DIVISION OF QUANTITATIVE BIOLOGY

Professor
AOKI, Kazuhiro

Assistant Professor:	*KONDO, Yohei*
	GOTO, Yuhei
Technical Staff:	*BINO, Takahiro*
Postdoctoral Fellow:	*ITO, Reina*
	NAKAMURA, Akinobu
Research Staff:	*MIURA, Haruko*
	KOMATSUBARA, Akira
SOKENDAI Graduate Student:	*TANII, Ryosuke*
	MUKAI, Masaya
	YAMAMOTO, Kei
Visiting Graduate Student:	*UDA, Youichi*
	MARYU, Gembu
Visiting Scientist:	*ODA, Shigekazu*
Technical Assistant:	*EBINE, Emi*
	GOTO, Yoko
Secretary:	*ONODA, Kaori*

Living cells act as input-output (I/O) units, in which their environment and/or internal states are recognized on the cell surface and processed within the cell, thus leading to an adaptive response (Figure 1). This cellular information processing is mainly controlled by intracellular signal transduction, which is comprised of a series of chemical reactions such as protein-protein interaction and protein phosphorylation. Dysregulation of cell signaling by gene mutation is widely known to result in pathological diseases, such as malignant tumors.

The intracellular signaling pathway has been extensively studied over the past few decades. As a result, many proteins and regulations have been identified, which has resulted in an increase in the pathway's complexity. Computer-assisted simulation is one of the most promising approaches in 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 can be solved numerically by computers. Several signaling pathway simulation models have been reported to date. However, most of the kinetic parameters utilized for these simulation models have not been measured experimentally, but have been assumed by being simply arbitrarily determined. Consequently, there are substantial differences in the kinetic parameters among these studies, thereby making it difficult to quantitatively evaluate these simulation models.

To address these issues, we are currently focusing on the development of research tools that enable us to (1) visualize, (2) quantify, and (3) manipulate intracellular signaling pathways.

I. Visualization of cell signaling

With the advent of fluorescent proteins, it has become possible to visualize kinetic reactions at the single-cell level. Förster (or fluorescence) resonance energy transfer (FRET) is a non-radiative process in which the excitation energy of a donor fluorophore is transferred to a nearby acceptor fluorophore. Taking advantage of this principle, FRET-based biosensors allowed us to detect PKA, ERK, Akt, JNK, PKC, and S6K's kinase activity in living cells with a high temporal and spatial resolution (Komatsu N, Mol. Biol. Cell, 2011). By using a FRET biosensor, we have been able to reveal the role played by ERK activation dynamics in cell proliferation (Aoki K, Mol Cell, 2013) and collective cell migration (Aoki K, Dev Cell, 2017).

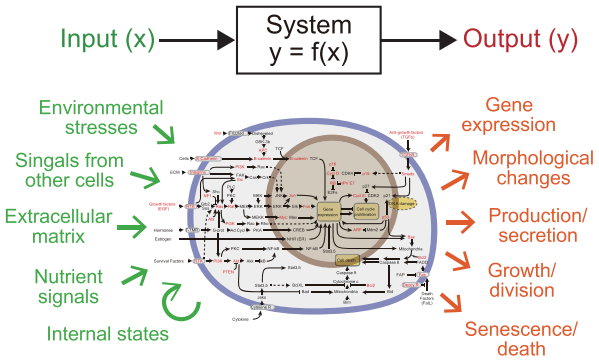

Figure 1. Information processing by intracellular signaling devices and networks.

Although FRET biosensors enable us to monitor cell signaling events, FRET imaging is not suitable for multiplexed imaging, because the biosensors are composed of two different fluorophores in many cases. To circumvent this limitation, we are currently developing single-fluorophore based biosensors. We recently developed a red-fluorescence dopamine (DA) reporter. DA, a neuromodulator, is involved in many neuronal functions. To monitor DA in living cells

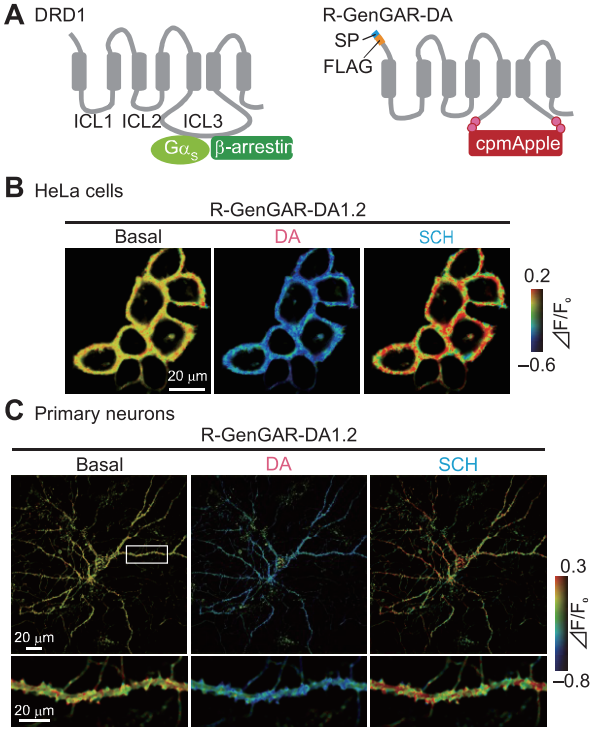

Figure 2. Development of a genetically encoded red fluorescent dopamine (DA) reporter. (A) Schematic representation of a dopamine receptor, DRD1 (left), and Red DA reporter, R-GenGAR-DA. (B and C) HeLa cells (B) or mouse primary hippocampal neurons (C) expressing R-GenGAR-DA were stimulated with DA, followed by a DRD1 antagonist, SCH.

and animals, we designed the red-fluorescent DA reporter with circular-permutated mApple (cpmApple), a red fluorescent protein; cpmApple was inserted into the third intracellular loop of a DA receptor, DRD1 (Figure 2A). The linker sequences between DRD and cpmApple were optimized by random mutagenesis and subsequent live-cell screening. Finally, we picked up the best performance reporter, and named the red genetically encoded GPCR activation reporter for DA, R-GenGAR-DA. The red fluorescence intensity decreased in response to DA, and returned to the basal level upon a DRD1 antagonist treatment (Figure 2B and 2C). In addition, by using previously reported green fluorescence norepinephrine (NE) reporter, we succeeded in simultaneously visualizing DA and NE in living neurons (Nakamoto C, Goto Y, *et al*., bioRxiv, 2020).

II. Quantification of cell signaling and physical parameters

Kinetic parameters such as protein concentration and dissociation constant, Kd, have been measured by conventional *in vitro* biochemical analyses. However, some kinetic parameters could 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 to 2 (Sadaie W, *et al*., MCB, 2014). Therefore, it is critical to measure kinetic parameters in living cells. To this end, we combined CRISPR/Cas9-mediated genome editing techniques with quantitative fluorescence imaging techniques.

First, we developed an efficient knock-in system, and tagged endogenous proteins with fluorescent proteins by Cas9-mediated genome editing (Figure 3A). Next, we quantified their concentrations and Kd value by fluorescence correlation spectroscopy (FCS) and fluorescence cross-correlation spectroscopy (FCCS) (Figure 3B). These analyses revealed temporal changes in Kd values of the binding between ERK2 and RSK2 in response to EGF. Our approach provides a robust and efficient method for quantifying endogenous protein concentrations and dissociation constants in living cells (Komatsubara AT, Goto Y, *et al*., JBC, 2019).

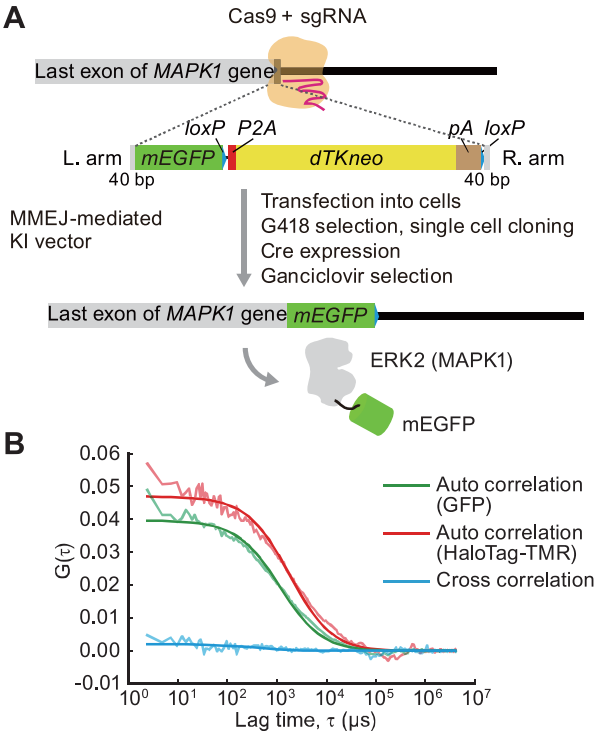

Figure 3. Quantification of protein concentration and dissociation constant of endogenous proteins. (A) Schematic illustration of gene knock-in with an MMEJ-mediated KI vector at MAPK1 locus. (B) Auto- and cross-correlation functions of ERK2-mEGFP and RSK2-HaloTag. *mEGFP* and *HaloTag* genes were knocked-in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

III. Manipulation of cell signaling

Artificial manipulation of biochemical networks are useful in the 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. Of these two, the LID system is beneficial in terms of temporal and spatial manipulations. The photo-responsive proteins derived from fungi, cyanobacteria, plants, and modified fluorescent proteins are used in the LID system.

Among these, we focus on the phytochrome B (PhyB)-PIF LID system. Upon red-light illumination, PhyB binds to PIF, and the PhyB-PIF complex dissociate from each other by far-red light exposure (Figure 4A). The reversible control of association and dissociation by light is a unique feature in the PhyB-PIF system, because other LID systems can only control association or dissociation by light. One drawback is that covalent attachment of a chromophore, phycocyanobilin (PCB), is required for the light-responsive function of PhyB-PIF. To overcome this issue, we have developed a method for biosynthesis of PCB in mammalian cells by introduction of the gene products of *HO1*, *PcyA*, *Fd*, and *Fnr* into mitochondria (Uda Y, *et al*., PNAS, 2017) (Figure 4A). Recently, we further improved the system for PCB synthesis, which allowed establishing stable cell lines synthesizing PCB and light-induced control of protein localization at the cell population level (Figure 4B and 4C).

To take full advantage of this, we applied the genetically encoded PCB synthesis system to *S. pombe* and *C. elegans*, both of which are incapable of having purified PCB delivered to them. As we expected, the expression of *HO1*, *PcyA*, *Fd*, and *Fnr* genes induced PCB biosynthesis in *S. pombe*

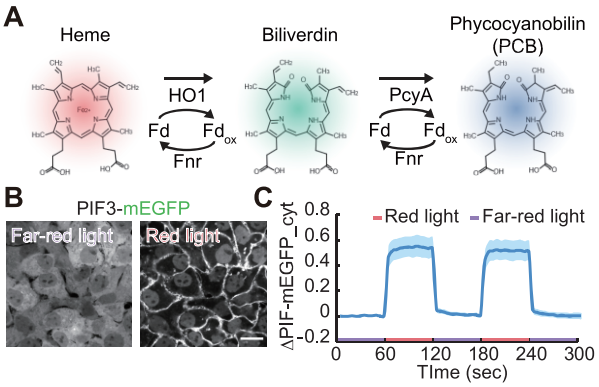

Figure 4. PhyB-PIF light-inducible dimerization (LID) system. (A) The metabolic pathway of phytochrome chromophores, PCB. (B and C) Light-induced translocation of PIF3-mEGFP to the plasma membrane, where PhyB is localized (B). Membrane translocation of PIF3-mEGFP is quantified (C).

and *C. elegans*. Using this system, we developed optogenetic control of the cell cycle in two ways: the Opto-G2/M checkpoint triggered G2/M cell cycle arrest in response to red light, and Opto-SAC induced a spindle assembly checkpoint (SAC) in response to red light and then quickly released the SAC by far-red light (Figure 5). The genetically encoded system of PCB synthesis would 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.

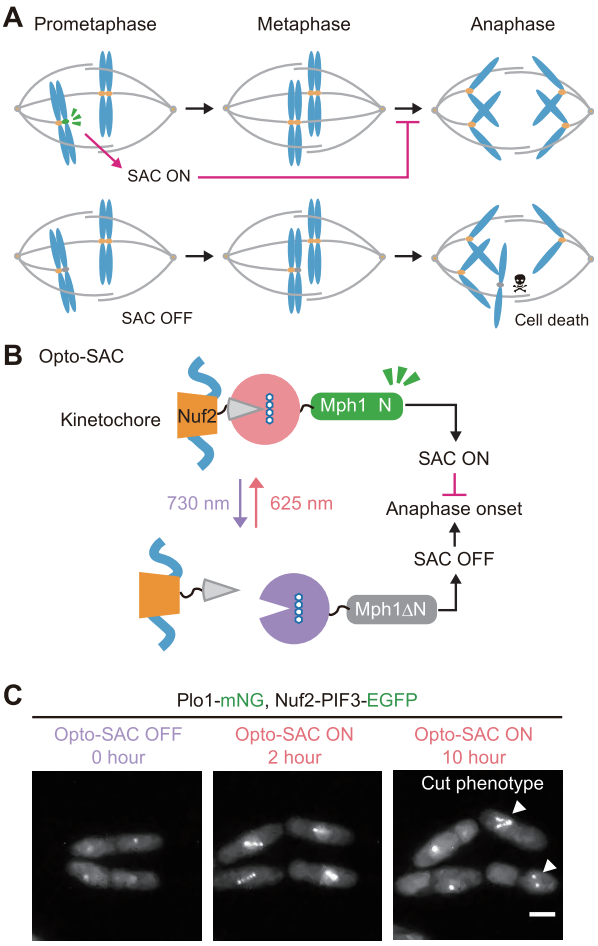

Figure 5. Optogenetic control of spindle assembly checkpoint (Opto-SAC). (A) Schematic of the spindle assembly checkpoint (SAC), which ensures the faithful chromosome segregation and anaphase onset by monitoring kinetochore-microtubule attachment. (B) Schematic of the design of Opto-SAC. Mph1ΔN, which lacks the kinetochore binding domain, is fused with PhyB (PhyB-mph1ΔN). Endogenous Nuf2 is fused with PIF3 (nuf2-PIF3) as a kinetochore localizer. Upon red light illumination, PhyB-Mph1ΔN is recruited to kinetochores through the binding to Nuf2-PIF3, and SAC turns ON. Far-red light exposure induces dissociation of PhyB-Mph1ΔN from kinetochores, leading to the inhibition of activated SAC. (C) Representative cells undergoing a failure of mitosis under long-term metaphase arrest by Opto-SAC.

Publication List:

〔Original papers〕

- Komatsubara, A.T., Goto, Y., Kondo, Y., Matsuda, M., and Aoki, K. (2019). Single-cell quantification of the concentrations and dissociation constants of endogenous proteins. J. Biol. Chem. *294*, 6062-6072. doi: 10.1074/jbc.RA119.007685
- Yagi, H., Yagi-Utsumi, M., Honda, R., Ohta, Y., Saito, T., Nishio, M., Ninagawa, S., Suzuki, K., Anzai, T., Kamiya, Y., Aoki, K., Nakanishi, M., Satoh, T., and Kato, K. (2020). Improved secretion of glycoproteins using an N-glycan-restricted passport sequence tag recognized by cargo receptor. Nat. Commun. *11*, 1368. doi: 10.1038/s41467-020-15192-1

〔Original paper (E-publication ahead of print)〕

- Nakamura, A., Oki, C., Kato, K., Fujinuma, S., Maryu, G., Kuwata, K., Yoshii, T., Matsuda, M., Aoki, K., and Tsukiji, S. Engineering orthogonal, plasma membrane-specific SLIPT systems for multiplexed chemical control of signaling pathways in living single cells. ACS Chem. Biol. 2020 Mar 20. doi: 10.1021/acschembio.0c00024