Probes: FRET sensor design and optimization

Vivien Marx

Trial, error and the art of optimizing 'molecular rulers' that sense molecules or interactions.

Because of the way light diffracts in a conventional microscope, a single fluorescent protein (FP) two nanometers in diameter will look like a blurry disk around 250 nanometers wide, says biophysicist Jin Zhang from the University of California at San Diego (UCSD). If two proteins carrying fluorophore labels move closer together than this distance, the colors will overlap. That shows colocalization, but an experimenter will still be uncertain about whether the two proteins are touching or interacting. Förster resonance energy transfer (FRET) sensors, however, are tools for studying such nuances because they report quantitatively on behavior.

"The beauty of FRET is that it only happens when two fluorescent proteins are less than ten nanometers apart," says Zhang. "FRET is like a tiny ruler that lets us more accurately measure distances between molecules—if they are close enough that you see FRET, then you can be fairly confident they are interacting." This "molecular ruler" aspect of FRET gives researchers views beyond the interaction of two proteins and, she says, "that's why FRET sensors are such an incredibly powerful tool for cell biology."

Always on

A small number of biologists have long used FRET, the theory of which emerged in the late 1940s, but major advances in FRET-based tool development had to await the "marriage" of green fluorescent protein (GFP) and FRET, says Atsushi Miyawaki, RIKEN researcher and probe developer. Sensors were to be, in the words of Nobel laureate Roger Tsien, "molecular spies." Tsien developed GFP and generated GFP variants that widened the spectral range and popularity of these 'spies' for investigating cellular events up close and with minimal intrusion[1]. In the mid-1990s, as a member of the Tsien lab, Miyawaki engineered sensors that show large changes in FRET efficiency[2].

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FPs are a little like light bulbs, with an outer shell surrounding a delicate inner filament that gives off light, says UCSD's Jin Zhang.

FRET sensors are not lights switching on or off; to some extent they are always 'on', which makes them a little like car dashboard dials, says Stanford University researcher and probe developer Michael Lin. Behavior, such as two proteins interacting, changes the sensor's fluorescence intensity (see **Box 1**, "FRET sensors: donors, acceptors").

Visible metabolism

FRET sensors can help labs study metabolism, such as metabolic flux of the enzyme nicotinamide adenine dinucleotide phosphate (NADH) at the single-cell and subcellular levels. Kinetic information about NADH and NADPH is tough to come by with chromatography or enzymatic cycling assays.

Leveraging the traits of a sensor called SoNar, a group of researchers at several institutions in China including the Institute of Neuroscience and the Chinese Academy of Sciences in Shanghai developed iNap, a sensor for tracking small changes in cellular NADP+ to NADPH *in situ*. The scientists showed that ratiometric fluorescence increases ninefold when NADPH binds to iNAP and the probe is exposed to excitation light. Andreas Wiederkehr of the Ecole Polytechnique Fédérale de Lausanne and Nicolas Demaurex of the University of Geneva point out such data reflect the redox state of cells and tissues, which can play a role in disease[3].

Having genetically encoded sensors for NAD+/NADH such as Frex, Peredox, RexYFP and SoNar, as well as those for NADP+/NAPH such as iNap and Apollo-NADP+, will help labs better understand kinetic changes. And as Wiederkehr and Demaurex note, the sensors enable a "new era" for monitoring transient changes in the redox network.

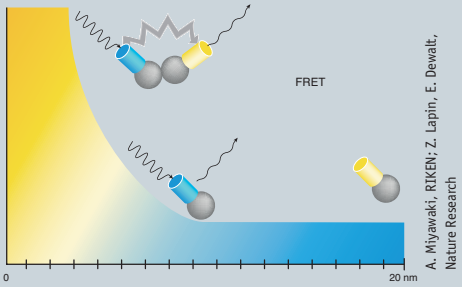
Membrane events

Katharina Gaus of the University of New South Wales and her colleagues, including postdoctoral fellows Yuanqing Ma and Jesse Goyette, developed MCS+, a FRET sensor for tracking changes to membrane charge in live cells[4]. The sensor stays attached to the membrane, which dampens interference signals from molecules in the cytosol and enhances the sensor's signal-to-noise ratio. The team uses the sensor for studying the immunological synapse, where an antigen and a T cell interact.

The team wants to explore how membrane charge regions develop, says Goyette.

BOX 1 FRET SENSORS: DONORS, ACCEPTORS

FRET sensors can be bimolecular or unimolecular. Bimolecular constructs are better for characterizing protein–protein interactions, whereas unimolecular ones are used for studying conformational changes of proteins, says RIKEN researcher Atsushi Miyawaki.



For bimolecular FRET sensors, a protein of interest carries a fluorophore such as cyan-emitting fluorescent protein (CFP), and a separate fluorophore, perhaps yellow FP, is on a different protein. With a unimolecular sensor, a molecule has two fluorophores attached.

Upon interaction or conformational change and when the two fluorophores are less than ten nanometers apart, energy transfer takes place between one fluorophore that acts as a donor and the other fluorophore as an acceptor. This energy transfer via a dipole–dipole link leads donor light emission to be quenched and the acceptor molecule to emit light. The FRET sensor output is typically ratiometric and based on this changed emission of donor and acceptor.

Sources: Michael Lin, Stanford University; Atsushi Miyawaki, RIKEN; Jin Zhang, UCSD.

And there are links to explore related to membrane charge and T cell activation, says Ma, such as a decrease in membrane charge, disassociation of the CD3 ζ-chain from the membrane, and CD3 ζ-phosphorylation state, which has been proposed as a 'safety on' model in T cell activation.

MCS+ delivers a quantitative readout of events. Energy transfer between donor and acceptor fluorophore decreases as the interaction between a molecule and the membrane decreases. The sensor's design builds on R-pre, a fluorescence-intensity-based sensor with one fluorophore. The team got the idea after a seminar by R-pre developer Sergio Grinstein of the University of Toronto. He described the challenge of measuring membrane charge and phosphatidylserine levels during phagocytosis both with R-pre and with the PS sensor Lact-C2 GFP. With R-pre, when membrane charge at the cell plasma membrane decreases, the sensor moves from plasma membrane to the cytosol. This lowers R-pre's fluorescence intensity, which is hard to image with microscopes that do not allow optional sectioning, such as traditional epifluorescence microscopes, says Ma. Once R-pre moves away from the membrane, it does not reattach immediately. Membrane charge shifts have to be measured at the membrane continuously or at a one-nanometer distance, he says, so that is why they wanted MCS+ to stay membrane-associated.

The lab is still optimizing the sensor—for example, its dynamic range in live cells, says Ma. The FRET signal change with MCS+ in live cells is likely more complicated than the well-controlled conditions of an "*in vitro* membrane lawn experiment," he says. Local pH and ionic activities are difficult to predict in live cells. FRET sensor design decisions emerge from the biology that a lab is probing, says Goyette. Few design aspects "can be truly generalized."

Designed, but capricious

Colleagues often ask for his help to make FRET sensors, says Wolf Frommer, a sensor developer at the Heinrich Heine University Düsseldorf. The basic steps are not hard: PCR, insertion into a suite of vectors, purification of proteins after bacterial expression and fluorescence measurement of the sensors[5]. But, he says, "nobody has a clue how they really work, so the construction is empirical." Unexpected events occur even with single-molecule genetically encoded calcium sensors such as GCaMPs. "All that means is, make lots of constructs and see which ones work best," he says.

As Zhang explains, when inserting a switch component into a scaffold, researchers can never be sure how well it will work. Sensor tweaking is therefore necessary. A number of labs are working on ways to test many different biosensor variations, she says, which would accelerate design and optimization, also for her new FLINC sensors[6].

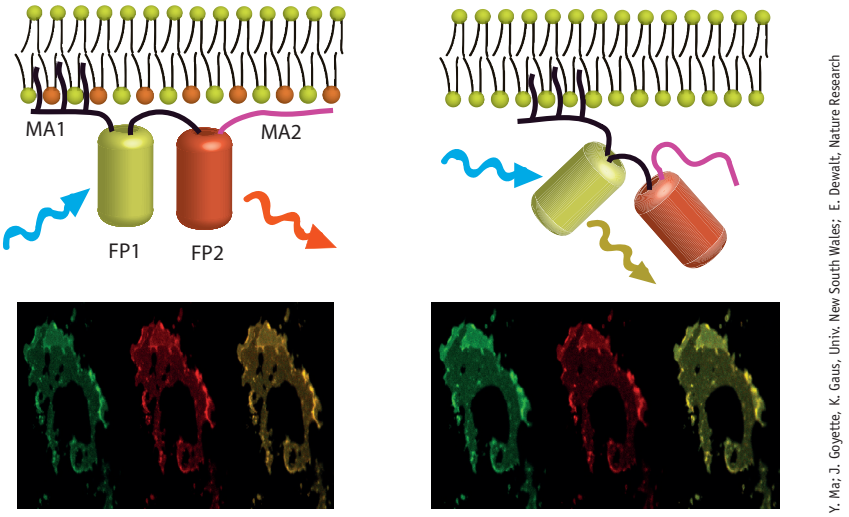
Together with Tsien, Miyawaki codeveloped FRET sensors of Ca^{2+} called cameleons that are tandem fusions of a blue- or cyan-emitting mutant of GFP, calmodulin, calmodulin-binding peptide M13 and an enhanced green- or yellow-emitting FP. They offer good dual-emission ratiometric responses. Cameleons have many "offspring," says Miyawaki, who named them due to how unpredictable the design of most of the constructs was. "They were really capricious," he says.

In "nice drawings" a sensor might appear to work fine, says Frommer. The scaffold might bind glucose and change conformation. On paper, researchers might also draw the fusion to two FPs and expect "the classic change": the closer the labeled molecules, the more FRET.

But a cell contains more than the probed single molecules, and FPs are connected by linkers with flexible domains. Hence, says Frommer, "we actually measure many molecules." Extended imaging times and the fact that each molecule can take many poses make it hard to predict the actual sum of all sensor structures. Linkers can vary in length and composition, leading to many options at each position. "We use a battery of constructs with slight variations to make libraries and screen them," he says.

When designing and optimizing FRET sensors, he recommends that labs hone their sensors' signal-to-noise ratio and sensitivity. "And this means empirical improvements," says Frommer. He and his team recently developed Matryoshka sensors with a platform they believe can be generalized for creating dual ratiometric FP biosensors[7]. Named after the Russian nested dolls, the sensor has a nested single FP cassette called Green-Orange Matryoshka. It comprises green- and orange-emitting FPs: a stable reference FP—large Stokes shift LSSmOrange—nested within a reporter FP of circularly permuted GFP.

The team believes the design offers greater sensitivity, dynamics and detection range with a better signal-to-noise ratio to help experimenters exclude expression or instrument artifacts when acquiring quantitative data *in vivo*. The team tested their MatryoshCaMP6s for monitoring biological processes in *Arabidopsis* seedlings



Y. Ma; J. Goyette, K. Gaus, Univ. New South Wales; E. Dewalt, Nature Research

MCS+ is a FRET sensor for tracking membrane charge in live cells.

and the researchers see opportunities for applying this strategy for sensing small molecules such as sugars, amino acids, neurotransmitters or hormones.

As Frommer explains, there's much to heed when optimizing sensors. Adding glucose to cells changes cell shape so that the apparent change in FRET signal can be explained by an experimenter looking at a different imaging slice. FRET biosensors face some limitations such as the fact that the detection range tends to be limited to two orders of magnitude of analyte concentration; there are limits to dynamic range due to the size of the FP barrel structure and the way it constrains how close the chromophores can get; and rotational averaging with the linkers leads to signal loss. When performing careful quantitative measurements with FRET sensors including Matryoshka sensors, researchers will want to be sure the reference fluorophore or YFP is stable, says Frommer. "And controls, controls, controls," he says. Ideally those controls are affinity mutants.

Even in the absence of a one-scaffold-fits-all FRET sensor design, labs can follow some general principles, says Lin. They will want to maximize the distance between fluorophores and minimize FRET in one state and minimize the distance between the fluorophores to maximize FRET in the other state.

BOX 2 FRET SCALE-UP

Fluorescence Innovations (FI) is a company that Gregory Gillispie, a former academic, founded to commercialize the high-speed spectrophotometer he developed for analyzing fluorescence lifetime decay curves. He moved FI from Bozeman, Montana, to Minneapolis to work more closely with University of Minnesota biosensor developer and biologist David Thomas. Thomas co-founded a company, Photonic Pharma, in 2015 that plans to scale up FI's FRET-based platform for high-throughput live-cell screens in drug discovery. When fully operational, the system will measure several plate wells per second, says Gillispie.

"We don't employ time-correlated single-photon counting, which is at the core of nearly all FLIM systems," says Gillispie. Instead, the instrument's software analyzes the fluorescence lifetime decay waveforms of every pulse of laser light that hits a sample. By recording the response of many photon events in a given time interval, the platform moves beyond the speed constraints of time-correlated single-photon counting.

Gillispie likes how "exquisitely sensitive" FRET is to the distance between donor and acceptor. Harnessing this sensitivity is a way to discover an interaction, possibly a binding event or structural change involving a compound under investigation and a target. "FRET won't work for every drug target of interest," he says, but it is the right choice for a "goodly number of targets." Their approach, he says, can expand the number of cases for which a FRET assay can reasonably be contemplated.

If there are two sensing domains that bind to each other in the presence of the signal, this can be achieved with a long linker between the two sensing domains, as has been shown in the Matsuda lab at Kyoto University, says Lin. In his view, labs might modulate the affinity between fluorophores to help them bind to one another after they are first brought close together by conformational changes in the sensory domains. That can further improve FRET in the high-FRET state. "It may be useful to try a few different fluorophore pairs, too," he says.

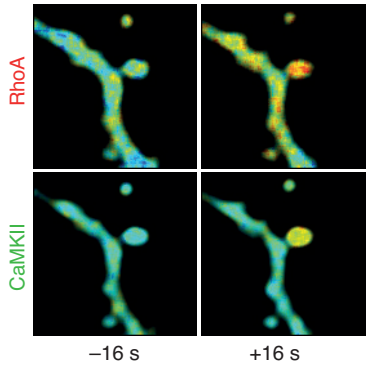
Imaging decisions

A high FRET signal tells a researcher two fluorophores are close—around ten nanometers apart (see **Box 1**). Fluorescence lifetime imaging microscopy (FLIM) adds to that information with the percentage of donors undergoing FRET, giving labs better quantitative information. FRET can be leveraged for drug discovery (see **Box 2**, "FRET scale-up").

Lin's team, along with Ryohei Yasuda of the Max Planck Florida Institute, developed a FRET pair that can show two biochemical events simultaneously, for use with FRET–FLIM or in regular FRET[8]. A group of researchers at several institutes in Europe used *in vivo* FRET–FLIM to show how labeled transcription factors in the *Arabidopsis* root form complexes that influence cell-fate segregation in neighboring cells[9].

Commenting generally on FRET–FLIM, Miyawaki says that the technique is "extremely useful particularly for intermolecular FRET." When weighing the choice of lifetime versus color change measurements, he says that FLIM is more quantitative than intensity-based measurement. As Ma explains, FRET–FLIM's greater sensitivity for FRET signals versus ratiometric imaging comes at the cost of longer image acquisition time. Theoretically, both techniques are insensitive to protein concentration, but photomultiplier-based detectors can respond in a nonlinear fashion to changes in fluorescence intensity, which can lead to artifacts with ratiometric imaging.

One aspect to keep in mind, says Goyette, is that complex fluorescence decay curves for donors with FLIM-based methods can complicate analysis. "Selection of a donor with a single exponential fluorescence lifetime is a good idea," he says. FRET–FLIM also requires specialist equipment that is less common



Dual FRET–FLIM reporting allows simultaneous measurements in dendritic spines in mouse hippocampal brain tissue.

than the microscopy setup for ratiometric methods.

FRET–FLIM is great for detecting protein–protein interactions in live cells, says Ma. But these data often lack information about the molecules' spatial distribution. Super-resolution microscopy can offer this information at nanometer resolution. Two-color super-resolution imaging has been used to detect protein–protein interactions, for example, but one must trade off with temporal information because the sample is fixed.

A striking image that captures a biological principle "sticks in your memory in a way a bar chart can't," says Goyette. But the seductive power of pretty pictures can lead to poor applications of new microscopy techniques, he says. Super-resolution microscopy is best chosen when it is suited to the biological question of interest.

Super-resolution view

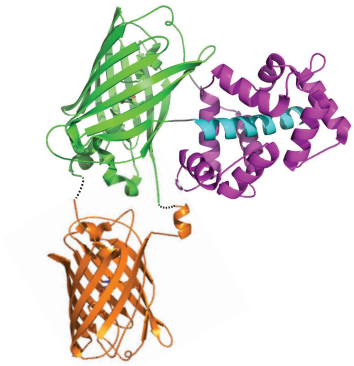
Nanodomains are intriguing, confined cellular locales, with clusters of a few proteins that control many biochemical processes in cells, says UCSD's Zhang. To study these reactions takes sensors, but nanodomains are too small to be imaged with confocal microscopy. Super-resolution microscopy is the alternative but it offered few ways to watch nanodomain reactions as they occurred. This led her to develop FLINC sensors to see the spatial organization of biochemical activities in super-resolution.

FPs are a little like light bulbs, with an outer shell surrounding a delicate inner filament that gives off light, says Zhang, and the filament is sensitive to changes in the outer shell's protective environment. FLINC has two FPs: Dronpa, a green FP, and TagRFP-T, a red FP. On its own, TagRFP-T fluoresces brightly and steadily. When Dronpa is so close that the outer shells touch, the shell of TagRFP-T gets a little distorted, its emitted light dims and starts to flicker.

When recorded as a movie, these fluctuations look like spots randomly blinking on and off with each movie frame showing a different subset of blinking spots. By mathematically analyzing the spots and their blinking across the length of the movie, scientists can quantify the fluctuations at each spot and pinpoint the locations with high fluctuations. No two spots blink at the same time, which lets experimenters discern spots that are too close to discern with conventional fluorescence microscopy.

The resulting super-resolution microscopy image and its wealth of detail could not be captured with classic FRET sensors, because they lack these fluorescence fluctuations, says Zhang. Even with classic FRET sensors that have good temporal resolution, "there's a limit to how much spatial detail you can make out."

To design FLINCs, Zhang and her team drew on the somewhat generalizable FRET sensor approach: a pair of FPs are placed on either side of another protein fragment, which acts like a switch, opening and closing in response to a biochemical process in the cell. The opening and closing is what controls the distance between the two FPs. Since both FRET and FLINC can take place only when the two FPs are close, FRET or FLINC signal changes indicate when a specific biochemical process is occurring. "If you change the identity of the protein switch, you can detect a different biochemical process," she says. "This 'interchangeable parts' design is one of the



Matryoshka sensors are named after Russian nested dolls. The sensor has a nested single-FP cassette called Green-Orange Matryoshka.

things that makes these sensors so powerful and useful," she says.

With its fluorescence lifetime measurements, FRET–FLIM renders FRET very quantitative, says Zhang, but FRET–FLIM may be difficult to partner with super-resolution imaging. In the future, it might be possible to combine classic FRET biosensors with super-resolution imaging, she says. But FRET is highly useful even without this partnership.

FRET sensors have opened up ways to study many processes inside living cells, says Zhang, but the field can still grow. Current biosensors are good for studying individual biochemical processes happening in isolated cells, but all processes in a complex organism involve interaction among multiple biochemical processes in a given cell. There are interactions between multiple cells in a given tissue or organ and sometimes interactions between multiple tissues or organs. "To really unravel how all of these complex processes work, we need to have tools that allow us to 'see' many different biochemical reactions at once in real time and to be able to track these processes as they happen in an intact tissue or live animal," she says.

FRET sensors have enabled this 'seeing' but limitations include that fact that FRET sensors typically contain two different-colored FPs, says Zhang. It's hard to image multiple FRET sensors in the same cells and keep their fluorescence signals from overlapping and becoming hard to tell apart, she says. Also, in many cases, experimental FRET signals are small; "any new and enhanced biosensors that have higher sensitivity or are easier to use for simultaneous, or 'multiplexed', activity imaging would be really important advances."

Vivien Marx is technology editor for *Nature Methods* (v.marx@us.nature.com).

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In the version of this article initially published, the captions and image credit information for images from the Frommer lab and the Lin lab were swapped. The image from the Frommer lab was also truncated. The errors have been corrected in the PDF and HTML versions of this article.