Analyzing single protein molecules using optical methods
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Studies on single protein molecules have advanced from mere proofs of principle to insightful investigations of otherwise inaccessible biological phenomena. Recent studies predict a tremendous number of possible future applications. The long-term vision of biologists to watch single molecular processes in real time by peering into a cell with three-dimensional resolution might finally be realized. Another fascinating perspective is the identification and selection of single favorable variants from complex libraries of diverse biomolecules.

Introduction
Scientific discoveries are usually preceded by the invention of new scientific tools. The enormous progress in the life sciences in recent years is a direct result of the emergence of new powerful bioanalytical technologies. Single-molecule analyses are among the most striking of these, paving the way for new applications in biophysics and biotechnology. The most ambitious goal is probably the investigation of single protein molecules inside the living cell. Another significant challenge is the development of methods to trace, manipulate and select biomolecules with respect to their individual properties. The requirement for only minute amounts of analytes without the need to synchronize their dynamics, and the possibility to distinguish subpopulations of molecular species, which in bulk analysis would be hidden by a statistic mean, open up great opportunities for miniaturized analytical methods and screening techniques.

Single-molecule analysis is presently dominated by two different kinds of approach: optical methods based on fluorescence [1,2•] and, more recently, Raman spectroscopy [3,4•]; and atomic force microscopy (AFM), that is, imaging and manipulating single molecules using microscopic cantilevers (see [5,6] for reviews). The AFM technique yields extremely high spatial resolution and is the most direct way to make contact with molecules. On the other hand, optical techniques are not restricted to surfaces and, therefore, are more versatile with respect to the molecular environment. Optical techniques offer a particularly attractive approach to probe molecules in thermodynamic equilibrium, as they cause minimal interference with the biological system. The scope of this short review will be limited to optical studies, in particular those based on fluorescence methods, reported over the past two years; specific focus is given to protein analysis. For a broader view on the topic, the reader is referred to several excellent review articles highlighting the systematic and historic contiguity of this fascinating field of research [1,2•,7–9].

Optical detection of single biomolecules
The increasing importance of fluorescence-based techniques for single-protein analysis can be attributed not only to the rapid development in laser and detector technology, but also to the availability of a large variety of highly efficient fluorescent labels in the visible spectral range. Protein autofluorescence of tryptophan residues or intrinsic chromophores is scarcely sufficient for a direct analysis of single units [10••,11]. Of particular importance, especially for in vivo applications, are genetically encoded tags such as green fluorescent protein and its mutants or homologs that can be fused to proteins at specific sites [12,13]. Appropriate labels should not only exhibit large absorption cross-sections and fluorescence quantum yields, but also high photostability (i.e. the potential to withstand strong laser intensities), as required for large signal-to-background ratios. It is assumed that a conventional dye molecule used for single-molecule analysis emits about $10^5$ to $10^6$ photons before being irreversibly photobleached [7].

The primary measurement parameters obtained from fluorescence spectroscopy are the wavelength and intensity of emitted light (i.e. the energy and emission rate of photons). Pioneering work on single-molecule spectroscopy, mainly at low temperatures, showed that it is possible to record full fluorescence spectra for individual dye molecules and even to trace the dynamics of spectral jumps [1]. Most applications in biology, however, sacrifice spectral resolution in order to enhance the overall photon detection yield. Combinations of specific interference filters in the detection pathway are often sufficient to suppress background light, such as Rayleigh and Raman scattering, and allow spectral identification of probe molecules. Other parameters with increasing relevance for optical single-molecule measurements are the fluorescence lifetime [14–16] (i.e. the average decay time of the first excited [fluorescent] state), which is sensitive to the local microenvironment of the probe, and fluorescence polarization anisotropy [17,18,19•,20], which is strongly dependent on rotational mobility of the molecular dipoles. At present, there is a general tendency to simultaneously acquire as many of these parameters as possible, in so-called multidimensional approaches [2•,16,17,20].

Abbreviations
AFM atomic force microscopy
FCS fluorescence correlation spectroscopy
FRET fluorescence resonance energy transfer

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A spectroscopic principle that has recently advanced to become a prominent tool for single molecule studies is fluorescence resonance energy transfer (FRET) [21,22••,23,24•,25••,26–28•] between two spectrally distinguished labels. The labels can be attached to two interacting species [22••,30••] or to different sites of a single protein molecule [22••,23,25••,26–28]. Depending on the spectral properties, distances and relative orientations of these labels, thought of as molecular dipoles, radiationless energy transfer from the excited donor dye to the non-excited acceptor dye is facilitated leading to a decrease in donor fluorescence and an increase in acceptor fluorescence. Therefore, the proper design of fluorescence assays with specifically labeled proteins with different tags at different positions allows the relative distances between the labels to be studied as a FRET pair by a visible change in their relative emission. FRET efficiency can thus be considered a molecular ruler [21] for distances in the range of several nanometers, enabling studies of protein–protein colocalization and/or interaction [22••,30••] and allowing conformational changes within a single protein to be analyzed [23,28]. Although fluorescence as a reporter of the molecular environment has little potential to reveal structural properties, the combination of smart labeling with elaborate detection schemes provides the basis for a ‘dynamic structural biology’ [8].

**Measurements on diffusing single molecules**

An important task for single-molecule detection is the reduction of background noise, preferably accomplished by spatial restriction of the sample volumes. One of the most versatile detection schemes with effective volume elements of less than 10^{-15} L is the open confocal setup, which can easily be adapted to freely diffusing molecules in any transparent environment [14••,24•,25••]. The measurement volume in the radial direction is defined by the diffraction-limited spot of a laser beam focused by a high-resolution objective; a pinhole in the image plane establishes the depth of focus, and thus limits the volume axially. A graphic representation of this so-defined open volume element is given in Figure 1. Photons emanating from single molecules traversing the illuminated region (red trace) are detected by an extremely sensitive avalanche photodiode. If the measurement volume is thought to be empty for most of the time, the arrival and limited dwell time of fluorescent molecules within this region is represented by so-called ‘bursts’ of the measured fluorescence count rate. If photobleaching of the fluorophores is prevented at appropriate power levels, these burst intervals or ‘detection time windows’ are limited only by molecular mobility. Selective analysis of certain fluorescence parameters and characteristic fluctuation time constants can then be performed on single bursts in solution [1,14••,15], in cells [31] or in membranes [32]. Subpopulations, that is, groups of molecules with different mobility [33–35] or different spectral features, such as brightness [36,37,38•], fluorescence lifetime [14••] or anisotropy [17], can be distinguished. Burst analysis on subpopulations of different FRET efficiency, indicating different intramolecular distances of labeled residues, was successfully used to study pathways of protein folding at the single-molecule level [25••]. Both the static and dynamic heterogeneity of processes occurring on timescales faster than the average residence times can be resolved.

An elegant method with high statistical confidence to derive characteristic time constants of single-molecule dynamics is fluorescence correlation spectroscopy (FCS) [33–35,39••]. Here, the fluctuating intensity signal recorded from any fluorescent sample is subject to a mathematical procedure called autocorrelation analysis. Applied to highly dilute samples, FCS provides direct access to fast intramolecular dynamics that modulate the fluorescence intensity [40,41] or lifetime [42] and allows many different modes of molecular mobility to be distinguished, particularly in the cellular environment [33–35]. The two-color variant of FCS, cross-correlation spectroscopy or, more generally, coincidence analysis [39••,43,44], is a sensitive tool to follow the association or dissociation of different molecular species. It has been used to follow the specific cleavage of a double-labeled DNA substrate by a restriction endonuclease at the single-molecule level in real time [39,44], with sampling intervals of less than 100 ms [44]. Recently, two-color coincidence analysis has even enabled diagnostics of sparse pathological prion protein aggregates in cerebrospinal fluid [45•].

**Imaging and dynamic analysis of fixed molecules**

Temporal resolution in open confocal setups is extremely high and only limited by the detector dead time. By averaging large

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**Figure 1**

The principle of confocal single-molecule detection: fluorescent particles are analyzed during their residence in the illuminated focal spot. Measurement parameters are wavelength (λ), fluorescence lifetime (τ), translational mobility (D_{trans}), rotational mobility (D_{rot}), fluorescence fluctuations (ΔF), coincidence or cross-correlation (CC) and FRET. Molecular interactions can be analyzed by changes in mobility and/or anisotropy (D_{trans}, D_{pol}, C.C. or FRET). Intramolecular dynamics are best studied by recording intensity fluctuations (ΔF) resulting from changes in τ or FRET efficiency.
numbers of molecular transit events or bursts, the measurement statistics are usually very good, and by allowing the molecules to diffuse freely the interference of the detection process with the biological system is minimized. On the other hand, processes on larger timescales than the mobility-restricted residence times in the focal spot cannot be followed. For this reason, increasing numbers of investigations are being carried out on immobilized single molecules attached to glass surfaces [20, 22, 26, 28, 29] or embedded in buffer-filled pores of polymers (e.g., polyacrylamide or agarose gels [10, 27, 46]). Molecules are located either using sample-scanning and confocal detection or by widefield illumination in conjunction with extremely sensitive charge-coupled device (CCD) cameras. Lateral resolution down to 50 nm can be obtained [47].

To gain better axial resolution and to suppress the background above and below the focal plane, many researchers [26, 30, 48] implement an illumination strategy using total internal reflection (TIR) of the laser light at a glass surface (e.g., a coverslip). The penetration depth of light into samples on the other side of this surface is only several hundred nanometers. Near-field scanning microscopy [49] is another elegant approach to increase resolution, but has considerable disadvantages if complex sample topologies are to be studied. For analyses in living cells, a particularly important tool is the use of two-photon excitation of fluorophores at wavelengths of approximately twice their absorption maxima [34, 39, 50]. This technique provides dramatically enhanced background suppression, owing to the limitation of the excitation to the focal plane, and has the additional crucial advantage of accessing multiple labels by a single excitation wavelength [39].

Some fascinating studies on single enzymes have been carried out by combining imaging and time-resolved detection on fixed molecules. In measurements on cholesterol oxidase, which is naturally fluorescent in its oxidized form but nonfluorescent in its reduced form, enzymatic turnovers could be observed as an on/off blinking behavior of the immobilized molecules [10]. By recording turnover distributions, the validity of the Michaelis–Menten mechanism could be tested. The most striking outcome of these and similar studies [51] is the finding of dynamic disorder, indicating memory effects of the enzyme (i.e., the activity and dynamics of an enzyme are dependent on previous substrate turnover). Applying FRET and polarization anisotropy measurements to single immobilized copies of staphylococcal nuclease, Ha et al. [22] recorded conformational dynamics and were able to relate the characteristic timescales of FRET efficiency fluctuations to enzyme activity. The principle of studying conformational transitions by FRET has been applied to the folding and unfolding of single proteins [26, 28] and recently also to ribozymes [29], where a folding intermediate was discovered.

In the above-mentioned and other studies [48, 52], much information is derived from changes in the overall fluorescence capacity of single molecules. It is therefore crucial to rule out dye-induced dynamics that have nothing to do with protein activity or conformational changes. Recent measurements on immobilized or free single fluorophores report blinking induced by light or other environmental factors [40, 41, 46] and act as an important corrective to unmask disadvantageous labeling systems and other sources of potential artifacts to be suppressed by data analysis [23].

Spatial tracking of mobile molecules

To investigate certain processive molecules, such as motor proteins [9, 53] or transcription enzymes [54], or to observe functional proteins in their native cellular environment, spatial and temporal information need to be combined. In order to track these molecules over large enough time intervals, but with sufficient spatial and temporal resolution to record colocalization [30, 55] and characteristic dynamics [56, 57, 58], successive imaging of a whole plane or space in conjunction with elaborate image analysis is required. Fluorescence widefield illumination and detection with sensitive CCD cameras have been successfully employed for this purpose. This approach has been used to map single receptor molecules in three-dimensions on cell membranes [56] and to track slow diffusion of single green fluorescent protein molecules through the pores of polyacrylamide gels [57]. Implementing objective TIR, Sako et al. [30] succeeded in unraveling the early events, such as dimerization and autophosphorylation, in the signal transduction of epidermal growth factor (EGF) receptors on live cell surfaces. They tracked single fluorescent spots representing labeled EGF bound to receptors. This landmark study, which provided direct evidence for dimerization before EGF binding, demonstrated that single-molecule investigations are indeed possible in living cells and can provide us with information that cannot be derived from ensemble studies on many molecules.

Conclusions: implications for biotechnology

Besides their impact on basic research, one of the most promising features of methods for single protein detection and analysis is their compatibility with ultra-high-throughput screening strategies. This compatibility could be utilized in the proteomics field, for drug screening purposes, or for the screening-based directed evolution of proteins. Screening applications take advantage of several of the above-mentioned aspects: with access to molecular distributions, rather than the statistical mean, and with high temporal resolution, unique protein characterization is facilitated. Furthermore, the high sensitivity leads to inherent miniaturization and thereby to more compact formats, higher throughput and less consumption of materials [59, 60]. The perspectives of single-molecule-based approaches for the discrimination and sorting of biomolecules with desired properties were envisaged at an early stage [61]. The combination of optical single-protein analysis with corresponding miniaturized sorting devices (e.g., based on microfluidic structures [62, 63]) or other approaches for particle manipulation [64] may constitute the next generation of tools for evolutionary protein design: libraries of gene fragments with their gene product
linked to them could be probed molecule by molecule, classified, and sorted with respect to the protein phenotype. Selected genes would then undergo the next step of variation and expression before being sorted once more, leading cycle by cycle to optimized protein functions.

References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

• of special interest
•• of outstanding interest


A novel approach of polarization-resolved imaging convincingly demonstrates the impact of anisotropy studies for single-molecule analysis on membranes. Anisotropy appears to be a sensitive measure to probe different lipid environments.


The first application of intramolecular FRET to study conformational dynamics and characterize the activity of fixed single enzyme molecules. This study was based on earlier ones of the same group demonstrating the principle of single-molecule structural analysis by FRET, and triggered a large number of similar approaches.


The first piece of work to demonstrate the identification of subpopulations of different FRET efficiency in open confocal volumes with freely diffusing pair-labeled DNA molecules of variable length. Enzymatic cleavage of DNA is shown to shift the FRET efficiency distribution.


The potential of resolving distance heterogeneity of single FRET pairs is discussed for protein folding studies. By observing discrete peaks in the FRET efficiency histograms, rather than a continuous shift, a two-state unfolding mechanism could be verified.


Although carried out on ribozymes rather than proteins, this pioneering study gives a fascinating perspective for the accessibility of intermediate states in single-molecule folding and unfolding.


A comprehensive study that highlights the impact of single-molecule analysis in unravelling the pathways of signal transduction in situ. By imaging and tracking of single receptors using fluorescence coincidences and FRET, the chronological order of ligand binding, aggregation and autophosphorylation could be derived.

Protein technologies and commercial enzymes


