Bioengineering

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The Alpha Beta Rearrangement of the Asp-Gly Sequence

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The protein proopiomelanocortin (POMC) is a precursor several nerve peptide hormones, including MSHs, ACTH, CLIP, LPH, β-endorphin, and the Joining peptide (JP). The biological activity of POMC derived peptides have been well studied except for JP. To investigate the biological function of JP, we chemically synthesized JP by an Fmoc solid phase method. However, the yield of the synthesized JP was poor and a highly efficient α/β-rearrangement in the Asp-Gly sequence was observed. Therefore, we evaluated the rearrangement of the Asp-Gly sequence under several conditions to estimate the biological activity and the stability of the correct configuration of JP.

For this purpose, JP was chemically synthesized by ordinary Fmoc and Boc solid phase methods. After deprotection, JP was separated and identified by reverse-phase HPLC and MALDI-TOF/MS, respectively. The rearrangement of the Asp-Gly moiety was observed in the case of the Fmoc method but was not significant in the case of the Boc method.

To estimate the stability of the Asp-Gly moiety, JP was treated with several buffers in the pH range of 1-8. The α/β-rearrangement was gradually increased in a pH-dependent manner and was significantly observed under strongly acidic conditions. In addition, salt effects for the rearrangements were also estimated. The results will be discussed in this paper.

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Photo-Regulation of Small G Protein Normal and Oncogenic K-Ras using Photochromic Molecules

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Ras is one of the small G-proteins known as a molecular switch mediating cellular signalling. Switching ON state of Ras is induced by exchange of bound GDP for GTP and OFF state is by hydrolysis of GTP to GDP. Interestingly, the core nucleotide-binding motif of Ras is considerably conserved with the ATP driven motor proteins, myosin and kinesin. Therefore, it is believed that these nucleotide requiring proteins share common molecular mechanism utilizing nucleotide hydrolysis cycle. Previously, we have incorporated photochromic molecules, 4-phenylazophenyl maleimide (PAM), into the functional site of kinesin as a photo-switching nano device and succeeded to regulate kinesin ATPase activities reversibly upon visible light (VIS) and ultra-violet (UV) light irradiation. Therefore, it is expected that Ras can be also regulated using photochromic molecules.

In this study, we performed basic study to control the function of Ras using photochromic molecules upon VIS and UV light irradiations. We prepared normal and oncogenic Ras mutants which have a single cysteine at functional sites and modified with photochromic molecules of azobenzene and spiropyran derivatives stoichiometrically. The GTPase activities of PAM-Ras were reversibly altered upon VIS and UV light irradiations. In order to monitor the effect on GTPase kinetic pathway by the photoisomerization of PAM, we synthesized fluorescent GTP analogue, NBD-GTP. The Kinetic studies suggested that the initial binding step of NBD-GTP to Ras and the dissociation step of NBD-GDP from Ras were regulated by the photoisomerization of PAM.

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Harnessing the Dynamical Movement of OmpG Loops for Protein Sensing

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Oligomeric protein nanopores with rigid structures have been engineered for the purpose of sensing a wide range of analytes including small molecules and biological species such as proteins and DNA. We chose a monomeric β-barrel porin, OmpG, as the platform from which to derive the nanopore sensor. OmpG is decorated with several flexible loops that move dynamically to create a distinct gating pattern when ionic current passes through the pore. Biotin was chemically tethered to the most flexible one of these loops. The gating characteristic of the loop's movement in and out of the porin was substantially altered by analyte protein binding. The gating characteristics of the pore with bound targets were remarkably sensitive to molecular identity - even providing the ability to distinguish between homologues within an antibody mixture. A total of five gating parameters were analyzed for each analyte to create a unique fingerprint for each biotin binding protein. Our exploitation of gating noise as a molecular identifier may open new possibilities for more sophisticated sensor design while OmpG's monomeric structure greatly simplifies nanopore production.

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Multicolor Monomeric Near-Infrared Fluorescent Proteins

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The genetically encoded near-infrared fluorescent probes are preferable for non-invasive in vivo imaging. In the near-infrared spectral region (650-900 nm) mammalian tissues are relatively transparent to light because the combined absorption by hemoglobin and water is minimal.

Previously, we have developed five spectrally distinct fluorescent proteins, iRFP670, iRFP682, iRFP702, iRFP713 (aka iRFP) and iRFP720, from bacterial phytochromes. As a chromophore, iRFPs use a heme derivative, called biliverdin, abundant in mammalian cells. All iRFPs incorporate endogenous biliverdin efficiently and autocatalytically, do not require its exogenous supply and, therefore, can be used as easily as GFP-like proteins. iRFPs are dimers and can mainly serve for labeling of organelles and whole cells. iRFPs have enabled multicolor imaging of deep tissues in living animals.

Here we report a palette of monomeric iRFPs suitable for protein tagging, which also do not require external biliverdin. To engineer these proteins we first used rational design to monomerize the proteins and engineer spectral shifts. Then we applied directed molecular evolution with the high-throughput screening for selection of mutants, which incorporate biliverdin efficiently and specifically. As the result, we have engineered three spectrally different monomeric iRFPs, named miRFP670, miRFP703 and miRFP709. miRFPs are characterized by high effective brightness in mammalian cells, high pH stability and high photostability. We demonstrated that miRFPs perform well as fusion tags for cellular proteins.

The set of miRFPs should enable imaging of several tagged proteins in living mammals, and thus will be useful in cell and developmental biology and biomedicine. The developed molecular evolution approaches could be used for optimization of genetically encoded tools derived from other photoreceptors including flavoproteins and opsins.

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Design and Characterization of Force-Sensitive DNA Origami Components

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Scaffolded DNA origami is powerful design and fabrication tool for the creation of nanoscale objects via bottom up self-assembly. These objects have ~nm level geometric complexity and spatial accuracy, which is comparable to biological machinery. DNA origami has been used to create different a wide range of objects such as drug delivery containers or platforms to guide molecular robots. Current applications of DNA origami exploit the large stiffness of bundles of dsDNA to create structures that maintain a well-defined and static geometry. However, DNA origami nanostructures with mechanically functional components, such as springs or actuators have remained largely unexplored. We aim to make DNA origami devices that are responsive to force magnitudes typically seen in biomolecular system (~picoNewtons). We have currently developed a binary approach to make force-sensitive DNA origami components and demonstrated this approach through the design of a binary force sensor. This force sensor incorporates structures similar to DNA hairpins into DNA origami designs. The hairpin-like structures undergo a conformational change at a specific force threshold. We have characterized the conformational change dynamics of this force sensor using different experimental methods including single-molecule total internal reflection fluorescence microscopy, transmission electron microscopy and magnetic tweezers. We have shown that such dynamics can be tuned according to the design to meet the requirement of a wide range of applications. An analog force sensor is also in development using similar approaches. Ultimately we aim to use these devices to measure forces of molecular interactions in cellular systems, for example cellular traction forces applied during cell migration.

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Remodeling Protein Interfaces to Regulate Recognition

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Protein affinity reagents play an important role across a wide-range of life science applications. Efforts to enhance affinity reagents through protein

engineering typically focus on improved affinity or stability. Here, we examine methods to engineer coupled equilibria that are linked to the protein binding event, which result in protein variants that can be regulated reversibly, based on environmental conditions. Using single domain (VHH) antibodies as model affinity reagents, along with combinatorial libraries, which provide a route to screen new and wild-type residues across a significant amount of interface surface area, we explore the creation of either pH- or metal ion-dependent protein recognition. The resulting protein variants have been analyzed to evaluate the structural and thermodynamic consequences of the remodeled interfaces on protein regulation and stability. The results suggest the ability to introduce new function, such as a reversible linked binding event, is likely to scale with the complexity/size of the protein-protein (ligand) interface. Furthermore, the combinatorial approach to introduce new function should be generally transferable to other protein affinity reagent scaffolds.

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A Continuous-Flow C. elegans Sorting System with Integrated Optical Fiber Detection and Laminar Flow Switching

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Sorting of C.elegans genotypes is a routine task in most C.elegans research labs. Conventionally, the worms are sorted manually which is both labor intensive and slow. In recent years microfluidics has become a useful and important tool for biologists to study C.elegans, including sorting. Although they have shown successful sorting results, the immobilization of worms which was adopted in most of these works causes aversive stimulation to the worms and their behavior will potentially be altered when carrying out post-sorting characterizations.

In this work, both worm detection and switching are achieved without any intervention of the continuous worm flow. The genotypes of the worms are detected by integrated optical fibers based on their fluorescence, without the need for immobilization. Switching is based on the steering of laminar flow boundaries. A novel design that integrates two control inlets dynamically switches the fluidic flow to desired outlets by changing the relative pressure in the control inlets, which cause the two laminar flow boundaries to steer.

Compared to previously reported microfluidic C. elegans sorting devices, sorting in this system is conducted in a continuous flow environment without any immobilization technique or need for multilayer mechanical valves to open and close the outlets. The continuous flow sorter not only increases the throughput but also avoids any kind of invasive or possibly damaging mechanical or chemical stimulus. We have characterized both the detection and the switching accuracy of the sorting device at different flow rates, and efficiencies approaching 100% can be achieved with a high throughput of about 1 worm/s. To confirm that there was no significant damage to C. elegans following sorting, we recovered the sorted worms, finding no differences in behavior and propagation compared to control.

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Discovering Emergent Behavior of Host-Microbiome Interactions with Biomimetic Robotics

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Although underlying dynamics governing host-microbiome interactions are poorly understood, recent studies have shown that commensal bacteria play an important role in regulating the health and behavior of their host. In order to better elucidate this interaction, we designed a biomimetic robotic host platform comprised of an onboard synthetically engineered microbiome. By computationally simulating engineered gene networks in these commensal communities, we discovered complex emergent behaviors in the host, such as stalk-and-strike hunting patterns, dependent exclusively on biochemical network dynamics. This simulation models behavior at multiple scales from the molecular kinetics of genetic transcription to the physical actuation of robotic components. Taken as a whole, this study provides both a computational tool for understanding inter-kingdom communication while presenting a design for a biomimetic system capable of translating genetic based cellular behavior into macro-scale robotic locomotion.

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Regulation of Cell Function via Extracellular Biophysical Environment: A Theoretical- Experimental Approach

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Regulatory promise of electric field (EF) as a non-pharmacological, non-invasive tool to control cellular functions is of great therapeutic interest. However, biophysical mechanisms for the cell-EF interactions are not understood. We developed a theoretical-experimental approach to investigate EF effects on cells in electrode-free physiologically-relevant configuration, i.e. with cells attached to a substrate. Cell is modeled as a membrane-enclosed hemisphere with realistic parameters. Our numerical results demonstrated that EF frequency is the major parameter that controls the mechanism of EF interactions with cells in realistic environment. Non-oscillating or low-frequency EF leads to charge accumulation on the cell surface membrane and results in field screening from the cytoplasm, suggesting that in this regime, cell responses are regulated by EF interactions with the surface membrane receptors. In contrast, high-frequency EF penetrates the cell membrane and reaches cell cytoplasm, where it may directly activate intracellular responses. Theoretical simulation predicts the non-uniform distribution of the induced field within the cell membrane, which depends on the applied EF frequency. Importantly, substrate properties significantly affect both magnitude and distribution of the induced field on the cell membrane, underscoring the need for a comprehensive, physiologically-relevant modeling approach for EF-cell interactions. These theoretical predictions were confirmed in our experimental studies of the effects of applied EF on responses of vascular cells. Results show that non-oscillating EF increases vascular endothelial growth factor (VEGF) expression while field polarity controls cell adhesion rate. High-frequency, but not low-frequency, EF provides differential regulation of cytoplasmic focal adhesion kinase and VEGF expression depending on the substrate, with increased expression in cells cultured on RGD-rich synthetic hydrogels, and decreased expression for basement membrane (matrigel) culture. These results advance our understanding of complex mechanisms underlying cell-EF interactions and may contribute to future EF-based therapies.

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Mechanobiology of mRNA Localization in Breast Cancer Cells

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Introduction: Metastasis occurs when cancer cells form secondary tumors in distant areas of the body. Localization of RNAs in lamellipodial regions has been proposed to play an important role during metastatic progression. In one pathway, the tumor suppressor protein, adenomatous polyposis coli (APC) targets RNAs to cell protrusions. APC -associated mRNAs have been implicated in cellular migration and metastatic progression. Therefore, localization or not of these mRNAs has functional significance in cellular migration and metastasis. Additionally, It has been shown that cancer cells modulate their gene expression in response to the mechanical properties of the substrate. Furthermore, mRNA localization at adhesion sites is influenced by mechanical tension, which is adjusted by cells as a function of the mechanical properties of the cell environment. Therefore, mechanical properties of tissues may play a role during metastasis by modulating localization of mRNAs. As a result, this study investigates APC- associated mRNA localization as a function of substrate stiffness.

Methods: We used the MCF10A cell series, a breast cancer progression model composed of cell lines representing pre-malignant to invasive transformation to investigate mRNA localization. By using in situ hybridization to fluorescently label mRNAs, and micropatterned polyacrylamide gels of varying stiffness, we observed APC associated mRNA localization. Glu-tubulin and vinculin were immunostained to study their relationship to mRNA localization.

Results: On stiffer substrates (280kPa), we observed increased mRNA localization compared to softer substrates (0.87kPa). Staining of cytoskeletal elements such as Glu-tubulin and vinculin showed a correlation between the abundance and location of the proteins and APC-associated mRNAs. These