The Cat That Caught the Canary: What To Do with Single-Molecule Trapping

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ABSTRACT It has recently become possible to trap individual fluorescent biomolecules in aqueous solution by using real-time tracking and active feedback to suppress Brownian motion. We propose areas of investigation in which anti-Brownian electrokinetic (ABEL) trapping of single molecules is likely to lead to significant new insights into biomolecular dynamics.

One day in June 1827, Robert Brown peered through his microscope at a sample of pollen. He reported, "While examining the form of these particles immersed in water, I observed many of them very evidently in motion." At first suspecting that the pollen was swimming, he convinced himself otherwise through an exhaustive search of other finely divided objects, ranging from a piece of the Sphinx to "the mucous coat interposed between the skin and muscles of the haddock." He concluded that random motion was a fundamental property of all microscopically divided matter immersed in water.[1]

The jiggling continues today, a consequence of random collisions with thermally agitated water molecules.[2] Yet two recent papers report bringing this motion under control, at least for a single molecule for a few seconds, through improvements to the anti-Brownian electrokinetic (ABEL) trap.[3,4] The idea is simple: one watches the Brownian motion of a particle using fluorescence microscopy. Whichever way the particle moves, one pushes back in the opposite direction with electrokinetic forces. The key to successful trapping is to perform this feedback as quickly and accurately as possible, and it took several years to progress from the initial concepts to the present implementations.

ABEL traps now operate near the information limits imposed by diffraction and shot noise: photons only reach the detector at a finite rate, and each photon carries imperfect information about the location of its source, due to diffractive blurring. The recent generation of traps build on the work of the Mabuchi lab[5] to apply sophisticated statistical filtering, implemented in real-time digital hardware, to squeeze maximal information from every detected photon. These traps act upon this information with negligible delay. Wang and Moerner[3] trapped short fluorescently labeled DNA oligonucelotides, and we recently trapped individual fluorophores.[4] What now? How can we apply these traps to gain new insights into biomolecular dynamics and interactions? What additional improvements in instrumentation or conceptual advances are needed to enable new applications? In this Perspective, we present a vision for the future of single-molecule trapping in solution.

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What Can We Learn from a Trapped Molecule?

The recent work by Wang and Moerner, described in this issue of *ACS Nano*, hints at how the ABEL trap can reveal previously hidden aspects of molecular dynamics. Their system uses the information encoded in the series of photon arrival times and feedback voltages to estimate, in real time, the diffusion coefficient, D, and electrokinetic mobility, μ, of the particle in the trap. The diffusion coefficient measures the strength of the random jiggling, and depends inversely on the hydrodynamic radius and the local viscosity. Time-dependent changes in D could arise from conformational transitions, binding and unbinding with other species in solution, or changes in local viscosity. The electrokinetic mobility reflects the hydrodynamic drag on the particle, but also its charge. Ionization events or covalent modifications might significantly alter μ. There is not currently any

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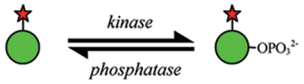
TABLE 1. Free-Solution Processes Amenable to Study in the ABEL Trap[a]

Reaction Type		Affected Observables
Protein Folding		E_{FRET}, D, μ, θ
Phosphorylation		μ
Binding		D, μ, θ, I, τ_{FL}
Conformational Change		E_{FRET}, D

[a] In each case, several spectroscopic techniques may be used to follow the dynamics. E_{FRET}, FRET efficiency; D, diffusion coefficient; μ, electrophoretic mobility; θ, polarization anisotropy; τ_{FL}, excited state fluorescence lifetime.

satisfactory method to measure changes in D or μ on time scales between milliseconds and minutes, and the new generation of ABEL traps opens this possibility.

A key advantage of the ABEL trap over surface-immobilization techniques is that the ABEL trap avoids possibly perturbative interactions with the surface. Thus, ABEL trapping is well poised to study fragile samples that might otherwise be disrupted by surface interactions. Processes associated with dynamic changes in transport coefficients are also uniquely suited to study *via* the ABEL trap. We propose several such examples (Table 1).

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Protein Folding. The folding process is exquisitely sensitive to weak intramolecular interactions, and unfolded proteins expose sticky hydrophobic residues. Studies of protein folding in molecules bound to a surface are fraught with confounding artifacts. To study protein folding in the ABEL trap, one needs a means to initiate the folding/unfolding transition reversibly. Photoinduced electron transfer[6] or temperature jump[7] experiments are one option, and photogenerated surfactants are another.[8] Alternatively, one could go to partially denaturing conditions, and study the equilibrium fluctuations between folded and unfolded states.[9,10]

One also needs a readout of the folding state of the protein. This readout could come from any combination of time-dependent changes in the transport coefficients (D and μ), or by more conventional single-molecule spectroscopies, such as fluorescence resonance energy transfer (FRET) or excited state lifetime analysis. Ultimately, one would like to study protein folding in the context of chaperonins, to understand how the process functions in a cell. Initial steps toward that goal have already been taken by the Moerner lab.[11]

Phosphorylation. Addition and removal of phosphate groups from proteins plays a key role in intracellular signaling,[12] and misregulation of phosphorylation is often associated with cancer. Owing to its medical relevance, many techniques have been developed for measuring ensemble-averaged phosphorylation in cells and in purified proteins. Single-molecule measurements might provide deeper mechanistic understanding of how and when phosphorylation happens, yet there are currently no single-molecule techniques that are sensitive to this process. Ideally, one would like an assay capable of probing a wide range of kinases and substrates, including both wild-type isoforms and oncogenic mutants, in the presence or absence of known and putative kinase inhibitors.

The negative charge associated with a phosphate measurably alters the electrophoretic mobility of some protein targets of phosphorylation.[13] The trapping of fluorescently labeled protein in the presence of unlabeled kinases may reveal the dynamics of the elementary steps of kinase binding, reaction, and unbinding, through their effects on D and μ. To study these processes under quasi-static conditions, one should include phosphatases in the solution to reverse the process. In a few cases, phosphorylation induces conformational changes in the substrate that are large enough to be detected by

FRET, in which case that too could be used as a readout.

Transient Binding. Many techniques have been developed to measure intermolecular interactions, including pull-down assays, yeast two-hybrid screens, electrophoretic mobility shift, surface plasmon resonance, and optical techniques based on FRET or colocalization. However, there is a lack of techniques for quantifying weak and/or transient interactions.

Binding of a small fluorescently labeled molecule to a larger unlabeled molecule may measurably alter the mobility and diffusion coefficient of the labeled molecule. These changes are undetectable in surface-immobilized molecules. The ABEL trap is well suited to the study of binding of proteins to fluorescently labeled nucleic acids, for instance. We recently demonstrated this principle by studying the binding of RecA to DNA.[4] Similar strategies could be used to study binding of an enzyme to a fluorescently labeled substrate, or weak protein–protein interactions.

Nanoscale Physics. The ABEL trap is essentially an implementation of a Maxwell's Demon,[14] in that its interaction with a molecule is conditional on the random thermal motion of that molecule. The connection between entropy and the information contained in position measurements is subtle and the subject of much study. Recent experiments with the ABEL trap have begun to probe this topic experimentally.[15] Other researchers have used ABEL-like traps to study and to control inorganic nanoparticles. Demonstrations include placement of quantum dots for experiments in quantum optics,[16] and measurements of the angle-dependent scattering spectrum of gold nanoparticles.[17]

The ABEL trap may enable fundamental studies of electrokinetics in confined geometries, an important topic for future nanofluidic systems. For instance, one can ask: Is the mobility of a simple particle really constant, or does it fluctuate rapidly due to fluctuations in the ionic atmosphere around it or transient interactions with the surface? When an electric field is applied, does a particle respond instantaneously, or does the motion take some time to develop? How do mobility and diffusion coefficient depend on the depth of the sample cell, the details of the surface chemistry, and the ionic composition of the medium? While some of these questions could, in principle, be answered in bulk measurements, the ABEL trap enables highly precise measurements in the absence of broadening due to heterogeneity in particle size, shape, or composition.

Limitations of the ABEL Trap. The ABEL trap is not a panacea for the challenges of single-molecule spectroscopy. Several factors constrain the choice of systems to study:

Low concentrations of the fluorescently labeled species, typically a few picomolar, are needed to avoid multiple molecules entering the trapping region simultaneously and confusing the tracking system. Thus the ABEL trap is not suited to studying intermolecular processes in which both species are fluorescent.

Low conductivity buffer is important when trapping very small objects (less than 10 nm hydrodynamic diameter). Small objects require large electrokinetic velocities, and hence large feedback voltages, to achieve stable confinement. These conditions lead to heating and deleterious electrochemical byproducts if the buffer is too conductive. Buffer conditions must be optimized for each system.

A bright, photostable fluorophore is essential to achieving high photon count rates and accurate feedback. The requirements on brightness and photostability become more stringent as the trapped particle gets smaller. Brief blinks may interrupt the feedback for enough time for the particle to exit the trap. Trapping of the smallest molecules has only been demonstrated with far-red fluorophores. Trapping using light in other parts of the spectrum, or GFP homologues, will require additional technical development.

Prevention of sticking is a challenge in some ABEL trap experiments. Although the molecule is not bound to the surface, fused silica or glass walls constrain the molecule to a thin film, ~800 nm deep. The molecule collides with the confining surfaces hundreds of times per second; without proper consideration of surface chemistry, the molecule may stick.

How Can the ABEL Trap Be Improved? The ABEL trap will be most useful when additional spectroscopic modalities are layered on top of the optics used for trapping. Then, one could apply the repertoire of single-molecule spectroscopic techniques to the trapped molecule or complex. We expect the next generation of ABEL traps to include the following techniques.

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FRET. Energy transfer provides a sensitive probe of separation between a donor and acceptor fluorophore. To implement FRET analysis, one would use two spectrally distinct detectors; photons from both would be used for tracking and feedback, while the two channels would be considered separately for measurements of time-dependent FRET. We expect FRET to be most useful for intramolecular distance measurements, for example, of protein folding or conformational transitions, or of assembly and folding of DNA nanostructures.

Polarization and Lifetime Analysis. Photons carry information

about their source in their polarization and precise arrival time at the detector. Polarization indicates the orientation of the transition dipole in the emitter at the moment of emission. For small objects, one may use polarization anisotropy to estimate the molecular tumbling time, which depends sensitively on the hydrodynamic radius ($t \propto r^3$), while for larger objects the polarization fluctuations directly yield this information. Excited-state lifetime may be probed by illumination with a high-repetition-rate pulsed laser. The lifetime provides information on the local rigidity of the environment around the chromophore, and on nonradiative decay pathways.

Rapid Mixing and Sample Introduction. A key challenge in current implementations of the ABEL trap is the ~30 min required for sample introduction. This delay limits studies to quasi-static processes, or to processes that can be triggered by a laser flash. Ideally, one would like to change buffer conditions or to introduce additional components while maintaining a single molecule in the trap. One could then probe dynamic responses to changing conditions. One can also envision an ABEL trap as an analytical component on the end of a sample preparation and fractionation apparatus, just as ion traps are used in mass spectrometry. One might like to analyze molecules directly from a cell, or coming off a microfabricated capillary electrophoresis channel. These applications will require designs that integrate the ABEL trap sample cell with other micro- and nanofluidic components.

The Future of Single-Molecule Studies in Nanostructures. The ABEL trap is just one of several technologies under development to facilitate studies on single molecules in free solution.[18] Significant information can be obtained by confining molecules between parallel walls,[19] or in thin capillaries,[20] lipid vesicles,[21] nanofabricated zero-mode waveguides,[22] or water-in-oil hydrosomes.[23] These devices achieve confinement through purely mechanical means, with the attendant decrease in complexity relative to the ABEL trap, but also a loss of high-resolution electrokinetic data.

There are still many unexplored options at the nexus of nanofabrication and single-molecule biophysics that will enable increased insight into the dynamics and interactions of biological molecules. In his treatise *Theory of Heat*, Maxwell wrote, "If we conceive a being whose faculties are so sharpened that he can follow every molecule in its course, such a being... would be able to do what is at present impossible to us."[14] In the coming years, we expect many more reports of experiments Maxwell would have thought impossible.

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