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Single-Molecule Imaging of AMPA and Nicotinic Acetylcholine Receptors in Mammalian Cells

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Here we present our recent advances in single molecule imaging of two membrane receptor proteins: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs) and nicotinic acetylcholine receptors (nAChRs). AMPARs have roles in synaptic plasticity and post-stroke neuron survival. nAChRs play a key role in nicotine addiction. Both receptors are composed of multiple subunit types, the stoichiometry which may change in response to changing conditions. We succeeded in imaging both receptors in mammalian cells using total internal reflection fluorescence microscopy (TIRFM). To image AMPARs, we transfect COS-7 cells with pHlourin-GluR2 (pHluorin is a pH-sensitive GFP mutant; pHlourin-GluR2 receptors are homotetrameric AMPARs with pHlourin in the extracellular domain). At the end of the secretory pathway, AMPARs move to the cell surface in vesicles with a low pH lumen, which lowers pHluorin fluorescence. When vesicles fuse with the cell membrane, the pH increases, and pHluorin's fluorescence brightens. To reduce fluorophore density so as to easily distinguish individual fluorescent receptors, we allow the visible fluorophores to photobleach under TIRFM laser excitation, followed by resting the cells for 2 minutes. During this time, new AMPARs are transported to the cell membrane via small vesicle transport. The cells are again imaged using TIRFM, identifying individual fluorescent receptors. This technique also has the advantage of reducing autofluorescent background from the cell. Similarly, we image nAChRs using HEK-293 cells transfected with chimaeric α7-5HT3 subunits, which form homopentameric nAChRs. We label the channels on the cell surface using tetramethyl-rhodamine-bungarotoxin, which binds tightly to nAChRs. We again use our photobleach-rest-image technique, but in this case new fluorescent nAChRs appear by diffusing from the top cell surface to the bottom, TIRFM-illuminated surface. We are now in the process of looking for multi-step photobleaching events that should correspond to the number of receptor subunits.

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Single-molecule Imaging of ATP-driven Stepping Rotation of F_oF_1-ATP Synthase Reconstituted into Supported Membrane

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F_oF_1-ATP synthase (F_oF_1) is a complex of two rotary motors and ATP hydrolysis/synthesis reaction catalyzed by water-soluble F_1 motor is tightly coupled with proton transport by membrane-embedded F_o motor. In this study, a novel method for single-molecule rotation assay of F_oF_1 was developed. F_oF_1 from *Escherichia coli* was reconstituted into the supported membrane (>10 μm in diameter) formed on the NiNTA-modified coverglass and immobilized via histidine-tags in c-ring of F_o. Rotation was visualized by streptavidin-coated 200nm latex beads attached to the biotinylated β subunits of F_1 and recorded at time resolution up to 0.5 ms.

Using this system, we observed rotation of F_oF_1 that has mutation in putative proton channel of the a-subunit of F_o (Angevine, et. al., PNAS 2003, JBC 2007). The mutant F_oF_1 showed much lower ATP hydrolysis, proton transport, and ATP synthesis activities as compared with those of wild type in ensemble-molecule assay. In single-molecule experiment, rotation rate of mutant F_oF_1 at high ATP concentration was much lower than that of wild type. Furthermore, small steps in rotation were frequently observed. Analysis of the pair-wise distance indicated minimal step-size of ~36°, consistent with number of the c-subunit (c_{10}) in a rotor ring of F_o motor (Jiang et al., PNAS 2001). These results indicate that proton translocation through the a-subunit of F_o sector can be rate-limiting factor of ATP-driven rotation of F_oF_1.

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How is the Temperature Sensitive (TS) Reaction of F_1-ATPase Coupled with its Rotation?

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F_1-ATPase (F_1) is a rotary molecular motor in which γ subunit rotates against $\alpha_3\beta_3$ cylinder. So far, 80° and 40° substeps in rotation of F_1 have been observed, and kinetic analysis has shown that the 80° substep is initiated by concomitant ATP binding and ADP release, and the 40° substep occurs after ATP hydrolysis and inorganic phosphate release. Temperature sensitive (TS) reaction of F_1 has found recently at the ATP binding angle at the temperature below 10 °C, and it has been suggested to correspond to ADP release (Watanabe et al., EMBO report 2008). In this study, TS reaction of βE190D mutant, that has much slower ATP hydrolysis rate than wild type, was found below 20 °C. Q10 factor of TS reaction of βE190D mutant was 16, that is comparable to that of wild type. Previous single-molecule rotation observations of the hybrid F_1 that has only one mutant β subunit, $\alpha_3\beta(WT)_2\beta(E190D)\gamma$ have shown that the β subunit binds ATP at 0°, cleaves ATP at 200°, and release ADP and inorganic phosphate after cleavage of ATP (Ariga et al., NSMB 2007). In contrast to our expectation, observation of hybrid F_1 $\alpha_3\beta(WT)_2\beta(E190D)\gamma$ rotation at 18 °C has revealed that TS reaction occurs at ATP binding angle(0°) to mutated β subunit. Furthermore, simultaneous observation at the video rate (30 frames per seconds) showed that Cy3-ATP bindings and steps in rotation occur concomitantly. We will discuss about the reaction schemes that can explain these results.

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Towards A New Generation Of Single Molecule High Resolution Sensors

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We present here the methodology that we have developed in the transdisciplinary Joliot Curie laboratory in partnership with the laboratory of physics of Ecole Normale Supérieure Lyon (France) during the past four years for the preparation of sensor chips allowing the detection of single biomolecule interactions (DNA-DNA, DNA-proteins). Three confined measurement methods have been implemented, namely atomic force microscopy in liquid media which is used to capture the structural and mechanical properties of DNA and DNA-protein assemblies, interfacial impedance spectroscopy which probes the surface resistivity and capacitance of adsorbed DNA or lipidic layers, and high resolution surface plasmon microscopy which allows the detection of single biological molecules or assemblies of nanometer size such as DNA plasmids, DNA protein complexes (nucleosomes). Combining these three techniques we have afforded the possibility of direct in-situ synthesis and characterization of DNA-protein complexes such as nucleosomes, and the dynamical study of their stability in time, depending both on DNA sequence and its mechanical properties and on the ionic strength, buffer composition and pH of the liquid medium in contact with these complexes. The possibility of recording the dynamical response in liquid medium of a single molecular assembly under an external constraint modification, without need of a fluorescent marking is very challenging in the domain of biosensors since it should allow a definite improvement of the sensitivity and selectivity of DNA and protein sensor chips. We will discuss this assertion, based on experimental evidences and theoretical estimations of the sensitivity limits of the three experimental methods undertaken in this project.

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Cooperative Three-step Motions In Catalytic Subunits of F_1-ATPase Drive 80° And 40° Substep Rotations

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Detecting conformational changes in enzymes is crucial for understanding their functions. Concerning F_1-ATPase, the smallest rotary molecular motor ever known, domain motions in three catalytic β-subunits coupled with transitions between chemical states during ATP hydrolysis are assumed to be responsible for rotation of the central shaft γ-subunit. Nevertheless, the extent and timing of motions in β in rotating F_1-ATPase still remain largely unknown. Here we directly observe motions in β and rotation of γ simultaneously in the same single molecules; changes in lateral orientation of a single fluorophore fixed to the C-terminal helix of β and substep rotations of beads attached to γ were detected. Furthermore, asymmetric stepping patterns of γ in hybrid F_1-ATPase containing one or two mutant β-subunits enabled identification of chemical steps coupled to these motions. The 80° substep of γ is caused by cooperative bending motions of two β-subunits; a ~40° counterclockwise motion of the first β upon ATP-binding and a clockwise ~20° motion of the second β upon ADP-release (viewed from F_o side). Meanwhile, the 40°substep of γ is driven by another ~20° clockwise motion of the third β after ATP-cleavage. Thus three-step cooperative bending of β-subunits causes two substep rotations of γ. Moreover, these results indicate that the initial crystal structure mimics the conformation in the catalytic dwells, and that the conformation in the ATP-waiting dwells contains a novel set of β-subunits; Open, Closed, and partially closed β-subunits. Thus the present study bridges the gap between chemical steps and mechanical work in motor proteins. The present approach to illustrate the enzymatic functions through detection of motions will give insights into mechanisms of diverse enzymes.