# A triple threat to single molecules

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Three single-molecule methods promise to increase the time resolution of experiments, to allow better access to sparsely populated molecular states and to permit combinatorial high-throughput analysis.



Complex biomolecules, such as proteins, sample many structural states that are not necessarily well characterized by analyzing the average properties over a population of molecules. Single-molecule spectroscopy tackles this problem directly and could become the premier tool for protein folding studies if three nagging problems can be solved: single-molecule spectroscopy has low time resolution, such analysis easily misses rarely populated states that are often of greatest biological interest, and it is hard to apply in high throughput so one can actually sample all those states. As reported in last month's issue and in the current issue of *Nature Methods*, three groups have made important progress in regard to each of these three problems[1–3]. All demonstrate the advantages for single-molecule fluorescence resonance energy transfer (FRET) experiments, in which a dye hooked up to a protein is excited by light and then either fluoresces or transfers its energy to another dye that fluoresces with a different color. Protein dynamics alters the distance between dye labels, changing the FRET efficiency and hence the balance of emission wavelengths, which is detected.

Campos *et al.*[1] tackle the speed problem with their method. Many protein functional and folding motions are fairly fast, ranging from nanoseconds to microseconds. For example, current ensemble relaxation experiments with nanosecond time resolution have revealed that once a protein is 'primed to fold,' it takes only a microsecond to cross the transition region between the denatured and folded state[4,5]. Current single-molecule experiments can get just below a millisecond, not quite enough to resolve such fast motions[6]. The payoff for reaching these time scales in single-molecule experiments is well worth the effort: the complex nature of a protein hopping rapidly between various structures on the energy landscape could be revealed in much more detail by single-molecule experiments.

In a single-molecule spectroscopy experiment, the production of photons via the regular shuttling of the dye molecules between the ground and excited singlet states is occasionally interrupted by 'blinking', such that the dye is temporarily stuck in a triplet state. This puts the experiment on hold; the halting of rapid-fire shuttling results in lower time resolution (**Fig. 1a**). The humble oxygen molecule is the key culprit, but it is also part of the solution to better time resolution. Oxygen can help prevent blinking because its low-energy triplet state efficiently quenches the dye back to the ground state. Nonetheless, researchers performing single-molecule experiments usually eliminate the $O_2$ by adding antioxidants. Why? The high-energy singlet form of $O_2$ and other reactive species generated from $O_2$ chew up dye molecules, silencing or 'bleaching' them permanently.

Campos *et al.*[1] report a simple but powerful idea: leave the oxygen in but add cysteamine to specifically get rid of unwanted singlet oxygen and other reactive oxygen derivatives. Including Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) as an additional triplet quencher in the mixture, they got close to the best expected shuttling rate for Alexa Fluor dyes, which allowed them to study microsecond folding dynamics of the small proteins BBL and α-spectrin SH3 domain.

Gambin *et al.*[2] have another fix for the speed problem. They devised a method to populate transient, higher energy states of single molecules by combining relaxation, a technique usually used in ensemble measurements[7], with single-molecule detection. In relaxation measurements, a solvent parameter like pH, temperature or concentration is suddenly 'jumped', and the structural changes that result are followed by a detection method. Under such conditions, states can be transiently populated that are not accessible with high probability by structural fluctuations in equilibrium, the usual *modus operandi* of single-molecule studies. The difficulty lies in doing a jump that is compatible with single-molecule detection. For example, microfluidic streams that rapidly mix the solvent conditions usually move too fast for efficient single-molecule detection.

Gambin *et al.*[2] designed a microfluidic device to mix the single molecules with two other streams to jump the solvent condition, and then used a carefully crafted splitting of the mixed stream to decelerate the stream of single molecules for fluorescence-based detection (**Fig. 1b**). The carefully shaped microfluidic channels yield results with 200 microsecond time resolution.

They applied their system to study the folding kinetics of the intrinsically disordered protein α-synuclein, the culprit in Parkinson's disease[8]. By jumping the concentration of SDS, a detergent that promotes formation of helical structure when it binds to α-synuclein, they captured an intermediate state, which they detected with higher FRET efficiency than either the unfolded or folded state. The FRET experiment indicated that the intermediate is in a jack-knifed helical state and that reversing the process under different conditions results in the protein following a different folding pathway.

With their method, Kim *et al.*[3] tackle the throughput problem. To really probe the heterogeneity of single-molecule processes, it would be nice if one could examine single molecules under as many experimental conditions as possible. There are many variables to pick from, such as pH, salt concentration or

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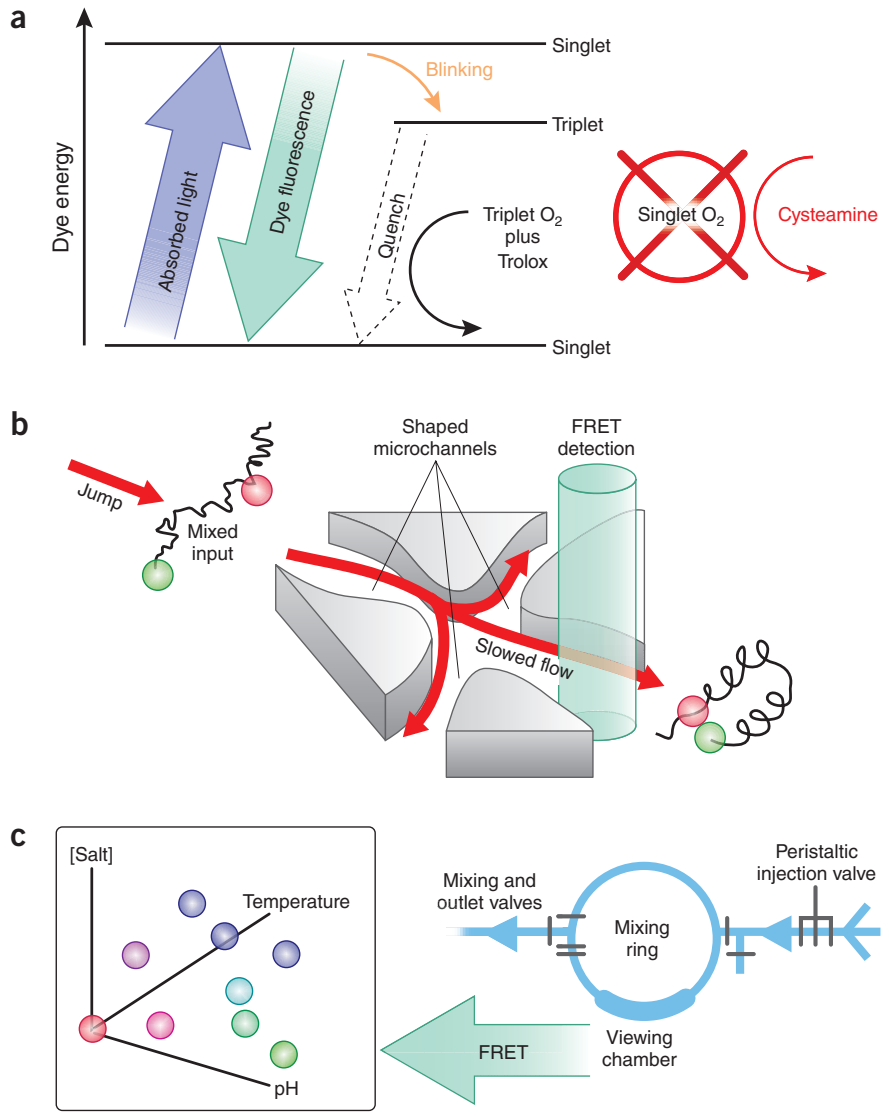


**Figure 1** | Three improved single-molecule techniques. (**a**) Dyes 'blink' into triplet states, taking them out of the absorption-fluorescence cycle. $O_2$ can quench the triplet state and restart the cycle, but singlet $O_2$ bleaches the dyes permanently. Keeping $O_2$ in, adding Trolox for additional triplet quenching and getting rid of singlet $O_2$ with cysteamine improves the time resolution of single-molecule imaging. (**b**) Carefully shaped flow channels take a single-molecule stream that was rapidly mixed to change solvent conditions; the flow is then slowed down to be compatible with FRET imaging. (**c**) A peristaltic valve distributes input from seven independent channels into a storage-detection ring, where other valves process the single-molecule stream. A multidimensional space of solvent conditions (three dimensions are shown) can be scanned rapidly with this device.

temperature, to name just three. Progress has been made in this direction already[9], but Kim *et al.* have adapted a particularly ingenious ring-mixing technique[10] for multidimensional scanning of single-molecule energy landscapes and enzymatic activity. The miniature ring geometry with microfluidic valves (**Fig. 1c**) permits the efficient detection of photons from single molecules in a 'viewing chamber', can run through three-dimensional condition scans in no time and has volume injection errors of 0.0001%, better than the best-pipetting graduate student.

Their experiments with the ring mixer highlight the new capabilities: using polythymine RNA as a probe for mRNA synthesis by RNA polymerase, they show that glutamate can up- or downregulate transcription as a function of polymerase and glutamate concentration. They also scanned single-stranded DNA hybridization as a function of multiple salt and polyadenine concentrations, creating a three-dimensional map of DNA subpopulations that would be difficult to resolve in bulk experiments. Multiparameter resolution of molecular subpopulations under many experimental conditions is key for being able to use single-molecule experiments to extract information about biomolecular energy landscapes.

The 'triple threat' of techniques discussed here improves single-molecule experiments by sharpening their time resolution, detecting high-energy intermediates inaccessible via equilibrium fluctuations and enabling fast access to multiple solvent conditions. The better quenching cocktail specifically targets FRET experiments, whereas the new instruments adding relaxation and multiparameter scanning to single-molecule experiments could also be combined with other detection techniques.

The devices described here are flexible open platforms for further development. For example, the design of Kim *et al.*'s[3] ring mixer enables stochastic sampling protocols for solvent parameters rather than scanning parameters linearly. Many more parameter combinations then could be sampled in a given amount of time. If combinatorial capabilities could be combined with fast relaxation, entirely new applications open up. For example, a genetic algorithm then could search automatically for optimal single-molecule folding conditions, such as minimizing the concentration of an undesirable folding intermediate that leads to aggregation.

Rather than just studying single molecules, we will learn to manipulate them.

**COMPETING FINANCIAL INTERESTS**
The author declares no competing financial interests.

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