 

*Award Accounts*

**The Chemical Society of Japan Award for 2005**

# Methods of Analysis for Imaging and Detecting Ions and Molecules

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For imaging molecular processes in living cells, we developed novel intracellular fluorescent and bioluminescent indicators for second messengers, protein phosphorylation, protein/protein interactions, and protein localizations that work in single living cells. Key molecules and steps of cellular signaling pathways were visualized in target live cells under fluorescent microscopy using developed fluorescent indicators. A second new approach to molecular imaging is also described. Chemically modified STM tips (molecular tips) were developed for molecular imaging at the interfaces. The molecule-specific imaging is based on an increase in a tunneling current due to the overlap of electronic wave functions induced by the chemical interactions between tip and sample molecules. Chemically selective imaging using STM molecular tips can be described as intermolecular tunneling microscopy.

The development of methods for imaging and detecting ions and molecules has been the focus of the work in my laboratory. In other words, "seeing what was unseen" in single live cells and interfacial molecular recognition chemistry is the focus of our research.

Methods for low invasive analysis of cellular signaling processes in live cells have been explored extensively in our laboratory over the past several years. The approach has been to develop genetically encoded fluorescent and bioluminescent indicators to pinpoint each cellular process in single living cells.

Chemistry-facilitated intermolecular electron tunneling is another approach to molecular imaging that we have made. Chemical imaging was possible because of a distinctive chemical affinity between the STM molecular tip and the analyte molecule that alters the tunneling current. This was achieved by chemically modifying the STM metal tips.

## 1. Illuminating Molecular Processes in Single Living Cells

We have developed genetically encoded intracellular fluorescent and bioluminescent optical probes, which include second messengers, such as guanosine 3′,5′-cyclic monophosphate (cGMP),[1] inositol 1,4,5-trisphosphate ($IP_3$),[2] lipid second messengers,[3,4] and nitric oxide (NO),[5] protein phosphorylations,[6–8] protein–protein interactions,[9–14] protein localizations,[15–19] and nuclear receptor ligands,[20,21] for monitoring intracellular signaling. These probes are not only for fundamental biological studies, but also for assay and screening of possible pharmaceutical or toxic chemicals that inhibit or facilitate cellular signaling pathways.

An amplifier-coupled fluorescent indicator for NO was developed to visualize physiological nanomolar dynamics of NO in living cells (detection limit of 0.1 nM) (Fig. 1).[5] A previously developed cGMP fluorescent indicator, CGY, was combined with a soluble guanylate cyclase, sGC, for the amplified detection of NO in living cells. This amplifier-coupled fluorescent indicator was named "NOA-1." NOA-1 binds with a single NO molecule and generates a large number of cGMP molecules in single living cells. This increased amount of cGMP is in situ detected by a cGMP FRET sensor which is built in NOA-1. NOA-1 not bound to cGMP does not emit the FRET signal. This genetically encoded, highly sensitive indicator showed that approximately 1 nM of NO, which is enough to relax blood vessels, is generated in vascular endothelial cells even in the absence of shear stress. A nanomolar range of basal endothelial NO appears to be fundamental to vascular homeostasis.

Phosphatidylinositol-3,4,5-trisphosphate ($PIP_3$) regulates diverse cellular functions, including cell proliferation and apoptosis, and has roles in the progression of diabetes and cancer. However, little is known about its production. We developed fluorescent indicators for $PIP_3$ based on fluorescent resonance energy transfer (FRET).[3] These novel $PIP_3$ indicators are composed of two distinctly colored mutants of green fluorescent protetin (GFP) and a $PIP_3$-binding domain. The $PIP_3$ level is observed by dual-emission ratio imaging, which allows for stable observation with high accuracy. Furthermore, the indicators are fused to localization sequences to direct them to the plasma membrane or endomembranes, allowing localized analysis of $PIP_3$ concentrations. Using these fluorescent indicators, we analyzed the spatio-temporal regulation of $PIP_3$ production in single living cells (Fig. 2). An indicator for another lipid second messenger, diacylglycerol, was also reported.[4]

In addition, we developed genetically encoded fluorescent indicators for visualizing protein phosphorylations in living cells.[6,7] In this approach, a substrate domain for a kinase pro-

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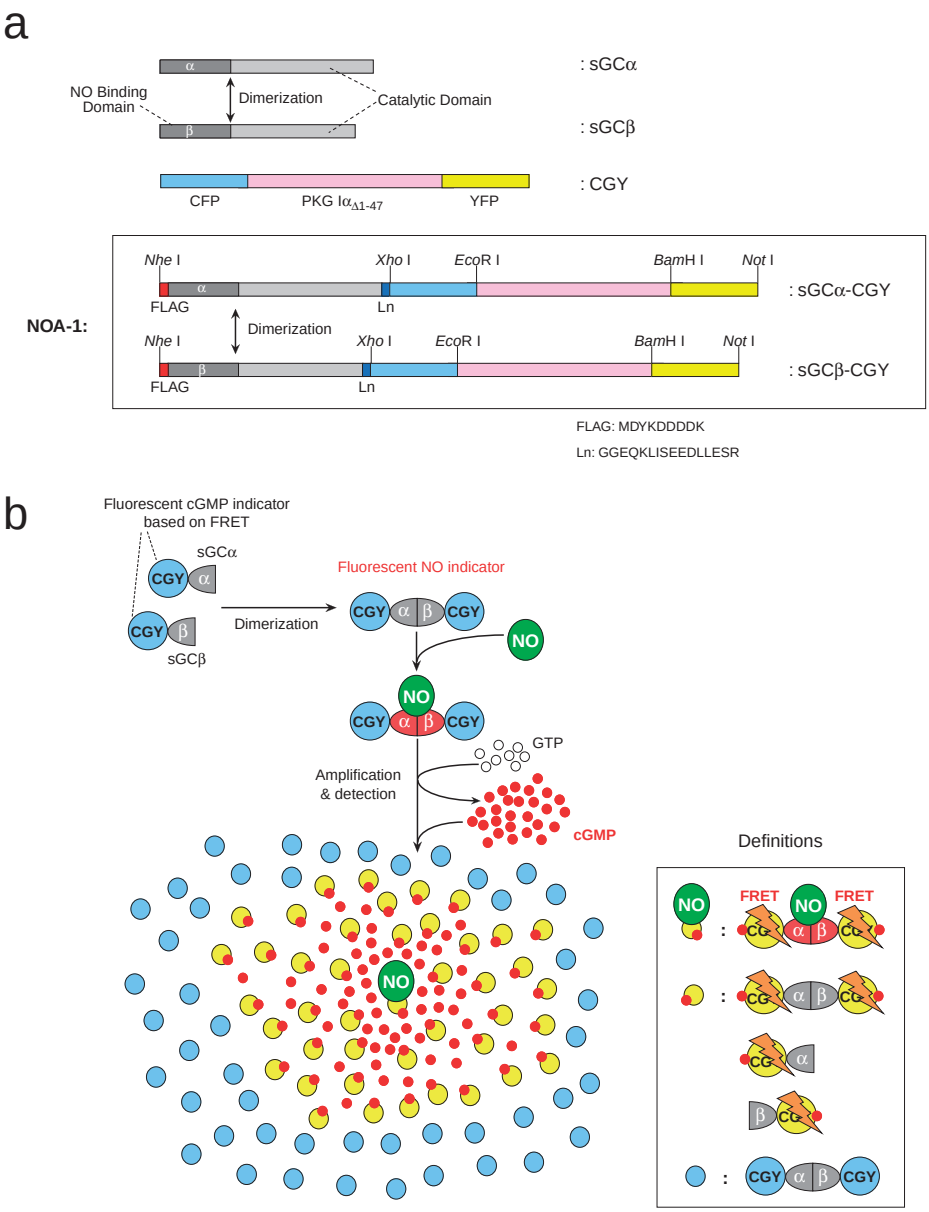


Fig. 1. An amplifier-coupled fluorescent indicator for visualizing NO in single living cells. (a) Schematic representations of domain structures of sGC$\alpha$, sGC$\beta$, CGY, sGC$\alpha$-CGY, and sGC$\beta$-CGY. The amino acid sequence of FLAG tag and linker (Ln) is shown at the bottom. The heterodimer of sGC$\alpha$-CGY and sGC$\beta$-CGY was named "NOA-1." (b) Principle of the present NO indicator, NOA-1. sGC$\alpha$-CGY and sGC$\beta$-CGY spontaneously associate to form a matured heterodimer, that is, NOA-1. NOA-1 binds with NO and generates cGMP at the rate of 3000–6000 molecules min$^{-1}$. Thus, generated cGMP binds to the CGY domain in NOA-1 make NOA-1 emit a FRET signal. Approximately 99.9% of cGMP molecules thus generated diffuse and bind to NO-free NOA-1. As a result, even a single NO molecule can trigger a large number of NOA-1 to emit FRET signals. Even if sGC$\alpha$-CGY and sGC$\beta$-CGY exist as monomers, the monomers also emit FRET signals upon binding with generated cGMP.[5]

tein of interest is fused with a phosphorylation recognition domain via a flexible linker sequence. The tandem fused unit consisting of the substrate domain, linker sequence, and phosphorylation recognition domain is sandwiched with two fluorescent proteins of different colors, cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP), which serve as the donor and acceptor fluorophores for FRET. As a result of the phosphorylation of the substrate domain and subsequent binding of the phosphorylated substrate domain with the adjacent phosphorylation recognition domain, FRET is induced between the two fluorescent units, and phosphorylation-dependent changes in the fluorescence emission ratios of the donor and acceptor fluorophores are observed. Upon activation of the phosphatases, the phosphorylated substrate domain was dephosphorylated, and the FRET signal decreased (Fig. 3). To monitor protein–protein interactions (PPIs), a new method with general applicability was developed based on protein splicing.[9,10] In this process, an intervening protein sequence is excised, and the flanking protein fragments are spliced together. In this splicing system, the flanking pieces are the

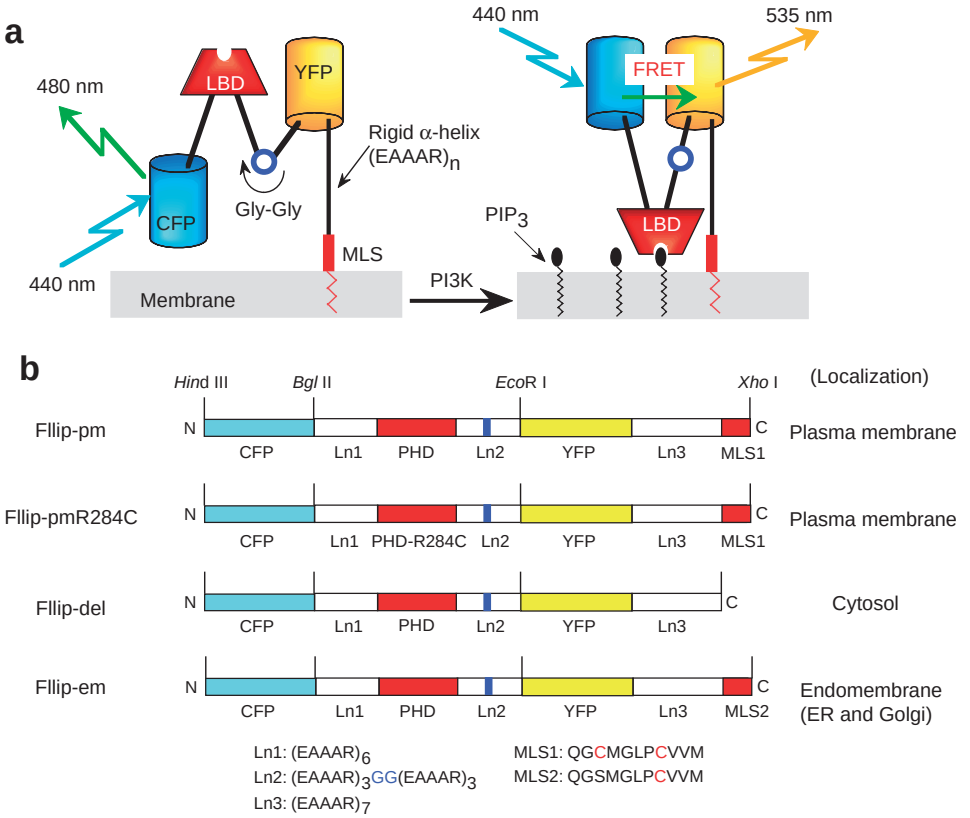


Fig. 2. Fluorescent indicators for $PIP_3$ (fllip) in single living cell. (a) Principle of fllip for visualizing $PIP_3$. CFP and YFP, which have different colors, are mutants of green fluorescence protein from *Aequorea victoria* with mammalian codons and additional mutation. Upon binding of $PIP_3$ with the PHD within fllip, a flip-flop-type conformational change of fllip takes place, which changes the efficiency of FRET from CFP to YFP. (b) Schematic representations of domain structures of the present fllips. Shown at the top of each bar are the restriction sites. PHD is derived from human GRP1 (261–382) and selectively binds with $PIP_3$. PHD-R284C is a mutant PHD, in which the arginine 284 is replaced with a cysteine not to bind with $PIP_3$. Ln1, Ln2, and Ln3; linkers, the amino acid sequences of which are shown in the bottom. MLS1 and MLS2; membrane localization sequences to the plasma membrane and the endomembranes, respectively, the amino acid sequences of which are also shown at the bottom.[3]

N- and C-terminal fragments of split GFPs. The intervening sequence is DnaE, which is a splicing protein derived from *Synechocystis*. Interaction between protein A and B brings the two parts of DnaE, close enough to fold together properly and initiate the splicing and linking of the two GFP halves with a peptide bond. Reconstitution of the split GFPs is then monitored by its fluorescence. Unlike an earlier protein interaction assay, the split-GFP system involves the reconstitution of GFP and does not require that the PPIs occur near the cell nucleus and reporter genes or that an enzyme substrate be present. This makes the method generally more useful and enables the screening of the interactions in the cytosol or at the inner membrane level.

One of the most distinct features of eukaryotic cells, in particular mammalian cells, is different compartmentalization of each protein. A method was developed that allows rapid identification of novel proteins compartmentalized in intracellular organelles, such as mitochondria or endoplasmic reticulum, by screening large-scale cDNA libraries (Fig. 4).[15,16] The principle is based on reconstitution of split GFP by protein splicing with DnaE. Nucleocytoplasmic trafficking of functional proteins plays a key role in regulating gene expressions in response to extracellular signals. We developed a genetically encoded bioluminescent indicator for monitoring the nuclear trafficking of target proteins in vitro and in vivo.[17] The principle is based on reconstitution of split fragments of *Renilla* luciferase (Rluc) by protein splicing with a DnaE intein. A target cytosolic protein fused to the amino-terminal half of Rluc is expressed in mammalian cells. If the protein translocates into the nucleus, the Rluc moiety meets the carboxy-terminal half of Rluc, which is localized in the nucleus with a fused nuclear localization signal, and full-length Rluc is reconstituted by protein splicing. Bioluminescence is thereby emitted with coelenterazine as the substrate (Fig. 5). This approach is an extension of an earlier method to identifying mitochondrial proteins.[15] Cell-based screening with the genetically encoded indicator can provide a quantitative measure of the extent of nuclear-translocation of androgen receptor upon stimulating with various chemicals (Fig. 6).

## 2. Chemistry-Facilitated Intermolecular Electron Tunneling

Molecular tips in scanning tunneling microscopy can directly detect intermolecular electron tunneling between sample and tip molecules and show tunneling occurs through chemical interactions that provide overlap of respective electronic wave

functions, that is, hydrogen-bond,[23–29] metal-coordination-bonds,[30] and charge-transfer interactions.[22] Nucleobase molecular tips were prepared by chemical modification of underlying metal tips with thiol derivatives of adenine, guanine, cytosine, and uracil, and the single nucleobase adsorbate probes intermolecular electron tunneling to or from a sample nucleobase molecule (Fig. 7). We found that electron tunneling between a sample nucleobase and its complementary nucleobase molecular tip was more efficient than that with its noncomplementary counterpart. The complementary nucleobase tip was thereby capable of electrically pinpointing each nucleobase (Fig. 8).[24]

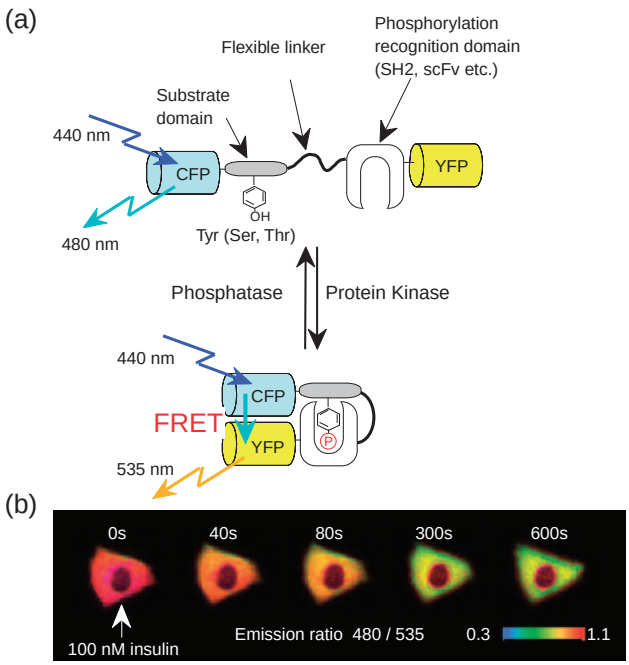


Fig. 3. Fluorescent indicators (phocus) for protein phosphorylation in living cells. (a) Principle of phocus for visualizing protein phosphorylation. CFP and YFP are different colored mutants of green fluorescent protein (GFP) derived from *Aequorea victoria* with mammalian codons and additional mutations. (b) The CFP/YFP emission ratio is pseudocolor images of the CFP/YFP emission ratios before (time 0) and at 40, 80, 300, and 600 s after the addition of 100 nM insulin, obtained from the CHO-IR cells expressing phocus in which a nuclear export signal peptide attached next to YFP.[6]

A fullerene molecular tip was used to detect electron tunneling from a single porphyrin molecule.[22] Electron tunneling was found to occur locally from an electron-donating moiety of the porphyrin to the fullerene through charge-transfer (Fig. 9). In addition, electron tunneling within the single fullerene–porphyrin pair exhibited rectifying behavior in which electrons can be driven only from the porphyrin to the fullerene. We demonstrated that localized electron tunneling enables us to spatially visualize the frontier orbital of the porphyrin involved in electron tunneling. In addition, rectification demonstrates that the fullerene–porphyrin pair constitutes a molecular rectifier. We believe that molecular tips bring insight into intermolecular electron transmission toward realization of molecular electronics as shown here (Fig. 10).

Chemically selective imaging using molecular tips can be described as "intermolecular tunneling microscopy," and is of general significance for novel molecular imaging of chemical identities on membrane and solid surfaces.[22,24]

We have developed methods for visualizing and detecting key signaling molecules and processes in living cells.[31] We have also invented molecular tips for visualizing molecular recognition processes at membrane solid interfaces. These remarkable techniques will become key methods for studying chemistry and biology, i.e., for seeing what was otherwise unseen.

The work summarized here was achieved by the dedicated efforts of staffs and students, Analytical Chemistry Laboratory, the University of Tokyo, during the tenure of Professor Yoshio Umezawa. The credit is given to each co-worker in the authorship of relevant references (vide infra).

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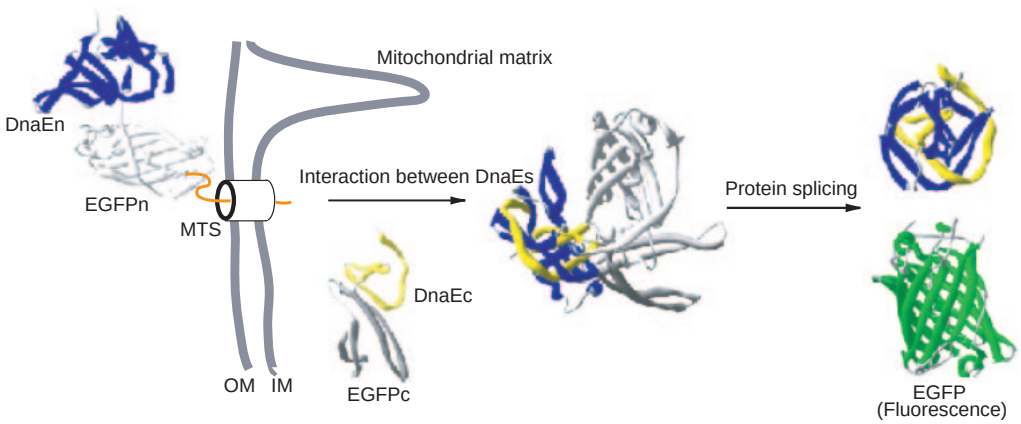


Fig. 4. Scheme showing how EGFP is formed by protein splicing of DnaEs when a test protein is localized in the mitochondrial matrix. 3D structures colored blue and red represent DnaEn and DnaEc, while light and dark gray represent amino- and carboxy-terminal halves of EGFP, respectively. Orange strand means MTS. OM and IM are mitochondrial outer and inner membranes, respectively.[15]

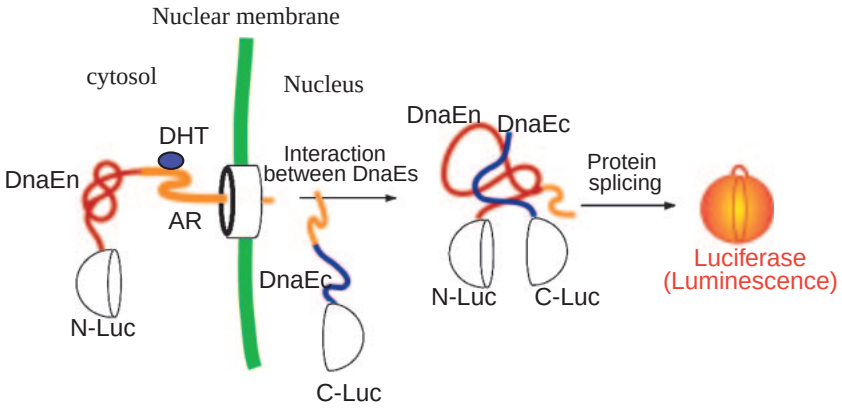


Fig. 5. When AR is bound to 5$\alpha$-dihydrotestosterone (DHT), it translocates into the nucleus, and brings the N- and C-terminal halves of DnaEs close enough to fold correctly, thereby initiating protein splicing to link the concomitant Rluc halves with a peptide bond. The C-terminal half of split Rluc was located beforehand in the nucleus by a fused nuclear localization signal. The cells containing this reconstituted Rluc allow one to monitor nuclear translocation of AR with its luminescence by coelenterazine as the substrate.[17]

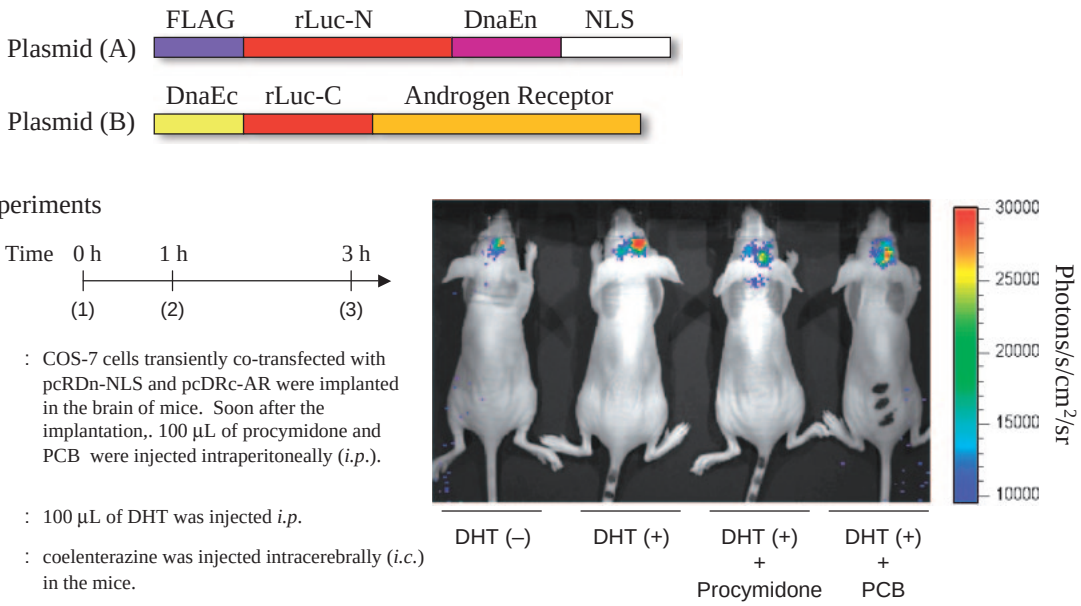


Fig. 6. Effects of inhibitors on AR translocation into the nucleus in the mouse brain. PCB and procymidone were found to have an ability to pass through the blood-brain barrier, to reach the brain and to inhibit the AR signal transduction in the organ.[17]

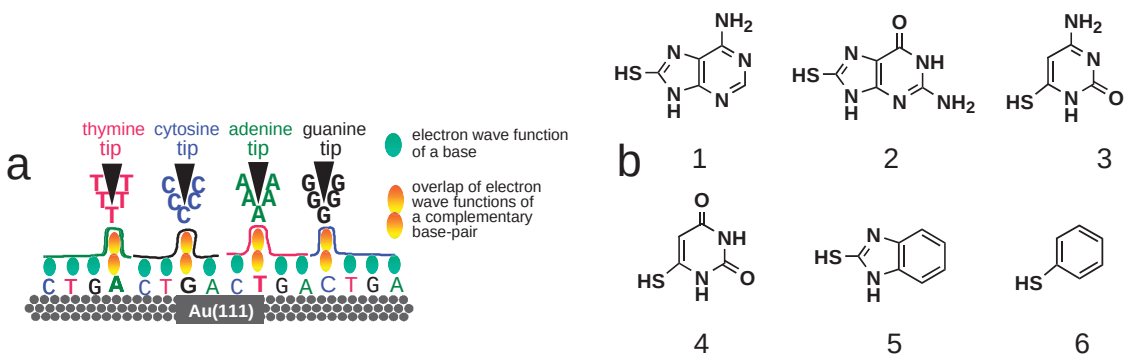


Fig. 7. A nucleobase tip pinpoints its complementary nucleobase based on base-pair facilitated electron tunneling. (a) Formation of the complementary base-pairs between the nucleobase tip and the sample nucleobases leads to greatly facilitate electron tunneling in STM. Nucleobase tips can thus pinpoint the corresponding complementary nucleobases. (b) The chemical structures for thiol derivatives of adenine (**1**), guanine (**2**), cytosine (**3**), and uracil (**4**) are shown, together with benzimidazole-2-thiol (**5**) and benzenethiol (**6**).[24]

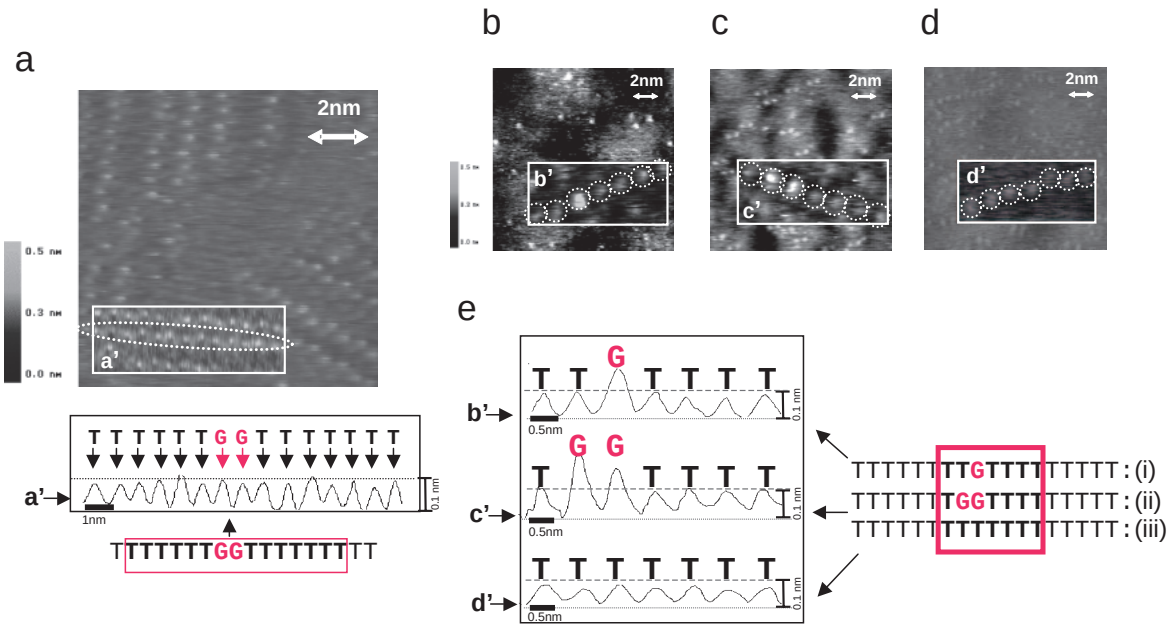


Fig. 8. SNP typing in 18-mer single-stranded peptide nucleic acids. (a) An STM image ($10 \times 10\,nm^2$) with an unmodified tip of single-stranded eighteen-mer PNAs, the sequence of which is TTTTTTTGGTTTTTTTTT. A cross-sectional profile along the strand circled by white dots in the inset a′ ($3.5 \times 10\,nm^2$) is also shown. White arrow drawn in the image pinpointed one end of a PNA strand. (b, c, d) STM images ($15 \times 15\,nm^2$) with cytosine tips of three kinds of PNA (peptide nucleic acid) strands; TTTTTTTTGTTTTTTTTT, TTTTTTTGGTTTTTTTTT, and TTTTTTTTTTTTTTTTTT, are shown in (b), (c), and (d), respectively. The magnified images ($2.0 \times 5.0\,nm^2$) of image (b), (c), and (d) are shown in the inset b′, c′, and d′, respectively. (e) Cross-sectional profiles of a row of base images along the PNA strand circled by white dots in the inset b′, c′, and d′, respectively. The *y*-axis of the cross-sectional profiles represents the extent of electron tunneling along the strands.[24]

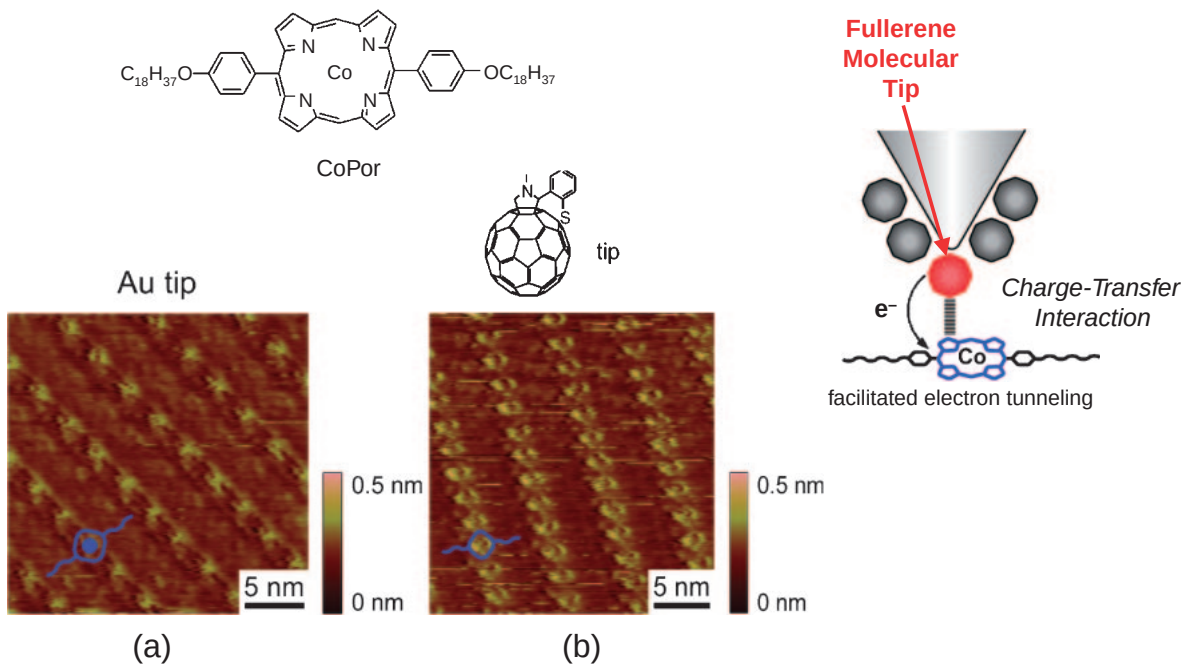


Fig. 9. A fullerene molecular tip can detect localized and rectified electron tunneling within a single fullerene–porphyrin pair. STM images of CoPor monolayers physisorbed onto HOPG. The insets schematically show the molecular arrangement in the images. White rings, blue circles, and bars represent pyrrole moieties, $Co^{II}$ ions, and alkyl chains, respectively. The cross-sectional profiles measured along with the lines in the STM images were presented below. Blue arrows in the images and cross-sectional profiles indicate the position of $Co^{II}$ ions. (a) Observed with an unmodified gold tip. Bias voltage, −1.30 V (sample negative); tunneling current, 0.30 nA. (b) Observed with the fullerene molecular tip. Bias voltage, −1.25 V; tunneling current, 0.30 nA. Localized electron tunneling (ET) spatially visualizes the frontier orbital of the porphyrin involved in ET.[22]

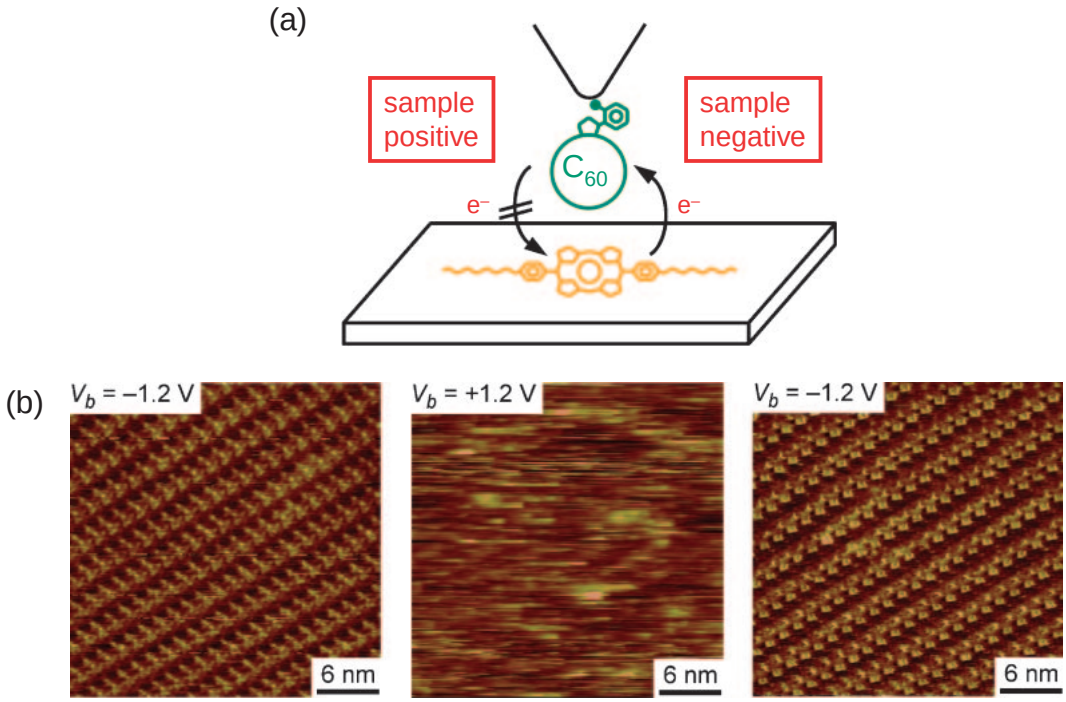


Fig. 10. Electron tunneling within the single fullerene–porphyrin pair constitutes a molecular rectifier. Polarity dependence in STM observation of CoPor. (a) Schematic illustration of the electron flow between a porphyrin (orange) and fullerene derivative tip (green). All of the atomic labels were omitted for simplicity in the chemical structures of the tip and sample molecules. (b) STM images of CoPor monolayers physisorbed onto HOPG observed with MPF tips and different bias voltage (Vb). These images were successively observed with the same tip and the same sample region. Tunneling currents were 0.3 nA (Left and Center) and 0.35 nA (Right). Total *z*-scale ranges were 0.25 nm (Left), 0.5 nm (Center), and 0.35 nm (Right). The insets schematically show the molecular arrangement in the images. White rings, blue circles, and bars represent pyrrole moieties, $Co^{II}$ ions, and alkyl chains, respectively.[22]

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