Single Molecule Detection, Analysis, and Manipulation

Nils G. Walter
University of Michigan, Ann Arbor, USA

Single molecule detection represents the ultimate goal in analytical chemistry. Both confocal and wide-field microscopes are now widely used to detect, analyze, and manipulate single molecules in samples ranging in complexity from controlled in vitro conditions to the inside of living cells. Conformational rearrangements and chemical reactions of single molecules can be monitored in real time to detect rare and/or short-lived intermediates and molecular heterogeneity. The molecular motion of single molecules or particles can be followed at high temporal and spatial resolution. Individual molecules can be repeatedly manipulated and their mechanical properties measured using calibrated forces. The current status of the analytical chemistry of single molecules is surveyed here, including fluorescence microscopy, optical tweezers, and atomic force microscopy (AFM).

1 INTRODUCTION

The ultimate goal of analytical chemistry is the detection, analysis, and manipulation of single molecules. In addition to the ability to extract novel, biologically and technologically critical information on the behavior of materials, the direct, real-time observation of single molecules offers broad inspirational appeal to even the scientific layman. The era of single molecule analytical chemistry is in full swing and will likely further expand to profoundly change our fundamental understanding of the physics, chemistry, and biology of matter. With numerous reviews and textbooks now available,[1–19] this survey will, by necessity, only provide a glance at some of the exciting advances of this burgeoning field.

2 HISTORY AND BREADTH OF APPLICATIONS

Over the last decades of the twentieth century, Feynman's suggestion that ''there's plenty of room at the bottom''[20] inspired the quest for techniques that reach the single molecule detection limit. Perhaps the first analytical (bio)chemistry study of single molecules was the one in 1961 observing single β-galactosidase molecules trapped in microdroplets and acting on a fluorogenic substrate.[21] In the 1980s, successful near-field approaches were developed, including scanning tunneling microscopy (STM) and AFM, which use sharp, nanometer-scale tips to probe and manipulate atoms or molecules on surfaces using tunneling electrons and molecular forces, respectively.[22] Modern STM techniques even allow chemical synthesis at the single molecule level,[4] while AFM has been developed into a tool to image and change the conformational states of single nucleic acid and protein biopolymers.[6,11] Complementarily, optical microscopy of single quantum systems was developed to probe ''at a distance'', often resulting in smaller perturbations of the molecule under study at the expense of lower spatial resolution.[1] Among the first implementations, single atomic ions confined

Encyclopedia of Analytical Chemistry R.A. Meyers (Ed.)

in electromagnetic Paul or Penning traps were probed, confirming fundamental physical predictions of quantum mechanics.[23–25] Hans G. Dehmelt and Wolfgang Paul (together with Norman F. Ramsey) shared the Physics Nobel Prize in 1989 for their development of these ion traps, while Steven Chu, Clause Cohen-Tannoudji and William D. Phillips shared a second Physics Nobel Prize in 1997 for their development of atom traps. Around the same time as these developments occurred, optical spectroscopy was combined with near-field probing in near-field scanning optical microscopy (NSOM). In NSOM, a subwavelength probe acts as a ''stethoscope'' to map, at a resolution higher than the diffraction limit, the near field generated by a sample, such as a single molecule on a surface, upon illumination.[26–28] Soon thereafter, wide-field absorption measurements on single pentacene molecules trapped in a *p*-terphenyl crystal at the temperature of liquid helium were realized under conditions of suppressed sample scattering,[29,30] followed by the fluorescence detection of both similarly crystalline samples[31] and fast flowing sample streams that minimize the observed volume.[32] The red-shifted fluorescence emission can be separated from the excitation light so that the necessary signal-to-noise ratio for single molecule detection is reached by straightforward optical filtering. Importantly, in this approach Abbe's law (also sometimes referred to as *Rayleigh's resolution limit*), which intuitively postulates that optical resolution is impossible below ~200 nm, is circumvented by using a dilute sample that separates individual fluorophores by more than the diffraction limit. The same dilution approach was applied to single enzyme molecules in a capillary and led to the discovery of heterogeneities in catalytic activity.[33] It also enabled numerous wide-field fluorescence video microscopy applications that now routinely image single immobilized molecules in condensed matter under ambient conditions.[1–3,7,8,12–14,17–19,34,35] The drawback is that each single molecule appears much larger than its physical dimensions since it is imaged as a diffraction-limited Airy disk, but recent advances are beginning to overcome this limitation.[15,19,36–48] Perhaps the most immediate impact on bioanalytical technology may be expected from the ultimate miniaturization and multiplexing of biological assays afforded by single molecule approaches such as those applied in DNA sequencing.[49] Not surprisingly then, a number of companies are currently commercializing single molecule sequencing.

A separate set of approaches for single molecule detection is based on confocal microscopy. Confocal fluorescence correlation spectroscopy (FCS) limits the illumination and detection volumes through the use of a diffraction-limited laser focus and a small detection pinhole, respectively, so that single diffusing molecules are registered as a sequence of stochastic fluorescence bursts that can be correlated with one another. Developed initially in the 1970s as a way to measure the kinetics of chemical reactions,[50–52] FCS has found its major application in the analytical chemistry of molecular interactions by evaluating diffusion constants and location coincidence.[53–56] In combination with fluorescence resonance energy transfer (FRET) as a molecular ruler, confocal fluorescence microscopy of freely diffusing molecules has also been used to report (bio)polymer folding.[57] Furthermore, FCS in combination with FRET has been applied to single immobilized molecules to access fast rate constants of conformational change.[58] Finally, laser scanning confocal fluorescence microscopy has been utilized to reconstruct a larger field of view of immobilized molecules and zoom in on a single molecule to observe fast enzymatic dynamics.[19,59]

A focused laser light beam results in significant momentum exchange with matter. The resulting 3-D trapping potential is used in optical tweezers to levitate microscopic particles without any physical contact. Starting in the early 1970s, optical tweezers were developed as a noninvasive tool to manipulate polarizable objects such as glass and plastic beads with refractive indices distinct from their environment.[60–63] If a single molecule is suspended between two trapped beads similar in dimension to the wavelength of light, or between a bead and a surface, it can be manipulated (stretched and bent) with forces of up to 300 pN at sub-piconewton resolution or, conversely, forces that molecular motors exert on it can be measured.[5] Modern bioanalytical applications can control the extension of, for example, a single DNA molecule to angstrom resolution, enabling the detection of the incorporation of one nucleotide by a transcribing single RNA polymerase molecule,[64] with potential applications in single molecule sequencing.[65]

In the following, more specific information is given on the experimental implementation of and corresponding requirements for each of these modern single molecule tools.

3 PREPARATION AND HANDLING OF THE FEW

Typical solutions used in ensemble experiments are millimolar to micromolar in concentration of the molecule of interest. A solution with one molecule in a typical volume for single molecule detection, 1 fL, corresponds to a 60 nM solution, which can be diluted further if enrichment of the molecules by affinity capture is employed. Such small concentrations are readily obtained by serial dilution; however, special precautions need to

be taken to prevent adsorptive loss of precious material to vessel walls. Silylation or other types of surface passivation of all materials in contact with the dilute solution becomes important to ensure good control over the actual concentration used for single molecule analysis. Depending on the specific single molecule tool, various approaches can be chosen to prepare molecules suitable for the measurement.[19]

3.1 Preparation of Single Fluorescent Molecules or Particles

Molecules can be labeled with fluorophores either during or after their chemical or enzymatic synthesis.[66–69] Ligation techniques, available for both nucleic acids and proteins, can be used to attach shorter labeled to longer unlabeled segments.[70–72] Macromolecular complexes can be noncovalently assembled from their components, some of which can be specifically fluorophore labeled. Organic dye molecules are most widely used for sensitive single molecule fluorescence detection because of their small size, wide choice of spectral properties, and high stability and quantum yield, but intrinsic fluorophores (such as genetically appended fluorescent proteins)[73–75] and larger, but even more photostable quantum dot labels[76] (or inert nanoparticle scatterers) are gaining popularity. In principle, a single fluorophore can be used for labeling with minimal perturbation of the target molecule, or multiple identical fluorophores for enhanced signal (although at some point self-quenching will limit the emission quantum yield). If even less perturbation is desired, a label-free approach can be employed wherein a single, unlabeled molecule of interest displaces or somehow changes a fluorophore-labeled marker molecule to yield a detectable signal. If distance measurements between two points on the molecule or particle of interest are desired, two distinct fluorophores of suitably overlapping spectral properties for FRET or of sufficiently distinct spectral properties for coincidence or colocalization analysis can be employed. Depending on the fluorescence detection technique, immobilization or trapping of the molecule may be necessary for enhanced sensitivity and/or an extended observation window.[19]

3.2 Immobilization or Trapping of Single Molecules for Fluorescence Detection

Detection techniques such as FCS are often used on diffusive molecules, eliminating the risk of artifacts through surface immobilization. Specific binding to sufficiently passivated surfaces is possible, for example, through the biotin–streptavidin interaction on aminosilylated and pegylated surfaces, allowing for extended observation times and improved signal-to-noise detection through the use of a prism or objective-type total internal reflection fluorescence microscopy (TIRFM).[77,78] Gel[79] and vesicle trapping[80] as well as dielectrophoretic[81] and electrokinetic trapping[15,82] in solution have recently been developed to further safeguard against surface immobilization artifacts, especially in the case of sticky proteins and macromolecular complexes.

3.3 Preparation of Single Molecules or Particles for Mechanical Probing and Manipulation

A different approach is possible when (bio)polymers are probed by optical tweezers or AFM. Sufficiently large or extendable molecules can be coupled through covalently attached small molecule handles such as biotin or digoxigenin to (sub)micrometer-sized polystyrene or silica beads that are handled micromechanically by optical tweezers.[14,83,84] Specific or nonspecific adsorption to the atomic scale tip of an AFM can be used to manipulate single molecules adsorbed or covalently coupled to a flat surface, while the same molecules can be imaged and probed in AFM by steric (nonadsorptive) and chemical tip interactions, respectively, as the tip raster-scans over the surface.[11,85] An advantage of AFM is the fact that it does not require attachment of a fluorophore label or small molecule handle, but the necessary surface adsorption of nucleic acids to like-charged mica through bridging divalent metal ions, for example, as well as the mechanical forces exerted by the AFM tip may lead to structural and functional distortion.[19]

3.4 Microfluidics for Handling and Manipulating Single Molecules

Microfluidics are increasingly used to manipulate small amounts of liquids, typically in the nanoliter range, making them useful for handling and mixing dilute solutions of single cells and molecules.[86–88] In their simplest incarnation, a microfluidic device can be assembled by hand from a fused silica slide, a coverslip, and a double-sided sticky tape to immobilize a dilute solution of molecules on the surface of the coverslip.[77,88] Through fast buffer exchange, reactions can be conveniently induced upon addition of a necessary reactant or cofactor to the immobilized molecules.[89] Microfluidic flow systems can be used to mechanically stretch single (bio)polymers[90–92] or to control their exposure to agents inducing specific reactions, for example, when trapped by optical tweezers.[93] Microfluidic systems have the advantage of low reagent consumption associated with small volumes, but surface adsorption may become a greater problem because of the larger surface-to-volume ratio. Another microfluidic approach entails the use of microdroplets that contain single molecules and can be fused with droplets that contain specific reagents.[21,94]

4 SINGLE MOLECULES IN FOCUS: FLUORESCENCE MICROSCOPY

Optical observation of single fluorescent molecules or particles typically requires efficient fluorophores, detectors, and microscopes. The most commonly used components and approaches are described in the following.

4.1 Ultrasensitive Fluorescence Detection: Fluorophores and Detectors

Detection of single molecules in a fluorescence microscope is ultimately limited by the total number of photons emitted by the detected fluorophore. Since a molecule can, in principle, emit a light quantum every 10 ns based on a typical excited-state lifetime, a photon flux of up to 100 million/s might be expected from a single molecule. However, two problems limit the total photon yield in single molecule detection. First, the fluorophore typically behaves nonideally. Most fluorophores either blink, i.e. visit nonfluorescent dark states, or photobleach, i.e. irreversibly react with typically oxygen in the excited state to give nonfluorescent products, after a few hundred up to a million cycles of excitation and emission. Dark states are often reversible, which can be exploited to repeatedly photoswitch fluorophores to improve spatial resolution in fluorescence microscopy by repeatedly determining the fluorophore position and reconstructing an enhanced image.[19,43,44,46] Photobleaching and, to some extent, dark states can be suppressed by the addition of oxygen scavenger systems.[77,95–97] Second, microscope optics and geometries are nonideal. Only a fraction of the emitted photons will be collected by the optics and some will be lost in the necessary optical filtering of the excitation light from the Stokes (red-)shifted fluorescence photons before they reach the detector.

Inorganic nanocrystals or quantum dots have been proposed as potential replacements for organic dye fluorophores.[76] These nanometer-sized clusters of, for example, zinc sulfide–capped cadmium selenide have broad excitation spectra, while their narrow emission peaks can be tuned through their size, facilitating multiplexed applications. Advantages of quantum dots are their high extinction coefficient and prolonged photostability; disadvantages are their bulkiness, challenging surface functionalization for attachment to (bio)polymers, limited shelf life, and potential toxicity in live cell and animal applications. However, residual blinking can be suppressed and fine-tuned by increasing their shell thickness.[98]

A preferred detector for single-point measurements of single fluorescent molecules, such as in FCS and laser scanning confocal fluorescence microscopy, is the single-photon counting avalanche photodiode (SPAD).[10] Single-photon counting is possible because of the low photon arrival rate in single molecule detection so that individual photoelectron impulses can be discerned. SPADs have the advantage over photomultiplier tubes of higher quantum efficiency and lower required voltage, while their smaller active area is fully sufficient for detection of confocal photons.[10] For detection of single molecules in larger sample areas, an intensified charge coupled device (ICCD) or electron-multiplied charge coupled device (EMCCD) is the detector of choice. A CCD camera is not per se a photon counting device, but an integrating detector with good quantum efficiency. The noise in each pixel of a cooled CCD chip does not significantly increase with integration time, making a longer integration time a route to improved signal-to-noise ratio.[10] Modern CCD cameras use image intensifier tubes with photocathode, microchannel plate, and phosphor screen (in an ICCD) or on-chip multiplication through clock-induced, spurious impact ionization of photoelectrons (in an EMCCD) for the most sensitive fluorescence detection. Fluorescence imaging of the diffraction-limited Airy disk of a single molecule allows measurement of its position at nanometer precision as well as of changes in that position over time, i.e. during molecular-scale movement.[15,19,38–40,43,44,46]

4.2 Near-field Scanning Optical Microscopy

NSOM is based on a simple principle but is nontrivial in its implementation.[10] Using an optical glass fiber drawn to a fine tip (typically 80 nm in diameter), a highly localized excitation is directed toward a surface bound sample. The sides of the fiber are aluminum coated so that only a small fraction of light coupled into the larger distal end of the fiber is transmitted through the tip as an evanescent field. The tip is raster-scanned over the surface using equipment similar to an AFM. As only a very small surface area is excited, there is no need for a confocal aperture and the resolution obtained is determined by the diameter of the fiber tip, rather than the optical resolution limit. NSOM has so far only found limited application since the detection of single molecules often requires specific surface preparation protocols, the raster-scanning is slow, and the fiber tip is very fragile.

4.3 Wide-field Microscopy for Single Molecule Detection

Wide- (or far-)field fluorescence microscopy is attractive for single molecule detection since it uses an area detector that monitors, at reasonably high temporal resolution, the large number of single molecules (up to several hundred)

that are spatially resolved in an optical field of view of the sample.[10] Using an ICCD or EMCCD camera, temporal resolutions of commonly 100 ms and down to a few milliseconds can be obtained. For particularly low light levels, integration over several image frames can be used, leading to a corresponding decrease in temporal resolution. Standard epifluorescence can be used if the sample is thin (for example, a monolayer of cultured living cells) so that there is little or no signal from molecules outside the focal plane.[10] More typical for *in vitro* studies (or studies of cell membrane bound molecules) is the reduction of the illuminated volume through the use of a thin evanescent field generated by prism- or objective-type total internal reflection on a fused silica–sample interface.[10,19] Fluorophores that are within ~150 nm of the interface are excited by the evanescent field that falls off exponentially with distance from the surface. The emission from these typically surface-immobilized fluorophores is collected by an objective, filtered to remove unwanted excitation light, and imaged on a CCD. Typically the same objective is used for excitation and emission collection in objective-type TIRFM, leading to easier alignment but somewhat higher background from aberrant reflections. Color information can in principle be added by using a true color camera, but typically at the price of much lower sensitivity. More efficient is the use of optical filters that can easily split the emission into two or four colors or polarizations and project them on neighboring fields of the CCD for FRET and/or anisotropy analysis. The recent advent of techniques that overcome the optical resolution limit by fitting the diffraction-limited Airy disk of a single molecule with a Gaussian distribution to obtain position measurements over time at nanometer precision has led to a further surge in the popularity of wide-field video microscopy.[15,19,38,39] In some clever applications that exploit the independent photobleaching or blinking behavior of organic dye molecules, the number of and positions of multiple fluorophores within the resolution limit can also be determined at nanometer precision.[15,19,40,43,44,46,99]

4.4 Laser Scanning Confocal Microscopy

Laser scanning confocal microscopy is based on confocal illumination and fluorescence detection.[10] The laser excitation volume is raster-scanned across the sample and the light collected by the objective is focused on a pinhole to pass only light from the desired small and open confocal volume and reject light from the outside. A single-point detector such as an SPAD is then used to measure the emission from each point of the sample. The associated time resolution on a single spot is high (tens of microseconds), but scanning over a surface takes time (typically minutes). The arguably most successful applications of this technique in the single molecule field have involved the focal detection of single, immobilized or trapped (bio)polymers after locating them in a sample scan.[79]

4.5 Fluorescence Correlation Spectroscopy: A Quasi-single Molecule Technique

There are basically two strategies for the observation of single molecules: one embeds them in or on some sort of matrix, whereas the other studies them directly in solution. Free solution detection requires dilution of the molecules and miniaturization of the observation volume. This has been achieved by monitoring solutions of fluorescing molecules within a diffraction-limited open confocal volume, i.e. in an observation volume of less than 1 fL.[10,100] The velocity of a single molecule migrating through the observation volume is measured by detecting it at time t and asking whether it is still present after a time interval τ. Mathematically, this is described by the correlation function (Equation 1):[100]

$$G(t) = \sum I(t) \times I(t + \tau) \tag{1}$$

where τ is the waiting time.

$G(t)$ is related to the Brownian motion of the molecule and therefore can be used to calculate the diffusion constant of the molecule, which in turn yields a molecular weight estimate. This FCS approach may be used to measure the binding of a smaller fluorescent molecule to an at least threefold larger nonfluorescent molecule, as diffusion of the larger complex will slow down. The correlation analysis not only allows measurement of the absolute migration velocities (provided that the confocal detection volume is calibrated), but also of the ratio of free to bound molecules, i.e. the binding constants according to the mass action law. Since one needs to correlate the signals from a sufficient number of molecules and molecular complexes for this analysis, FCS is not a true single molecule technique, but suitable for small concentrations and detection volumes. A closely related technique that yields additional information on the number and fluorescence intensity distribution of single molecules in the detection volume is the photon counting histogram analysis of fluorescence fluctuation spectroscopy.[101]

5 BUILDING SINGLE MOLECULE SUSPENSION BRIDGES: OPTICAL TWEEZERS

Optical tweezers experiments are typically designed to use two attachment sites on a (bio)polymer to suspend and

extend it, or one attachment site to stretch it out linearly via fluid flow. The strong biotin–streptavidin interaction is most commonly used to couple one end of the (bio)polymer to a polysterene bead that has a sufficient size (500–700 nm) and a refractive index sufficiently distinct from an aqueous solution to generate appropriate trapping forces with infrared optical tweezers. The other end of the target molecule is either bound to a surface or a second bead. The second bead can then be controlled by either a glass micropipette or a second optical trap for an assay format in the shape of a dumbbell.[14,102] The dumbbell dual-beam trap affords lower mechanical drift and higher beam-pointing stability, but requires a longer tether length than the single-beam trap. If particularly high manipulation forces (up to 300 pN) are needed, a single-trap dual-beam experiment can be performed where two laser beams converge to one overlapping focus (of ~1μm diameter) that traps a polysterene bead with a single molecule suspended toward a fixed surface or micropipette held bead.[103] Optical tweezers are commonly used to measure force–extension curves as the suspended single (bio)polymer is pulled apart or, conversely, changes its length during a process of interest such as the conversion of a single-stranded into a double-stranded DNA or vice versa. When the extended (bio)polymer generates a counterforce, the tethered trapped bead will be displaced from the center of the tweezing laser focus. This movement is readily detected and the associated force calculated by video microscopy or back-focal-plane interferometry wherein the intensity distribution of the trapping laser after passage through the bead is measured by a quadrant photodiode.

6 SINGLE MOLECULE MECHANICS: DIRECT MANIPULATION WITH THE ATOMIC FORCE MICROSCOPE

In AFM, a several tens of micrometer long cantilever (made by photolithography from materials such as silicon nitride) with a sharp probing tip at its end (ideally narrowing down to a single atom) is raster-scanned over a flat surface. In the imaging mode, a surface contour map is generated with a vertical resolution of a up to 1 Å and a lateral resolution of ~5 Å as the probe sterically interacts with the scanned surface. Typical flat samples are DNA molecules or biomolecular complexes adsorbed onto a mica surface[6] or planar supported lipid bilayers with inserted membrane proteins.[11] AFM imaging is used to study the conformation, assembly, oligomeric state, and reactivity of single (bio)polymers in such samples.[19] To minimally perturb soft biological specimens, the probe is typically gently tapped up and down during scanning (tapping mode). Depending on its chemical properties and mechanical design, the AFM probe can also be used to physisorb or chemisorb a single component of the sample and mechanically extend it by pulling back from the surface. In such a force spectroscopy experiment, the applied pulling force induces a stepwise unfolding of the (bio)polymer's secondary structure.[104,105] The bending of the cantilever during both imaging and force spectroscopy experiments is readily amplified and detected by deflection of a laser beam onto a quadrant photodiode to generate a contour map and force extension curve, respectively.

7 QUO VADIS: WHERE DOES THE FUTURE LEAD?

Current and future advances in the underlying technology of single molecule analysis focus on three broad areas:[19]

1. improving the spatial and temporal resolution limit of single molecule microscopy by breaking Abbe's law and using faster detectors, brighter fluorophores, and improved contrast generation;[15,36–48]
2. combining the single molecule tools of fluorescence microscopy with optical tweezers[106–108] or AFM manipulation[109] to overcome their individual limitations and correlate structural changes directly with the applied mechanical forces;
3. combining single molecule experimental tools with computational ("in silico single molecule")[110] as well as ensemble experimental tools such as nuclear magnetic resonance (NMR)[111] to facilitate interpretation and cross-validation of both.

It is expected that, in combination, all of these past to future developments will be truly transformative in our understanding of the world around us, will enable breakthrough discoveries in all sciences ranging from systems biology in living cells to materials design, and will ensure that analytical chemistry continues to be a critical component in the modern sciences.

ABBREVIATIONS AND ACRONYMS

AFM	Atomic Force Microscopy or Microscope
EMCCD	Electron-multiplied Charge Coupled Device
FCS	Fluorescence Correlation Spectroscopy
FRET	Fluorescence Resonance Energy Transfer
NSOM	Near-field Scanning Optical Microscopy
SPAD	Single-photon Counting Avalanche Photodiode

STM	Scanning Tunneling Microscopy or Microscope
TIRFM	Total Internal Reflection Fluorescence Microscopy

RELATED ARTICLES

Clinical Chemistry **(Volume 2)**
Nucleic Acid Analysis in Clinical Chemistry

Forensic Science **(Volume 5)**
Fluorescence in Forensic Science • Microspectrophotometry in Forensic Science

Nucleic Acids Structure and Mapping **(Volume 6)**
DNA Molecules, Properties and Detection of Single • Optical Mapping in Genomic Analysis

Electronic Absorption and Luminescence **(Volume 12)**
Fluorescence Lifetime Measurements, Applications of

REFERENCES

1. W.E. Moerner, M. Orrit, 'Illuminating single molecules in condensed matter', *Science*, **283**, 1670–1676 (1999).
2. S.H. Leuba, J. Zlatanova, *Biology at the Single Molecule Level*, Pergamon Press, New York, 2001.
3. C. Zander, J. Enderlein, R.A. Keller, *Single Molecule Detection in Solution: Methods and Applications*, Wiley-VCH, Hoboken, NJ, 2002.
4. S.W. Hla, K.H. Rieder, 'STM control of chemical reaction: single-molecule synthesis', *Annu. Rev. Phys. Chem.*, **54**, 307–330 (2003).
5. M.J. Lang, S.M. Block, 'Resource Letter: LBOT-1: Laser-based optical tweezers', *Am. J. Phys.*, **71**, 201–215 (2003).
6. H.G. Hansma, K. Kasuya, E. Oroudjev, 'Atomic force microscopy imaging and pulling of nucleic acids', *Curr. Opin. Struct. Biol.*, **14**, 380–385 (2004).
7. F. Kulzer, M. Orrit, 'Single-molecule optics', *Annu. Rev. Phys. Chem.*, **55**, 585–611 (2004).
8. E.J. Peterman, H. Sosa, W.E. Moerner, 'Single-molecule fluorescence spectroscopy and microscopy of biomolecular motors', *Annu. Rev. Phys. Chem.*, **55**, 79–96 (2004).
9. K.O. Greulich, 'Single-molecule studies on DNA and RNA', *Chem. Phys. Chem.*, **6**, 2458–2471 (2005).
10. J.R. Lakowicz, *Principles of Fluorescence Spectroscopy*, 3rd Edition, Springer, New York, 2006.
11. D.J. Muller, K.T. Sapra, S. Scheuring, A. Kedrov, P.L. Frederix, D. Fotiadis, A. Engel, 'Single-molecule studies of membrane proteins', *Curr. Opin. Struct. Biol.*, **16**, 489–495 (2006).
12. A. Smith, C. Gell, D. Brockwell, *Handbook of Single Molecule Fluorescence Spectroscopy*, Oxford University Press, Oxford, 2006.
13. C. Wang, C. Bai, *Single Molecule Chemistry and Physics: An Introduction*, Springer, Berlin, 2006.
14. W.J. Greenleaf, M.T. Woodside, S.M. Block, 'High-resolution, single-molecule measurements of biomolecular motion', *Annu. Rev. Biophys. Biomol. Struct.*, **36**, 171–190 (2007).
15. W.E. Moerner, 'New directions in single-molecule imaging and analysis', *Proc. Natl. Acad. Sci. U.S.A.*, **104**, 12596–12602 (2007).
16. N.G. Walter, 'Future of biomedical sciences: Single molecule microscopy', *Biopolymers*, **85**, 103–105 (2007).
17. M. Sauer, J. Hofkens, J. Enderlein, *Handbook of Fluorescence Spectroscopy and Imaging: From Ensemble to Single Molecules*, Wiley, Hoboken, NJ, 2008.
18. P.R. Selvin, T. Ha, *Single-Molecule Techniques – A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 2008.
19. N.G. Walter, C.Y. Huang, A.J. Manzo, M.A. Sobhy, 'Do-it-yourself guide: how to use the modern single-molecule toolkit', *Nat. Methods*, **5**, 475–489 (2008).
20. R.P. Feynman, 'There's Plenty of Room at the Bottom', in *Miniaturization*, ed. H.D. Gilbert, Reinhold Publishing Corporation, New York, 282–296, 1961.
21. B. Rotman, 'Measurement of activity of single molecules of beta-D-galactosidase', *Proc. Natl. Acad. Sci. U.S.A.*, **47**, 1981–1991 (1961).
22. P.K. Hansma, V.B. Elings, O. Marti, C.E. Bracker, 'Scanning tunneling microscopy and atomic force microscopy: application to biology and technology', *Science*, **242**, 209–216 (1988).
23. W. Neuhauser, M. Hohenstatt, P. Toschek, H. Dehmelt, 'Optical-Sideband Cooling of Visible Atom Cloud Confined in Parabolic Well', *Phys. Rev. Lett.*, **41**, 233–236 (1978).
24. W. Nagourney, G. Janik, H. Dehmelt, 'Linewidth of single laser-cooled Mg ion in radiofrequency trap', *Proc. Natl. Acad. Sci. U.S.A.*, **80**, 643–646 (1983).
25. W.M. Itano, J.C. Bergquist, D.J. Wineland, 'Laser Spectroscopy of Trapped Atomic Ions. Science', *Science*, **237**, 612–617 (1987).
26. D.W. Pohl, W. Denk, M. Lanz, 'Optical Stethoscopy – Image Recording with Resolution Lambda/20', *Appl. Phys. Lett.*, **44**, 651–653 (1984).
27. A. Lewis, K. Lieberman, 'The Optical near-Field and Analytical-Chemistry', *Anal. Chem.*, **63**, A625–A62 – (1991).

28. R.J. Hamers, 'Scanned probe microscopies in chemistry', *RNA*, **100**, 13103–13120 (1996).
29. W.E. Moerner, L. Kador, 'Optical-Detection and Spectroscopy of Single Molecules in a Solid', *Phys. Rev. Lett.*, **62**, 2535–2538 (1989).
30. W.E. Moerner, L. Kador, 'Finding a Single Molecule in a Haystack – Optical-Detection and Spectroscopy of Single Absorbers in Solids', *Anal. Chem.*, **61**, A1217–A1223 (1989).
31. M. Orrit, J. Bernard, 'Single Pentacene Molecules Detected by Fluorescence Excitation in a Para-Terphenyl Crystal', *Phys. Rev. Lett.*, **65**, 2716–2719 (1990).
32. E.B. Shera, N.K. Seitzinger, L.M. Davis, R.A. Keller, S.A. Soper, 'Detection of Single Fluorescent Molecules', *Chem. Phys. Lett.*, **174**, 553–557 (1990).
33. Q.F. Xue, E.S. Yeung, 'Differences in the Chemical-Reactivity of Individual Molecules of an Enzyme', *Nature*, **373**, 681–683 (1995).
34. R.M. Dickson, D.J. Norris, Y.L. Tzeng, W.E. Moerner, 'Three-dimensional imaging of single molecules solvated in pores of poly(acrylamide) gels', *Science*, **274**, 966–969 (1996).
35. X.S. Xie, J. Yu, W.Y. Yang, 'Living cells as test tubes', *Science*, **312**, 228–230 (2006).
36. M. Schrader, U.G. Hofmann, S.W. Hell, 'Ultrathin fluorescent layers for monitoring the axial resolution in confocal and two-photon fluorescence microscopy', *J. Microsc.*, **191**, 135–140 (1998).
37. M.G. Gustafsson, D.A. Agard, J.W. Sedat, 'I5M: 3D widefield light microscopy with better than 100 nm axial resolution', *J. Microsc.*, **195**, 10–16 (1999).
38. R.E. Thompson, D.R. Larson, W.W. Webb, 'Precise nanometer localization analysis for individual fluorescent probes', *Biophys. J.*, **82**, 2775–2783 (2002).
39. A. Yildiz, J.N. Forkey, S.A. McKinney, T. Ha, Y.E. Goldman, P.R. Selvin, 'Myosin V walks hand-over-hand: single fluorophore imaging with 1.5-nm localization', *Science*, **300**, 2061–2065 (2003).
40. X. Qu, D. Wu, L. Mets, N.F. Scherer, 'Nanometer-localized multiple single-molecule fluorescence microscopy', *Proc. Natl. Acad. Sci. U.S.A.*, **101**, 11298–11303 (2004).
41. A. Egner, S.W. Hell, 'Fluorescence microscopy with super-resolved optical sections', *Trends Cell Biol.*, **15**, 207–215 (2005).
42. M.G. Gustafsson, 'Nonlinear structured-illumination microscopy: wide-field fluorescence imaging with theoretically unlimited resolution', *Proc. Natl. Acad. Sci. U.S.A.*, **102**, 13081–13086 (2005).
43. E. Betzig, G.H. Patterson, R. Sougrat, O.W. Lindwasser, S. Olenych, J.S. Bonifacino, M.W. Davidson, J. Lippincott-Schwartz, H.F. Hess, 'Imaging intracellular fluorescent proteins at nanometer resolution', *Science*, **313**, 1642–1645 (2006).
44. M.J. Rust, M. Bates, X. Zhuang, 'Sub-diffraction-limit imaging by stochastic optical reconstruction microscopy (STORM)', *Nat. Methods*, **3**, 793–795 (2006).
45. K.I. Willig, R.R. Kellner, R. Medda, B. Hein, S. Jakobs, S.W. Hell, 'Nanoscale resolution in GFP-based microscopy', *Nat. Methods*, **3**, 721–723 (2006).
46. M. Bates, B. Huang, G.T. Dempsey, X. Zhuang, 'Multi-color super-resolution imaging with photo-switchable fluorescent probes', *Science*, **317**, 1749–1753 (2007).
47. B. Huang, W. Wang, M. Bates, X. Zhuang, 'Three-dimensional super-resolution imaging by stochastic optical reconstruction microscopy', *Science*, **319**, 810–813 (2008).
48. S. Manley, J.M. Gillette, G.H. Patterson, H. Shroff, H.F. Hess, E. Betzig, J. Lippincott-Schwartz, 'High-density mapping of single-molecule trajectories with photoactivated localization microscopy', *Nat. Methods*, **5**, 155–157 (2008).
49. T.D. Harris, P.R. Buzby, H. Babcock, E. Beer, J. Bowers, I. Braslavsky, M. Causey, J. Colonell, J. Dimeo, J.W. Efcavitch, E. Giladi, J. Gill, J. Healy, M. Jarosz, D. Lapen, K. Moulton, S.R. Quake, K. Steinmann, E. Thayer, A. Tyurina, R. Ward, H. Weiss, Z. Xie, 'Single-molecule DNA sequencing of a viral genome', *Science*, **320**, 106–109 (2008).
50. D. Magde, W.W. Webb, E. Elson, 'Thermodynamic Fluctuations in a Reacting System – Measurement by Fluorescence Correlation Spectroscopy', *Phys. Rev. Lett.*, **29**, 705–708 (1972).
51. D. Magde, E.L. Elson, W.W. Webb, 'Fluorescence correlation spectroscopy. II. An experimental realization', *Biopolymers*, **13**, 29–61 (1974).
52. E.L. Elson, 'Quick tour of fluorescence correlation spectroscopy from its inception', *J. Biomed. Opt.*, **9**, 857–864 (2004).
53. E. Van Craenenbroeck, Y. Engelborghs, 'Fluorescence correlation spectroscopy: molecular recognition at the single molecule level', *J. Mol. Recognit.*, **13**, 93–100 (2000).
54. R. Rigler, E.L. Elson, *Fluorescence Correlation Spectroscopy: Theory and Applications*, Springer Publisher, Berlin, 2001.
55. M. Gosch, R. Rigler, 'Fluorescence correlation spectroscopy of molecular motions and kinetics', *Adv. Drug Deliv. Rev.*, **57**, 169–190 (2005).
56. K. Bacia, S.A. Kim, P. Schwille, 'Fluorescence cross-correlation spectroscopy in living cells', *Nat. Methods*, **3**, 83–89 (2006).
57. A.A. Deniz, T.A. Laurence, M. Dahan, D.S. Chemla, P.G. Schultz, S. Weiss, 'Ratiometric single-molecule studies of

freely diffusing biomolecules', *Annu. Rev. Phys. Chem.*, **52**, 233–253 (2001).

58. H.D. Kim, G.U. Nienhaus, T. Ha, J.W. Orr, J.R. Williamson, S. Chu, 'Mg2+-dependent conformational change of RNA studied by fluorescence correlation and FRET on immobilized single molecules', *Proc. Natl. Acad. Sci. U.S.A.*, **99**, 4284–4289 (2002).
59. X.S. Xie, J.K. Trautman, 'Optical studies of single molecules at room temperature', *Annu. Rev. Phys. Chem.*, **49**, 441–480 (1998).
60. A. Ashkin, 'Acceleration and Trapping of Particles by Radiation Pressure', *Phys. Rev. Lett.*, **24**, 156–159 (1970).
61. A. Ashkin, J.M. Dziedzic, J.E. Bjorkholm, S. Chu, 'Observation of a Single-Beam Gradient Force Optical Trap for Dielectric Particles', *Opt. Lett.*, **11**, 288–290 (1986).
62. A. Ashkin, J.M. Dziedzic, 'Optical trapping and manipulation of viruses and bacteria', *Science*, **235**, 1517–1520 (1987).
63. A. Ashkin, J.M. Dziedzic, T. Yamane, 'Optical trapping and manipulation of single cells using infrared laser beams', *Nature*, **330**, 769–771 (1987).
64. E.A. Abbondanzieri, W.J. Greenleaf, J.W. Shaevitz, R. Landick, S.M. Block, 'Direct observation of base-pair stepping by RNA polymerase', *Nature*, **438**, 460–465 (2005).
65. W.J. Greenleaf, S.M. Block, 'Single-molecule, motion-based DNA sequencing using RNA polymerase', *Science*, **313**, 801 (2006).
66. N.G. Walter, J.M. Burke, 'Fluorescence assays to study structure, dynamics, and function of RNA and RNA-ligand complexes', *Methods Enzymol.*, **317**, 409–440 (2000).
67. N.G. Walter, 'Structural dynamics of catalytic RNA highlighted by fluorescence resonance energy transfer', *Methods*, **25**, 19–30 (2001).
68. L.J. Kricka, 'Stains, labels and detection strategies for nucleic acids assays', *Ann. Clin. Biochem.*, **39**, 114–129 (2002).
69. N.G. Walter, 'Probing RNA structural dynamics and function by fluorescence resonance energy transfer (FRET)', *Curr. Protoc. Nucleic Acid Chem.*, **11.10**, 11.10.11–11.10.23 (2002).
70. W.C. Kurschat, J. Muller, R. Wombacher, M. Helm, 'Optimizing splinted ligation of highly structured small RNAs', *RNA*, **11**, 1909–1914 (2005).
71. V. Muralidharan, T.W. Muir, 'Protein ligation: an enabling technology for the biophysical analysis of proteins', *Nat. Methods*, **3**, 429–438 (2006).
72. M.R. Stark, J.A. Pleiss, M. Deras, S.A. Scaringe, S.D. Rader, 'An RNA ligase-mediated method for the efficient creation of large, synthetic RNAs', *RNA*, **12**, 2014–2019 (2006).
73. R. Yuste, 'Fluorescence microscopy today', *Nat. Methods*, **2**, 902–904 (2005).
74. J. Yu, J. Xiao, X. Ren, K. Lao, X.S. Xie, 'Probing gene expression in live cells, one protein molecule at a time', *Science*, **311**, 1600–1603 (2006).
75. J. Elf, G.W. Li, X.S. Xie, 'Probing transcription factor dynamics at the single-molecule level in a living cell', *Science*, **316**, 1191–1194 (2007).
76. X. Michalet, F.F. Pinaud, L.A. Bentolila, J.M. Tsay, S. Doose, J.J. Li, G. Sundaresan, A.M. Wu, S.S. Gambhir, S. Weiss, 'Quantum dots for live cells, *in vivo* imaging, and diagnostics', *Science*, **307**, 538–544 (2005).
77. T. Ha, 'Single-molecule fluorescence resonance energy transfer', *Methods*, **25**, 78–86 (2001).
78. R. Roy, S. Hohng, T. Ha, 'A practical guide to single-molecule FRET', *Nat. Methods*, **5**, 507–516 (2008).
79. H.P. Lu, L. Xun, X.S. Xie, 'Single-molecule enzymatic dynamics', *Science*, **282**, 1877–1882 (1998).
80. E. Boukobza, A. Sonnenfeld, G. Haran, 'Immobilization in surface-tethered lipid vesicles as a new tool for single biomolecule spectroscopy', *J. Phys. Chem. B*, **105**, 12165–12170 (2001).
81. J. Voldman, R.A. Braff, M. Toner, M.L. Gray, M.A. Schmidt, 'Holding forces of single-particle dielectrophoretic traps', *Biophys. J.*, **80**, 531–541 (2001).
82. A.E. Cohen, W.E. Moerner, 'Method for trapping and manipulating nanoscale objects in solution', *Appl. Phys. Lett.*, **86**, 093109 (2005).
83. C. Bustamante, Z. Bryant, S.B. Smith, 'Ten years of tension: single-molecule DNA mechanics', *Nature*, **421**, 423–427 (2003).
84. I. Tinoco, P.T.X. Li, and C. Bustamante, 'Determination of thermodynamics and kinetics of RNA reactions by force', *Q. Rev. Biophys.*, 1–36 (2006).
85. P. Hinterdorfer, Y.F. Dufrene, 'Detection and localization of single molecular recognition events using atomic force microscopy', *Nat. Methods*, **3**, 347–355 (2006).
86. E.A. Ottesen, J.W. Hong, S.R. Quake, J.R. Leadbetter, 'Microfluidic digital PCR enables multigene analysis of individual environmental bacteria', *Science*, **314**, 1464–1467 (2006).
87. J. Melin, S.R. Quake, 'Microfluidic large-scale integration: the evolution of design rules for biological automation', *Annu. Rev. Biophys. Biomol. Struct.*, **36**, 213–231 (2007).
88. L.R. Brewer, P.R. Bianco, 'Laminar flow cells for single-molecule studies of DNA-protein interactions', *Nat. Methods*, **5**, 517–525 (2008).
89. S. Liu, G. Bokinsky, N.G. Walter, X. Zhuang, 'Dissecting the multistep reaction pathway of an RNA enzyme

by single-molecule kinetic ''fingerprinting''', *Proc. Natl. Acad. Sci. U.S.A.*, **104**, 12634–12639 (2007).

90. T.T. Perkins, D.E. Smith, R.G. Larson, S. Chu, 'Stretching of a single tethered polymer in a uniform flow', *Science*, **268**, 83–87 (1995).
91. A.M. van Oijen, P.C. Blainey, D.J. Crampton, C.C. Richardson, T. Ellenberger, X.S. Xie, 'Single-molecule kinetics of lambda exonuclease reveal base dependence and dynamic disorder', *Science*, **301**, 1235–1238 (2003).
92. J.T. Mannion, H.G. Craighead, 'Nanofluidic structures for single biomolecule fluorescent detection', *Biopolymers*, **85**, 131–143 (2007).
93. M. Spies, I. Amitani, R.J. Baskin, S.C. Kowalczykowski, 'RecBCD Enzyme Switches Lead Motor Subunits in Response to chi Recognition', *Cell*, **131**, 694–705 (2007).
94. J. Tang, A.M. Jofre, G.M. Lowman, R.B. Kishore, J.E. Reiner, K. Helmerson, L.S. Goldner, M.E. Greene, 'Green fluorescent protein in inertially injected aqueous nanodroplets', *Langmuir*, **24**, 4975–4978 (2008).
95. I. Rasnik, S.A. McKinney, T. Ha, 'Nonblinking and long-lasting single-molecule fluorescence imaging', *Nat. Methods*, **3**, 891–893 (2006).
96. D.J. Crawford, A.A. Hoskins, L.J. Friedman, J. Gelles, M.J. Moore, 'Visualizing the splicing of single pre-mRNA molecules in whole cell extract', *RNA*, **14**, 170–179 (2007).
97. C.E. Aitken, R.A. Marshall, J.D. Puglisi, 'An oxygen scavenging system for improvement of dye stability in single-molecule fluorescence experiments', *Biophys. J.*, **94**, 1826–1835 (2008).
98. B. Mahler, P. Spinicelli, S. Buil, X. Quelin, J.P. Hermier, B. Dubertret, 'Towards non-blinking colloidal quantum dots', *Nat. Mater.*, **7**, 659–664 (2008).
99. M.P. Gordon, T. Ha, P.R. Selvin, 'Single-molecule high-resolution imaging with photobleaching', *Proc. Natl. Acad. Sci. U.S.A.*, **101**, 6462–6465 (2004).
100. E. Haustein, P. Schwille, 'Fluorescence correlation spectroscopy: novel variations of an established technique', *Annu. Rev. Biophys. Biomol. Struct.*, **36**, 151–169 (2007).
101. Y. Chen, J.D. Muller, P.T. So, E. Gratton, 'The photon counting histogram in fluorescence fluctuation spectroscopy', *Biophys. J.*, **77**, 553–567 (1999).
102. J.R. Moffit, Y.R. Chemla, S.B. Smith, C. Bustamante, 'Recent advances in optical tweezers', *Annu. Rev. Biochem.*, **77**, 205–228 (2008).
103. M.J. McCauley, M.C. Williams, 'Mechanisms of DNA binding determined in optical tweezers experiments', *Biopolymers*, **85**, 154–168 (2007).
104. F. Oesterhelt, D. Oesterhelt, M. Pfeiffer, A. Engel, H.E. Gaub, D.J. Muller, 'Unfolding pathways of individual bacteriorhodopsins', *Science*, **288**, 143–146 (2000).
105. A.P. Wiita, R. Perez-Jimenez, K.A. Walther, F. Grater, B.J. Berne, A. Holmgren, J.M. Sanchez-Ruiz, J.M. Fernandez, 'Probing the chemistry of thioredoxin catalysis with force', *Nature*, **450**, 124–127 (2007).
106. A. Ishijima, H. Kojima, T. Funatsu, M. Tokunaga, H. Higuchi, H. Tanaka, T. Yanagida, 'Simultaneous observation of individual ATPase and mechanical events by a single myosin molecule during interaction with actin', *Cell*, **92**, 161–171 (1998).
107. M.J. Lang, P.M. Fordyce, A.M. Engh, K.C. Neuman, S.M. Block, 'Simultaneous, coincident optical trapping and single-molecule fluorescence', *Nat. Methods*, **1**, 133–139 (2004).
108. S. Hohng, R. Zhou, M.K. Nahas, J. Yu, K. Schulten, D.M. Lilley, T. Ha, 'Fluorescence-force spectroscopy maps two-dimensional reaction landscape of the holliday junction', *Science*, **318**, 279–283 (2007).
109. R.J. Owen, C.D. Heyes, D. Knebel, C. Rocker, G.U. Nienhaus, 'An integrated instrumental setup for the combination of atomic force microscopy with optical spectroscopy', *Biopolymers*, **82**, 410–414 (2006).
110. S.E. McDowell, N. Spackova, J. Sponer, N.G. Walter, 'Molecular dynamics simulations of RNA: An in silico single molecule approach', *Biopolymers*, **85**, 169–184 (2007).
111. K.A. Henzler-Wildman, V. Thai, M. Lei, M. Ott, M. Wolf-Watz, T. Fenn, E. Pozharski, M.A. Wilson, G.A. Petsko, M. Karplus, C.G. Hubner, D. Kern, 'Intrinsic motions along an enzymatic reaction trajectory', *Nature*, **450**, 838–844 (2007).