DIVISION OF QUANTITATIVE BIOLOGY

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Living cells act as input-output (I/O) units, in which environment and/or internal states are recognized on the cell surface and processed within a cell, leading to the adaptive response to these changes. This cellular information processing is mainly controlled by intracellular signal transduction, which is comprised of a series of chemical reactions, most commonly protein phosphorylation. Importantly, dysregulation of the signal transduction by gene mutation results in pathological diseases such as cancer.

The intracellular signaling pathway has been extensively studied over the last few decades, and most of its proteins and regulations have been identified, causing the perceived increase in the pathway's complexity. Computer-assisted simulation is one of the most promising approaches for the understanding of the signal transduction pathway as a system. Indeed, chemical and physical reactions constituting the signal transduction can be described by a set of ordinary differential equations, and solved numerically by computers. A number of simulation models of signaling pathways have, in fact, been reported to date. However, most of the kinetic parameters utilized for the simulation models were not measured experimentally, but rather were assumed by fitting the experimental data with the simulation model or simply determined arbitrarily. Consequently, there are substantial differences in the kinetic parameters between these studies, thereby making it difficult to evaluate these simulation models quantitatively.

To address these issues, we currently focus on the development of research tools enabling us to (1) visualize, (2) quantify, and (3) manipulate the intracellular signaling pathways with fluorescence imaging techniques and computational approaches.

I. Visualization of cell signaling

With the advent of fluorescent proteins, it has been possible to visualize kinetic reactions at the single cell level. Förster (or fluorescence) resonance energy transfer (FRET) is a non-radiative process, in which excitation energy of donor fluorophore is transferred to a nearby acceptor fluorophore. Taking advantage of the principles, FRET-based biosensors allowed us to detect kinase activity of PKA, ERK, Akt, JNK, PKC, and S6K in a living cell with high temporal and spatial resolution (Komatsu N, Mol. Biol. Cell, 2011) (Figure 1).

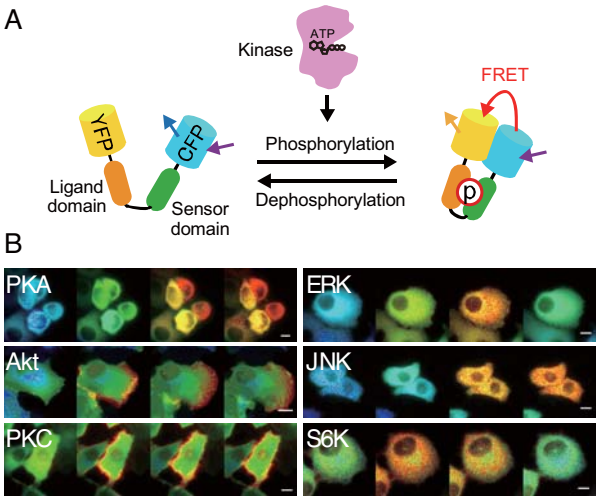

Figure 1. Visualization of kinase activity in living cells. (A) Schematic representation of intramolecular FRET biosensor of kinases. (B) PKA, ERK, Akt, JNK, PKC and S6K activities by FRET imaging. HeLa cells expressing FRET biosensors for the indicated kinases were stimulated with each ligand.

By using a FRET biosensor, we revealed that intercellular propagating waves of ERK activation determined the direction of the collective cell migration (Figure 2). Cell migration is a fundamental process in many physiological and pathological contexts. Collective cell migration refers to a movement of cell groups with physical and functional cell-to-cell connections, and is inherently involved in the processes of embryonic development, wound healing, and cancer invasion. However, it remains unclear how cells in a group organize physical and chemical clues into synchronized directionality and migration to facilitate coordinated collective migration. We visualized ERK activity in collectively migrating Mardin-Darby canine kidney (MDCK) cells by an ERK FRET biosensor, EKAREV, and found the

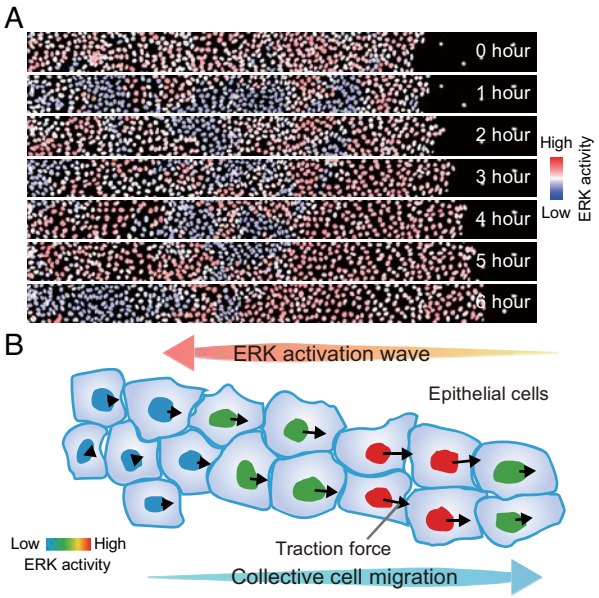

Figure 2. ERK activation wave and collective cell migration. (A) Propagating waves of ERK activation in wound healing of MDCK cells. (B) Schematic representation of collective cell migration driven by ERK activation propagation.

intercellular propagating waves of ERK activation (Figure 2A). Interestingly, MDCK cells collectively migrated against the direction of the ERK activation wave. The inhibition of ERK activation propagation suppressed collective cell migration. The ERK activation wave spatiotemporally controlled actomyosin contraction and cell density. Furthermore, an artificially-generated ERK activation wave by optogenetics reproduces induction of the collective cell migration. These data provide new mechanistic insights into how cells sense the direction of collective cell migration (Aoki K, Dev Cell, 2017).

Although FRET biosensors enable us to monitor cell signaling events, FRET imaging is not suited for multiplexed imaging, because these biosensors are authentically composed of two different fluorophores in many cases. To circumvent this limitation, we are currently developing biosensors based on the principle of the kinase translocation reporter (KTR) system; the reporter is translocated from nucleus to cytoplasm when it is phosphorylated by its target kinase (Figure 3). This approach yields several kinase reporters, e.g., ERK, Akt, p38, and JNK, and will provide clues as to how cells emerge all-or-none and irreversible cellular events such as cell cycle progression and apoptosis (Maryu G, Cell Strut, Funct, 2016; Miura H, bioRxiv, 2017) (Figure 3).

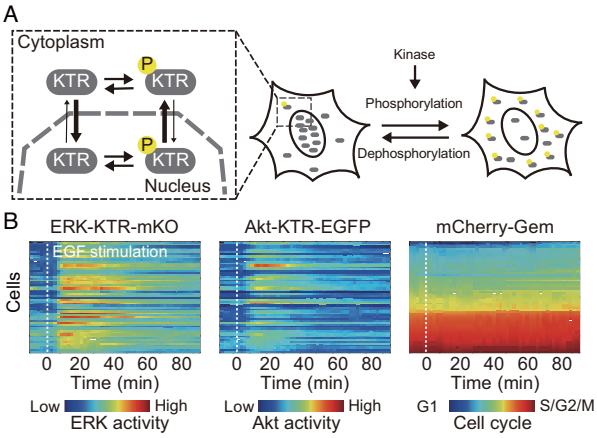

Figure 3. Multiplexed imaging of ERK and Akt activities and cell cycle state. (A) Schematic representation of the principle of KTR system. (B) Shown here are heat maps of ERK activity (left), Akt activity (middle), and cell cycle (right) obtained by KTR systems.

II. Quantification of cell signaling

Classically, kinetic parameters such as protein concentration and dissociation constant, Kd, have been measured by *in vitro* biochemical analyses. However, some kinetic parameters might significantly differ between *in vitro* and *in vivo*. For instance, the Kd values measured *in vivo* were higher than the *in vitro* Kd values by an order of 1 or 2 (Sadaie W, MCB, 2014). Therefore, it is critical to measure kinetic parameters in living cells.

To this end, we launched a research project of quantitative cell cycle modeling in fission yeast *Schizosaccharomyces pombe* and mammalian cultured cells. *S. Pombe* offers many advantages for studying the cell cycle; this organism shares most of the genes used for the cell cycle by mammalian cells and shows efficient labeling of endogenous genes with conventional knock-in techniques. We established several *S. Pombe* lines expressing cyclin and cyclin-dependent kinase fused with GFP and RFP, respectively, and quantified these expression levels during the process of the cell cycle by live cell imaging. In mammalian cells, we developed an efficient knock-in system with CRISPR/Cas9-mediated genome editing techniques (Figure 4), and combined with fluorescence correlation spectroscopy (FCS) and fluorescence cross-correlation spectroscopy (FCCS) to quantitatively determine the protein concentration and the dissociation constant of endogenous protein (Komatsubara A, bioRxiv, 2017)(Figure 4).

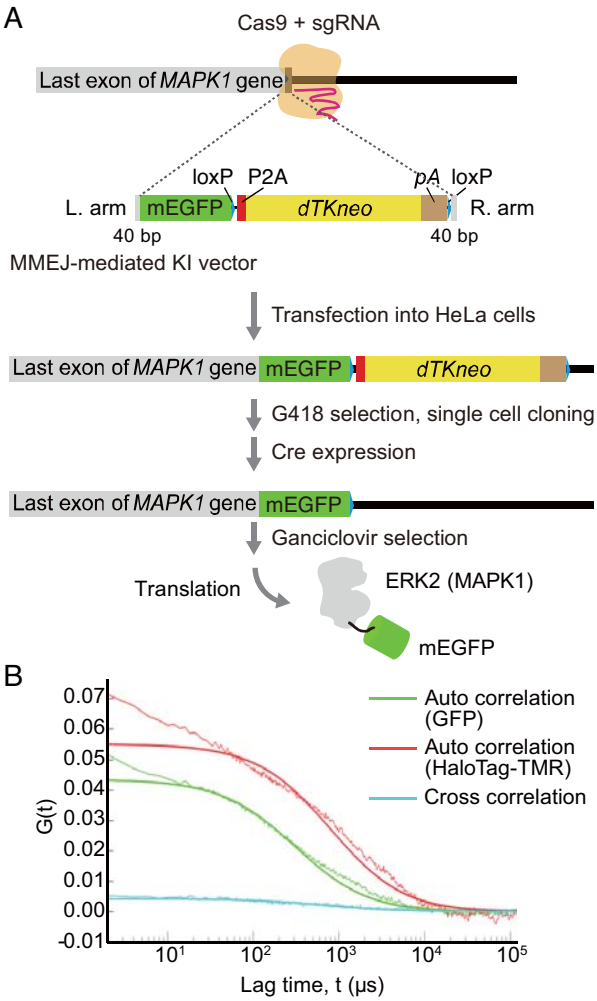

Figure 4. Quantification of protein concentration and dissociation constant of endogenous proteins. (A) Schematic illustration of gene knock-in with an MMEJ-mediated KI vector at MAPK1 locus. (B) Auto- and cross-correlation functions of ERK2-mEGFP and RSK2-HaloTag. *mEGFP* and *HaloTag* genes were knocked-in at the site of 3' *ERK2* and *RSK2* genes, respectively.

III. Manipulation of cell signaling

Artificial manipulation of biochemical networks would be useful for quantitative understanding of biological systems. Multiple methods have been suggested for controlling signal transduction, including light-induced dimerization (LID) and chemically induced dimerization (CID) systems. Among them the LID system has strong advantages in terms of temporal and spatial manipulations. The photo-responsive

proteins derived from fungi, cyanobacteria and plants, and modified fluorescent proteins are used in this system.

We focus on the phytochrome B (PhyB)-PIF LID system: Upon red-light illumination, PhyB binds to PIF, and the PhyB-PIF complex dissociates from each other by infra-red light exposure (Figure 5A). The reversible control of association and dissociation by light is a unique feature in the PhyB-PIF system, because either association or dissociation is regulated by light in other LID systems. One drawback is that covalent attachment of a chromophore, e.g. phycocyanobilin (PCB), is required for the light-responsive function of PhyB-PIF. To overcome this issue, we developed a method for synthesis of phycocyanobilin in mammalian cells by introduction of the gene products of *HO1*, *PcyA*, *Fd*, and *Fnr* into mitochondria (Uda Y, PNAS, 2017) (Figure 5B). The amount of synthesized PCB was comparable to externally-delivered PCB. We could demonstrate light-induced ERK activation with the PhyB-PIF LID system combined with endogenously synthesized PCB, showing the artificial oscillation of ERK activation (Figure 5C and 5D). The genetically encoded system of PCB synthesis will provide a potential advantage for establishing transgenic animals that stably synthesize PCB endogenously, thereby enabling the optogenetic manipulation of cell signaling in deeper tissues without injecting PCB.

Publication List:

〔Original papers〕

- Aoki, K., Kondo, Y., Naoki, H., Hiratsuka, T., Itoh, R.E., and Matsuda, M. (2017). Propagating wave of ERK activation orients collective cell migration. Dev. Cell *43*, 305–317 e5.
- Uda, Y., Goto, Y., Oda, S., Kohchi, T., Matsuda, M., and Aoki, K. (2017). Efficient synthesis of phycocyanobilin in mammalian cells for optogenetic control of cell signaling. Proc. Natl. Acad. Sci. USA *114*, 11962–11967.

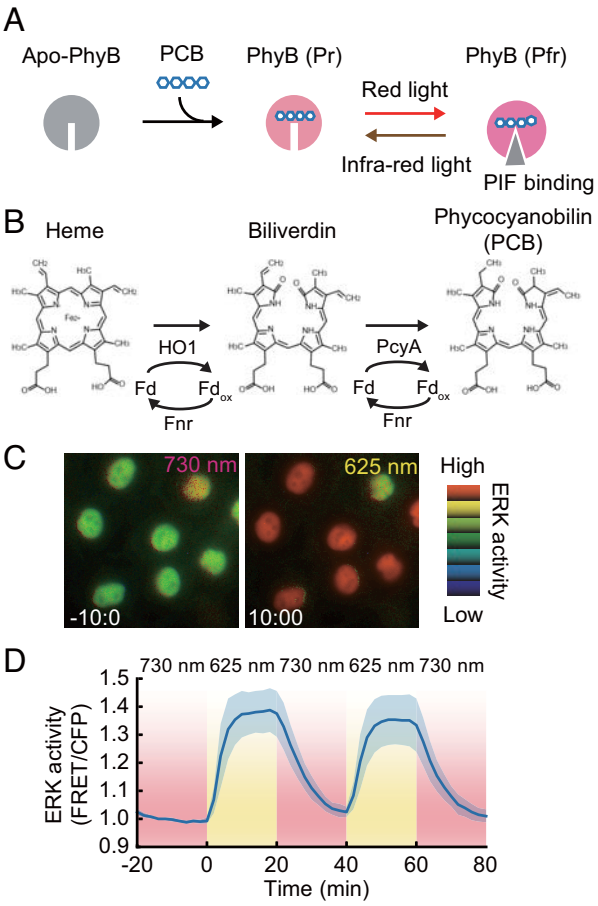

Figure 5. PhyB-PIF light-inducible dimerization (LID) system with PhyB-PIF and its application to the manipulation of cell signaling. (A) Apo-PhyB covalently attaches its chromophore, which is phycocyanobilin (PCB) to produce holo-PhyB. There are two holo-PhyB forms, PhyB (Pr) and PhyB (Pfr), which change over in manner dependent on the chromophore status. Only PhyB (Pfr) associates with PIF. (B) The metabolic pathway of phytochrome chromophores. (C) Light-induced activation of ERK with PhyB-PIF LID system. Representative images of ERK activity at the nucleus in HeLa cells. (D) The average ERK activity (normalized FRET/CFP ratio) is plotted as a function of time with the standard deviation.