

International Symposium on

# “Artificial Cell Reactor Science and Technology”

5 - 6 April, 2018

Ito International Research Center,  
The University of Tokyo



## **GREETING and PREFACE**



Welcome to ImPACT International Symposium on  
Artificial Cell Reactor Science and Technology

As the symposium chair and as a program manager of “Implusing Paradigm Change through Distructive Technologies Program (ImPACT Program)” organized by Cabinet Office, Government of Japan, and The Japan Science and Technology Agency (JST), I would like to welcome you all to the International Symopjium “Artificial Cell Reactor Science and Technology”.

ImPACT Noji Program entitled “Artificial Cell Reactor Technology for an Enriched and Secure Society and New Bioengineering” is conducted to promote Artificial Cell Reactor (ACR) technology based on microfabrication technology. The ACR technology globally attracts a large attention not only from academia but also from industries as to be a next generation platform for several bioassays because of its broad range of applications such as high-sensitivity digital counting diagnostics, high throughput screening of functional molecules, and synthetic biology.

On the occasion of the symposium, we invite top scientists as speakers from all over the world active in the relevant fields such as synthetic biology, chemical biology, and micro/nano device technology, to learn state of the art technology. The symposium will give a fruitful discussion on remaining tasks and future plans toward the implementation of the ACR technology among participants.

I am looking forward to many participants having excellent discussion and communication through this symposium.

April 5, 2018

Noji Hiroyuki  
Program Manager, ImPACT program  
Professor, The University of Tokyo

**Program**

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## **Presentation Day 1**

- Artificial cell reactor array technology—
- Membrane protein (GPCR)—
- Biomass—

## Oral Presentation -1

### Artificial cell reactor array technology for directed evolution



Hiroyuki Noji

Graduate School of engineering The University of Tokyo, Tokyo 113-8656, Japan

The recent decades have seen great success in directed evolution technology that allows screening of enzymes with enhanced functionalities from random mutant library. However, there are still technical challenges to be addressed such as accurate identification of enzymes with enhanced functions in ultra high-throughput manner and the suppression of protein expression variance among *in vivo* or *in vitro* reactors.

Here, we introduce *in vitro* artificial cell reactor array (ACRA) as a new platform for directed evolution of enzymes. Typical ACRA devices display a million of micron-sized droplets of which volume is 10-40 femto ( $10^{-15}$ ) liter in approximately 10 mm<sup>2</sup> surface of a device. DNA molecules encoding enzyme gene are stochastically encapsulated in digital format, zero or one molecule per reactor. Thus, each reactor shows distinct gene expression activity: while the majority of reactors is empty with no gene expression activity, the remainings show distinct gene expression activity. One of advantageous features of ACRA is that the variance of gene expression among positive reactors is significantly small. Typical coefficient variance of gene expression is below 15-20 % that is mostly close to the theoretical limit of intrinsic gene expression noise. Another beneficial feature of ACRA is that it allows to measure the activity of enzymes with high accuracy. Due to the stable feature of ACRA, one can measure the enzyme activity from a time-course of a wide time range, longer than 12 hours.

Taking these advantages, we attempted single-shot selection of enhanced enzyme from random mutant libraries. Library of *E. coli* alkaline phosphatase (*EcALP*) was prepared by saturation mutagenesis or error-prone PCR. Library DNA molecules ( $10^{+5}$ ) were encapsulated at single molecule level with *in vitro* gene translation mixture and a fluorogenic substrate of *EcALP*. After 5-12 hour incubation, reactors with the brightest fluorescent signal were selected. DNA was recovered from the selected reactors with a PC-controlled micro glasspipette system. After DNA sequencing, recovered mutant *EcALPs* were investigated. Selected mutant *EcALPs* showed remarkably enhanced enzymatic activity, 5-10 time higher than the wild type. This demonstrates the highly accurate features of ACRA allowed identification of highly active mutant *EcALPs* in single-round selection, promising rapid and accurate selection of enzyme clones with enhanced functionalities.

#### Biography

Dr. Hiroyuki Noji is the project manager of ImPACT program 'Artificial cell reactor' and a professor in the Department of Applied Chemistry at the University of Tokyo. He received his PhD from Tokyo Institute of Technology in 1997. He has been conducting single-molecule biophysics of  $F_1$ -ATPase of ATP synthase to pursue the complete elucidation of the mechanochemical coupling mechanism of  $F_1$ -ATPase. In 2005, he invented femtoliter chamber array system for single molecule enzymatic analysis that was later developed as digital bioassays and artificial cell reactor technology presented in this talk.

## Oral Presentation -2

### De novo protein design and application to design of thermostable proteins



Nobuyasu Koga

Institute for Molecular Science, Japan

Protein design holds promise for applications ranging from novel therapeutic to biomaterial. Previously, we discovered a set of design rules for creating protein structures completely from scratch. The rules relate local structures to tertiary packing motif, which enabled us to design amino acid sequences that fold into unique tertiary structures of the various  $\alpha\beta$  and all- $\alpha$  structures with high thermal stability ( $T_m > 100^\circ\text{C}$ ), using the biomolecule design software Rosetta. However, our developed design protocol still requires iterative human try and error in the design process, for example, the design of amino acids compatible with local backbone geometry, and the design of core packing with hydrophobic amino acids. This is probably because of the imperfection of the Rosetta score function used for designing protein structures and the difficulty for conducting negative design by destabilizing myriad structures of unfolded state, not only stabilizing a folded structure. Here, to design "native-like" amino acid sequence with a little of human assistance, we developed a method for making sequence profile indicating favored amino acid types for each residue position depending on its backbone buriedness and torsion angle type based on database analysis for naturally occurring protein structures. Designing amino acids based on the sequence profile generated by our developed method, we readily designed proteins that have funnel-shaped energy landscapes *in silico*. Furthermore, we applied the method for increasing thermal stability of a beta glucosidase. Guided by the generated sequence profile using the beta-glucosidase backbone structure, small hydrophobic amino acids (Ala or Val), in the beta glucosidase were mutated to large hydrophobic amino acids (Ile, Leu, Phe, Tyr, or Trp) to increase VdW and hydrophobic interactions. In this talk, our development for de novo design protocol and the application for remodeling the naturally occurring protein, the beta glucosidase.

#### References

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#### Biography

Dr. Nobuyasu Koga is an associate professor at the Research Center of Integrative Molecular Systems at the Institute for Molecular Sciences at the National Institute for Natural Sciences. Dr. Koga studied on protein folding and molecular motors using coarse-grained molecular dynamics simulations at Kobe University during his PhD course. After receiving his PhD degree from Kobe University in 2006, he started to work on protein design in the Baker laboratory at the University of Washington as a postdoctoral researcher from 2007. Then, he joined the Institute for Molecular Science in Okazaki, Japan in 2014 to start the lab for protein design.

### Oral Presentation -3

#### A Library-based Approach to Realize Intraoperative Rapid Imaging of Tiny Tumors by Novel Fluorogenic Probes for Aminopeptidases

Yasuteru Urano

Graduate School of Pharmaceutical Sciences and of Medicine, The University of Tokyo, Tokyo 113-0033, Japan



We have succeeded to develop novel fluorogenic probes for various aminopeptidases based on our newly established rational design strategy with intramolecular spirocyclization [1]. For example, gGlu-HMRG, a novel fluorogenic probe for  $\gamma$ -glutamyltranspeptidase (GGT), which is well-known to be upregulated in various cancer cells, was developed. In mouse models of disseminated human peritoneal ovarian cancer, activation of gGlu-HMRG occurred within 1 min of topically spraying onto tissue surfaces that are suspected of harboring tumors, with high tumor-to-background contrast [2]. We also proved that tumor regions in real clinical specimen of breast cancer patients, even those smaller than 1 mm in size, could be easily discriminated from normal mammary gland tissues within 1-15 min after probe application [3,4].

Encouraged by these promising results, we prepared a library of fluorogenic probes composed of more than 300 probes for various aminopeptidases. These probes were applied on fresh biopsy samples from esophageal cancer patients, and we found the enzymatic activity of dipeptidyl peptidase-4 (DPPIV) was upregulated in tumor-positive biopsy samples, but not with tumor-negative biopsy samples. Indeed, cancer region in the resected human fresh specimens was clearly visualized by topically spraying DPPIV-activatable fluorescence probes within 10 min [5]. These findings clearly demonstrated that our fluorogenic probes should be a breakthrough in rapid detection of tumors during endoscopic and surgical procedures.

Another example of our recently developed probe is one for glutathione (GSH) in living cells. Alterations in redox state are well known to be associated with a variety of diseases and cellular functions. Among them, GSH is the most abundant redox-related biomolecule, however, existing fluorescent probes are unsuitable for live imaging of GSH dynamics due to irreversible fluorogenic mechanisms or slow reaction rates. Very recently, we have succeeded to develop first-in-class reversible and rapid fluorescent probes for intracellular GSH by utilizing the concept of intermolecular attack of nucleophiles to specific rhodamines. Our probes exhibited GSH concentration-dependent ratiometric fluorescence changes with reversible and rapid detection capabilities, and live-cell imaging of GSH dynamics with temporal resolution of seconds were successfully achieved [6].

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#### Biography

Yasuteru Urano is a Professor at Graduate School of Pharmaceutical Sciences and Graduate School of Medicine, the University of Tokyo (UT), JAPAN. Dr. Urano obtained BE, ME, and PhD degree (Pharmaceutical Sciences) in 1990, 1992, and 1995 from the UT, respectively. After a JSPS post-doctoral fellow from 1995 to 1997, he became an assistant professor in 1997, an associate professor from 2005 at Graduate School of Pharmaceutical Sciences, UT, and then a full professor at Graduate School of Medicine, UT from 2010. From 2013, he also became a professor at Graduate School of Pharmaceutical Sciences, UT. He is now assigned as a project leader of AMED CREST.

### Oral Presentation -4

#### Comprehensive characterization of enzyme superfamilies toward discovery and engineering of new enzymes

Nobuhiko Tokuriki

University of British Columbia, Canada



Enzymes are not only a keystone for biology, but also essential tools for biotechnology. The remarkable versatility of enzymes can be utilized for diverse applications including remediation of environmental pollutants and industrial biocatalysis (replacement of chemical synthesis). Over the last decade, the rapid transformation of sequencing technology has allowed us to obtain enormous amounts of sequence (genomic) information from various organisms and environmental samples. However, it is difficult to harness such sequence information into biochemical functions.

In this talk, I will present a methodology to and pipeline to effectively explore such vast sequence space, and identify unexplored potentials of enzyme superfamilies (a cluster of homologous enzymes that share similar sequence and structural features but exhibit diverse functions). I discuss our on-going investigation of two enzyme superfamilies that is very important for biology and biotechnological applications - the nitroreductase (NTR) and metallo-beta-lactamase superfamilies. First, I show comprehensive and large-scale computational and experimental characterizations of the NTR superfamily that has revealed >10 new sequence clusters (and possibly new functional families) in the superfamily. Second, I present the evolutionary relationships between enzymes in the metallo-beta-lactamases superfamily, in particular, how AHL lactonases independently evolved at three times within the superfamily. At last, I discuss the development of biotechnological tools to inhibit bacterial biofilm formations using these newly discovered quorum quenching enzymes.



## Oral Presentation -5

[Keynote]

### Evolving GPCRs to Favorable Biophysical Properties: Enabling Structural and Functional Studies

Andreas Plückthun

University of Zürich, Dept of Biochemistry, Zürich, Switzerland



G-protein coupled receptors (GPCRs) have enormous pharmacological relevance but our understanding of GPCR architecture and signaling mechanism has remained limited, as have the design features of agonists and antagonists. Two fundamental limitations have hampered progress: (i) Low expression levels, and (ii) low stability of solubilized GPCRs. We have devised several strategies to address these problems.

The first method is based on FACS and fluorescent ligands, thus enforcing receptor functionality, and increasing functional expression [1-4]. More recently, we have also developed a similar system in yeast [9]. In each case, the evolved mutants are much more stable in detergents. To select for detergent stability directly, we developed the CHES technology [5], such that a billion receptor mutants can be individually solubilized in situ, and those can be identified that are stable in short chain detergents. Finally, we have developed a strategy to select for functional receptors in the absence of ligand [8]. Using these and additional approaches, we have recently been able to determine crystal structures of several new receptors and use the stabilized mutants for fragment screening as well as computational docking [6,7,10,11], and recent progress in these areas will be reported.

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#### Biography

Andreas Plückthun studied at the University of Heidelberg (Germany) and obtained his PhD from the University of California at San Diego (USA). His postdoctoral training was at Harvard University. He became group leader at the Gene Center at the Max-Planck-Institute for Biochemistry (Martinsried near Munich, Germany). In 1993, he became Full Professor in the Dept. of Biochemistry, University of Zurich (Switzerland). He was elected member of the German Academy of Science and the European Molecular Biology Organization. He received the Young Investigator's Award of the German Chemical Industry Trust, the Karl-Heinz-Beckurts Award, the J. P. Morgan Chase Health Award, the Wilhelm-Exner Medal and the European Grand Prix for Innovation, and the 2016 Christian B. Anfinsen award of the Protein Society. He has authored over 415 publications with have been cited >36,000 times. He is cofounder of three companies, Morphosys (TecDAX MOR), Molecular Partners (SIX MOLN) and G7 Therapeutics (now Heptares Zurich).

## Oral Presentation -6

### Disruptive technology to create thermostabilized mutants of G-protein coupled receptors

Takeshi Murata

Graduate School of Science, Chiba University, Chiba, Japan



G-protein-coupled receptors (GPCRs), which are indispensable to life and also implicated in a number of diseases, construct important drug targets. For the efficient structure-guided drug design, however, their structural stabilities must be enhanced. Introduction of mutations can enhance their thermostability, but the stabilizing mutations are identified by experiments testing a number of trial mutants. Recently we have developed a theoretical method that allows us to treat all of the possible mutations of GPCRs. It employs a free-energy function (FEF) that takes into account the translational entropy of hydrocarbon groups within the lipid bilayer as well as the protein intramolecular hydrogen bonding using the 3D structural information [1]. We have also proposed a reliable strategy of finding key residues to be mutated and selecting their mutations, which lead to considerably higher stability. Representative single mutants predicted to be stabilizing or destabilizing were experimentally examined and the success rate was found to be remarkably high [2]. Using this method, we have identified the thermostabilized mutants of several human GPCRs such as muscarinic acetylcholine receptor (M2R) and prostaglandin E receptor 4 (EP4) [3], which were leading to the determination of new crystal structures for the two GPCRs. Furthermore, we are currently establishing a more efficient screening method to create thermostabilized mutants of GPCRs by combining the theoretical method with a molecular evolution engineering method.

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#### Biography

Dr. Takeshi Murata is a professor of Graduate School of Science at Chiba University. His research on V-ATPase first began at Tokyo University of Science as a graduate research student in 1994. After receiving his PhD degree, he joined John Walker's laboratory at MRC as a postdoctoral fellow in 2000. He focused on the structural studies of the V-ATPase and solved the crystal structure of the membrane rotor ring at 2005. He came back to Japan at RIKEN as a researcher in 2005, and moved to Kyoto University as an assistant professor in 2007. During these periods, he developed the experimental protocols to obtain monoclonal antibodies that recognize conformational epitopes of membrane proteins for co-crystallization, and solved crystal structures of membrane protein-antibody complexes. He became a faculty of Chiba University in 2009. His current focus is still on structural studies of membrane proteins including V-ATPases, GPCRs, rhodopsins, and transporters.

## Oral Presentation -7

### Evolutionary engineering and characterization of membrane proteins using liposome display

Tomoaki Matsuura

Department of Biotechnology, Graduate School of Engineering, Osaka University, Japan



An *in vitro* transcription-translation (IVTT) system produces proteins without using living cells. Based on this characteristic, IVTT has been used for various applications including *in vitro* protein evolution. As protein synthesis with IVTT is disconnected from cell growth, a wide range of proteins can be targeted. However, the proteins evolved to date using *in vitro* evolutionary methods have all been globular proteins (*e.g.*, antibodies and enzymes), and there have been no previous reports of *in vitro* evolution of membrane proteins. Membrane proteins account for 20–25% of all open reading frames in the genome, and more importantly, more than 50% of the current pharmaceutical targets are membrane proteins. Despite their importance, technical difficulties such as the expression of membrane proteins in heterologous host cell, and establishing a functional screening assay for each membrane proteins, have limited the usage of directed evolution for engineering membrane proteins [1,2]. We previously report the development of a method, named liposome display, where membrane proteins are synthesized inside cell-sized liposomes using IVTT and DNA encoding membrane protein of interest [2]. This method was used to modify the surface of cell-sized liposomes with various biological molecules. In this presentation, I will introduce the recent results obtained by liposome display technology, including *in vitro* membrane protein evolution [2,3] and asymmetric decoration of liposome surface with proteins of interest. Furthermore, how the method can be used to study the properties of membrane proteins will be presented [4,5].

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#### Biography

Tomoaki Matsuura, an Associate Professor at Osaka University, received his PhD from Osaka University (1999) and conducted postdoctoral studies in the Plückthun lab at the University of Zurich (Switzerland). His current interests are to develop a cell-free system that exceeds the performance of an *Escherichia coli* cell, and to engineer membrane proteins using the cell-free system using cell-sized liposomes.

## Oral Presentation -8

### Enzymes degrading cellulose: from molecular mechanisms to traffic simulator

Kiyohiko Igarashi<sup>1,2</sup>

<sup>1</sup>Graduate School of Agricultural and Life Sciences, the University of Tokyo, Japan

<sup>2</sup>VTT Technical Research Centre of Finland, Finland



Cellulose is a major component of plant cell wall and the most abundant biomass on earth. Efficient degradation of cellulose makes it possible to produce fuels and chemicals from plant resources for the achievement of Bioeconomy, although biochemical conversion of cellulose by cellulase is quite slow and the reaction becomes a bottleneck of the process. Cellobiohydrolases (CBHs) are types of cellulases hydrolyzing crystalline cellulose to soluble oligosaccharides, and one of the key enzymes in the cellulose biorefinery. CBHs share a common two-domain structure, cellulose-binding domain and catalytic domain, and these domains cooperatively function for the effective hydrolysis of crystalline cellulose. Since the reaction is carried out at the surface of insoluble substrate, it is not straightforward to analyze the reaction at a solid/liquid interface [1,2].

We recently reported the real-time visualization of crystalline cellulose degradation by individual cellulase molecules using a high-speed atomic force microscopy, having sub-second time resolution and nanometer space resolution [3-5]. Cellulose-degrading ascomycete *Trichoderma reesei* cellobiohydrolase I (*TrCel7A*) molecules were observed to slide unidirectionally along the crystalline cellulose surface, but at some points the movement of individual molecules was halted, leading to the appearance of traffic jams of enzyme molecules. From the comparison with GH family 6 cellobiohydrolase (*TrCel6A*) [6], the basidiomycete *Phanerochaete chrysosporium* cellobiohydrolases (*PcCel7C* and *PcCel7D*) [7,8] and chitinases [9], we discuss possible molecular mechanisms of these processive enzymes and the natural degradation of crystalline cellulose.

We will also introduce neutron crystallography [10] to clarify the detailed hydrolytic mechanisms of inverting cellulases and application of traffic simulator to the analysis of molecular behavior of cellulases to connect mesoscopic gap between biochemical and single molecular analysis.

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#### Biography

Kiyohiko Igarashi received PhD degree in 1999 from Graduate School of Agricultural and Life Sciences in the University of Tokyo. Before receiving PhD, he focused on the kinetic analysis of cellobiose dehydrogenase, extracellular flavocytochrome produced by filamentous fungi. After receiving the degree, he stayed in Uppsala University (Sweden) as a PostDoc from 2000 to 2001, and returned as an Assistant Professor. Currently he is doing kinetics of cellulase and other CAZymes combined with 3D structure. He is also focusing on the single molecular observations of cellulase and chitinase acting at the solid/liquid interface.

## Oral Presentation -9

### Interaction of oxidoreductases during biomass deconstruction

Roland Ludwig

BOKU – University of Natural Resources and Life Sciences, 1190 Vienna, Austria



Ninety percent of lignocellulose-degrading fungi contain genes encoding lytic polysaccharide monoxygenases (LPMOs). These enzymes catalyze the initial oxidative cleavage of recalcitrant polysaccharides after activation by an electron donor, which is essential for a fast and efficient degradation of lignocellulose. Understanding the source of electrons is fundamental to fungal physiology and will also help exploit LPMOs for biomass processing. Using genome data and biochemical methods, we characterized and compared different extracellular electron sources for LPMO: cellobiose dehydrogenase, phenols procured from plant biomass or produced by fungi, and glucose-methanol-choline (GMC)-oxidoreductases that regenerate LPMO-reducing diphenols [1]. Our study shows, that a number of enzymes which were so far connected to lignin degradation also participate in cellulose and hemicellulose degradation via their reaction products (e.g.  $H_2O_2$ ) or by redox mediating species. Cellobiose dehydrogenase and other GMC-oxidoreductases have been studied to investigate their catalytic mechanism and interaction with LPMO [2]. The cytochrome domain of cellobiose dehydrogenase forms the only currently known extracellular electron transfer chain, which specifically and with high efficiency transfers electrons to LPMO [3]. The availability of GMC-oxidoreductases or small molecular weight reductants is obligatory to activate fungal oxidative attack on polysaccharides.

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#### Biography

Dr. Roland Ludwig received his PhD in 2004 from BOKU and continued with postdoctoral studies at the Austrian Centre for Industrial Biotechnology (ACIB) and Lund University, Sweden. His research centers on basic and applied enzymology and is supported by an ERC Consolidator Grant since 2016. He is cofounder of DirectSens GmbH, a company developing and producing biosensors. Together with coworkers and collaborators he published 160 peer reviewed articles and holds several patents. He serves as the Head of the Study Commission which develops bachelor and master study programs at BOKU.

## Oral Presentation -10

### [Keynote]

### Development of microbial cell factories for consolidated bioprocessing by synthetic bioengineering platform

Akihiko Kondo<sup>1,2,3</sup>

<sup>1</sup>Graduate School of Science, Technology and Innovation, Kobe University,  
<sup>2</sup>Department of Chemical Science and Engineering, Graduate School of Engineering, Kobe University, 1-1 Rokkodai-cho, Nada-ku, Kobe, Hyogo 657-8501, Japan.  
<sup>3</sup>Cell Factory Research Team, Center for Sustainable Resource Science, RIKEN 1-7-22 Suehirocho, Tsurumiku, Yokohama, 230-0045, Japan



In order to construct sustainable biorefineries, which produce variety of biofuels and bio-based chemicals from non-food biomass, consolidated bioprocessing (CBP), which integrates enzyme production, saccharification and fermentation into a single process, is a promising strategy for effective bioproduction. One of the key technologies to develop microbial cell factories for CBP is cell surface engineering, which is a powerful tool to express enzymes on the cell surface without loss of their activities [1]. We have developed various cell surface display systems in yeast, bacteria and fungus and showed the direct fermentation of biomass. To construct artificial metabolic pathway, we have developed a novel synthetic biology platform to employ a combination of computer simulation [2] and metabolic analysis to design novel metabolic pathways suitable for target chemicals production. To efficiently re-write genome and construct cell factories, we have also developed the platform technologies such as genome editing and a large gene cluster synthesis systems and are going to integrate to set up the automated systems for efficient construction of microbial cell factories. By tethering the DNA deaminase activity to nuclease-deficient CRISPR/Cas9 system, a genome editing tool that enables targeted point mutagenesis [3,4] have developed (termed Target-AID). In addition, an efficient DNA assembly method, namely, Ordered Gene Assembly in *B. subtilis* (OGAB) method have developed. OGAB method can assemble more than 50 DNA fragments to construct up to 100 kb DNA in one-step using *B. subtilis* [5]. Combination of a cell surface displayed enzyme and an intracellular metabolic engineering is a very effective approach to develop cells with novel fermentation ability for industrial applications.

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#### Biography

Akihiko Kondo received his Ph.D. from Kyoto University in Chemical Engineering (1988). He was appointed as full professor of Kobe University in 2003 and also appointed as a team leader of the cell factory research team at RIKEN institute (Yokohama) in 2012. A. Kondo has developed various platform technologies such as cell surface display systems, metabolic pathway design tools, metabolic analysis technologies, genome editing and long chain DNA synthesis technologies and applied for construction of various microbial cell factories. A. Kondo has been appointed as editor or editorial board members of many Journals such as Journal of Biotechnology, Biotechnology for Biofuels, Bioresource Technology, FEMS Yeast Research etc. He has published more than 570 peer reviewed international papers.

## **Presentation Day 2**

- Synthetic biology —
- Artificial cell(1) —
- Artificial cell(2) —

## Oral Presentation -11

[Keynote]

### L-form bacteria as a model for proliferation of primitive cells and artificial bioreactors

Jeff Errington

Centre for Bacterial Cell Biology, Newcastle University, NE2 4AX, UK



The peptidoglycan cell wall is a defining structure of the bacteria. It is the target for our best antibiotics and fragments of the wall trigger powerful innate immune responses against infection. The genes for peptidoglycan synthesis are present in most bacterial lineages, suggesting that the wall emerged early in cellular evolution. Surprisingly, many bacteria can switch almost effortlessly into a cell wall deficient “L-form” state in which they become completely resistant to many cell wall active antibiotics, and which may be involved in persistence or recurrence of various infections. Remarkably, L-form growth is completely independent of the complex FtsZ-based division machine that is essential for proliferation of most bacteria. Proliferation occurs instead by a seemingly haphazard process involving membrane blebbing or tubulation and scission, leading to progeny of irregular size and shape. The switch to this mode of proliferation seems to require only the upregulation of membrane synthesis, leading to an increased surface area to volume ratio. Microfluidic methods have recently provided a useful method with which to probe more deeply into the requirements for L-form division. L-forms may provide insights into how primitive cells proliferated before the evolution of the cell wall. The L-form mode of proliferation may also provide the basis for self-replication of artificial cells.

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#### Biography

Jeff Errington has spent much of his research career studying fundamental questions about the structure and function of bacterial cells. Early on, he made important contributions to our understanding of the molecular biology underpinning endospore formation in *Bacillus subtilis*. More recently he has contributed substantially to understanding of chromosome replication and segregation, cell division and cell morphogenesis in bacteria. His lab was one of the pioneers in the application of digital fluorescence imaging methods to bacteria. He is presently Director of the Centre for Bacterial Cell Biology; the world's first major research centre focused specifically on the molecular and cellular biology of bacterial cells. His contributions to basic science have been recognized by election to various learned societies, including Fellowship of the Royal Society, EMBO, the UK Academy of Medical Sciences and the European and American Academies of Microbiology. His academic work is currently funded by major grants from the European Research Council and the Wellcome Trust.

## Oral Presentation -12

### Cell-free tool towards de novo genome synthesis

Masayuki Su'etsugu

College of Science, Rikkyo University, 171-8501, Japan



The recent advances of synthetic genomics allow to construct a genome-sized large DNA from scratch, providing a powerful approach for engineering living organisms. Traditional DNA amplification method, PCR cannot keep up with the length and fidelity required for the genome synthesis. So far, the synthesis of large DNA relies on conventional biological cloning using *Escherichia coli* and/or yeast, which is time-consuming and labor-intensive. In addition, some sequences are toxic or unstable in the living host cells. In order to overcome these constraints, we have developed a cell-free tool to synthesize genome-sized DNA molecules. Living organisms can propagate their large chromosome with great fidelity. We have thus reconstituted a replication cycle of the *Escherichia coli* circular chromosome using 25 proteins *in vitro* [1]. This reaction, termed RCR (Replication Cycle Reaction), propagates circular DNA molecules including a chromosomal origin (*oriC*) exponentially by autonomous repetition of the replication cycle form in an isothermal condition. Very large DNA up to 0.2 Mb is successfully propagated as a supercoiled monomer form with extremely high fidelity. We have further developed a novel DNA assembly reaction, termed RA (Recombination Assembly), in which multiple fragments with overlapping ends are efficiently ligated in a single-step isothermal reaction. The assembly reaction is subjected directly to the RCR propagation, so that the target circular DNA molecules but not linear intermediate molecules can be selectively propagated as a supercoil DNA. Using this two-step reaction, RA-RCR, we have successfully constructed a 27 kb plasmid from 50 fragments. RA-RCR thus provides a powerful cell-free tool to generate large circular DNA without relying on conventional biological cloning.

#### References

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#### Biography

Dr. Su'etsugu has received his PhD from Kyushu University, Japan in 2005, and continued his work on initiation regulation of chromosome replication in *Escherichia coli*, as Assistant Professor at the same university. He then joined Jeff Errington's lab as a postdoctoral researcher at Newcastle University, UK to study cellular dynamics of replication machinery of the *Bacillus subtilis* chromosome. After moving back to Japan, he became an Associate Professor at Rikkyo University, Tokyo in 2013, and started his laboratory. He was also JST-PRESTO researcher. His laboratory currently focuses on synthetic biology based on *in vitro* reconstitution of bacterial cellular systems, and is developing a genome-synthesis technology with the support of CSTI ImPACT.

## Oral Presentation -13

[Keynote]

### Design, Construction, and Analysis of a Synthetic Minimal Bacterial Cell

John I. Glass

J. Craig Venter Institute, CA 92037, USA



The minimal cell is the hydrogen atom of cellular biology. Such a cell, because of its simplicity and absence of redundancy would be a platform for investigating just what biological components are required for life, and how those parts work together to make a living cell. Since the late 1990s, our team at the Venter Institute has been developing a suite of synthetic biology tools that enabled us to build what previously has only been imagined, a minimal cell. Specifically, a bacterial cell with a genome that expresses only the minimum set of genes needed for the cell to divide every two hours that can be grown in pure culture. That minimal cell has about half of the genes that are in the bacterium on which it was based, *Mycoplasma mycoides* JCVI syn1.0, the so-called synthetic bacteria we reported on in 2010. We used transposon bombardment to identify non-essential genes, and genes needed to maintain rapid growth in *M. mycoides*. Based on those data, we designed and synthesized a reduced genome in eight overlapping segments. All segments were individually viable when combined with wild type versions of the seven other segments. Combinations of reduced segments that were not viable allowed us to identify synthetic lethal pairs of genes. These occur when two genes each encode an essential function. Those findings required re-design and re-synthesis of some reduced genome segments. Three cycles of design, synthesis, and testing, with retention of quasi-essential genes, produced synthetic bacterium JCVI-Syn3.0 (531 kb, 474 genes), which has a genome smaller than that of any autonomously replicating cell found in nature. Synthetic bacterium JCVI-Syn3.0 retains almost all genes involved in synthesis and processing of macromolecules. Surprisingly, it also contained 149 genes with unknown biological functions, suggesting the presence of undiscovered functions essential for life. This minimal cell is a versatile platform for investigating the core functions of life, and for exploring whole-genome design. Since it was initially reported in 2016, we have identified functions for more than 50 of the original 149 genes of unknown function and have developed a computational model of the cell.

This work was supported by Synthetic Genomics, Inc., DARPA Living Foundries contract HR0011-12-C-0063, and the J. Craig Venter Institute.

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#### Biography

John Glass completed his PhD in viral genetics at the University of North Carolina at Chapel Hill and postdoctoral studies at the University of Alabama at Birmingham. He was one of the first scientists to sequence and analyze a complete bacterial genome. He spent five years in the Infectious Diseases Research Division of the pharmaceutical company Eli Lilly, where he was involved in bacterial genomics and development of Incivek, one of the first drugs to cure hepatitis C virus. Currently, he is a Professor and Leader of the Synthetic Biology and Bioenergy Group at the J. Craig Venter Institute. There, in addition to his work producing the first synthetic and minimal bacterial cells, he has developed improved methods for rapidly producing influenza virus vaccine, worked on veterinary vaccines, minimizing the genome of the fast growing yeast, *Kluyveromyces marxianus*, and researched producing synthetic Human Artificial Chromosomes. He published over 70 papers.

## Oral Presentation -14

[Keynote]

### Manipulation of bacterial genomes by BGM vector: From synthesis to activate

Mitsuhiro Itaya

Laboratory of Genome Designing Biology, Institute for Advanced Biosciences, Keio University, Turuoka Japan



All cells/microbes possess their own genome DNA versatile in gene contents and GC contents. Recent technologies on giant DNA synthesis can reproduce existing genomes and related gene networks. We have long developed a unique genome handling system using *Bacillus subtilis* as a platform for large DNA synthesis. The system called BGM, Bacillus Genome Manipulations, resulted in the first successful case for a whole genome cloning of *Synechocystis* PCC6803 [1]. From the first chimera genome we are learning many fundamental biological issues previously obscure. At the same time we have been writing a general scenario in which all the procedures from synthesis to activate genomes can be manipulated by BGM system in simpler manner. I would like not only to recap our original systems [2-4] but also present scenarios with on-going works aiming at production of cells with designed genomes [5]. The new scenario should lead to new pipelines through which functional genomes are provided and tested for broad applications as manufactured microbes.

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#### Biography

Itaya, M has completed his PhD from University of Tokyo. After postdoctoral works at NIH (Bethesda, USA), basic researches on genome engineering using *Bacillus subtilis* were carried out at Mitsubishi-Kagaku Institute of Life Sciences (Machida, Tokyo Japan) since 1986. Major topics on genome synthesis and big DNA delivery are being continued and improved at Keio University (Tsuruoka, Yamagata Japan) since 2006.

## Oral Presentation -15

### Life-rebuilt in cell-free systems

**Michael C. Jewett**  
Northwestern University  
Department of Chemical and Biological Engineering  
Center for Synthetic Biology  
2145 Sheridan Road, Evanston IL 60208, USA



Imagine a world in which we could adapt biology to manufacture any therapeutic, material, or chemical from renewable resources, both quickly and on demand. Industrial biotechnology is one of the most attractive approaches for addressing this need, particularly when large-scale chemical synthesis is untenable. Unfortunately, current approaches to engineering organisms remain costly and slow. This is because cells themselves impose limitations on biobased product synthesis. It is difficult to balance intracellular fluxes to optimally satisfy a very active synthetic pathway while the machinery of the cell is functioning to maintain reproductive viability. Further, chemical reactions take place behind a selective barrier, the cell wall, which limits sample acquisition, monitoring, and direct control. In addition, cells are adapted to a relatively simple chemical operating system (i.e., a few common sugars, 20 amino acids), which presents researchers a limited set of accessible molecules with which to work. In this presentation, I will discuss my group's efforts to overcome these limitations and widen the aperture of the traditional model of biotechnology. In one direction, we seek to create a new paradigm for engineering biocatalytic systems using cell-free biology. In another area, we are catalyzing new directions to repurpose the translation apparatus for synthetic biology. Our new paradigms for biochemical engineering are enabling a deeper understanding of why nature's designs work the way they do, as well as opening the way to novel biobased products that have been impractical, if not impossible, to produce by other means.

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#### Biography

Michael Jewett is the Charles Deering McCormick Professor of Teaching Excellence, an Associate Professor of Chemical and Biological Engineering, and co-director of the Center for Synthetic Biology at Northwestern University. He is also an Institute Fellow at the Northwestern Argonne Institute for Science & Engineering. Dr. Jewett's lab seeks to re-conceptualize the way we engineer complex biological systems for compelling applications in medicine, materials, and energy by transforming biochemical engineering with synthetic biology. Dr. Jewett is the recipient of the NIH Pathway to Independence Award in 2009, David and Lucile Packard Fellowship in Science and Engineering in 2011, the DARPA Young Faculty Award in 2011, the Agilent Early Career Professor Award in 2011, the 3M non-tenured faculty grant in 2012, the Camille-Dreyfus Teacher-Scholar Award in 2015, and the ACS Biochemical Technologies Division Young Investigator Award in 2017.

## Oral Presentation -16

### Direct Evolution of Ribosome using Artificial Cell and Sorting Technologies

**Norikazu Ichihashi**  
Osaka University, Yamadaoka 1-5, Suita, Osaka, Japan



One of the largest challenges in the synthesis of reproducible artificial cell is in vitro assembly of ribosomes from in vitro synthesized rRNAs and proteins. The rRNAs require chemical modifications after transcription for the assembly, a complicated process in which many enzymes and several compounds participate. To circumvent the modification, we performed artificial evolution of 16S rRNA that forms the functional 30S subunit without chemical modifications using the liposome sorting technology. We encapsulated the integrated synthesis, assembly, and translation system into liposomes, and collected liposomes that contains highly active ribosomes with a cell sorter. After 15 rounds of selection cycles, we found one point mutation near the 3' terminus that significantly enhanced the reconstitution activity of the functional 30S subunit from unmodified 16S rRNA to a comparable level to that from native modified 16S rRNA. The effect of the mutation did not depend on the reconstitution scheme, orthogonal anti-SD sequences, or the target genes to be translated. The mutation we found in this study enabled reconstitution of the active 30S subunit without rRNA modification, and thus would be a useful tool for simple construction of self-reproducing ribosomes. In addition, by using this evolutionary method, we can obtain a protein-less primitive ribosome, which can be a directed evidence of the RNA world.

#### References

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#### Biography

N. Ichihashi has completed his PhD from the University of Tokyo and postdoctoral studies from Osaka university. He is an associate professor of the graduate school of information science and technologies of Osaka university. His ultimate goal is to understand the origin of life.

## Oral Presentation -17

[Keynote]

### Origin of metabolism from the spontaneous overcrowding of biopolymers in liposomes

**Pier Luigi Luisi**  
ETHZ, Zürich, Switzerland



While much of the prebiotic chemistry of the origin of life is still under debate, it is commonly accepted that at a certain point one has to consider the emergence of the first cells with DNA, RNA and proteins. There is here a main conceptual difficulty. If we assume that all basic components have first formed independently in a diluted form in an outside medium, then it is not obvious how all these components were incorporated all together and concentrated into a closed cellular compartment. Our later work offers a possible experimental solution to this basic difficulty. In fact, we can show that, given a dilute – and therefore non- reactive – aqueous solution of the macromolecular components for minimal life, the formation of liposomes *in situ* brings about their spontaneous entrapment with a very significant increase of the local concentration– which then permits protein synthesis. We discuss how this phenomenon (that we call spontaneous overcrowding) might be related to the origin of metabolism and cellular life.

#### Biography

Dr. Luisi, after his graduation at the Scuola Normale Superiore in Pisa, made his academic career at the Swiss Federal Institute of Technology in Zürich (ETHZ), Switzerland, where he became professor of Chemistry in the department of material sciences. Since 2003, till 2012, he has been working at the University of Rome3, Italy, as professor of Biochemistry. His research focussed in the origin of life, and the synthetic biology of minimal living cells. In Zurich he initiated the field of enzymology in reverse micelles, later the self-reproduction of micelles and vesicles. Author of over 500 reviewed papers, and a dozens of books, which include themes on philosophy of science and spirituality. The last books are resp. The systems view of life, with Fritjof Capra, and the second edition of his The emergence of life, both for Cambridge University Press..

At the ETHZ, he created (1985) the Cortona-week, an interdisciplinary residential week for graduate students, who are working with artists, psychologists, religious leaders, to open up their horizon and foster a new generation of world leaders with a more integrated and balanced view of life. See [www.cortona-week](http://www.cortona-week) and [www.cortona-india.org](http://www.cortona-india.org).

He has been associated from the start (1987) with the Mind and Life Institute, created by Francisco Varela and the Dalai Lama, devoted to the dialogue between modern science and Buddhism.

## Oral Presentation -18

### Toward reproduction of a bacterium from hybrid cell

**Kazuhito Tabata**  
Graduate School of engineering The University of Tokyo, Tokyo 113-8656, Japan



One major goal of synthetic biology is the design of an artificial cell. Prokaryotes make the easiest model, because they have a simple structure that contains only a cell wall, cell membrane and cytosol. However, these components are not enough, as mixing them into a liposome does not result in a cell regeneration. One reason is because the buffer we use to disrupt the cell and acquire its cytosolic content significantly decreases the concentration one or two magnitudes. A second reason is the function of the bilayer membrane used to enclose this content is insufficient. To overcome these problems, we have developed a lipid bilayer covered femto-Liter chamber.

To test our system, we introduced GFP-labeled E. coli protoplasts were introduced into the chamber, Fusion between the chamber and bacteria protoplast resulted in increased fluorescence and caused the cytoplasmic components of the bacteria to disperse throughout the chamber, and the membrane components bonded with the lipid bilayer, as described above. In other words, a hybrid cell was achieved. GFP fluorescent intensity of an each hybrid cell was changed with time. The average slope of the fluorescence intensity from the hybrid cells was slightly negative, a result likely due to protein degradation. However, if 5 mM ATP was added to the chamber in advance, the average slope was slightly positive. This result was attributed to protein synthesis caused by the ATP. We are currently conducting viability assessments of the hybrid system. We will present this reactor in detail and its application to the hybrid cell.

#### Biography

Dr. Kazuhito Tabata is a lecturer in the Department of Applied Chemistry at the University of Tokyo. He serves as an associate program manager in one of the projects awarded funding by ImpACT, a program designed by the Japanese cabinet office. He received his PhD from the Kanazawa University in 2001. He then did his post-doctoral research with prof. M. Yoshida and prof. H. Noji at Tokyo Institute of Technology, where he examined single molecule observation of FoF1-ATP synthase known as rotating molecule motor. After that, he accepted the position of assistant professor at the Osaka University in 2005. Since then, he has worked extensively on reconstitution of cell function in a micro reactor. He has been lecturer at the University of Tokyo since 2014. Now, he is investigating ways to exchange the endogenous genome with a synthetic one prepared by in vitro genome amplification system which developed a group of collaborators.



## Oral Presentation -19

### Unique Deformation Modes and Material Encapsulation of Giant Unilamellar Vesicles Encapsulating Biomacromolecules

Hiroaki Suzuki

Faculty of Science and Engineering, Chuo University, Japan



The shapes of giant unilamellar vesicles (GUVs) enclosing polymer molecules at relatively high concentration, used as a model cytoplasm, could differ significantly from those containing only small molecules [1-3]. We investigated the effects of the molecular weights and concentrations of polymers such as polyethylene glycol (PEG), bovine serum albumin (BSA), and DNA on the morphology of GUVs deflated by osmotic pressure. Although small PEG (MW < 1k) does not alter the mode of shape transformation even at > 10% (w/w), PEG with MW > 6k induces budding transformation at above 1% (w/w). Larger PEG frequently induced small buddings and tubulation from the membrane of mother GUVs. A similar trend was observed with BSA, indicating that the effect is irrelevant to the chemical nature of polymers. More surprisingly, long strands of DNA (> 105 bp) enclosed in GUVs induced budding transformation at concentrations as low as 0.01–0.1% (w/w). We expect that this molecular size dependency arises mainly from the depletion volume effect [2,3]. Our results showed that curving, budding, and tubulation of lipid membranes, which are ubiquitous in living cells, can result from simple cell-mimics consisting of the membrane and cytosolic macromolecules, but without specific shape-determining proteins. We additionally show that the molecular crowding in GUVs alter the mode of encapsulation of extremely large molecules, such as genomic DNA.

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#### Biography

Hiroaki Suzuki received M.S. and Ph.D. degrees in Mechanical Engineering from the University of Tokyo, Japan. From 2003 to 2007, he served as an Assistant Professor in Institute of Industrial Science (IIS), the University of Tokyo, and from 2007 to 2013 as an Associate Professor in the Graduate School of Information Science and Technology (IST), Osaka University. He became an Associate Professor in the Department of Precision Mechanics, Faculty of Science and Engineering, Chuo University, Japan in 2013, and promoted to a Professor in 2016. His research interests are micro self-assembly, microfluidics, biochips, and artificial cells.

## Oral Presentation -20

### Microfluidic formation of artificial cell membrane

Shoji Takeuchi

Institute of Industrial Science, University of Tokyo,  
4-6-1 Komaba, Meguro-ku, Tokyo 153-8505, Japan



This talk pertains to the reconstruction of a cell membrane integrated with membrane proteins in a microfluidic chip. Membrane proteins play very important roles in cells. They are also useful in various industrial fields, including next-generation diagnosis techniques, drug discovery, and so on. We have applied membrane proteins for highly sensitive and selective chemical sensors in a previous study, wherein we used a living cell expressing odorant receptors as the sensor that could distinguish multiple-target chemicals. This cell-based sensor can be integrated with a humanoid robot without any noise reduction systems. The sensor can be compact, and thus, easy to be incorporated into a portable device useful for environmental monitoring.

Moreover, we developed a simple method to use or analyze membrane proteins by incorporating them into artificially formed planar lipid bilayers. Lipid bilayers produced by conventional methods are often fragile, unsteady, and difficult to be reproduced. These weak properties reduce their usefulness in high-throughput systems. We thus developed a reproducible method, called the “droplet contacting method” for forming planar bilayers using simple fluidic control. This method is very simple and reproducible. Hence, it is now applied in various types of devices for membrane protein analysis and nowadays recognized as the “droplet interface bilayer (DIB)” widely used by many research groups. We are applying this lipid bilayer system to various fields, including chemical sensing, protein synthesis, drug kinetics studies, and artificial cells studies.

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#### Biography

Shoji Takeuchi received his B.E., M.E., and Dr. Eng. degrees in mechanical engineering from the University of Tokyo, Tokyo, Japan in 1995, 1997, and 2000, respectively. He is currently a professor in and the Director of the Center for International Research on Integrative Biomedical Systems (CIBiS), Institute of Industrial Science (IIS) at the University of Tokyo. He has authored more than 150 peer-reviewed publications and filed over 70 patents. He has been recognized with numerous honors, including MEXT Young Scientists' Prize in 2008, the JSPS prize in 2010, and the ACS Analytical Chemistry Young Innovator Awards in 2015. His current research interests include 3D tissue fabrication, implantable devices, artificial cells/lipid bilayer systems, and biohybrid MEMS.

# Poster Session Day 1

— Odd board numbers —

Poster Session **Board No.1**

**Screening of Mutated Alkaline Phosphatase Library by a Super-Arrayed Reactor**

Morito Sakuma<sup>1</sup>, Hiroshi Ueno<sup>1</sup>, Yoshiki Minagawa<sup>1</sup>, Yi Zhang<sup>2</sup>,  
Kentarō Miyazaki<sup>3</sup>, Kazuhito Tabata<sup>1</sup>, Hiroyuki Noji<sup>1,4</sup>

<sup>1</sup>Graduate School of Engineering, The University of Tokyo, Japan

<sup>2</sup>Japan Agency for Marine-Earth Science and Technology (JAMSTEC), Japan

<sup>3</sup>National Institute of Advanced Industrial Science and Technology (AIST), Japan

<sup>4</sup>Impulsing Paradigm Change through Disruptive Technologies Program (ImPACT, JST), Japan

Enzymes serve the critical function of catalyzing chemical reactions. Some enzymes have been applied to immunoassays, such as Enzyme-Linked Immunosorbent Assay (ELISA), and the production of biomass. Engineering high active, heat-stable and multifunctional enzymes is important to progress the reaction efficiently, while precise control of the enzyme activity by the mutation of DNA sequence is still challenging. Therefore, rapid and precise methods for evaluating extensive libraries of enzymes and recovering high active enzymes are beneficial for that purposes. In this experiment, DNA library of alkaline phosphatase (ALP) was encapsulated in a super-arrayed reactor, and high active ALP was recovered by a microinjection apparatus. On the reactor, fifty thousand micron-sized droplets could be formed at once, and single enzyme activity was quantitatively evaluated in the droplets. To evaluate ALP activity expressed from single-copy DNA within mutated DNA library, cell-free protein synthesis system (PURE system) and a fluorogenic substrate were encapsulated in the reactor (Figure 1(a)). Mutated ALP was expressed and degraded the substrates in the droplets, and the fluorescence intensity was gradually increased (Figure 1(b)). High active ALP could degrade many substrates, thus the fluorescence intensity in the reactors was high. By recovering DNA from the reactors with high fluorescence intensity, high active ALPs could be recovered. These results indicated that our method could evaluate vast mutated library and apply to rapid screening of enzymes.

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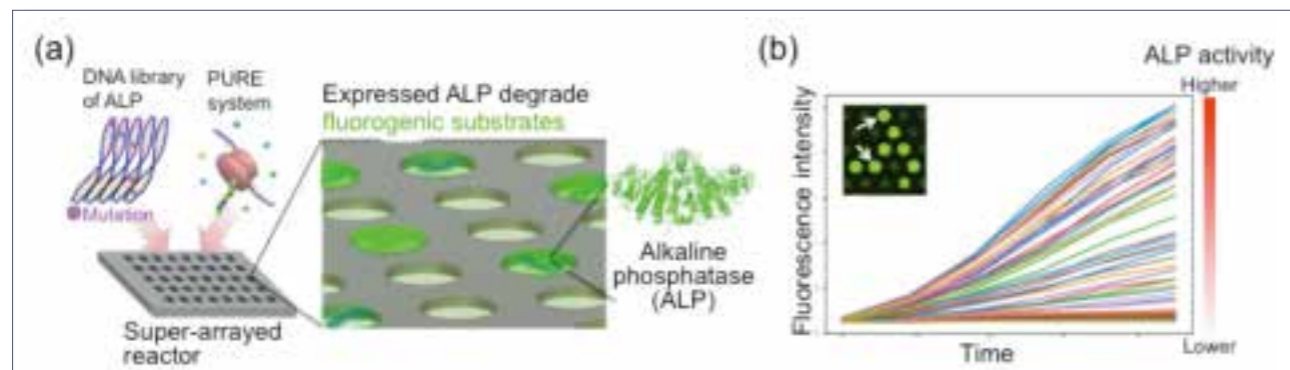


Figure 1. (a) Screening of high active alkaline phosphatase (ALP) by a super arrayed reactor. DNA library, cell-free protein synthesis system and fluorogenic substrate were encapsulated in the reactor. (b) Time-course of fluorescence intensity in the reactors. Inset image shows a part of the chambers, and white arrows do the chambers with ALP expression.

Poster Session **Board No.3**

**Robustness of an *Escherichia coli* protein translation system analyzed by computational modeling**

Tomoaki Matsuura<sup>1</sup>, Kazufumi Hosoda<sup>2</sup>, Yoshihiro Shimizu<sup>3</sup>

<sup>1</sup>Department of Biotechnology, Graduate School of Engineering, Osaka University, Japan

<sup>2</sup>Institute for Academic Initiatives, Osaka University, Japan

<sup>3</sup>Laboratory for Cell-Free Protein Synthesis, Quantitative Biology Center (QBiC), RIKEN, Japan

Robustness is one of the important and amazing feature of biological systems, however, not much has been done to investigate comprehensively the robustness or the sensitivity to external perturbation using models originated from existing reaction network that enumerate all detailed processes. To elucidate the dynamic features of a biologically relevant large-scale reaction network, we constructed a computational model of minimal protein synthesis<sup>[1]</sup> consisting of 241 components and 968 reactions that synthesize the Met-Gly-Gly (MGG) peptide based on an *Escherichia coli*-based reconstituted *in vitro* translation (IVT) system<sup>[2]</sup> (Figure 1). We performed a simulation using parameters collected primarily from the literature and found that the rate of MGG peptide synthesis becomes nearly constant in minutes, thus achieving a steady-state similar to experimental observations. We then attempted to utilize this simulator to clarify how each individual reaction affects the overall complex reaction network. We altered the kinetic parameter of the computational model globally and systematically to investigate the effect of these changes on the MGG peptide synthesis. Altering the kinetic parameter mimics the perturbation of the biological reaction networks by environmental changes and/or stimuli. We find that even changing the parameter by 100 to 0.01-fold, only 6% of the parameters affect the yield or the time of pre-steady state (defined as lag-time) by more than 1.5-fold. Conversely, even changing the parameter by 100 to 0.01-fold, more than 94% did not affect the reaction dynamics. Moreover, despite 100- to 0.01-fold changes in the kinetic parameter, none of the indicators (yield or lag-time) responded linearly to the changes, indicating the robustness and insensitivity of the reaction network to perturbation. Origin of these features, and its generality is discussed.

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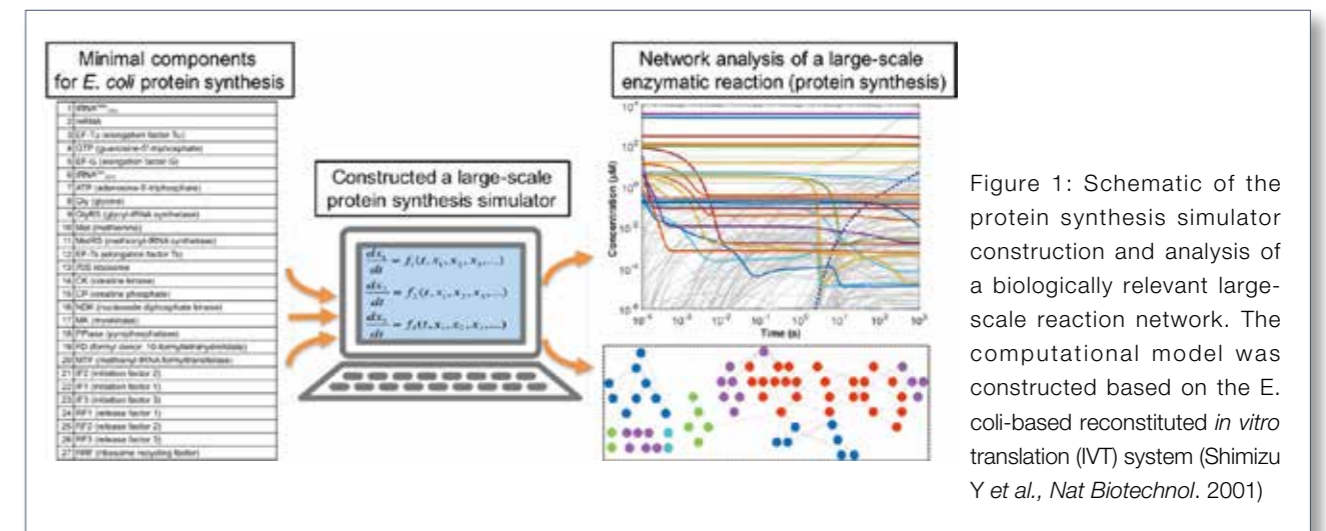


Figure 1: Schematic of the protein synthesis simulator construction and analysis of a biologically relevant large-scale reaction network. The computational model was constructed based on the *E. coli*-based reconstituted *in vitro* translation (IVT) system (Shimizu Y *et al.*, *Nat Biotechnol.* 2001)

Poster Session **Board No.5**

**Directed evolution of *Escherichia coli* alkaline phosphatase for enhanced activity**

Kentaro Miyazaki<sup>1</sup>, Junko Morijiri<sup>1</sup>, Shigemi Taguchi<sup>1</sup>, Ryoko Yaginuma<sup>2</sup>, Yi Zhang<sup>2,3</sup>, Hiroshi Ueno<sup>2</sup>, Hiroyuki Noji<sup>2,4</sup>

<sup>1</sup>AIST, Japan

<sup>2</sup>The University of Tokyo, Japan

<sup>3</sup>JAMSTEC, Japan

<sup>4</sup>JST ImPACT, Japan

We applied site scanning saturation mutagenesis (SSSM) to the entire region of the mature alkaline phosphatase sequence of *E. coli*. Degenerated primer was used to introduce random codons (coded by NNK) into each site and the resultant plasmid libraries (pUC-based) were transferred to *E. coli* DH5 $\alpha$  and grown on LB/Amp agar plates containing a chromogenic X-phosphate substrate. A total of 48 blue (bluer or bluest) colonies were selected from each library and grown in LB/Amp/IPTG broth at 37°C for overnight. The cell suspension was then used to screen for activity at various conditions (type of buffers, pHs, and substrates); variants exhibiting high activities relative to the wild-type enzyme were retrieved. Similarly, SSSM libraries were created in T7-based expression system. Combined *in vitro* cell-free transcription-translation-activity screening was performed in ultra high throughput femtoliter chamber array devices. We identified several variants exhibiting high activities by using fluorogenic substrate. Each DNA was retrieved from the hit chamber and mutation sites were determined by sequencing after PCR-amplification from a single DNA. Variants obtained by the above-mentioned procedures exhibited ~35-times activity that of the wild-type enzyme, which was well beyond that of the D101S mutant, a commercial enzyme currently used in diagnostic purposes.

Poster Session **Board No.7**

**Development of Novel Disease Diagnosis Platform based on Enzyme Activity Detection at Single Protein Level**

Shingo Sakamoto<sup>1</sup>, Toru Komatsu<sup>1,5</sup>, Rikiya Watanabe<sup>4,5</sup>, Yi Zhang<sup>4</sup>, Hiroyuki Noji<sup>4</sup>, Yasuteru Urano<sup>1,2,3</sup>

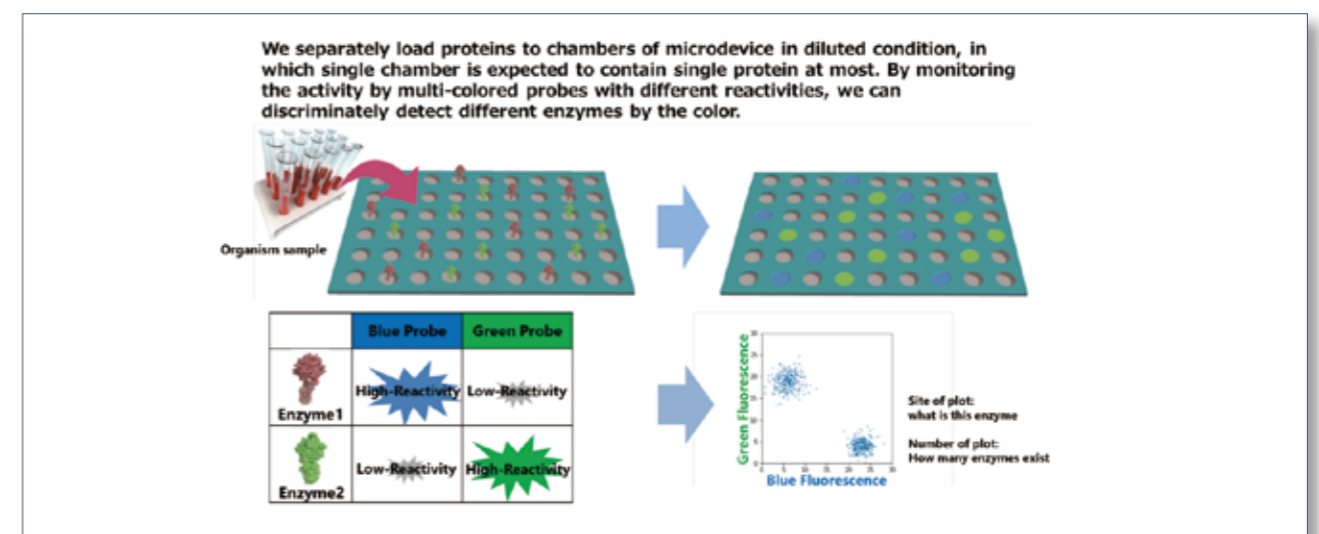
<sup>1</sup>Grad. Sch. Pharm. Sci., The Univ. Tokyo, Japan, <sup>2</sup>Grad. Sch. Med., The Univ. Tokyo, Japan,

<sup>3</sup>AMED CREST, Japan, <sup>4</sup>Grad. Sch. Eng., The Univ. Tokyo, Japan, <sup>5</sup>JST PRESTO, Japan

This research aims to develop a novel disease diagnosis system by directly monitoring the single molecular enzymatic activities of disease-related proteins in serum. It is known that various enzymes are present in living systems, and abnormal enzymatic activities are often related to the progression of various diseases. Enzymatic activity monitoring is routinely applied as a biochemistry-based disease diagnosis. However, the conventional diagnosis systems have some limitations when we want to apply them to develop more precise, early, and diverse diagnosis platform; (1) lack of sensitivity to detect lower amount of enzymes in the sample (2) inability to discriminate the activity of resembling enzymes, like isozymes. As a highly sensitive enzyme detection system, multi-well chamber type microdevice has been developed to enable the detection of enzymatic activities at single molecule level [1]. By separately detecting enzyme activity at single molecule level, we can acquire detailed information about what and how many enzymes are existing in the bio-sample of interest. However, this technology is with inherently lack of the way to know which enzyme is in each chamber since the enzyme loading into the chamber is a completely random event. We tried to overcome this obstacle by the way of discriminating the different enzymes in the chamber by the different reactivities toward fluorescent probes with multiple colors and reactive moieties. We focused on alkaline phosphatase (ALP), phosphate ester hydrolyzing enzymes having 6 isozymes in serum; the high activity of individual ALP isozymes in serum is known to reflect various diseases. We have developed ALP probes with multiple colors and reactive moieties and by using them, we achieved the discriminative single molecular counting of two ALP isozymes in mixture by different reactivities toward several probes. We also tried to detect and count specific ALP isozymes in serum derived from diabetes patients.

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Poster Session **Board No.9**

**Creation of high activity enzymes for biomass saccharification**

Taku Uchiyama<sup>1</sup>, Naoki Sunagawa<sup>1</sup>, Mako Kamiya<sup>2</sup>, Yasuteru Urano<sup>2</sup>, Kiyohiko Igarashi<sup>1</sup>

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Biomass is a natural and renewable material, and will be expected as a new carbon source in industrial process. Plant derived biomass consists of multifarious polysaccharide polymer. Among them, cellulose is a major component of plant biomass. Therefore, saccharification of cellulose is an important challenge of plant biomass utilization. In nature, cellulose is enzymatically degraded into glucose by cellulase and  $\beta$ -glucosidase. Accordingly, finding the novel cellulase and  $\beta$ -glucosidase is a key point for practical use of plant biomass. Numerous research studies have been conducted to find the novel cellulase and  $\beta$ -glucosidase from nature, however the enzymes which has sufficient performance for industrial use is not found yet. Evolutionary molecular engineering is one of the major methods to take the novel enzymes which has good characteristics beyond the natural one. To take the fine result from enzyme evolution, high throughput screening is a must. However, cellulose is an insoluble material, thus decomposition reaction by enzymes occurs at a solid-fluid interface. Hence, in situ detection of cellulase reaction is difficult. In many previous researches, to clear this problem, the cellulose mimic substrate which has soluble or high degradable character has been widely used. Therefore, it often occurs that the obtained enzymes well react to mimic substrates, but not work on the native cellulose.

In this study, to solve these problems, we design a new detection method of cellulase reaction according to combine the cellulose dehydrogenase (CDH) enzyme and fluorescent probe with cellulase. As a result of this new system, cellulase reaction can be shown as a fluorescent intensity. In addition, this fluorescent signal can be measured without removing undecomposed cellulose substrate, thereby we can estimate the cellulase activity in situ. Also the activity of  $\beta$ -glucosidase can be measured to use the fluorescent substrate.

Using these new measurement system and traditional assay system, now we are trying to get the novel cellulase and  $\beta$ -glucosidase. So far we can get slightly good engineered cellulase which has 1.3 times higher activity than wild type enzyme, and also try to take the good thermo-stable  $\beta$ -glucosidase. However, the character of obtained enzyme is insufficient for industrial use now. Therefore, we are taking the more preferred cellulase and  $\beta$ -glucosidase. In poster, we will introduce the current results of enzyme engineering.

Poster Session **Board No.11**

**Developing effective antimicrobials using novel Quorum Quenching Enzymes screened from Metagenomes**

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Given the growing threat of antibiotic resistant pathogens to healthcare, it is urgent to develop novel antibiotic and antivirulence compounds. Strategies to disrupt quorum sensing (QS) using quorum quenching (QQ) enzymes have attracted considerable attention as a new class of antimicrobial reagents to control bacterial physiology. However, specific QQ enzymes active on the autoinducer-2 (AI-2) and auto-inducing peptides (AIP) classes of QS signals, which are used by numerous gram-positive and negative pathogenic bacteria, have not been identified to date. Herein, we aim to develop new AI-2- and AIP-degrading enzymes derived from functional metagenomic screening. To screen for QQ enzymes, we designed biosensors using natural AI-2 and AIP QS signaling modules (transcription regulator-promoter pairs) that measure QQ catalytic activity. We will optimize the biosensors by systematic design of ribosome binding site (RBS) and 5'-untranslated region (UTR) to quantitatively regulate QS signal-dependent reporter gene expression. Additionally, we characterized few enzymes having anti-biofilm activity of bacteria, which used AI-2 signaling. To demonstrate the therapeutic potential as alternative antibiotics, we propose to establish *in vitro* and *in vivo* model systems such as human gut microbiome and plant root model. Finally, our findings will outline the mechanism of AI-2 and AIP QQ enzymes and provide novel insight into the anti-biofilm and anti-virulence strategies underlying pathogenic QS.

Poster Session **Board No.13**

**Identification of thermostabilizing mutations for G-protein coupled receptors:  
Rapid method based on statistical thermodynamics**

Satoshi Yasuda<sup>1,2,3</sup>, Yuta Kajiwara<sup>4</sup>, Yuuki Takamuku<sup>1</sup>, Nanao Suzuki<sup>2</sup>, Yosuke Toyoda<sup>5</sup>,  
Kazushi Morimoto<sup>5</sup>, Ryoji Suno<sup>5</sup>, So Iwata<sup>5</sup>, Takuya Kobayashi<sup>5</sup>, Takeshi Murata<sup>1,2,6</sup>, Masahiro Kinoshita<sup>3</sup>

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<sup>3</sup>Institute of Advanced Energy, Kyoto University, Japan, <sup>4</sup>Graduate School of Energy Science, Kyoto University, Japan,

<sup>5</sup>Graduate School of Medicine, Kyoto University, Japan, <sup>6</sup>JST, PRESTO, Japan

G-protein-coupled receptors (GPCRs), which are responsible for signal transduction pathways, construct important drug targets. For the efficient structure-guided drug design, however, their structural stabilities must be enhanced. Introduction of mutations can enhance their thermostability and stability in detergents, but the stabilizing mutations are currently identified by experiments. Here we develop a theoretical method that allows us to treat all of the possible mutations. It employs a free-energy function (FEF) that takes into account the translational entropy of hydrocarbon groups within the lipid bilayer as well as the protein intramolecular hydrogen bonding. First, the method is illustrated for the adenosine A2a receptor (A2aR) whose wild-type structure is known [1]. We propose a reliable strategy of finding key residues to be mutated and selecting their mutations, which will lead to considerably higher stability. Representative single mutants predicted to be stabilizing or destabilizing were experimentally examined and the success rate was found to be remarkably high. The melting temperature for two of them was substantially higher than that of the wild type. Then, we postulate that the wild-type structure of A2aR is unknown. We construct candidate models for the wild-type structure using the homology modeling and select the model giving the lowest value to the change in FEF on protein folding. The performance achieved is only slightly lower than that in the case where the wild-type structure is available [2]. Finally, the method is adopted for two other GPCRs [3]; muscarinic acetylcholine receptor 2 (M2R) and prostaglandin E receptor 4 (EP4). The crystal structures of M2R is available, but that of EP4 has not been reported yet. We found that they possess key residues at the same position (*i.e.* a hot-spot residue). Mutations of the hot-spot residue brought to substantially higher stability, leading to the determination of new crystal structures for the two GPCRs.

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Poster Session **Board No.15**

**Sequence profile for protein design based on database analysis of backbone  
en-vironment**

Shintaro Minami<sup>1</sup>, Nobuyasu Koga<sup>1,2</sup>

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Recently, technology of computational protein design from scratch has greatly developed and many de novo designed proteins were reported [1-3]. However, various manual adjustments for each individual design target by human experts are still indispensable. For example, the current standard software for protein design, Rosetta, generates many designs of which local sequences do not compatible with its local backbone geometry. (It is empirically known that the designs including such local sequence-structure unmatched regions do not stably fold [3].) In order to improve the de-signed sequences, human adjustments with trial and error were required. In this study, we propose a method for generating sequence profiles indicating favored amino acid types for each residue position based on its backbone environment with analysis of known structure databases. Guided by the profile, Rosetta can readily design sequences that compatible with local backbone structures. The key of our method is a database search with a novel representation of protein backbone structure environment for each residue position. Given a sequence design target backbone structure, the backbone environment for each residue position is represented by its backbone buriedness and torsion angle type as a query, and then the amino acid types for the similar backbone environments to the query in the database were statistically analyzed to predict the position-specific amino-acid preference for the position (sequence profile). In the presentation, we will introduce the detailed algorithm of the backbone environment search, and the test results of a new sequence design protocol with the predicted profile.

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[3] G.J. Rocklin, *et al.*, *Science* **357**, 6347 (2017).

## Toward design of thermostable beta-glucosidase with structure-based sequence profile

Naoya Kobayashi<sup>1</sup>, Shintaro Minami<sup>1</sup>, Taku Uchiyama<sup>2</sup>, Naoki Sunagawa<sup>2</sup>, Kiyohiko Igarashi<sup>2</sup>,  
and Nobuyasu Koga<sup>1</sup>

<sup>1</sup>Research Center of Integrative Molecular Systems, Institute for Molecular Science, National Institutes of Natural Sciences,  
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<sup>2</sup>Department of Biomaterials Sciences, Graduate School of Agricultural and Life Sciences, The University of Tokyo,  
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Enzymes are used in a wide range of industrial products such as pharmaceuticals and chemical products, biofuels and foods. Many of the enzymes are proteins, and in general, proteins are unstable to heat. Therefore, improving the thermal stability of enzymes is required for industrial applications. Here, we report an attempt toward the design of thermostable beta-glucosidase, expected to be industrially applied to the saccharification process of cellulosic biomass. Protein three-dimensional structure is mainly stabilized by hydrophobic interaction in the core region. Therefore, improving poorly packed hydrophobic core can stabilize proteins. To design thermostable mutants with a larger and well-packed hydrophobic core region, we focused on small side chain residues (Ala, Val, Leu, Ile, Ser, Thr, His) without forming hydrogen bonds in the core and boundary region of the target enzyme structure. We predicted sites that can be mutated from the small side chain residues to larger hydrophobic side chain residues (Leu, Ile, Phe, Tyr, Trp) based on the two different database analyses, (i) sequence profile<sup>[1]</sup> for a design target beta-glucosidase backbone structure, which indicates amino acid sequence preference for each residue position based on its main-chain dihedral angle pattern and its 3D-structural environment around the position, and (ii) multiple sequence alignment based on structure-based sequence alignment<sup>[2]</sup> of beta-glucosidase homologs derived from thermophilic bacteria. The predicted mutant structures were computationally modeled and were confirmed that they have no steric hindrance. Finally, 36 thermostable mutant candidates were selected. Our prediction method with the structure-based sequence profile is expected to be widely applicable to the stabilization of enzymes whose tertiary structures have been solved.

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## Poster Session Day 2

— Even board numbers —

Poster Session **Board No.2**

**Enzymatic construction of large circular DNA  
from over 50 overlapping fragments**

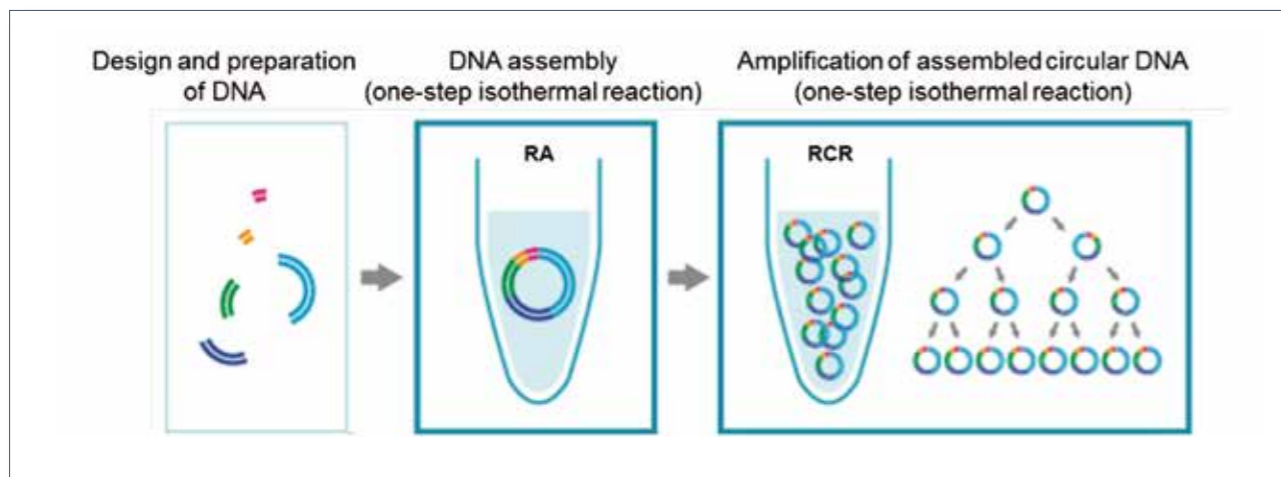
Tatsuaki Kurata, Masayuki Su'etsugu

Rikkyo University, Japan

DNA assembly methods are essential tools to construct a designed large length DNA for synthetic biology, particularly for synthetic genomics. Some new techniques are emerging to assemble multiple DNA fragments seamlessly in a single-step. For example, Gibson Assembly is able to assemble multiple DNA fragments via their overlapping ends by a mechanism including exonuclease processing and DNA annealing at 50°C. Recently, we have established a novel DNA amplification method by reconstituting a replication cycle of *Escherichia coli* circular chromosome [1]. This method, termed RCR (Replication Cycle Reaction) exponentially amplifies circular DNA harboring a replication origin of the *Escherichia coli* chromosome, *oriC*, even from a single DNA molecule in a single-step isothermal reaction. In the RCR method, circular forms of DNA are selectively amplified, and the linear forms are not amplified. One of remarkable features of RCR is maximum DNA length to be amplified. Circular DNA over 200-kb can be amplified as a supercoiled form. RCR thus appears to be valuable to amplify the large circular product selectively directly from the DNA assembly reaction. To test this idea, we originally developed a novel DNA assembly method involving multiple enzymes, termed RA (Recombination Assembly) to assemble multiple overlapping DNA fragments seamlessly in an isothermal reaction. By combining RA and RCR, we have successfully produced a 27-kb circular DNA from 50 fragments. RA-RCR is thus provides a powerful cell-free tool to produce a designed large circular DNA from multiple fragments.

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Poster Session **Board No.4**

***In vitro* amplification of circular DNA mediated by *oriC* transposon**

Seia Nara, Masayuki Su'etsugu

Rikkyo University, Japan

Amplification of DNA sequences is a fundamental technique in modern molecular biology. Replication Cycle Reaction (RCR) has been recently developed as a new method to amplify a large circular DNA up to 200 kb *in vitro*, by reconstituting the chromosome replication system of *Escherichia coli* using 25 purified proteins. A circular DNA template including a chromosomal replication origin, *oriC*, is amplified exponentially as intact circular molecules in an isothermal reaction. Although DNA amplification in RCR does not require oligonucleotide primers as in the case of polymerase chain reaction, it requires the *oriC* sequences (0.3 kb) inserted in the circular DNA template. In order to amplify various circular DNA templates lacking *oriC* in RCR even in the absence of their sequence information, we have here developed a rapid *in vitro* reaction to introduce *oriC* into the circular DNA template mediated by a transposon system. As a transposon DNA, we used an *oriC* fragment including transposase recognition sequences on its both ends. Transpososome complexes were formed by incubation of the *oriC* transposon DNA with the Tn5 transposase. Further incubation of the *oriC* transpososome complexes with the target circular DNA allowed *oriC* to be inserted into the target DNA. Subsequent amplification reaction using RCR, the target circular DNA was successfully amplified. Using this *oriC* transposon system, we were able to amplify a 15 kb non-*oriC* plasmid even from a very small amount (50 fg) of the starting template plasmid. The *oriC* transposon system thus provides a convenient tool to amplify non-*oriC* circular DNAs using RCR, and may be useful as a whole genome amplification tool to investigate environmental circular DNA.



Poster Session **Board No.6**

**Selective Pairing and Fusion of DNA-encapsulated Liposomes and Escherichia Coli Spheroplasts Using Dielectrophoretic Tweezers**

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We describe a method for selective pairing and fusion of DNA-encapsulated liposomes and Escherichia coli (E. Coli) spheroplasts using dielectrophoretic (DEP) tweezers toward transplantation of genome-size DNA into E. Coli. Cell-sized liposomes have been used to model physical and chemical property of cell membrane [1]. They can encapsulate micrometer-sized objects and can be fused to membrane of mammalian cells, which allows transplantation of the object to the cell [2]. However, no studies show fusion of cell-sized liposomes to bacteria such as E. Coli. A fusion method for cell-sized liposomes and E. Coli is needed for transplantation of genome-size synthetic DNA to the cell toward creation of artificial cells that can be fully designed for industrial use.

Here, we propose a method to selectively fuse liposomes that encapsulate large synthetic DNA to E. Coli using microfabricated DEP tweezers. First, we encapsulated large synthetic DNA (approximately 200 kb) to cell-sized liposomes of 1-10  $\mu\text{m}$  in diameter by emulsion transfer method. Next, we formed giant E. Coli of 1-10  $\mu\text{m}$  in diameter by combination of cephalixin-based elongation and lysozyme-based spheroplast formation for fusion with the liposomes. Finally, we utilized a microfabricated DEP tweezer [3] to selectively pair the liposome and E. Coli, then applied electric fields by handmade copper electrodes for electrofusion. We observed that the large synthetic DNAs were encapsulated in the cell-sized liposomes, and the liposomes can be paired and fused by the DEP tweezers and the electrodes. Giant E. Coli spheroplasts can be also paired by fused by the DEP tweezers and the electrodes. The cell-sized liposomes and the giant E. Coli can be selectively paired by the DEP tweezer. We believe our method will be useful in selective transplantation of genome-size DNA into E. Coli and investigation of electrofusion parameters for collecting living cells after fusion.

**References**

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- [2] A. C. Saito, T. Ogura, K. Fujiwara, S. Murata, S. M. Nomura, *PLoS One* **9**, e106853 (2014).
- [3] T. Kodama, T. Osaki, R. Kawano, K. Kamiya, N. Miki, S. Takeuchi, *Biosensors and Bioelectronics* **47**, 206 (2013).

Poster Session **Board No.8**

**Evolutionary arms-races between host-parasite RNA replicators in an artificial cell reactor**

Taro Furubayashi, Yohsuke Bansho, Daisuke Motooka, Shota Nakamura, Norikazu Ichihashi

Osaka University, Japan

Host-parasite relationships are universally observed in nature, in which host organisms are exploited by parasitic organisms, viruses, or transposons. Parasites could have been a big evolutionary driving force for their hosts through “evolutionary arms-races” [1], but it is still unclear how universal the arms-race phenomenon is, and how the arms-race influences evolution of their hosts.

In this study, we tried to reconstruct host-parasite evolutionary arms-races by evolving a simple host-parasite RNA replicator system [2] in an artificial cell reactor based on a water-in-oil emulsion using a cell-free translation system [3]. In this system, a host and a parasite RNA replicate using an RNA replicase encoded only in the host RNA. Both the host and the parasite RNA are capable of evolution through mutations introduced by replication errors and natural selection processes. We performed evolution experiments by encapsulating the Host-Parasite RNA replication system in the artificial cell reactor and repeating the process of RNA replication and feeding nutrients.

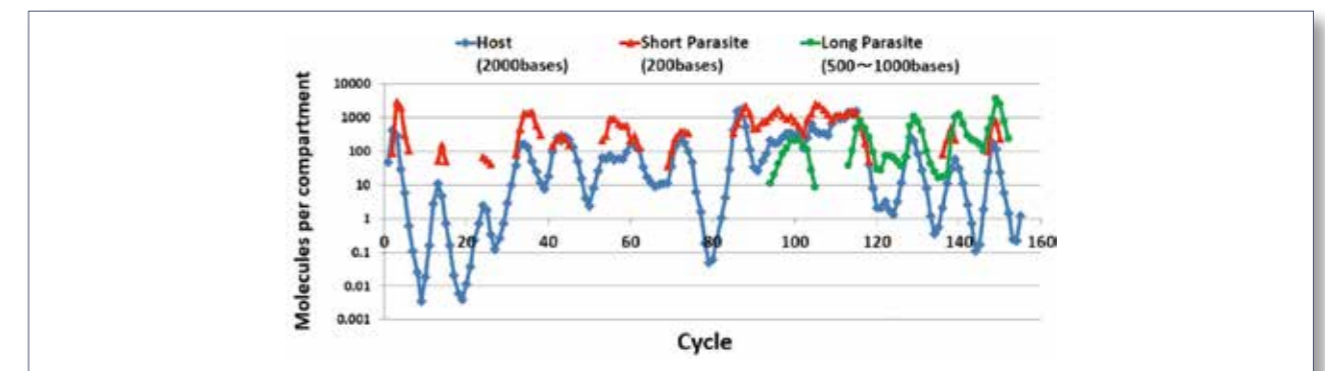
We found that

- 1) The number of the host and the parasite RNA oscillated and transitioned between oscillating competition phase and coexistent phase during the evolution experiments.
- 2) Novel parasite RNAs with different sequence lengths emerged from the middle stage of the evolution experiments.
- 3) The host RNA evolved faster in the presence of the parasite RNA.

These results indicate that evolutionary arms-races between the host and the parasite RNA really occurred, and that parasites can accelerate the evolution of their hosts.

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**Establishment of the fusion method between artificial and bacterial cells**

Gakushi Tsuji, Takeshi Sunami, Norikazu Ichihashi

Osaka University, Japan

**A** genome encodes whole information of life. Recently, genome sequences of many species have been determined. As a next important step after these 'reading genome' projects, many researchers are now trying to synthesize artificially-designed genomes. The expression of such artificial genomes in micro-bioreactors is considered to allow the custom-made organisms and therefore to solve various social problems. However, the reconstitution of the genome expression in micro compartment has not been succeeded yet. Recently, *in vitro* translation systems have been developed to express proteins from genes in phospholipid bilayer compartments called liposomes, but the expression from genomes remain to be done. In this study, we are attempting to develop an experimental scheme to handle and express genomes in a liposome.

**W**e propose three steps for achieving this goal (Fig. 1): (1) Fusing liposomes with *E.coli* to encapsulate the genome in a liposome directly without exposing to the extracellular solution, (2) forming pores on the liposome membrane temporarily by using streptolysin O to change the inner environment surrounding the genome, and (3) performing in-lipo genome expression. To date, we have succeeded in the fusion of *E.coli* cells with liposomes to obtain semi-synthetic artificial cell that contains the mixture of the cell lysate, the genome, and the inner solution of the liposomes. We also have succeeded in the formation of pores through that fluorescent-conjugated transferrin (80 kDa) can pass with streptolysin O. These methods would be a step forward to the genome expression method in liposomes.

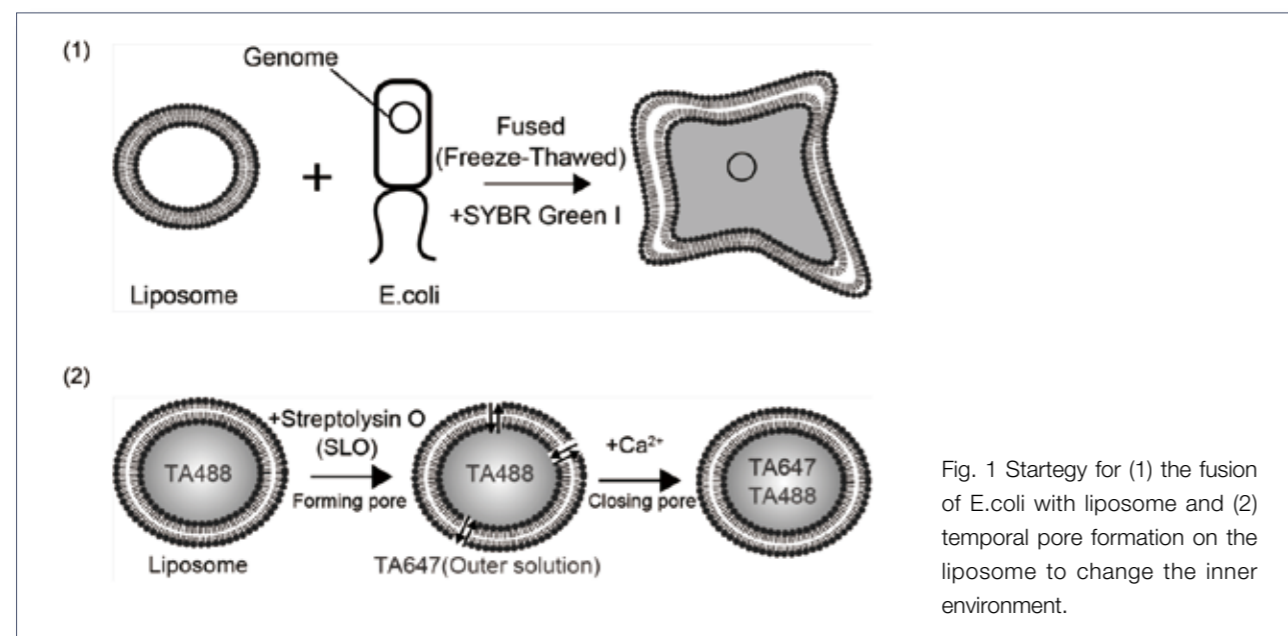


Fig. 1 Strategy for (1) the fusion of *E.coli* with liposome and (2) temporal pore formation on the liposome to change the inner environment.

**Repeated Substrate Supply to Biochemical Reactions Encapsulated in Giant Unilamellar Vesicles**

Taiji Okano, Hiroaki Suzuki

Chuo University, Japan

**M**icrodroplet or digital microfluidics technologies, which enable handling of biochemical reagents in minute volumes, found numerous applications in biology and biochemistry [1]. In these applications, it is often required to add some reagents to the solution for performing consecutive reactions. Previously, we demonstrated liquid handling operations such as mixing and aliquoting can be done by electrofusion and division of liposomes using a simple microfluidic device [2,3]. However, it was difficult to perform these operations repeatedly because all liposomes in the system are damaged or broken by electrical stimuli required for the fusion process. Here we developed a microfluidic device in which the electrical stimuli is localized in the fusion chamber, so that new liposomes can be provided from the reservoir as needed for repeated electrofusion (Fig. 1). Using this device, liposomes were properly stored in respective reservoirs with easy manual pipetting (Fig. 2). In this research, we loaded two types of liposome suspensions to the reservoirs; one contained  $\beta$ -galactosidase and the other contained its fluorogenic substrate. To perform electrofusion of liposomes, the enzyme-containing liposome and the substrate-containing liposome were brought into the fusion chamber with optical tweezers. After that, we applied AC electric field to align the liposomes by dielectrophoresis and three DC pulses to introduce their electrofusion (Fig. 3). As a result, the enzymatic reaction occurred in the fused liposome. After the reaction reached a plateau, we added some amount of substrate by fusing a new substrate-containing liposome, and we succeeded in restoring the reaction.

**References**

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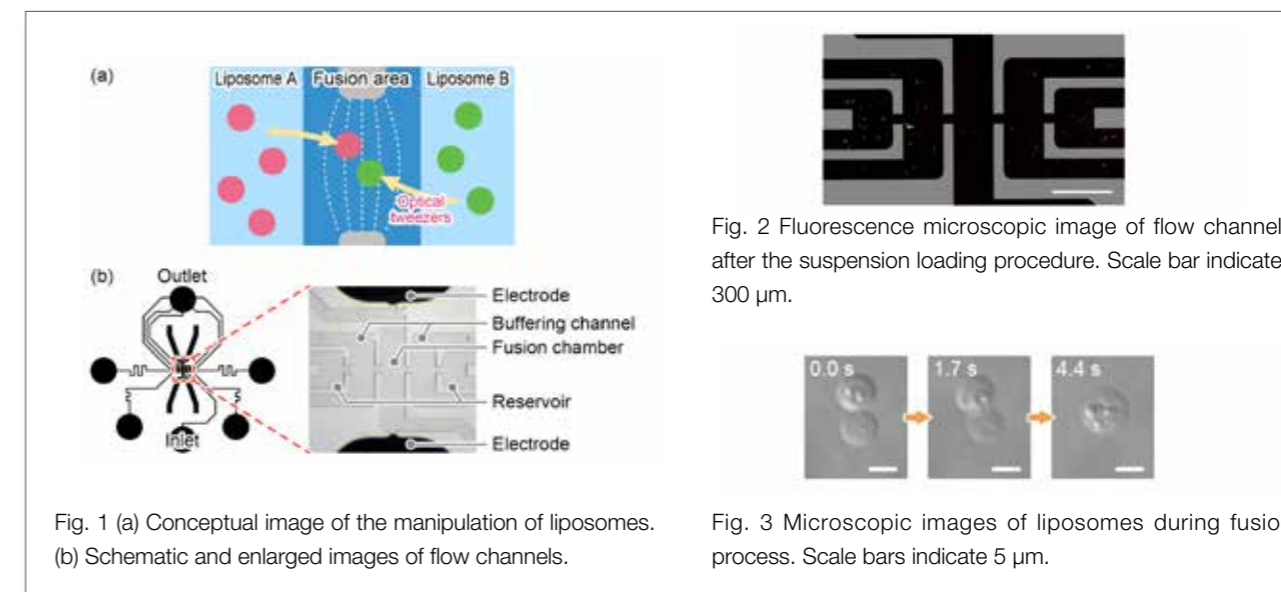


Fig. 1 (a) Conceptual image of the manipulation of liposomes. (b) Schematic and enlarged images of flow channels.

Fig. 2 Fluorescence microscopic image of flow channels after the suspension loading procedure. Scale bar indicates 300  $\mu$ m.

Fig. 3 Microscopic images of liposomes during fusion process. Scale bars indicate 5  $\mu$ m.

**A rapid digital counting assay for measurement of nucleic acid**

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TOPPAN PRINTING CO.,LTD., Japan

Recently, biomolecule detection with higher degree are required for accurate diagnosis of disease in medical field. In order to meet this needs, 'Digital Counting' is used, which quantifies absolute number of target molecules in the sample such as blood. The feature of 'Digital Counting' is that sample is distributed into many small compartments, and quantified by counting the compartments which include target molecule.

Digital polymerase chain reaction (dPCR) is typical technology using 'Digital Counting' for detecting DNA. dPCR is an assay that is based on PCR, to achieve target DNA quantification at high levels of sensitivity and specificity using tiny reaction compartments. However, the signal amplification reaction of the dPCR method takes a long time to detect, because PCR method rely on thermal cycling process which requires cycles of repeated heating and cooling many times. Therefore, in order to solve this problem, we are developing new 'Digital Counting' method, digital Invasive Cleavage Assay (digital ICA). Digital ICA can quantify DNA quickly because of two reasons. By using ICA, amplification of DNA is not required. And by using plates with femtoliter size holes, samples are divided into very small volume which minimizes reaction time.

Theoretically, digital ICA can detect not only DNA but also RNA and protein. Therefore digital ICA can detect many types of biomarkers in the future.

In this poster session, we will introduce mechanism of digital ICA, design and processing method of signature plate, result of EGFR gene mutation T790M detection, and future plan.

**Experimental investigation on encapsulation of isothermal nucleic acid amplification reaction into tiny chambers**

Teruya Enomoto, Ken Komiya

Tokyo Institute of Technology, Japan

Development of a sensitive and quantitative method for nucleic acids detection is a long-pending issue especially at the clinical sites. It is difficult to use the conventional polymerase chain reaction (PCR) or isothermal reactions at the points of detection including the clinical sites due to the problems caused by their high temperature conditions. For solving these problems, we constructed a novel isothermal nucleic acid amplification reaction, termed low-temperature amplification (L-TEAM) reaction, which amplifies pre-designed single-stranded DNA strands under a low-temperature condition. Encapsulating biochemical reaction into cellular-sized chambers allows digital detection of biomolecules<sup>[1]</sup>. In this study, we experimentally investigated the feasibility of encapsulating our L-TEAM reaction into the tiny chambers.

In our preliminary experiments monitoring the amplification via fluorescence measurement, we found the different amplification behavior of the reaction in the tiny chambers not only from that in bulk, but also from that in water-in-oil droplets whose sizes were similar to those of the chambers. To achieve efficient DNA amplification in the chambers, we intensively explored various experimental conditions, such as reaction components and their concentrations, additives for preventing non-specific adsorption to the chamber walls, and raw materials for the chambers. In the presentation, we report the results of the experiments described above and discuss the dependence of the optimal reaction conditions on the chamber materials. Further optimization based on these knowledges would achieve digital detection of nucleic acids.

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[1] S.H. Kim, S. Iwai, S. Araki, S. Sakakihara, R. Iino, H. Noji, *LabChip* **12**, 4986 (2012).

**A single virus digital bioassay by the use of smart phone fluorescence microscopy**Yoshihiro Minagawa<sup>1,2</sup>, Hiroshi Ueno<sup>1,2</sup>, Yuko Kawaguchi<sup>1,2</sup>, Kazuhito V. Tabata<sup>1,2</sup>,  
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Point of care testing (POCT) is a test that conducted near side of patient whenever medical care is needed. Therefore, rapid and simple diagnosis is desirable features for POCT. Digitalization of fluorogenic enzymatic assays through the use of femtoliter-volume chamber arrays is an emerging approach to realizing highly quantitative and rapid bioassays<sup>[1,2]</sup>. However, to observe the fluorescence signal in femtoliter-volume compartment, we require expensive and large microscope system. To apply the digital bioassay to POCT, it is necessary to develop portable and simple observation system. Here we demonstrate a single virus digital bioassay using smart phone fluorescence microscopy.

Firstly, we achieved a single influenza virus digital assay. When single influenza virus were encapsulated into the femtoliter-volume chamber arrays at a low ratio of less than 1 molecule pre chamber, each chamber showed a discrete fluorescence signal in an all-or-none manner, allowing the digital counting of the number of active influenza viruses.

Then, we developed a compact fluorescence observation system employing smartphone. This system involves a compact laser diode to illuminate femtoliter-volume chamber arrays with evanescent field, a long-pass thin film filter, and aspherical lens, and the mechanical translation stage for observing a large number of femtoliter-volume chambers. When the light originating from the laser diode undergoes total internal reflection within the glass on the femtoliter-volume chamber, the evanescent field is generated. We tested the imaging performance of this smartphone fluorescence microscopy system by conducting the single virus digital bioassay. We detected a discrete fluorescence signal in an all-or-none manner. For comparison, the same sample was imaged with conventional fluorescence microscopy. Both images were coincidence. This result suggests that the fluorescence microscopy employing smartphone permit the digital bioassay to POCT application.

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**Spontaneous Specific Localization of Actin and DNA in Cell-sized Water/Water Micro Droplet**Hiroki Sakuta<sup>1</sup>, Kingo Takiguchi<sup>2</sup>, Kanta Tsumoto<sup>3</sup>, Kenichi Yoshikawa<sup>1</sup><sup>1</sup>Doshisha University, Japan<sup>2</sup>Nagoya University, Japan<sup>3</sup>Mie University, Japan

Living cells maintain their lives through the generation of micro-compartments under crowding conditions of bio-macromolecules, such as nucleoplasm and cytoplasm. Intracellular micro-compartmentalization has generally been interpreted with phospholipid bilayer membranes [1]. On the other hand, it is known that aqueous medium crowding with mixed polymers generates segregation between two different bulk aqueous phases, i.e., aqueous two phase systems (ATPS). Here, we report the possible spontaneous occurrence of macro-compartments together with biologically important macromolecules. It has been shown that bio-macromolecules such as long DNAs and actin proteins are spontaneously entrapped inside the ATPS micro-compartments [2].

We report the results of our observation on a simple binary polymer (PEG/dextran) system in the presence of cytoskeletal protein, actin, and genetic coding polymer, DNA. It was found that the bio-macromolecules, actin and DNA, exhibit specific localization in cell-sized aqueous/aqueous micro droplets (CAMD) accompanied by the spontaneous micro-segregation of the binary polymers in an autonomous manner, when we adapt the polymer compositions near criticality of phase-separation. We show that the self-emergent micro droplets spontaneously capture polymerized actin, F-actin, while G-actin is distributed evenly. Here, we successfully control the state of actin by changing the concentrations of potassium and magnesium ions. It is found that both actin and DNA are spontaneously entrapped inside the cell-sized droplets. Interestingly, the distribution of actin inside the droplets changes markedly depending on the state of actin; G-actin, or disoersed F-actin, or bundled F-actin.

\* CAMD : Cell-sized Aqueous/Aqueous Micro Droplet**References**

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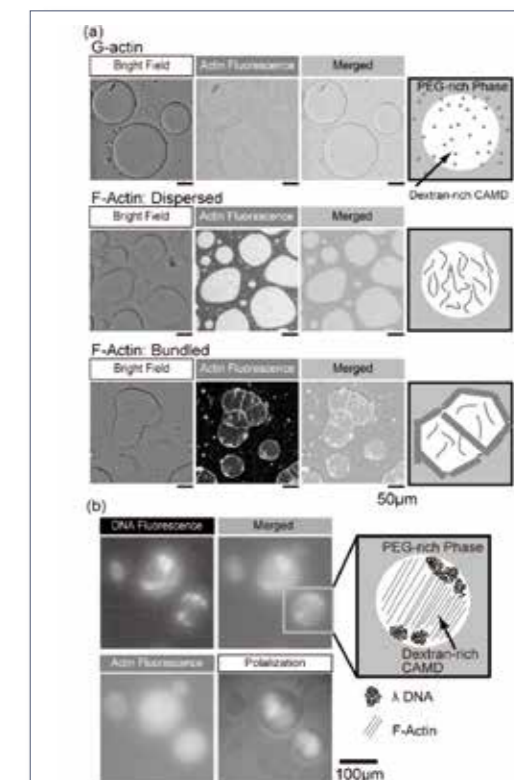
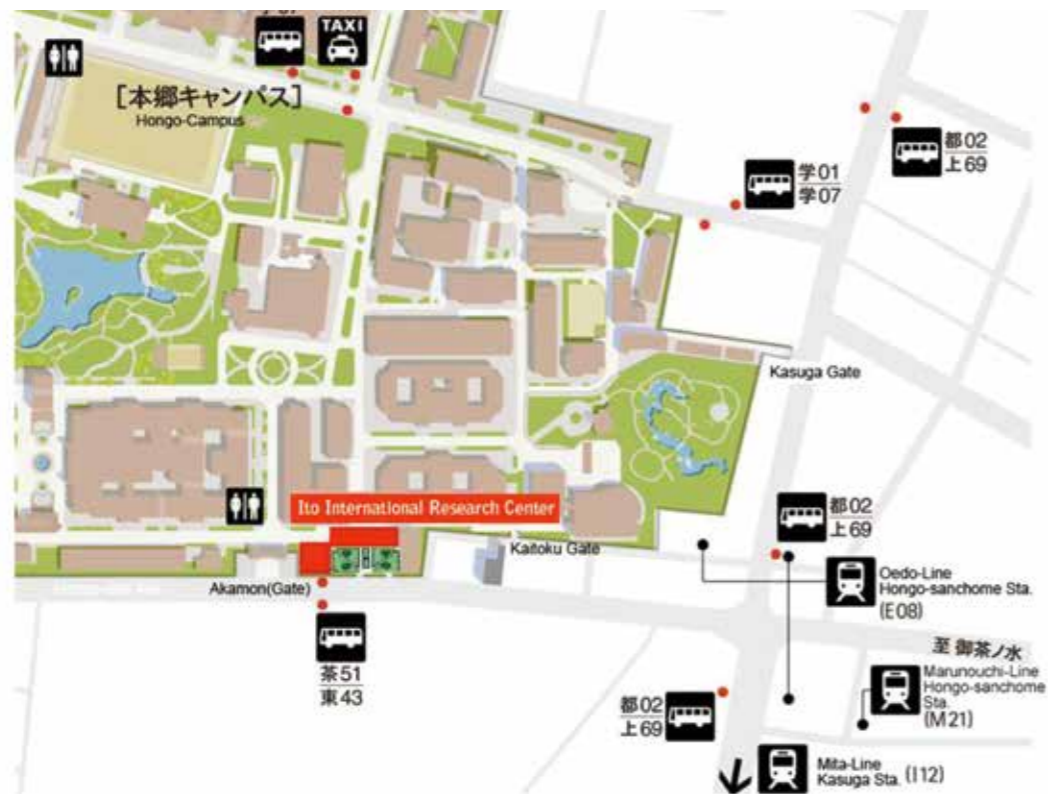


Fig.1 Specific localization of actin and long DNA in CAMD.(a) Different manner of localizations depending on polymerization actin states. (b) Segregation between DNA and F-actin. [3]

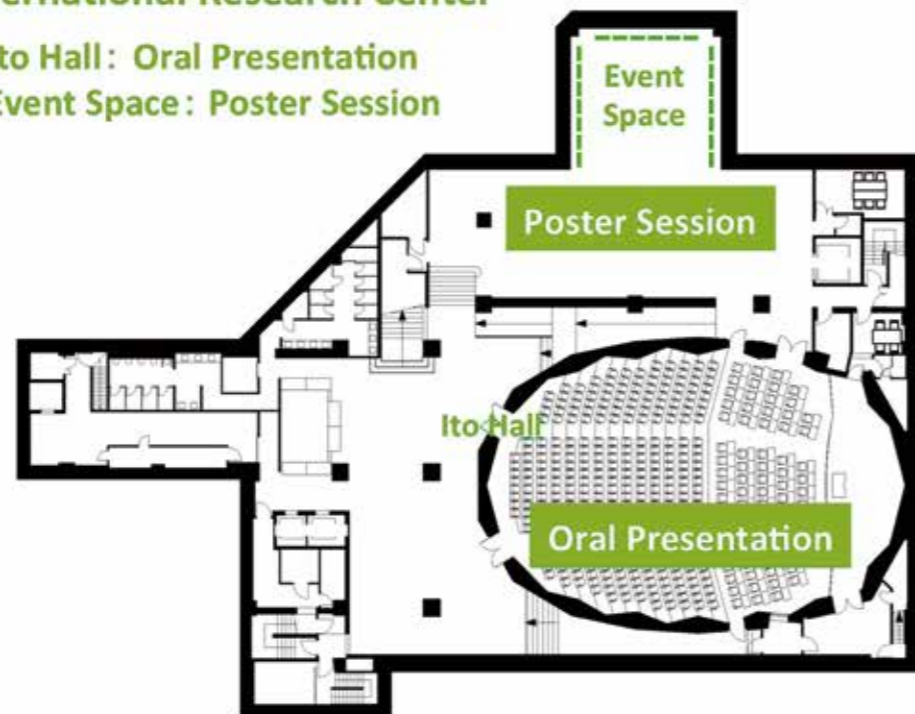
## **Floor Plan**

## Floor Plan

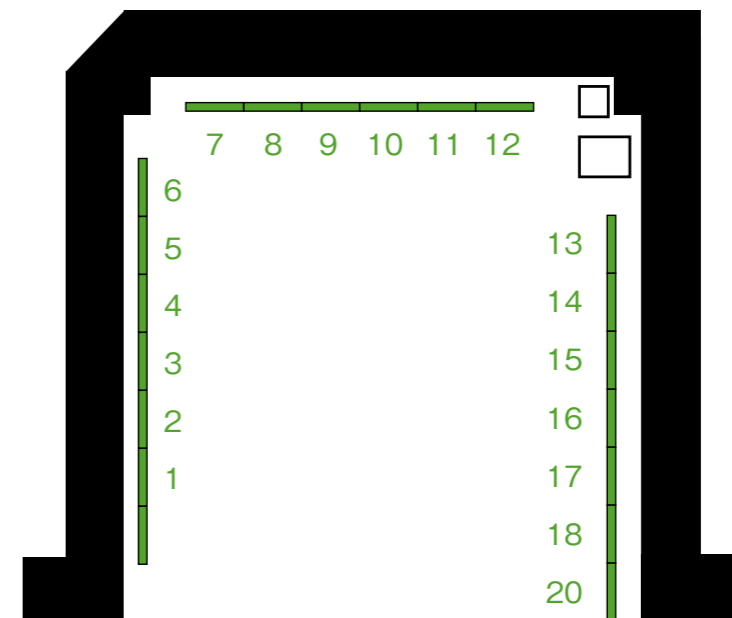


### Ito International Research Center

**B2F Ito Hall: Oral Presentation**  
**Event Space: Poster Session**



## Poster Presentation (Event Space)



### Day 1 (April 5) 14:50-15:50

- [1] Morito Sakuma  
Screening of Mutated Alkaline Phosphatase Library by a Super-Arrayed Reactor
- [3] Tomoaki Matsuura  
Robustness of an Escherichia coli protein translation system analyzed by computational modeling
- [5] Kentaro Miyazaki  
Directed evolution of Escherichia coli alkaline phosphatase for enhanced activity
- [7] Shingo Sakamoto  
Development of Novel Disease Diagnosis Platform based on Enzyme Activity Detection at Single Protein Level
- [9] Taku Uchiyama  
Creation of high activity enzymes for biomass saccharification
- [11] Sang-Soo Han  
Developing effective antimicrobials using novel Quorum Quenching Enzymes screened from Metagenomes
- [13] Satoshi Yasuda  
Identification of thermostabilizing mutations for G-protein coupled receptors: Rapid method based on statistical thermodynamics
- [15] Shintaro Minami  
Sequence profile for protein design based on database analysis of backbone environment
- [17] Naoya Kobayashi  
Toward design of thermostable beta-glucosidase with structure-based sequence profile

### Day 2 (April 6) 15:00-16:00

- [2] Tatsuaki Kurata  
Enzymatic construction of large circular DNA from over 50 overlapping fragments
- [4] Seia Nara  
In vitro amplification of circular DNA mediated by oriC transposon
- [6] Shotaro Yoshida  
Selective Pairing and Fusion of DNA-encapsulated Liposomes and Escherichia Coli Spheroplasts Using Dielectrophoretic Tweezers
- [8] Taro Furubayashi  
Evolutionary arms-races between host-parasite RNA replicators in an artificial cell reactor
- [10] Gakushi Tsuji  
Establishment of fusion method between artificial and bacterial cells
- [12] Taiji Okano  
Repeated substrate supply to biochemical reactions encapsulated in giant unilamellar vesicles
- [14] Y. Horiuchi  
A rapid digital counting assay for measurement of nucleic acid
- [16] Teruya Enomoto  
Experimental investigation on encapsulation of isothermal nucleic acid amplification reaction into tiny chambers
- [18] Yoshihiro Minagawa  
A single virus digital bioassay by the use of smart phone fluorescence microscopy
- [20] Hiroki Sakuta  
Spontaneous specific localization of actin and DNA in cell-sized water/water micro droplet