The darling of single-molecule techniques, smFRET has been used to study protein-protein interactions, RNA folding, and more. Jeffrey Perkel explores what’s in store to make smFRET more applicable for dynamic, multidimensional interactions.

At the 2009 Advances in Genome Biology and Technology conference in Marco Island, Fla., Life Technologies previewed its new third-generation sequencing technology. Unlike so-called next- or second-generation sequencing strategies—which rely on some form of add, pause, and read strategy—the new approach being taken by Life Technologies enables the DNA polymerase to run unfettered.

“The system runs at Mother Nature’s speed,” says Joe Beechem, head of research and development for SMS Advanced Research at Life Technologies. “What we are doing is taking a bird’s-eye view.”

Dubbed “Project Starlight,” the key to the new technology lies in its use of single-molecule fluorescence resonance energy transfer (smFRET). The system watches as the polymerase grabs nucleotide triphosphate after nucleotide triphosphate for incorporation into a growing DNA strand, recording each event as a burst of fluorescence energy. But this is no ordinary fluorescence; each burst represents an energy transfer from a fluorescent quantum dot–coupled polymerase (a FRET “donor”) to a nucleotide, whose gamma phosphate is tagged with one of four distinct FRET “acceptors.”

With 100,000 or so individual templates flickering on and off at the same time, the project’s name is apt. “It literally looks like you are looking at the night sky,” says Beechem.

DNA sequencing is merely the latest in a long line of applications for smFRET, which has already tackled conformational dynamics, protein-protein interactions, RNA folding, and more. “It really changed the landscape of structural biology,” says Shimon Weiss. Now a professor of chemistry, biochemistry, and physiology at University of California, Los Angeles (UCLA), Weiss’s lab at UC Berkeley was where smFRET was first developed in 1996. “It allows you to look at moving parts as opposed to static structures.”

A Little History

As the name implies, smFRET is a single-molecule variant of FRET. Say you have two fluorophores, such as Cy3 and Cy5. The former is optimally excited at 550 nm and emits at 570 nm; the latter excites at 650 nm and emits at 670 nm. Under normal circumstances, the two dyes behave independently, emitting light whose wavelength depends on which of the two fluorophores was excited (a fact that underlies most every multicolor fluorescence microscopy experiment).

But if the two molecules are brought very close together, have matching photophysical properties, and are oriented just so, then the excitation of one leads to emission from the other. Like a game of molecular hot potato, the FRET donor nonradiatively passes its energy to the FRET acceptor, thereby returning to a relaxed state without itself fluorescing. In that case, excitation of Cy3 causes Cy5 to fluoresce.

This effect is exquisitely distance- and orientation-dependent, like a very short range, directional antenna. In fact, fluoro-
phores separated by more than about 10 nm cannot do it. But as a result, even though FRET cannot reveal precise distances, it can yield relative information; the technique is therefore sometimes referred to as a “molecular ruler.”

FRET, of course, isn’t new. The concept was first detailed decades ago, and the principle is as old as time: it is essentially thanks to FRET that light harvesting complexes in plant leaves can funnel their captured energy to photosynthetic centers. Yet traditional FRET studies describe the average behavior of a bulk population of molecules—the proverbial roar of the molecular crowd, as it were. smFRET focuses on individual molecules, recording energy transfer events as single molecules flex.

Represented as plots of fluorescence intensity versus time, smFRET can be seen as spikes in acceptor emission mirrored by concomitant drops in donor emission. By reading those graphs, researchers have inferred the conformation of folded RNAs and the proximity of protein domains. In 2007, Sua Myong and colleagues in Taekjip Ha’s lab at the University of Illinois, Urbana-Champaign used the technique to measure the step size of a DNA helicase.

Taking FRET to the single-molecule level wasn’t an easy feat. In fact, it was so challenging that Ha, who developed the technique as a graduate student in Weiss’s lab, recalls thinking it would be effectively impossible for others to ever repeat.

But the concluding statement in the abstract of his seminal paper, which his graduate research advisors insisted upon, reads: “Monitoring conformational changes, such as rotations and distance changes on a nanometer scale, within single biological macromolecules, may be possible with single-pair FRET.” (1)

“I thought that was [BS],” Ha recalls, “but it turned out they were right. It turned out to be a general mechanism to study biomolecular interactions.”

smFRET has become more than merely reproducible. Thanks to technical advances such as more-sensitive detectors, improved fluorophores, and better passivation chemistry, it’s something of an everyman’s single-molecule technique these days. It is a method almost anyone with a good total internal reflection fluorescence (TIRF) microscope can use. According to the ISI Web of Science, Ha and Weiss’ original paper has been cited nearly 400 times.

Starlight, Starbright

In Starlight’s case, the FRET donor is a custom quantum dot nanocrystal attached to the back of a DNA polymerase like a snail’s shell; the acceptors are the individual deoxyribonucleotides, each bearing a different dye. To make this work—that is, to get FRET to extend as far as the dNTP gamma phosphate position—the system actually uses a variant FRET scheme called a “three-color cascade,” in which the transferred energy hops from the quantum dot to an intermediately placed dye, and then to the final acceptor. The reactions themselves are imaged via simple TIRF microscopy, which limits fluorescence to a narrow optical slice immediately above the slide surface.

The approach is, according to Beechem, “a great way to molecularly confine the [reaction], but not physically confine it.” In other words, between the TIRF illumination and FRET detection, the system can ignore all the fluorophores in the reaction except those that are actually interacting with the polymerase. By comparison, Pacific Bioscience’s third-generation technology relies on “zero-mode waveguides” and a tethered polymerase to restrict fluorescence from labeled nucleotides to the volume immediately around the enzyme.

Beechem says Starlight can read up to 300 bases per minute for several minutes, for each of the approximately 100,000 distinct templates immobilized on a surface. But it’s not commercially available yet; Starlight technology is still in the research phase of development, Beechem says.

Multidimensional FRET

For all its power, standard smFRET is limited to measuring one interaction at a time—a single vector in a complex
three-dimensional space. To really probe molecular dynamics, researchers need to pinpoint those dye molecules relative to at least one other point. “In my view,” says Ha, “the future lies in multidimensional single-molecule measurements.”

In 2004, Ha and team member Sungchul Hohng took the first step towards such multidimensional smFRET by using three dyes (Cy3, Cy5, and Cy5.5) to study the conformational acrobatics of a DNA complex called a Holliday junction. More recently, Hohng (now assistant professor of physics at Seoul National University, South Korea) and Ha extended smFRET again. By carefully positioning each of four dyes (Cy2 or Alexa488, Cy3, Cy5, and Cy7), they were able to measure six intramolecular distances concurrently, again in a Holliday junction.

Ha says the trick is to cycle between three different lasers, an approach called alternating laser excitation, or ALEX. ALEX is a technical variant that is used to directly probe the presence of active FRET donors and acceptors, and to untangle the FRET signals arising from the different possible intermolecular events. Here’s how it works in the case of four-color smFRET: first, Cy5 is excited and its distance to Cy7 is measured using a 633-nm red laser. Then, Cy3 is excited and its distance to Cy5 and Cy7 measured with a 532-nm green laser. Finally, Cy2 is excited and its position relative to everything else is measured with a 473-nm blue laser. “You can, in these three steps, deconvolve everything,” says Ha.

Unfortunately, that approach really isn’t scalable, says Achillefs Kapanidis, a university lecturer in biological physics at the University of Oxford, since the addition of each new color makes data collection and analysis more complex. “Five-color ALEX, for instance, would be horribly complicated,” he says.

Kapanidis, though, has devised an approach he thinks is relatively scalable: switchable FRET. “Switchable” fluorophores are dyes that can be turned on and off using different wavelengths of light and/or chemicals. By placing one FRET donor and two acceptors on a single biomolecule and then cycling between the different acceptors (that is, switching them on and off in sequence), Kapanidis and his team showed that they could probe the structure and dynamics of both a protein-DNA complex and a Holliday junction.

Now Kapanidis’ team is working to improve the method. “Given our current FRET resolution, we anticipate that extensions to more than two pairs will be straightforward,” he wrote (2). For instance, he continued, the technique could facilitate analysis of multimeric protein complexes, such as helicases.

Going In Vivo
Keith Weninger, associate professor of physics at North Carolina State University, is addressing another problem with smFRET: moving the technique from the in vitro to the in vivo world. “In vitro biochemistry has been very powerful and has added to our knowledge, but there’s a lot of evidence that the environment inside cells is important in controlling the function of many proteins,” says Weninger.

Indeed, Weninger’s interest may be summed up elegantly as “the dynamics of proteins in vivo,” but that’s easier said than done. smFRET in vivo is a tricky business. Among the problems are high
Dissecting enzymology. Using three FRET probes—a polymerase-based donor (green) and two promoter-based acceptors (red and bright red)—Kapanidis, Weiss, and colleagues determined that initial transcription by RNA polymerase proceeds through a “DNA scrunching” mechanism, in which the enzyme remains stationary on promoter DNA and pulls into itself downstream DNA.


Probing a protein-DNA complex with switchable FRET. Two identical photoswitchable acceptor fluorophores (Alexa Fluor 647) are attached to either subunit of catabolite activator protein (CAP, yellow). CAP binds to a specific DNA recognition sequence (gray), labeled with a donor fluorophore (Cy3B). Switchable FRET measures the two donor-acceptor distances in this complex by sequentially turning the two acceptors on and off. Adapted from Uphoff et al., 2010.

background fluorescence and a difficulty labeling molecules in vivo—this is because fluorescent proteins make for poor smFRET partners photophysically, and also because it’s difficult to control their concentration.

Weninger, though, has found a way to circumvent those issues. Earlier this year, he and graduate student John Sakon showed it was possible to use intramolecular FRET to capture the conformational dynamics of SNARE proteins during membrane fusion inside living cells. His solution: microinjecting prelabeled, recombinant protein. “We typically would inject about 15 cells in 30 minutes per plate,” he says. This enabled the team to track dozens of molecules per plate before the fluorophores fizzled out. To overcome the fluorescent background, Weninger and his team optimized both growth conditions and their microscopy protocols. For instance, they used fluorescent dyes that were particularly bright. And instead of standard TIRF, his team used “near-TIRF,” in which the sample is illuminated at very steep angles to probe different optical planes without overwhelming the system with light.

This is certainly not the first time FRET has been applied in vivo. The technique has been used for years to identify protein-protein interactions in living cells, but those experiments measure bulk populations, not individual molecules. In 2004, a Japanese team used smFRET to measure protein–small molecule interactions in vivo (3), but that was a colocalization experiment. “This is the first demonstration of single-molecule in vivo detection of conformational changes,” Weninger says.

Given the pace of developments in this field, it surely won’t be the last. And other developments are on the horizon, too. Ha, for instance, is developing a method he describes as the “third way” between in vitro and in vivo studies, to study the biochemistry of native cellular (as opposed to recombinant) complexes. “It’s working,” he says, “and I think you’ll see many more studies of this type in the future.” And though Life Technologies is keeping mum about a formal release date for Starlight, Beechem sees promise. “I think it’s the most advanced FRET system that’s ever been made.”

References


Written by Jeffrey M. Perkel, Ph.D.

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