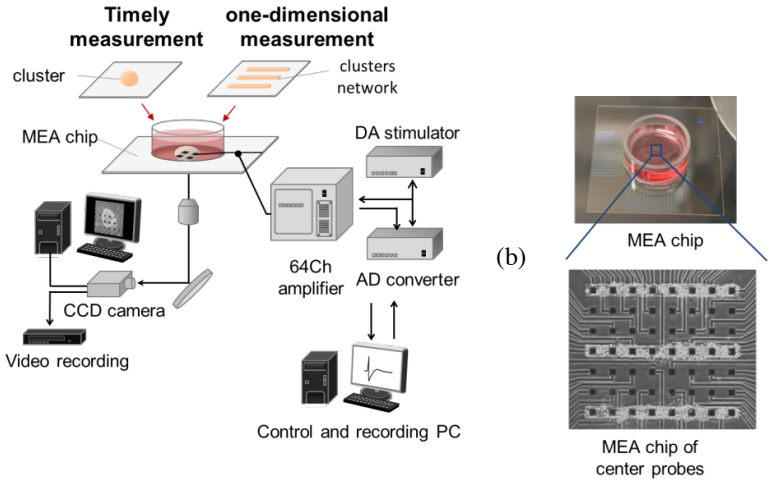

Figure 1. (a) Schematic of an on-chip multi electrode array system. (b) Schematic of a MEA chip. (c) Clusters of cardiac muscle cells which were lined-up on a surface of MEA chip.

Real time on-chip imaging cell sorter for shape recognition of target cells in droplets

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Automatic recognition of cells is an important challenge in numerous biological applications such as cluster cell analysis. Although fluorescence based methods can detect cells with accuracy, microfluidic on-chip imaging cell sorters offer several advantages in terms of recognition especially with regards to cells with complex morphologies. In this context we developed a label-free microfluidic droplet-sorting system based on image recognition of cells in droplets (Fig. 1). To test the applicability of this method, a mixture of two species with different morphologies (*Dunaliella tertiolecta* and *Phaeodactylum tricornutum*) were successfully identified and discriminated at a rate of 10 Hz (91 ± 4.5% and 90 ± 3.8%, respectively) (Fig. 2). The results indicate that imaging cell sorter can isolate single target cells from a mixture of cells with high accuracy without any staining.

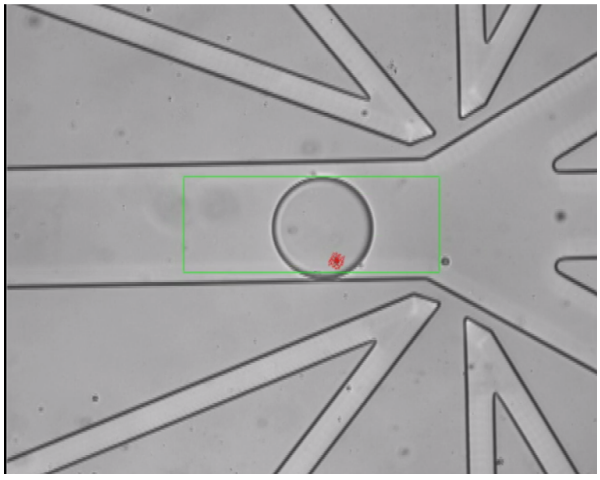

Fig. 1: Example of cell detection (red square) in the region of interest (green rectangle) before a droplet sorting process. Diameter of droplet is 80μm.

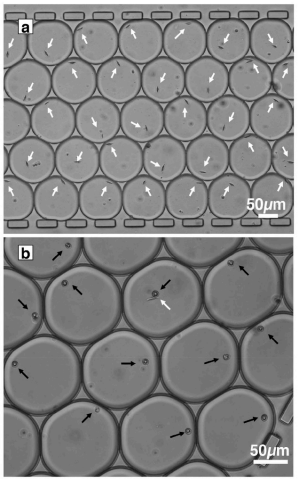

Fig.2: The panels a and b show parts of the collection channels after droplet screening process. White and black arrows show the droplets containing *Phaeodactylum tricornutum* and *Dunaliella tertiolecta*, respectively.

On-chip multi-imaging flowcytometry for non-labeled detection of cell clusters in blood with imaging biomarkers

Moe Iwamura[1)], Yuki Yamanaka[1)], Kento Iida[2)], Masao Odaka[3.4)], Akihiro Hattori[3.4)], Kenji Matsuura[3.4)],Hideyuki Terazono[3.4)] and Kenji Yasuda[1-4)]

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Detecting the circulating tumor cells (CTCs) in blood is important issue for understanding the metastasis as non-invasive liquid biopsy. However, conventional molecular biomarkers were not effective enough to identify the CTCs. Here, we have developed an on-chip multi-imaging cell analysis flowcytometry, in which target cells are identified by their morphological characteristics without any label of molecular biomarkers such as EpCAM antibody (Fig.1). Firstly, we fabricated poly-dimethylpolysiloxane (PDMS) microstructures in the cell sorting chip to evaluate the reproducibility, and found that the microstructures shrank at a constant rate of 80% at the first exposure step of SU-8 from photo-masks in the microfabrication process. Hence, we improved the procedure and succeeded in stably producing precise designed microchannel chips by designing the channel with compensations of the shrinkage. Secondly, we examined the efficacy of the system using the obtained blood samples of rats, in which prostate cancer cell line was implanted, and we detected that the frequency of CTC clusters increased exponentially over time after implantation (Fig.2). These results suggest that the function of the system is suitable for imaging biomarker identification with up to 10,000 samples/s resolution and was enough to measure the time course change of CTC clusters.

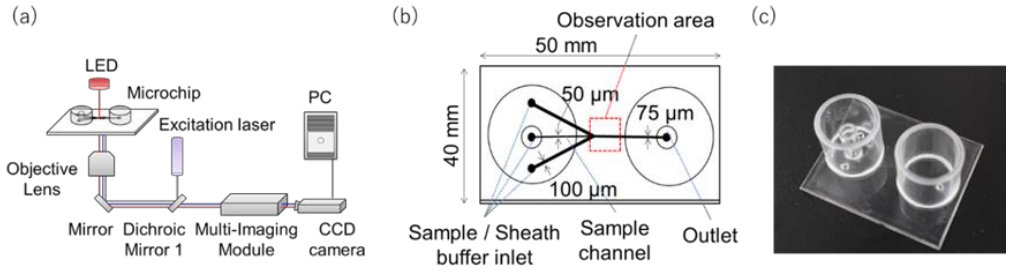

Figure 1. Overview of the on-chip multi-imaging cell analysis flowcytometry. (a) Schematic image. (b) Design of the microchip. (c) Image of the microchip.

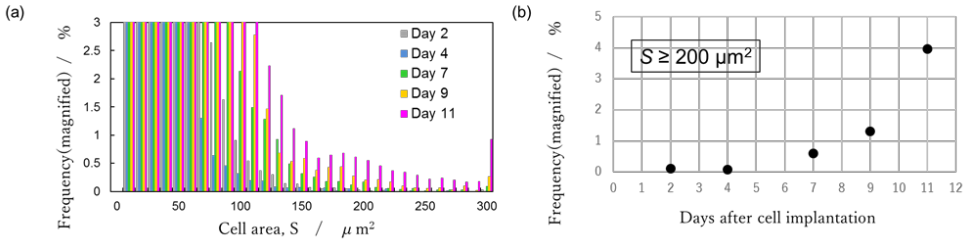

Figure 2. Frequency of cellular area. (a) Histograms of total cell area, *S*, of cancer cell-implanted. (b) The time course frequency of cells which have over 200 μm^2 areas.

Time course change of circulating tumor cell clusters in blood evaluated by imaging biomarkers of on-chip multi-imaging cell sorter

Masao Odaka[1,2)], Moe Iwamura[3)], Akihiro Hattori[1,2)], Kenji Matsuura[1,2)], Hideyuki Terazono[1,2)], Yuki Yamanaka[3)], Kento Iida[4)], and Kenji Yasuda[1-4)]

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Technology for analyzing diagnostic conditions and symptom of patients has rapidly advanced by liquid biopsy of blood, in which molecular and physical biomarkers are applied. Especially, it is expected to establish a diagnostic method accurately confirming cancer metastasis using a small volume of patient blood to evaluate the effectiveness of chemotherapy and post-surgery checking. Here, we have developed an on-chip imaging cell sorter to identify and evaluate their time course change of circulating tumor cells (CTCs) based on their morphological characteristics of clusters, which is the third and more precise index of identification of CTCs against conventional molecular or physical biomarkers (Fig. 1). As a practical application, we applied our imaging cell sorter for recognition of CTCs of human patient blood. We succeeded in identifying CTCs specifically as the clustered cells and found the correlation between the number of CTC clusters and chemotherapy effectiveness, which was not detectable for Cell Search® assay. Moreover, we confirmed the number of cell clusters in healthy blood and those of infection disease patients were negligible small. The results indicate the effectiveness and advantages of this non-labeled imaging biomarkers for practical CTC detections in chemotherapy and post-surgery checking.

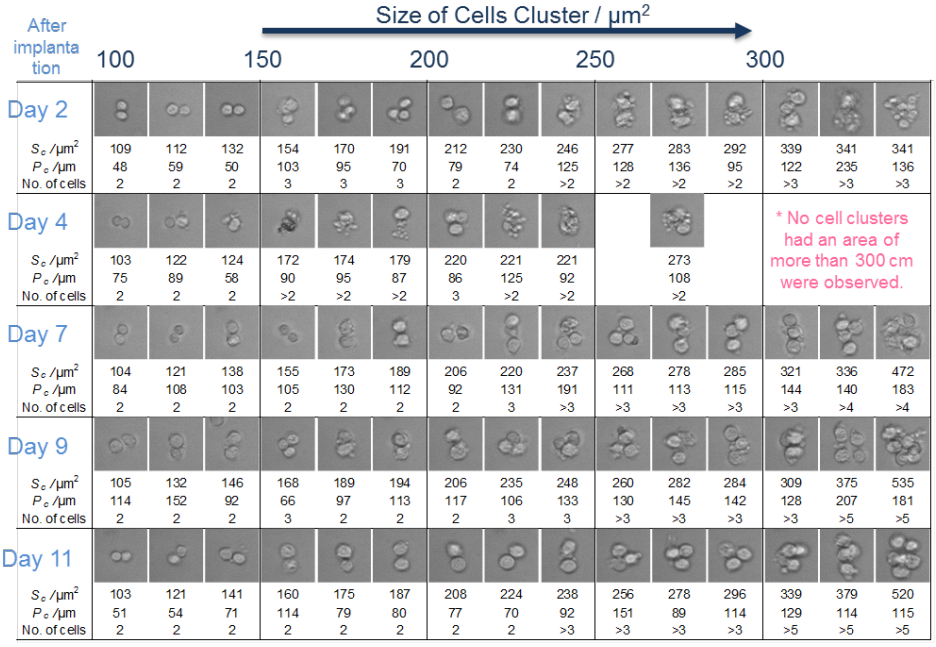

Figure 1. Typical time course change of CTC clusters in blood after implanted into rat. Size (S_C), cellular perimeter (P_C), and the number of cells in the cluster are indicated.

Origin of cardiomyocyte cluster beating rythms: Elucidation of selection rule of interbeat intervals of cardiomyocyte network from single cells

Naoki Takahashi[1)], Natuki Seki[2)], Hideyuki Terazono[3.4)] , Masao Odaka[3.4)], Akihiro Hattori[3.4)], Kenji Matsuura[3.4)] and Kenji Yasuda[1-4)]

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The synchronization behavior of cardiomyocytes is one of the most characteristic dynamics of the community effect of cell networks. However, the selection rule of interbeat interval (IBI) in the synchronization of cardiomyocytes has not been clarified. Here, we examined the hidden mechanism of IBI selection of the cell cluster from variety of different IBI of component cells during their clustering. First, we compared the IBIs and its stabilities (coefficient of variation; CV) of cardiomyocyte clusters of mouse embryo and those of isolated single cells, which were acquired from the cluster after its IBI measurement with trypsination. Some isolated cardiomyocytes showed faster IBI than the clusters. In addition, the IBI of the clusters has stability equivalent to the most stable isolated cells. These results showed experimentally that only the concept of conduction in cardiomyocyte network is not enough to explain IBI selection rule in a spontaneous beating cell networks. Furthermore, we suggest that the IBI of cardiomyocyte network is determined depending on the stability of the IBI of the component cells before synchronization.

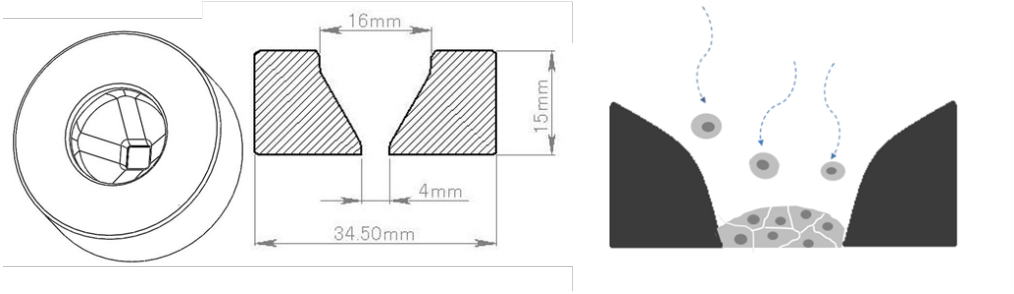

Figure 1. Dish holder for cell sedimentation.

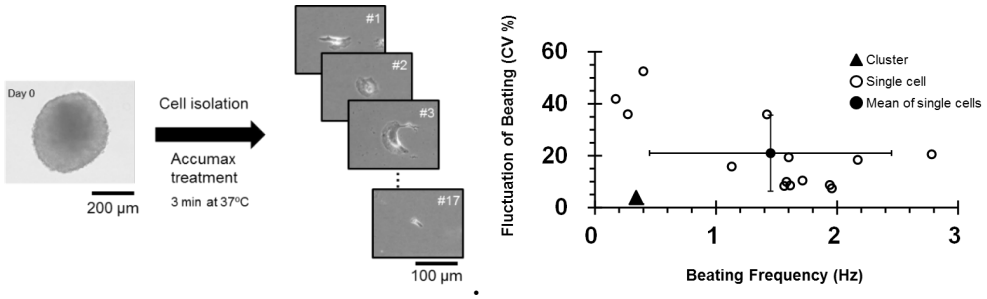

Figure 2. An example of beating properties of isolated single cardiomyocytes obtained from the beating cluster.

Minimum width of artificial neuronal circuit patterns for stable elongation of neurites

Takahito Kikuchi[1)], Shota Aoki[2)], Masao Odaka[3,4)], Akihiro Hattori[3,4)], Kenji Matsuura[3,4)], Hideyuki Terazono[3,4)], and Kenji Yasuda[1-4)]

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Development of microfabrication technology has enabled us to manipulate neurons and construct a living neuronal network in micropatterned structures as desired. Moreover, establishments of stepwise microfabrication technique have allowed us to control axon-dendrite polarity in cultured neurons and construct a living neuronal circuit. However, we still need to understand the characteristics of neurite elongation and differentiation for more precise and reliable control of polarity of neurites. Here, we have developed an *in situ* photothermal microfabrication system for stepwise and flexible micropatterning during cell cultivation, which consists of four major parts; a cultivation dish coated with an agarose thin layer, a 1064/1480-nm dual infrared laser, a phase-contrast microscope with a charge-coupled device (CCD) camera with image analyzer, and a semi-automated micropatterning computer (Fig. 1). We found the minimum requirement of micropattern width for neurite elongation: In 3 μm in width, 19% of neurites were elongated longer than 100 μm in the channel, whereas in 12 μm in width, 71% were elongated (Table1 and Fig. 2). The results indicate the existence of minimum microchannel width for steady neurite extension.

Table 1. Relation between width of channel and ratio of elongation.

Width of channel (μm)	Number of cells	Number of elongation	Percentage of elongation (%)
3.02 ± 0.64	31	6	19
6.58 ± 0.64	15	8	53
11.72 ± 0.80	7	5	71

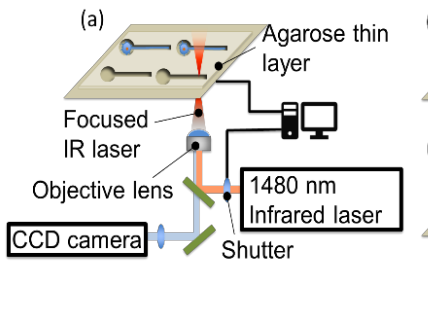

Fig. 1. Design (a) and procedures (b, c) of *in situ* photothermal microfabrication method. Microstructures of agarose are fabricated by spot heating of IR laser during cultivation.

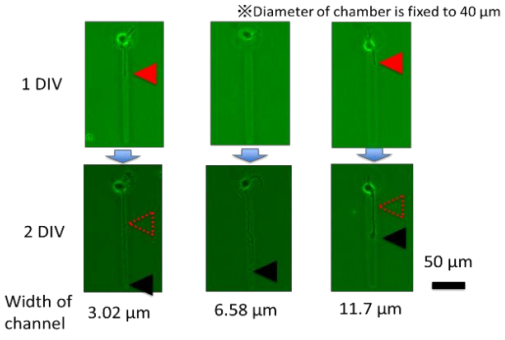

Fig. 2. Neurite elongation in each width of microchannel. Arrowheads, edges of elongated neurites.

Studies on spatiotemporal adaptive regulation mechanism of macrophage phagocytosis: response of multiple stimulations due to physical contacts with multiway optical tweezers

Yoshiki Nakata[1)], Yuya Furumoto[2)], Masao Odaka[3,4)], Akihiro Hattori[3,4)], Kenji Matsuura[3,4)], Hideyuki Terazono[3,4)], and Kenji Yasuda[1-4)]

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Macrophages have an intelligent function of recognition and response to antigens on their surface. However, their adaptive regulation mechanism in spatiotemporal viewpoint especially under competing condition due to multiple stimulations has not been investigated. Here, we examined a series of various time courses (temporal aspect) and contact positions (spatial aspect) of multiple stimulations for phagocytosis (Fig.1). We observed alveolar macrophages by on-chip single cell measurement assay, in which single cells are isolated within each microchamber in the cultivation microchip, and plurality of antigens can be handled simultaneously to contact to membrane surface of macrophages with two-way optical tweezers (Fig. 2). Consequently, a series of multiple phagocytosis by four antigens occurred according to antigen (zymosan) contacted order regardless of their positions (Fig. 3). Therefore, macrophages memorized the order of contacted zymosans at least 4 points. The results indicate that phagocytosis is not independent local phenomenon on cell membrane and macrophage memorizes the time course order of contacted antibody (at least 4 points).

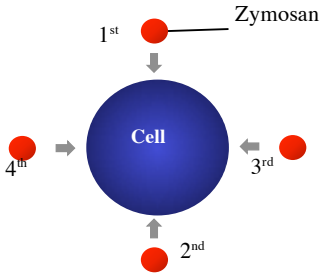

Fig.1. Spatiotemporal guiding of antigens. Order and contact positions of multiple stimulations.Strictly control the position and the timing of zymosan stimulation.

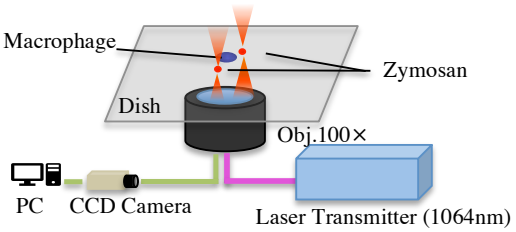

Fig.2. Two-way optical tweezers and observation assay. We caught a zymosan by optical tweezers, and carried on a macrophage.

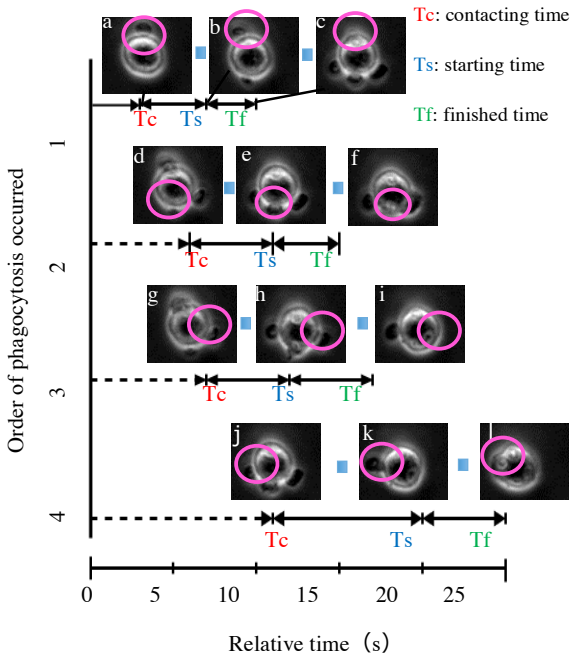

Fig.3. Order of phagocytosis images. The moment of antigens contact (a,d,g,j), starting phagocytizing (b,e,h,k), and finished (c,f,i,l).

Development of on-chip dual measurement system for *quasi-in vivo* screening of cardiotoxicity using extracellular field potential recording and optical displacement analysis of single cells

Akihiro Hattori[1, 2)], Naoki Takahashi[3)], Masao Odaka[1, 2)], Kenji Matsuura[1, 2)], Hideyuki Terazono[1, 2)] Natsuki Seki[4)] and Kenji Yasuda[1-4)]

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In cardiac toxic assessment in drug development, adding to the electrophysiological measurement is important methodology, quantitative evaluation of mechanophysiological responses in cardiomyocytes has becoming more important for precise prediction of cardiotoxicity especially in anticancer drug development. For the evaluation of correlation between electrophysiological effects and mechanophysiological effects of drugs simultaneously, we have developed the on-chip dual measurement assay that records extracellular field potentials and beating motions of one-dimensionally lined-up cardiomyocyte networks simultaneously. Human cardiomyocytes were applied into rectangular microchambers fabricated on the multi electrode array (MEA) chip, and labeled with fluorescent polystyrene beads to detect beating motion of cardiomyocytes network by 1/100 s high-speed camera. We succeeded in simultaneous recording of extracellular field potentials and motion of cardiomyocytes, and found that beating rate of cardiomyocytes and force generated by contraction of cardiomyocytes were changed significantly in the presence of drug. The results indicate that the compositive analysis of electrophysiological properties and mechanophysiological properties should become an effective biomarker of cardiotoxicity estimation.

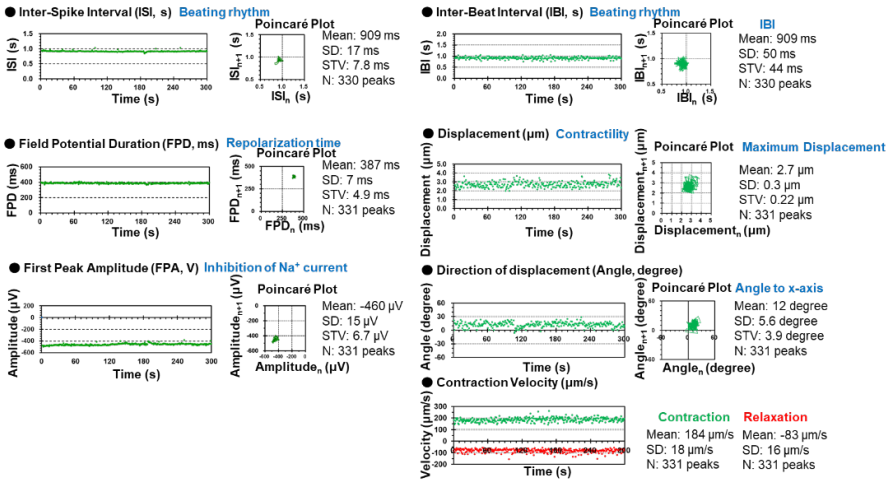

Fig. 1 Representative measurement parameters of the dual measurement system. (a) Analysis parameter of field potential (FP) recording. (b) Analysis parameter of optical imaging.

Molecular structure-based analysis of drug resistance of influenza virus B

Yusuke Kato[1,2], Haruka Hishiki[3], Yosuke Kameoka[4], Reiko Itoh[5], Tomohiro Someya[6], Nobue Inoue[7], Mana Haraki[8], Tomomichi Kurosaki[9], Shochi Suzuki[5], Tomoko Ogawa[10], Naruhiko Ishiwada[11], and **Kazuo Suzuki**[5, 12]*

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*Presentation

Neuraminidase inhibitors have been prescribed for the treatment of both influenza A and B viral infections in children. Infection with influenza virus occasionally displays prolonged period of the high fever despite the treatment with the neuraminidase inhibitors. Such prolonged fever suggests resistance of the viruses to the neuraminidase inhibitors. It has been reported that mutations in the genomes of influenza viruses are related with the drug resistance. Here, we studied association between prolonged fever and mutations in influenza B from child patients.

RNA was isolated from fixed nasopharyngeal swab of 206 patients with influenza in 2013/2014 season. The samples from ten patients having fever over 38ºC for more than 48hr were analyzed to determine neuraminidase sequences. Structural models reflecting the gene mutation of neuraminidase of influenza B virus (BNA) were built and analyzed.

Patients infected with influenza B exhibited longer period of fever on average than those with influenza A. Sequences of BNAs from the patients with prolonged fever period differed from that of the vaccine strain (B/Massachusetts/02/2012). Two of the BNA sequences contained mutations for potential drug resistance: S99N, T106I, K125T and S295R in the sample M2-1 and I262M, V271T, K/E272Q, E320K, D342G and M375K in the K41-1. Structural models of BNA with the mutations suggested that R295 of the M2-1 and Q272 and K375 of the K41-1 may be responsible for the drug resistance.

Drug-resistant mechanism of BNA was clarified by using structural model analysis. Proper use of neuraminidase inhibitors against influenza virus infection is necessary in clinical setting.

A microbead supported membrane-based fluorescence imaging assay reveals intermembrane receptor-ligand complex dimension with nanometer precision

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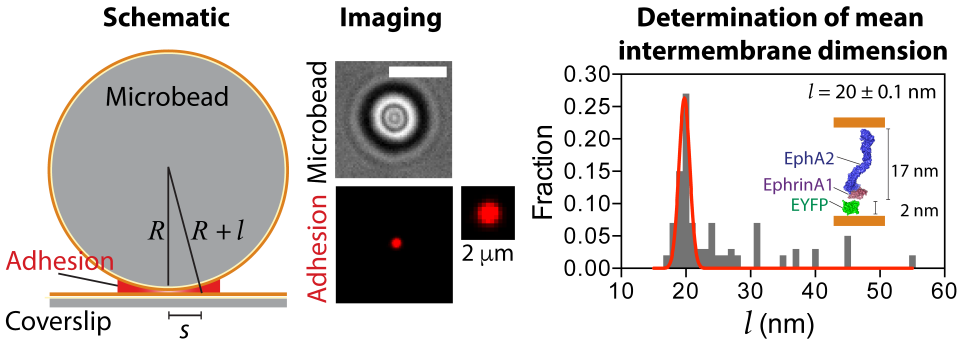
Abstract Body: Receptor-ligand complexes spanning a cell-cell interface inevitably establish a preferred intermembrane spacing based on the molecular dimensions and orientation of the complexes. This couples molecular binding events to membrane mechanics and large-scale spatial organization of receptors on the cell surface. Here, we describe a straightforward, epi-fluorescence-based method to precisely determine intermembrane receptor-ligand dimension at adhesions established by receptor-ligand binding between apposed membranes *in vitro*. Adhesions were reconstituted between planar and silica microbead supported membranes via specific interaction between cognate receptor:ligand pairs (EphA2:EphrinA1 and E-cadherin:anti-E-cadherin antibody). Epi-fluorescence imaging of the ligand enrichment zone in the supported membrane beneath the adhering microbead, combined with a simple geometrical interpretation, proves sufficient to estimate intermembrane receptor-ligand dimension with better than 1 nm precision. An advantage of this assay is that no specialized equipment or imaging methods are required.



Investigation of membrane interaction mechanism of Lti30 using Imaging Fluorescence Correlation Spectroscopy

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Dehydrins are intrinsically disordered plant proteins whose expression is upregulated under desiccation and cold stress conditions. Lti30 is a cold-induced dehydrin composed of six archetypical K-segments each with two adjacent histidines. This protein requires protonation of histidine residues to interact with the membrane so it is proposed that these histidine residues act as pH switch and influence Lti30's membrane binding abilities. Literature suggests that Lti30 becomes structured on interacting with membrane and it interacts actively with anionic membrane lipids via electrostatics and weakly interacts with zwitterionic lipids. In this work, the effect of Lti30, K-segment, and K-segment with two histidines on membrane diffusion has been studied with the help of Imaging Fluorescence correlation spectroscopy. Imaging FCS method allows measuring the effect of protein on hundreds of contiguous pixels and hence large areas on the membrane. Each measurement provides a diffusion and concentration map over the whole region of interest which can then be developed into a time-lapse movie. Effect of these protein/peptides have been studied on three supported lipid bilayer systems namely DOPC, DOPC: DOPS (4:1) and DOPC: DPPC (1:1) at four pH conditions of 5.8, 6.3, 7.4 and 9.0 as they are mechanistically important pH conditions for Lti30. With these experiments, for the first time, we show that Lti30 in addition to anionic lipids can interact actively with specific types of zwitterionic lipids as well. It is also found that on protonation Lti30 has a tendency to aggregate over the membrane which can have important implications for membrane stability.

Measuring Oxygen Concentrations in Bacterial Biofilms Using Transient State Monitoring by Single Plane Illumination Microscopy

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Biofilms are structured bacterial communities in which cells are held together within a matrix formed by self–secreted polymeric compounds. They are notoriously hard to remove once formed, and are responsible for up to 80% of all bacterial infections. Oxygen is required for respiration in aerobic bacteria and hence plays an essential role in the generation of energy, necessary for cell maintenance and growth. The distribution of oxygen within a biofilm can be heterogeneous and is expected to change during their life cycle. Most commonly, measurements of oxygen are carried out using electrodes which are invasive in nature and may alter the micromechanical properties of the sample, giving rise to artefacts.

Here, we apply Transient State (TRAST) monitoring, a non-invasive method that can measure the relative populations of molecules in the triplet states by averaging the fluorescence intensity under time-modulated illumination. We use a Single Plane Illumination Microscope (SPIM), which allows optical slicing, suitable for imaging thick samples such as biofilms (Figure 1a). We present, for the first time, a map of triplet relaxation times measured inside a *Pseudomonas* aeruginosa biofilm, at the *μm* range (Figure 1b). We further investigate the effect of microenvironmental factors (viscosity, oxygen uptake) on the behavior of fluorescence, to establish a protocol for quantitative oxygen concentration measurements. Our results indicate that completely anoxic zones lie within the colonies, and oxygen concentration gradients are formed near the boundaries of the biofilm that extend outside the areas of high cell density (Figure 1c).

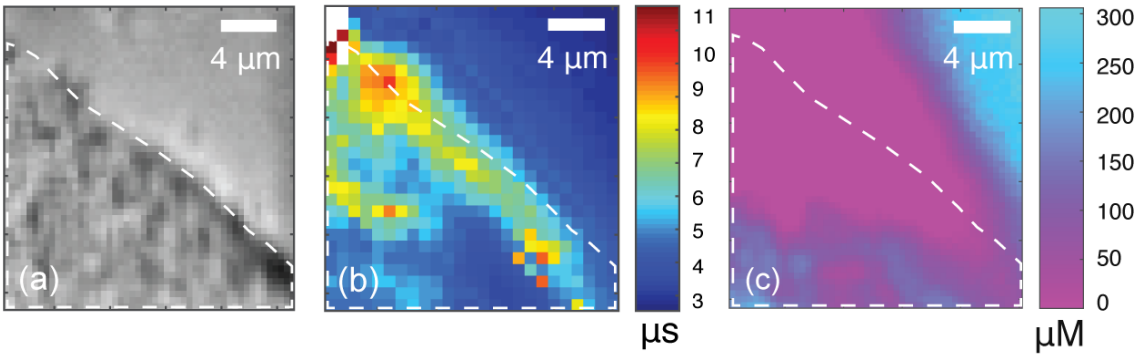

Fig 1. Imaging a P. aeruginosa biofilm microcolony (~80 h post–inoculation). (a) SPIM image. (b) Triplet relaxation time map. (c) Oxygen concentration map.

Plasma membrane domain confinement of secreted signaling protein Wnt3 revealed by SPIM-FCS in live zebrafish

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The embryonic development of a zebrafish is tightly regulated by multiple genes in a concerted manner. The WNT3 gene is one such gene that encodes the secreted signaling protein Wnt3 which activates the Wnt signaling pathway that is responsible for modulating vital developmental processes such as anterior-posterior axis specification and neurogenesis. Wnt3 is transported intracellularly to the cell membranes of Wnt-producing cells upon undergoing post-translational palmitoylation at the endoplasmic reticulum by membrane-bound O-acyltransferase, Porcupine. By comparing the amino acid sequence of Wnt3 with identified highly-conserved palmitoylation sites of other Wnt proteins, the lipid modifications of Wnt3 most likely occur at sites C80 and S212. Previously, our group demonstrated the importance of palmitoylation of Wnt3 by Porcupine for the membrane localization and secretion of Wnt3 as well as proper brain development in live Wnt3-EGFP transgenic zebrafish embryos through Porcupine inhibition experiments. In this study, we applied single plane illumination microscopy-fluorescence correlation spectroscopy (SPIM-FCS) on the same Wnt-EGFP transgenic line to determine the membrane distribution of Wnt3. SPIM-FCS conducts FCS measurements in an imaging approach that offers huge statistics of data due to its multiplexing capability which generates spatial maps of diffusivity and concentration. In addition, sub-resolution membrane organization of fluorescent probes was also deduced by performing the FCS diffusion law analysis on SPIM-FCS measurements which studies the spatial dependence of diffusion of the probes. Overall, our results illustrate the association of Wnt3 with cholesterol-dependent plasma membrane domains that is influenced by the inhibition of palmitoylation and the reduction of several lipid components via drug treatments in the cerebellum of live zebrafish embryos.

Theoretical and Simulation Studies for Radiation Damage from Free Radicals and Hydrogen Bubbles in Cryo-Electron Microscopy

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Abstract: Radiation damage is an important consideration for improving sample contrast and resolution in cryo-electron microscopy (cryoEM). It was proposed [1] that hydrogen bubbles are an important sign of sample damage in cryo electron microscopy: incident electrons dissociate H_2O molecules which produces hydrogen and hydroxyl radicals. The latter is expected to modify the hydrogen bonds in biomolecules, which produce hydrogen gas that nucleate into high-pressure bubbles. Knowing the pressure of such hydrogen bubbles, and how to reduce them are hence important for detecting and mitigating sample damage.

Here we investigate the claim that such hydrogen bubbles could have internal pressures of 1000 atm [2]. Furthermore, we explore the prospect of interpreting electron-induced damage of two-dimensional protein crystals from changes in their weak continuous diffraction intensities (i.e. inter-Bragg diffraction) [3].

Reference:

1. Glaeser, R. M., *Chapter Two – Specimen Behavior in the Electron Beam; Methods in Enzymology (eds.)* (Elsevier Inc., 2016).
2. R. Leapman and S. Sun, "Cryo-electron energy loss spectroscopy: observations on vitrified hydrated specimens and radiation Damage," Ultramicroscopy **59**, 71-79 (1995)
3. Kartik Ayyer *et al.*, "Macromolecular diffractive imaging using imperfect crystals," Nature **530**, 202-206 (2016)

Deconstructing the factors involved in the modulation of mobility of molecules in microcolonies of *Pseudomonas aeruginosa* biofilms

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Pseudomonas aeruginosa (PA) exists in two different life styles, which are the freely swimming unicellular planktonic form, and the surface attached multicellular form called biofilm. The bacterial cells in a PA biofilm are embedded in a self-produced matrix composed of different polysaccharides, DNA, proteins and phages. PA biofilm development has been categorized into three different stages. During the first stage, the bacteria attach itself to the substrate. During the second stage, the biofilms undergo maturation and form mushroom shaped microcolonies. During the last stage, the microcolonies disperse and release freely swimming planktonic bacteria. Cyclic diGMP has been identified as a soluble second messenger involved in bacterial communication during the dispersal stage. Single Plane Illumination Microscopy-Fluorescence Correlation Spectroscopy (SPIM-FCS) enables one to quantitate the diffusion coefficient at contiguous locations of a biofilm. Our results suggest that the diffusion coefficient of fluorescent probes decrease with increasing size of the microcolony. Only positively charged molecules localize into the biofilm and exhibit different mobilities at different locations of the biofilm. Phosphodiesterases that cleave cyclic diGMP have been shown to promote dispersal of biofilms. Hence using a bacterial strain with a phosphodiesterase gene under the control of arabinose induction, we have monitored the real-time changes in diffusion properties at contiguous locations of a microcolony before and after dispersal. Our results aid in deciphering the individual role of size, charge and bacterial cell physiology, in contributing to the diffusion limitation in PA biofilms.

Strain Maps Reveal Mechanical Properties of Embryos during Morphogenetic Movements

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The body plan of multicellular organisms is shaped through collective cell behaviors, and is known as morphogenesis, or morphogenetic movements. An important morphogenetic event during early embryo development is the convergence and extension at mid-gastrulation stage, where cells collectively move from the ventral side of the embryo towards the dorsal axis, underlying the neural tube formation and the somatogenesis. The biophysical and biochemical mechanisms of early development have been intensively studied at the cellular level, but due to the technical challenge for quantifying the force and deformation the global mechanics at the tissue level remains unknown. Here we combine in-toto imaging by light-sheet microscopy and computational methods to calculate the strain map of zebrafish development from 7.5-12 hpf, representing the tissue deformation, during mid-gastrulation. Key parameters for the dynamics include: 1. vector field of collective cell migrations, 2. mechanical curl of sheets of cells, which describes the domain rotation, and 3. strain, which measures the tissue deformation, have been calculated over the entire embryo. Furthermore, based on these results we have identified strain associated with convergent extension and pre-somatogenesis events. The strain maps are a first step in the quantitation of the mechanical forces that power morphogenesis.

A Method to Quantify Co-localization in Biological Images_

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Quantitative co-localization analysis with fluorescent microscopy is a common approach to assess the spatial co-ordination of molecules and thus to understand their functions in biological processes. However, the results of co-localization analysis might not be consistent due to various imaging conditions and it raises the problems in comparison and interpretation of the results from different methods We propose a novel method to separate a co-localization event into two aspects: co-occurrence and intensity correlation, which are usually combined as one parameter in other quantitative co-localization analyses. By examining co-localization through both co-occurrence and intensity correlation, the co-localization analysis provides accurate and interpretable results. Furthermore, the co-occurrence pixels can be visualized in an additional image channel to provide an intuitive impression of the quantity and locations of the co-localization events occurring.

Tension-dependent dynamics of adherens junction components during cell junction deformations

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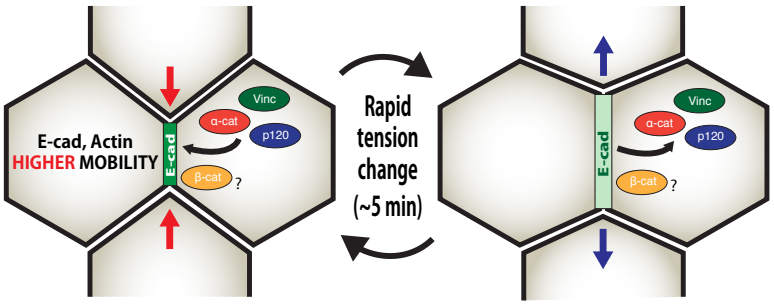
At the apical side of epithelial tissues, the cell-cell boundaries form framework of the tissue, and deformations in these boundaries, e.g. boundary contraction and elongation, and associated forces form the mechanical basis of tissue morphogenesis. In such highly mechanical process, the adherens junction (AJ) plays important roles for maintenance of tissue integrity, force transmission to neighbors, and regulation of cell shape changes or rearrangements. Although it has been studied that the AJ has ability to react against applied forces, we less know that how the AJ structures behave in boundary deformations that dynamically evolve in time and in space. To understand the AJ's mechanotransduction mechanism and answer how the AJ dynamics is involved in tissue morphogenesis, elucidations of mechanics underlying different boundary dynamics, tracking their transitions, and observations of responces of the AJ molecules are essential.

Using amnioserosa cells, which exhibit rapid cell boundary oscillations (contraction and elongation; 1 period = 2~5 min) during *Drosophila* dorsal closure, we demonstrated that cell junctional tensions closely correlated with boundary dynamics and shapes. Based on the correlations, we developed a method to non-invasively estimate the junctional tension over time, and found that the tension dynamically fluctuates during boundary oscillations. Furthermore, we showed that an adherens junction (AJ) molecule, vinculin, dynamically accumulates to or dissociates from oscillating boundary in a junctional-tension-dependent manner (Hara et al., *Curr. Biol.*, 2016).

Interestingly, our recent observations indicate that the other AJ components (E-cadherin, α-catenin, etc.) also dynamically fluctuate its density and stability in response to the junctional tension changes. It suggests that the AJ molecules have abilities to respond rapidly against tension changes in order to modify the mode of cell boundary deformation or reorganize cell-cell junctions during tissue morphogenesis.

In this presentation, I will provide an overview of mechanics underlying cell boundary deformations in *Drosophila* epithelium, and highlight the short-term dynamics of AJ components responding to rapid tension changes.



Higher junctional tension
(e.g. contraction)

Lower junctional tension
(e.g. elongation)

Basolateral protrusion and apical contraction cooperatively drive *Drosophila* germ-band extension

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Throughout development, tissues undergo complex morphological changes, resulting from cellular mechanics that evolve over time and in three-dimensional space. During *Drosophila* germ-band extension (GBE), cell intercalation is the key mechanism for tissue extension, and the associated apical junction remodelling is driven by polarized myosin II-dependent contraction. However, the contribution of the basolateral cellular mechanics to GBE remains poorly understood. Here, we characterize how cells coordinate their shape from apical to basal side during rosette formation, a hallmark of cell intercalation. Basolateral rosette formation is driven by cells mostly located at the dorsal/ventral part of the rosette. These cells exhibit actin-rich wedge-shaped basolateral protrusions and migrate towards each other. Surprisingly, the formation of basolateral rosettes precedes that of the apical rosettes. We further show that basolateral rosette formation is independent of apical contractility, but requires Rac1-dependent protrusive motility. Furthermore, we identified Src42A as a regulator of basolateral rosette formation. Our data show that in addition to apical contraction, active cell migration driven by basolateral protrusions plays a pivotal role in rosette formation and contributes to GBE.

(Nature Cell Biology 19(4), 375-383, 2017)

Geometric constraints induce cellular skew and apical-to-basal neighbor exchange in curved epithelial tissues

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Organ and tissue formation are complex three-dimensional processes involving cell division, growth, migration, and rearrangement, which all occur within physically constrained regions. However, analyzing such processes in three dimensions *in vivo* is challenging. Here, we focus on the process of cellularization in the anterior pole of the early *Drosophila* embryo to explore how cells compete for space under geometric constraints. Using microfluidics combined with fluorescent microscopy, we extract quantitative information on the three dimensional epithelial cell morphology. We observe a cellular membrane rearrangement in which cells exchange neighbors along the apical-basal axis. Such apical-to-basal neighbor exchanges are observed more frequently in the anterior pole than in the embryo trunk. Further, cells within the anterior pole skew toward the trunk along their long axis relative to the embryo surface, with maximum skew on the ventral side. We construct a vertex model for cells in a curved environment that reproduces the observed cellular skew and demonstrates that geometric constraints can induce an asymmetric force on cells in the anterior. Overall, cell deformation and rearrangements appear to play important roles in cell packing in the highly curved three-dimensional environment of the *Drosophila* embryo

in toto Quantitative Imaging Tools to study *Drosophila* Organogenesis

A. Singh, S. Tlili, K. Karkali, J. Munoz, E. Martin-Blanco and T. E. Saunders

How complex 3D organs are formed in a live organism is poorly understood- particularly in terms of its control on size and function. Understanding its dynamic behavior, its shape and complex functions in living organism brings many challenges. We use *Drosophila* embryos as a model organism and *in toto* quantitative imagining tools to study organogenesis - in particular, we focus on the ventral nerve cord formation during embryogenesis. The ventral nerve cord is made from two types of cells, neurons and glial cells. The glial cells provide mechanical support to neurons and they maintain ion-homeostasis. The ventral nerve cord spans nearly the entire embryo length and to access information about its three-dimensional topology requires *in toto* imaging tools, such as light-sheet microscopy. We use quantitative image analysis – specifically three-dimensional PIV - to extract the dynamic changes in the ventral nerve cord shape along its entire length in both wildtype and mutant backgrounds. Such an approach is allowing us to build up one of the first complete atlases of internal, three-dimensional organ formation.

Influence of the biomechanical signalling on apico-basal polarisation

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Aims and/or Background: Cell capacity to answer to biochemical, topographical and rheological parameter of the environment has been extensively studied in the past years. One of its observable behaviour is the cell polarity. Depending on the cell type, the cell is able to change its cell shape in order to create actin structure allowing the cell, for example, to migrate in a preferential direction (mesenchymal cell line) or to extend its membrane to increase surface contact with the external media (epithelial cell line). Polarised epithelial cells form three different types of contact with their surrounding environment. At the basal side, facing the tissue, transmembrane protein called integrins create link between the extracellular matrix (ECM) and the actin cytoskeleton (focal adhesion). On the side, transmembrane cadherins family maintain the integrity of the tissue by connecting the cell with their neighbours (cell-cell junction). At the very tip of the cell-cell junction near the lumen, a special structure called tight junction made by the interaction of transmembrane claudins is in charge of the control of transport of ion within the intercellular space. The tight junction is connected to an actin reinforced structure called actin belt via the ZOs proteins. A bad location of one of the protein in the tight junction can lead to different diseases going from bacterial or virus infection to cancer (1).

The goal of this study is to understand better the tight junction establishment and the biochemical and mechanical cues influencing its formation via a bottom-up approach using biocompatible tune-able microniches, and laser-based micropatterning technics.

Methods: Using laminated microniches, we were able to study the effect of biochemical and mechanical cues on apico-basal polarization. We manage, by coating with laser double patterning technics in 2D or by incubating separately the protein on the bottom and the side of the microwell, to distinguish the effect of ligand (Laminin, E-cadherin) signalling, combined with dimensionality, on the tight junction formation. The read-out that we choose to observe is the localisation of different apical markers (Podocalyxin, Moesin), tight junction markers (Claudin-3, Par-3, ZO-1, N-WASP), and some structural markers (F-Actin, E-cadherin) on all those different configurations.

Results: We observed, by comparing apico-basal polarity markers and tight junction markers localisation in 2D and 3D with the same kind of ligand signalling, that the dimensionality is important to recruit Claudin-3, Par-3 and ZO-1 on the side of the microwell (Figure). We hypothesized that this result may be due to a difference of tension on the cell edge between 2D and 3D configuration. Experiments with magnetic micro-beads has been planned in order to pull on the apical pole of the cell and see the effect on protein localisation.

We also observed than the double signalling (ECM, E-cadherin) in 3D microniches is essential to obtain a recruitment of Par3 on the extremity of the lateral side of the cell. However, we failed to observe any good recruitment of N-WASP on those systems.

Conclusion: With this study, we can conclude than the double signaling in a 3D environment is essential to obtain a good localization of Par-3 on the edge of the cell. We also observe the importance of three-dimensionality to control good tight junction markers localization.

(1) Forster & al, *HistoChem Cell Biol* (2008)

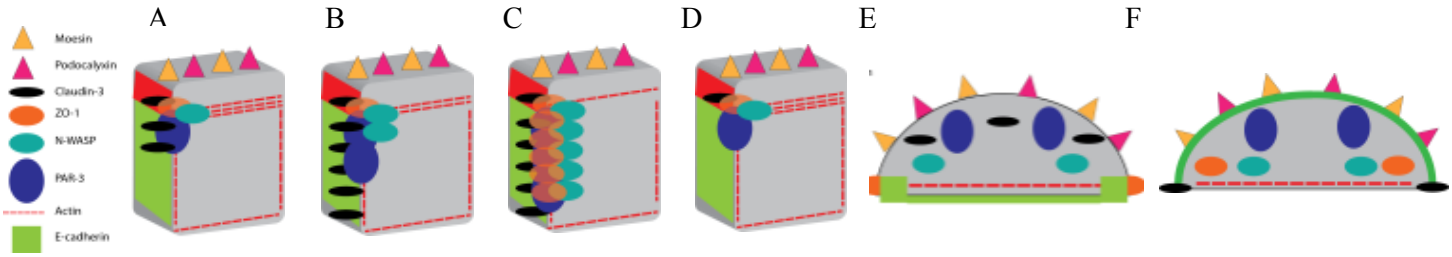

Figure 1: Protein localization depeding on the types of cues that the cell is experiencing. A 3D microwell with Ecadherin on the side, Laminin at the bottom. B 3D microwell with Ecadherin everywhere. C 3D microwell with Laminin everywhere. D Epithelial cell in a monolayer. E 2D micropattern of Ecadherin. F 2D micropattern of Laminin.

A novel DNA binding mode of H-NS drives chromosome compaction and gene silencing in single cells

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Nucleoid-associated proteins (NAPs) facilitate chromosome organization in bacteria, but the precise mechanism remains elusive. H-NS is a NAP that also plays a major role in silencing pathogen genes. H-NS possesses two functional domains, an N-terminal oligomerization domain and a C-terminal DNA binding domain separated by a flexible linker. The linker is suspected to play an important role in promoting H-NS and DNA interaction. We compared the localization of wt H-NS and a linker deletion mutant ΔL with the nucleoid in bacteria cells using super-resolution microscopy (single-molecule localization microscopy; SMLM). It was observed that in the ΔL linker mutant, the H-NS protein does not localize with the nucleoid, while the wt H-NS form foci that overlap with the nucleoid. Using DBSCAN, we show that the sizes of the clusters are 273 ± 108 nm, with 26.4% of the H-NS inside these clusters. The cell lengths of the ΔL linker mutant are longer, with larger nucleoids, suggesting that nucleoid compaction is affected. Next, we applied single-particle tracking-photoactivatable localization microscopy (sptPALM) in live cells to quantitate the amount of binding between wt H-NS / ΔL with the nucleoid. We showed that 95% of the wt H-NS are bound to the nucleoid, while in the ΔL linker mutant, only 21% are bound, 55% having weak interaction and 24% not bound. These observations identify the linker as being essential for the binding of H-NS to DNA and for the appearance of foci in super-resolution images. Supported by the RCE in Mechanobiology from the Ministry of Education, Singapore

Remodeling of adhesion and modulation of mechanical tensile forces during apoptosis in *Drosophila* epithelium

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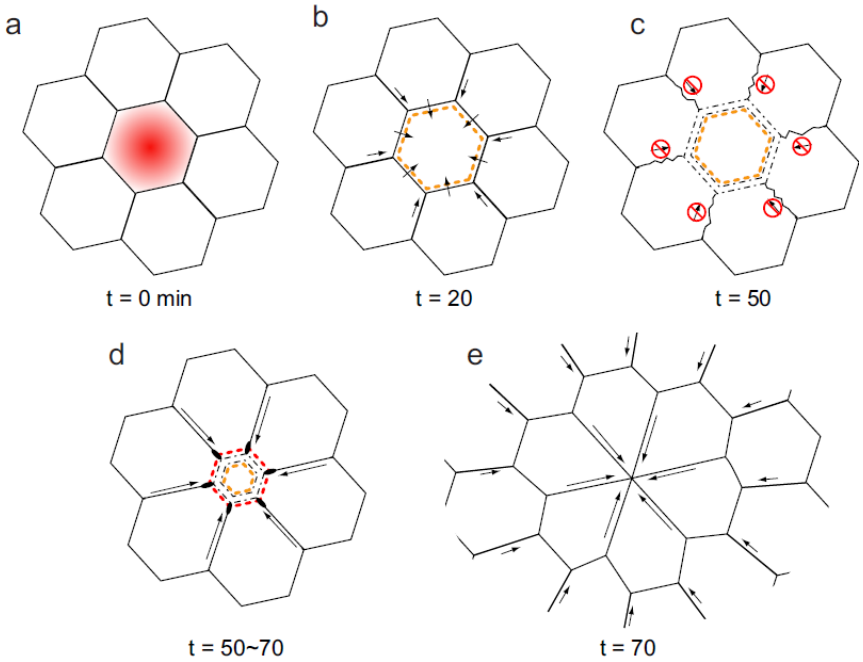
Apoptosis is a mechanism of eliminating damaged or unnecessary cells during development and tissue homeostasis (a). During apoptosis within a tissue, the adhesions between dying and neighboring non-dying cells need to be remodeled so that the apoptotic cell is expelled. In parallel, contraction of actomyosin cables formed in apoptotic and neighboring cells drives cell extrusion (b). To date, the coordination between the dynamics of cell adhesion and the progressive changes in tissue tension around an apoptotic cell is not fully understood. Live imaging of histoblast expansion, which is a coordinated tissue replacement process during *Drosophila* metamorphosis, shows remodeling of adherens junctions (AJs) between apoptotic and non-dying cells, with a reduction in the levels of AJ components, including E-cadherin. Concurrently, surrounding tissue tension is transiently released (c). Contraction of a supra-cellular actomyosin cable, which forms in neighboring cells (d), brings neighboring cells together and further reshapes tissue tension toward the completion of extrusion (e). We propose a model in which modulation of tissue tension represents a mechanism of apoptotic cell extrusion.



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