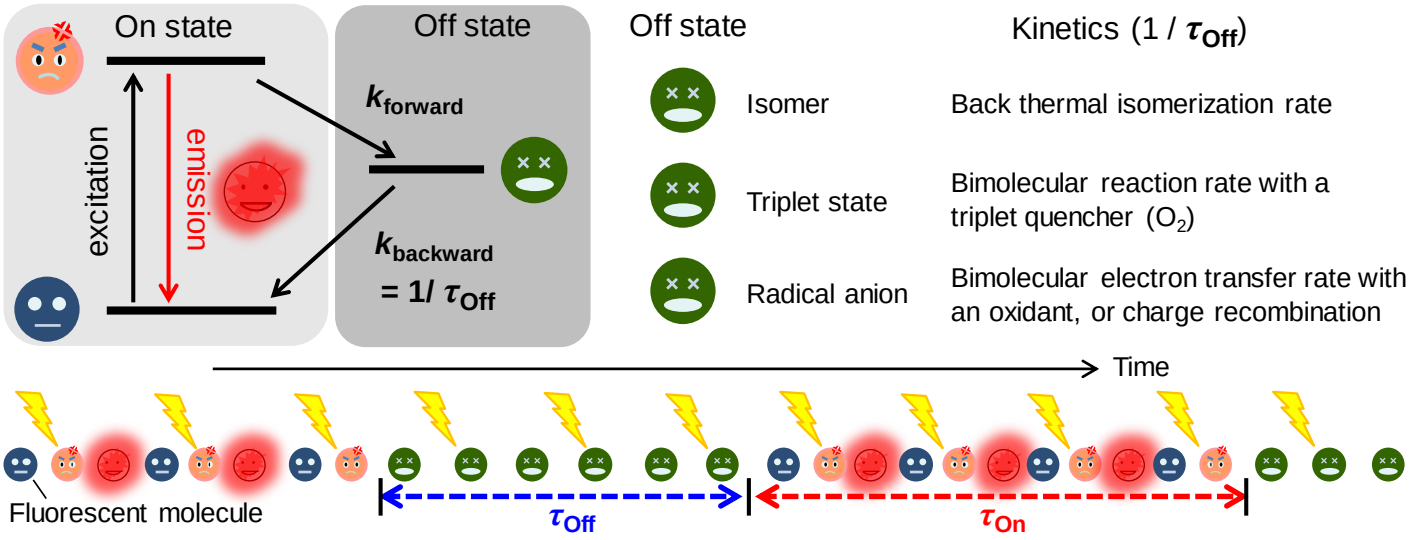
Investigation of various biological dynamic phenomena based on the development of single-molecule level analytical methods.



We are interested in biological phenomena taking place in a relatively short time range (microsecond to second (10^{-6} - 10^{0} sec.)). To access such phenomena, conventional approaches such as transient absorption measurements or NMR usually require substantial amount of sample (> 1 nmol). In order to achieve ultralow detection limit, one strategy would be to focus on a detection method that relies on the properties of molecules that become highlighted when we look at molecules at the single-molecule level. Among such phenomena, we have focused on the fluctuating emissions between bright “on” and dark “off” states of fluorescent molecules, so-called “blinking”. During the repetitive cycles of excitation and emission, fluorescent molecules may occasionally enter non-fluorescent off states, such as a triplet state, a radical ion state, and an isomerized state. Reversible formation of such off states causes a blinking of the fluorescence. By measuring the duration of the on time (τ_{ON}) and off time (τ_{OFF}) of the blinking, we can investigate various biological phenomena with sub-microsecond time resolution at the single-molecule level. The changes in the surrounding local microenvironment that modulate blinking would also be approaced at the single-molecule level by **K**inetic **A**nalysis based on the **C**ontrol of the fluorescence **B**linking (**KACB** method).

Review: Chem. Eur. J., **26**, 7740-7746 (2020).
Accounts Chem. Res., 54, 1001-1010 (2021).

・Fluorescent blinking triggered by *trans-cis* isomerizaition of a fluorophore Cy3

Cyanine dyes such as Cy3 are widely used as a fluorescent probe to investigate various biological phenomena. It is known to undergo *trans-cis* photo-isomerization and successive *cis-trans* back thermal-isomerization by rotation around the C–C bonds of the poly-methine chain. This *cis-trans* isomerization causes the blinking of the fluorescence. We focus on the fact that the size of the Cy3 is just about the same as the width of the triple helix. Since the *cis-trans* isomerization efficiency is

considered to be strongly dependent on the steric effects that impact the rotation of the molecule, we hypothesized that Cy3 may exhibit a triple helix-specific blinking. We incorporated Cy3 into DNA double helix, or triple helix conformation and investigated the differences in blinking behavior by Fluorescence Correlation Spectroscopy (FCS). The duration of the on time (τ_{ON}) and off time (τ_{OFF}) of the blinking, which reflect the inverse of the *trans-cis* photo-isomerization rate and the *cis-trans* back isomerization rate, respectively, were highest in the triple helix conformation. These results suggest that Cy3 can be used to track the presence of the triple helix conformation at the single molecule level.
Chem. Commun. **51**, 4861-4864 (2015).

・Fluorescent blinking triggered by formation of the triplet excited state of a fluorophore R6G

Bimolecular reaction can be slowed down by shielding a fluorophore from the solvent, *i.e.*, decreasing the solvent accessibility of the fluorophore. We attempted to monitor the changes in the microenvironment that perturbed the extent of the solvent exposure of the fluorophore. The bimolecular reaction rate between O_2 and the fluorophore R6G in the excited triplet state (^{3}R6G*) was tracked by monitoring the triplet blinking. The τ_{OFF} value corresponds to the lifetime of ^{3}R6G*. ^{3}R6G* quenching reaction with O_2 proceeded more slowly when it was buried in a DNA duplex than at the hairpin region exposed to the solvent. This enabled the analysis of the molecular-beacon type probe, allowing the detection of the target DNA strand at the sub-nanomolar level.
Chem. Commun **50**, 10478-10481 (2014).

・Fluorescent blinking triggered by the redox reaction of a fluorophore R6G (rKACB)

Despite the large microenvironment changes in the hairpin-duplex conformational transition, the observed difference in the τ_{OFF} value in the triplet blinking was smaller than 2-fold. We came to the conclusion that the small size of the O_2 would be a major obstacle to the sensitive observation of the changes in the extent of the solvent exposure of a fluorophore. We focused on the control of redox blinking to monitor the microenvironment of the fluorescent probe R6G. By adding ascorbic acid 2-phosphate (VcP) as a reductant, the triplet state was converted to the radical anion off-state ($R6G^{\bullet-}$). The τ_{OFF} value corresponds to the lifetime of $R6G^{\bullet-}$. Here, under an oxygen scavenging condition, a bulky oxidant, diethylenetriaminepentaacetic acid iron(III) (FeDTPA), was utilized to regenerate the intact R6G at the ground state. Owing to the much larger size of FeDTPA compared to O_2, the bimolecular reaction rate between the R6G radical anion ($R6G^{\bullet-}$) and FeDTPA changed dramatically along with the changes in microenvironment of R6G. Observation of the redox blinking enabled sensitive detection of subtle conformational changes around the R6G caused by single nucleotide alternations in the DNA sequence.
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By using rKACB, the structural switching dynamics of RNA between hairpin loop and stem structures were studied.
Angew. Chem. Int. Ed., **56**, 15329-15333 (2017).

We tested various fluorescent molecules for rKACB, and rKACB was adapted to the discrimination between B-form and A-form helixes, and investigation of antigen-antibody interactions at the single molecule level.
Chem. Eur. J., **24**, 6755-6761 (2018).

・Triplet–Triplet energy transfer (TTET) to investigate the dynamics of biomolecules

Cyclooctatetraene (COT) was used as both a triplet acceptor and a photo-stabilizing agent to control and observe the blinking at the single-molecule level. By using DNA as a platform, we demonstrated that triplet blinking of the fluorescent molecule ATTO 647N can be controlled by the collision reaction between COT. Dynamics of biomolecules can be investigated by measuring the blinking. COT and ATTO 647N were attached to the molecular beacon type probe which allowed us the detection of model biomarker miR-155 at the single-molecule level.
Angew. Chem. Int. Ed., 60, 12941-12948 (2021).
https://resou.osaka-u.ac.jp/en/research/20210414_1

・Fluorescent blinking triggered by the charge separated state in DNA using a fluorophore ATTO 655

By utilizing the transient absorption measurements, we previously showed that charge transfer dynamics in DNA is strongly affected by the DNA sequence. We showed that DNA sequence information including the data on single nucleotide polymorphisms (SNPs) can be read-out by measuring the charge transfer kinetics. However, the measurement requires a significant amount of sample (>1 nmol) and thus cannot be used for diagnosis. We focus on that, by using a fluorophore as a photosensitizer to generate charges on DNA, the charge-separation, charge-transfer, and charge-recombination dynamics in DNA can be monitored as the fluorescence blinking. In this case, the blinking was caused by the successive reduction and re-oxidation cycles of the fluorescent molecule ATTO 655. The τ_{OFF} value corresponds to the lifetime of the charge-separated state. Based on the fact that the charge-transfer dynamics in DNA is strongly affected by the DNA sequence, we demonstrated that single-nucleotide differences in DNA that modulate the charge-recombination kinetics can be detected by monitoring the blinking of the fluorescence.
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・Increasing the Hole Transfer Rate through DNA by Chemical Modification

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・SNPs detection by measuring the hole transfer rate through DNA

Chem. Eur. J., 11(13), 3835-3843 (2005).
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・Mechanisms of photosensitized DNA damage

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・DNA dynamics studied by formation of pyrene dimer radical cation

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・Effect of hydrogen bonding on the photo-oxidation of DNA

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Doctor thesis

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