Single molecules

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At first an experimental challenge, the ability to conduct experiments with single molecules has been strongly connected with progress in experimental techniques and instrumentation. Scanning probe techniques, such as the scanning tunneling microscope or the atomic force microscope (AFM), use sharp tips in close proximity (10^{-9} m) to a sample to measure tunneling currents or weak mechanical forces that, in turn, allow generation of a real-space "image" of a single atom or molecule (1). In another approach, macromolecules can be clamped between an AFM tip and a substrate to determine the forces needed to stretch a single polymer chain. Similar experiments are feasible by use of optical tweezers, where a macromolecule is attached to a tiny bead and a substrate. The light force acting on the bead can be used to translate it against a force generated by the macromolecule (2). In the optical domain, the fluorescence emission of single molecules (or more general single fluorophores) in some condensed-phase environments can be imaged by advanced optical microscopies, such as scanning confocal microscopy or near-field scanning optical microscopy (3). At low temperatures, single fluorophores can also be isolated by a frequency selective technique that uses the fact that the sharp optical transition frequencies of dopant molecules are different because of imperfections of the environment (4). These optical techniques allow for detailed spectroscopic investigations at the single-molecule level, taking advantage of spectral, time-resolved, and polarization information.

Using the above-mentioned methodologies, we now can count, image, identify, manipulate, modify, move, switch, sort, and trace single atoms, molecules, and nanoparticles. Although the elimination of ensemble averaging is crucial, there are more arguments for single-molecule investigations. By looking at the individual members of a population, we can determine distributions of physical quantities rather than measure moments of the distributions (average values), which contain less information. Such measurements can reveal heterogeneities or physical effects usually hidden in the ensemble average. Temporal fluctuations, which in ensemble experiments would need a very difficult-to-accomplish external synchronization, can be registered, as well as correlations between fluctuating properties. Some examples that corroborate the foregoing statements are the observations of photon antibunching in single-molecule fluorescence (5) and of fluorescence blinking in semiconductor nanocrystals (6) or conjugated polymers (7). Heterogeneities of various origin are abundant in complex biological systems, where studies with single fluorophore labels have revealed RNA folding dynamics (8) and variations in the turnover rates of single enzymes (9). In general, a large amount of current activity is directed toward applications of single-molecule methodologies to biological systems (2, 10).

Probing Single Molecules in Single Living Cells. Recent research has shown that single fluorescent molecules can be used to study complex molecular processes in living cells (11). In contrast to clean and well-controlled conditions *in vitro*, the intracellular environment contains a broad collection of biological macromolecules and fluorescent materials such as porphyrins and flavins. As a result, the observed background fluorescence is higher than that *in vitro* (e.g., buffer solution), but this background is continuous and stable and does not significantly interfere with the measurement of single-molecule photon bursts. By using fluorescently labeled transferrin (an iron transport protein that undergoes receptor-mediated endocytosis), single-molecule photon bursts are readily observed by focusing a laser beam into a cultured human HeLa cell (Fig. 1). As in free solution, the observed photon bursts should correspond to single transferrin molecules moving in and out of the laser beam inside the cell.

We envision that further developments will include real-time single-molecule tracking and two-color correlation measurement in single living cells (12, 13). Such capabilities will allow the direct observation of key intracellular events, such as the transport of gene therapy vectors, hybridization of antisense oligos to mRNA, and ligand-receptor binding and internalization. Also, fluorescence resonance energy transfer (10), expression of green fluorescent fusion protein (14) and molecular beacon studies (15) may be carried out in living cells, which would provide new opportunities to follow chemical reactions and observe molecular conformational changes *in vivo*.

AFM Studies of Single-Molecule Mechanics. The AFM is a simple instrument capable of imaging single macromolecules with submolecular resolution and measuring the forces required to unfold a single protein domain. The AFM functions like a miniature phonograph. A sharp tip mounted on a cantilever interacts with the sample, causing minute deflections that can be calibrated as a force (Fig. 2). The AFM can be used to study the mechanical properties of native or mutant fragments of proteins engineered as tandem repeats that are easily identified in the force–extension curves generated by the AFM recordings (16–18). In a typical experiment, the protein sample is placed on a gold-coated coverslip that is attached to a piezoelectric positioner. Protein segments are then picked up randomly by adsorption to the AFM tip and stretched for up to several hundred nanometers (Fig. 2*B*). These measurements generate a characteristic sawtooth pattern in the force–extension relationship measured by the AFM. The sawtooth pattern has been shown to contain a wealth of information that can be related to the molecular mechanisms underlying the mechanical stability of the protein being studied. These measurements can be made with single amino acid resolution and provide a direct view of the molecular determinants of the mechanical stability of proteins.

Abbreviation: AFM, atomic force microscope.

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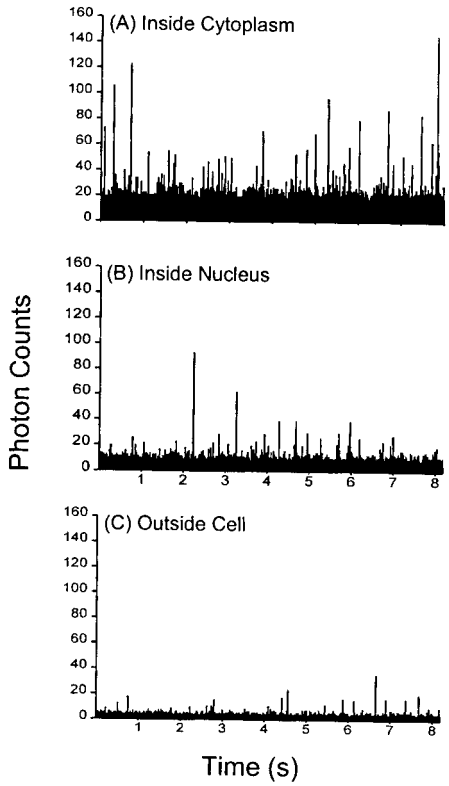

Fig. 1. Fluorescence detection of single transferrin–tetramethylrhodamine (TMR) molecules in the (*A*) cytoplasm and (*B*) nucleus of a cultured human cancer cell (HeLa). The cells were incubated with 6.25×10^{-3} M transferrin–TMR for 6–8 h. Repeated washing was used to remove transferrin–TMR molecules that were not transported into the cells by receptor-mediated endocytosis. The result of this procedure was verified by the absence of single-molecule fluorescence in the medium outside the cell (*C*). Excitation wavelength, 514.5 nm; laser power, 0.5 mW; integration time per data point, 1 ms.

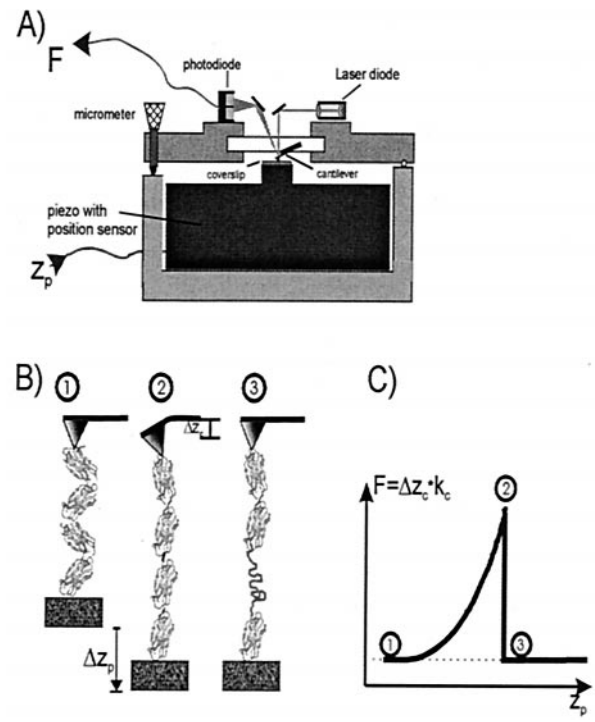

Fig. 2. (*A*) Schematics of a custom-built AFM. The AFM was constructed by using an AFM detector head mounted on top of a single axis piezoelectric positioner. (*B*) Stretching and unfolding protein domains with AFM. (1) An unstretched polypeptide with four individually folded domains adsorbed to an AFM tip; (2) stretching the protein requires force, which is monitored as a deflection of the cantilever; (3) the unfolding of a domain increases the protein length, relaxing the cantilever back to its resting position. (*C*) Idealized force–extension curve for the stretching of a multidomain polypeptide and the subsequent unfolding of a single domain. As the protein is stretched, the force increases in a nonlinear (non-Hookean) fashion; this is the energy required to decrease the degrees of freedom of the polypeptide. If the protein is further stretched, the probability for the unfolding of individual domains becomes very high. When a domain unfolds, the AFM tip travels back to its resting position, and the force goes back to zero (*B* and *C*). The numbers correspond to the stages marked in *B*.

For example, single-molecule measurements have shown evidence for intermediate (19) and misfolded states (20) that, because they are rare, cannot be observed with standard bulk measurement techniques such as x-ray or NMR.

Single-molecule AFM can also be applied to the examination of the conformations of other types of molecules, such as polysaccharides, where it has been recently shown that by applying a force, the pyranose ring of the polysaccharide molecules can be forced from the chair to the boat or inverted chair (21) conformations. Furthermore, the AFM has also been applied to the study of the molecular mechanics of DNA at high resolution (22). The observation of mechanically driven conformations in single molecules opens up the new field of mechanochemistry, with important implications for understanding the mechanical assembly of biological systems.

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