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ABSTRACT

We review some of the progress of single-molecule optical experiments in the past 20 years and propose some perspectives for the coming years. We particularly focus on methodological advances in fluorescence, super-resolution, photothermal contrast, and interferometric scattering and briefly discuss a few of the applications. These advances have enabled the exploration of new emitters and quantum optics; the chemistry and biology of complex heterogeneous systems, nanoparticles, and plasmonics; and the detection and study of non-fluorescing and non-absorbing nano-objects. We conclude by proposing some ideas for future experiments. The field will move toward more and better signals of a broader variety of objects and toward a sharper view of the surprising complexity of the nanoscale world of single (bio-)molecules, nanoparticles, and their nano-environments.

I. INTRODUCTION

Optical microscopy's spatial resolution is limited by diffraction to a few hundred nanometers. Therefore, its application to nanoscience is not as commonplace as that of electron microscopy, whose resolution is limited by the wavelength of electrons down to a fraction of a nanometer, or that of scanning probe microscopies STM and AFM, whose resolution is essentially determined by atomic-sized tips. However, optical photons interact very specifically with particular excited states of atoms, molecules, and solids so that individual localized electronic states, and thereby individual atoms, molecules, and defects in solids, can be detected and studied optically. Moreover, under proper conditions, the diffraction barrier of optics can be lifted by means of various super-resolution techniques.[1–3] Even more importantly, optics have very specific advantages compared to scanning probes and to electron microscopy: most optical methods are non-invasive, and they are not limited to the surfaces of solid bodies, but enable in-depth probing of liquids and solids, provided that these are transparent enough for the used wavelengths. Optical microscopy is compatible with a wide range of conditions, from cryogenic to ambient and high temperatures and from ambient to high pressures. It requires no vacuum and applies to complex materials ranging from catalyst nanoparticles all the way to living matter. Finally, the resonance of photons at optical frequencies with electronic levels of atoms and molecules enables spectroscopic observations, which sensitively report on these electronic states and, therefore, on the structure and dynamics of matter down to molecular scales. These decisive advantages explain the spectacular expansion of single-molecule optical methods in the past 20 years, despite the limited spatial resolution of optical microscopy images.

Single-molecule optics commonly relays a signal from a single localized electronic state (which we henceforth call a molecule, but which could be an atom, ion, nanoparticle, defect in a solid, etc.) directly from the nanoscale to the laboratory scale. It requires that only one optically interacting molecule sits in the diffraction-limited detection volume and that this molecule emits, or interacts with, enough photons to produce a reliable signal above background and noise. Historically, the first extinction experiment of Moerner and Kador[4] was succeeded by fluorescence experiments, providing a much improved signal-to-background ratio.[5] The selection of single molecules is made possible by resonance of the exciting photons with a molecular excited state. Although Raman scattering and fluorescence obey similar selection rules, the continuous transition from one to the other demonstrated in pre-resonant Raman scattering highlights the spectacular enhancement due to

resonance. Because of resonance, fluorescence is several orders of magnitude brighter than non-resonant Raman scattering. After fluorescence or photoluminescence, other optical methods have been developed to reach single-molecule sensitivity. Photothermal microscopy is based on absorption, i.e., on the dissipation of optical energy into heat as opposed to extinction of the incident light beam. Photothermal detection rejects background through high-frequency modulation of a heating beam and lock-in detection of the variations of scattered intensity of a probe beam.[6] This method reaches single-molecule sensitivity under favorable conditions.[7] Efficient subtraction of background in space and/or time makes it even possible to detect extinction changes produced by individual nanoparticles[8–10] or by single protein molecules.[11–14] The latter technique, often dubbed iSCAT, requires specific signatures from the particles to be detected, in space, time, or other property, to distinguish them from other scattering objects in their environment, such as impurities, defects of glass slides, and organelles of cells.

Beyond the technical challenge of detecting individual molecules optically, it is instructive to reflect on the specific advantages of single-molecule measurements vs conventional ensemble measurements. The complete removal of ensemble averaging offers unique benefits. It provides direct access to inhomogeneous static or quasi-static distributions. As a consequence, it is much more forgiving to imperfections, impurities, or heterogeneities in sample preparation; indeed, a single molecule is always 100% pure. In kinetics experiments, a single molecule does not require any synchronization step before a sequence of states can be measured and assigned;[15] indeed, a single molecule is in a single, possibly unknown, state at every single time. This property is particularly attractive for biomolecules and for soft-matter systems in which very subtle interactions and conformational changes determine the molecular state because these interactions cannot be precisely controlled in time and/or space. Fleeting intermediates in reactions, such as transition states upon barrier crossing, which may exist during very short time intervals only, appear in a long single-molecule trajectory even in the midst of a long uneventful period. The detection of such intermediates in a large ensemble is much more challenging. Another example of kinetic information uniquely accessible on single molecules is the sequence of states occupied by single proteins, which directly appears from a single-molecule trajectory,[16] whereas time-tagged information on a sequence of states is all but hidden in unsynchronized ensembles. These unique advantages explain why single-molecule spectroscopy has developed steadily in the past 30 years and has found applications in many fields ranging from heterogenous catalysis and materials science to bio-physics and bio-chemistry.

The present Perspective updates an earlier perspective written by one of us some 20 years ago,[17] which focused on single-molecule fluorescence at cryogenic temperatures. In the intervening time, single-molecule spectroscopy has developed in several exciting and unexpected directions, which include quantum optics and single molecules in interaction with plasmonic structures and with optical micro- and nano-cavities. Other entirely new branches have sprouted from the study and applications of new emitters, such as color centers; from the search for new methods, such as label-free absorption and scattering; and from the many super-resolution methods and their applications to bioscience questions. Single molecules have been applied more widely to explore complex and heterogeneous systems at molecular scales, free from ensemble averaging. In the following lines, we intend to review some chosen subjects and reflect on our best hopes for future developments in single-molecule optics.

II. RECENT ADVANCES IN SINGLE-MOLECULE SPECTROSCOPY

The explosive growth of single-molecule fluorescence in the 1990s has led several groups to search for more general optical methods to detect single-molecule signals, which may complement fluorescence and remedy some of its weak points: (i) its need for fluorescent labels, a rather restricted class of conjugated molecules that may interfere with the phenomena to be studied; (ii) the limited photostability of fluorescent labels, which may blink and bleach, thereby aborting signal acquisition on the one molecule under study; and (iii) the comparative weakness of fluorescence signals related to the fluorescence lifetime of some nanoseconds and to the limited collection efficiency of this multidirectional emission. A simple but general scheme for an optical experiment on a small object, molecule, or nanoparticle requires excitation with an incident wave, most often by a diffraction-limited tightly focused beam and in other cases with a collimated beam (see Fig. 1). Under excitation by the incident wave, the molecule emits a "secondary" optical wave into the far field, which may be at the same exciting wavelength or spectrally shifted away from it. This emitted wave bears information about the molecule that we are interested in so that we strive to detect it with the highest possible sensitivity. The weakness of single-molecule signals implies that we must efficiently filter the desired signal from the strong background of the exciting wave and of other waves reflected, transmitted, or scattered by the sample.[18] In single-molecule fluorescence [see Fig. 1(a)], as the secondary wave's color differs from the excitation's, it can easily be separated by means of sharp color filters (often multi-dielectric filters), which reject the excitation light with a high rejection ratio of several, typically 6–10, orders of magnitude. Some emitters have long photoluminescence lifetimes, which enables time-resolved separation of their luminescence from a pulsed excitation. Other characteristics of the emitted photons, such as their propagation direction and/or their polarization, can, in principle, be used for the isolation of the secondary wave, but rejection of the excitation is often too poor to rely entirely on these separation methods alone.

Non-fluorescent objects may interact with excitation light but will not emit spectrally shifted light. In that case, the only available optical wave has the same wavelength as the excitation. (We ignore Raman scattering and the weakly spectrally shifted Rayleigh–Brillouin scattering because of their weak intensity.) The most general detection method in this case is scattering, which can be applied in two versions. The first one, dark-field scattering, requires observation in directions where excitation light is absent [Fig. 1(b)]. The ultramicroscope invented by Siedentopf and Zsigmondy in 1902[19] and excitation by total internal reflection (TIR) of gold nanoparticles[20] are good examples of dark-field scattering with high rejection of the exciting wave. However, as the scattered intensity scales as the square of the volume of the particle, scattering by small particles cannot be easily distinguished from weak but omnipresent scattering by other uninteresting impurities and

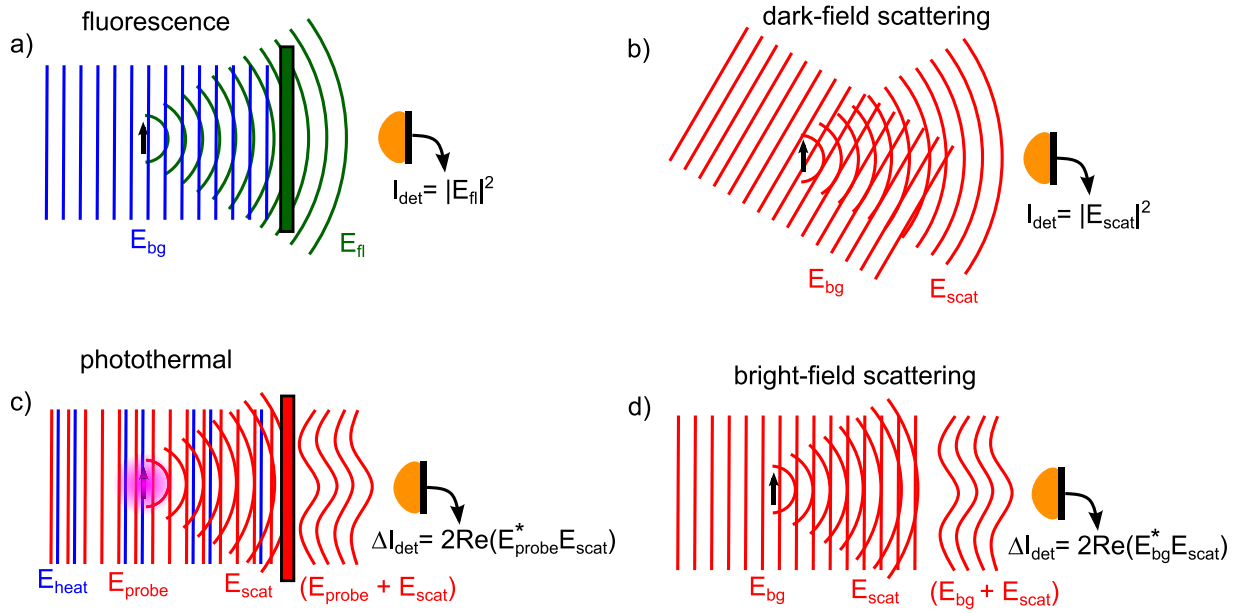

FIG. 1. Schematic representations of fundamental principles of (a) fluorescence microscopy, (b) dark-field scattering microscopy, (c) photothermal microscopy, and (d) bright-field microscopy or interferometric scattering (iSCAT) microscopy. (a) In fluorescence microscopy, a single molecule or particle here represented as a single dipole emits fluorescence (green) as a spherical wave, which is filtered from the background (blue) by using a dichroic filter. The detected fluorescence signal is the square of the amplitude of the fluorescence electric field. (b) In dark-field scattering microscopy, with the background wave and the spherical wave scattered by the single dipole having different angles, the detector can selectively be positioned to collect the scattered signal only. The detected intensity is thus only the square of the scattered electric field. (c) In photothermal microscopy, two lasers are used: a heating laser (blue) and a probe laser (red). The heating laser is intensity-modulated, while the probe laser is continuous. A thermal lens (magenta) is formed surrounding the single dipole after the non-radiative relaxation of the absorbed heating photons. The wave scattered by the thermal lens usually dominates scattering by the dipole itself. The wave scattered by the thermal lens interferes with the reflected or transmitted probe beam. The interference signal, i.e., the real part of the product of the electric fields of the probe and scattered beams, is afterward detected as a change of the detection signal by rejecting background through high-frequency modulation. (d) In bright-field scattering microscopy, the spherical wave scattered by a single dipole interferes with the reflected or transmitted probe wave and the interference signal is detected as the real part of the product of the probe and scattered electric fields. In this case, the interference signal is detected as a change of the detection signal by rejecting background by spatial and temporal filtering.

defects. Thus, dark-field scattering is too weak, in practice, for the detection of metal particles below 30 nm and of dielectric particles below 50 nm in diameter. The second scattering method, bright-field scattering, relies on the detection of *changes* of the scattered intensity and is better suited for very small particles. In this case, the scattered field interferes with the excitation field so that the change in intensity scales linearly with the volume of the particle, thus more favorably than dark-field scattering [Fig. 1(d)]. Also known as iSCAT,[21] bright-field scattering requires a careful subtraction of the background of unscattered excitation light. Extinction, i.e., the measurement of the change of the transmitted intensity due to scattering and absorption by a small object, is a special case of bright-field scattering. Indeed, according to the optical theorem,[18] the scattered wave interferes with the incident wave so as to exactly produce the attenuation of the incident wave measured as extinction in conventional ensemble experiments.

Finally, the absorption of light itself can be detected specifically, even when photoluminescence is extremely weak. Absorption-based methods apply, for example, to metal nanoparticles, semiconductor nanocrystals, and conjugated polymers. The absorption of light is not detected through the missing absorbed photons, i.e., through extinction, as is done in a standard spectrophotometer. Indeed, this weak signal would be difficult to isolate from the photon noise of the, usually weak, unabsorbed beam. Instead, it is interesting to detect a consequence of the absorption process itself, which only arises upon photon absorption, but not upon photon scattering. Examples of such consequences are the production of heat detected through scattering changes[22] [Fig. 1(c)] or changes of other optical signals due to a change of electronic state upon absorption of one or more photons, as used in coherent Raman scattering and derived methods.[23] Only in exceptional cases is the absorbed optical energy re-emitted as (photo-)luminescence that is then often distinguishable from the exciting wave by its frequency, giving rise to fluorescence or photoluminescence. In this case, as mentioned earlier, this emission is very easy to separate from excitation with suitable spectral filters with very efficient throughput and excellent rejection of the excitation light. However, absorption-based methods apply to a much broader class of objects, even though the signal-to-noise ratio is usually lower than that of fluorescence methods.

Figure 1 represents the four possible detection schemes discussed above: (i) fluorescence, (ii) dark-field scattering,

(iii) photothermal contrast, and (iv) bright-field scattering. In summary, the optical excitation gives rise to different effects, which can be used for optical detection. First, when a significant luminescence is emitted by the objects to be detected [Fig. 1(a)], usually at a different wavelength or at a delayed time, spectral or time-resolved separation by suitable optical elements enables excellent rejection of the excitation light, leading to the powerful fluorescence or photoluminescence methods for single-object optical detection. Very strong scatterers such as medium-sized plasmonic particles can be imaged directly in the dark field [Fig. 1(b)]. Photothermal detection and other pump–probe methods make use of a second auxiliary beam [Fig. 1(c)] to probe a consequence of the excitation, which indirectly reports on the absorption of the first beam. Finally, one can monitor the scattered wave through its interference with a reference wave [Fig. 1(d)] both at the same frequency as the excitation (or very close to it in the case of Rayleigh and Brillouin scattering). The iSCAT method detects this interference with a reference wave, which can be the transmitted or reflected beam or even light scattered by another object than the one of interest.

In the following, we review recent progress in single-molecule optics, starting with fluorescence-based methods, then absorption-based photothermal detection, and finally scattering-based detection.

A. New fluorescent emitters

Since the early 1990s, fluorescence has remained the workhorse method in single-molecule optics and has led to totally new insights and approaches in the study of complex systems in cell biology and materials science. Yet, important progress has been done in the last 20 years in the techniques, labels, and applications of fluorescence-based single-molecule spectroscopy. In the next paragraphs, we highlight some of the directions in single-molecule fluorescence, which have experienced much change in the past few years.

Historically, the first molecules to be detected individually were first rigid aromatic molecules in rigid matrices at low temperature and soon after that dyes at room temperature in polymer films and aqueous solutions. The latter dye molecules were typically laser and fluorescent dyes, which were often water-soluble and could be employed in biochemistry and cell biology. Even in this relatively old field, new designs have been proposed to improve the fluorescence properties, reduce bleaching and control blinking, and extend the range of colors accessible to fluorescence microscopy. New dyes were proposed not only for multiplexing and superresolution[24] but also for vibrational imaging by coherent Raman scattering.[25] The spectroscopy of individual emitters has been extended to more complex systems, such as individual conjugated polymers,[26–29] and to inorganic quantum dots,[30–34] including more recently single perovskite nanocrystals.[35–38] Novel architectures and materials open the road to controlling bleaching and blinking, so as to offer stable signals over long integration times. Spectacular quantum optical and magnetometry experiments have appeared with color centers in high-bandgap materials, such as the NV center in diamond,[39–43] which greatly benefited from the synthesis of high-quality diamond. The spectroscopy and photophysics of those color centers resemble those of molecules on many points, but their triplet ground state endows them with sensitivity to magnetic interactions. Sensitive magnetometry at nanometer scales and dynamical decoupling with hyperfine interactions have all been demonstrated with single NV color centers, which remain unique testbed systems for quantum information treatment. Plasmonic metal nanoparticles, which will be discussed below, also rather surprisingly emerged as useful photoluminescent labels.[44,45] Carbon dots are graphitic nanoparticles obtained by pyrolysis of organic compounds, which present bright blue emission. They often contain aromatic nitrogens, which shift their absorption and emission toward the red.[46] A summary of photophysical properties of some important classes of emitters is presented in Table I.

B. Superresolution

The use of single molecules for superlocalization, i.e., the localization of sources of a train of fluorescence photons, has led to superresolution fluorescence imaging.

Techniques such as photo-activated localization microscopy (PALM), stochastic optical reconstruction microscopy (STORM), and point accumulation for imaging in nanoscale topography (PAINT) have led to a revolution in fluorescence microscopy, mostly applied to biological samples. They yield images with resolutions that can become comparable to that of cryo-electron microscopy. We do not review this field here as it extends beyond single-molecule observations. Stimulated emission depletion, STED,[76] in fact, does not rely on single molecules at all, but on point-spread-function engineering and photophysical saturation. Single-molecule superlocalization leads to superresolution images when the density of molecules localized is high enough. Several schemes have been designed: PALM,[77] STORM,[78] and fluorescence-PALM[79] control switching from the dark to bright state by a photo-activation laser. The same switching process occurs spontaneously and randomly in direct-STORM,[80] which relies on blinking due to chemically assisted transitions to and from dark states. Here, the control parameters are concentrations, pH, and redox potentials of the sample solution. It is even possible to do away with activation completely in PAINT and derived methods[81,82] by exploiting transient binding of dyes on the structures to be imaged: transient immobilization of the dye at a given position enables superlocalization at this position, whereas freely diffusing dyes only contribute to background and are removed from the image. Different immobilization schemes have been designed, for example, DNA hybridization in DNA-PAINT.[83]

C. Cryogenic single-molecule spectroscopy

At cryogenic temperature, the spectral linewidth of a single molecule is very narrow, typically lifetime-limited. Such a sharp spectral line is sensitive to changes in the local environment or to interactions of the molecule with external electromagnetic fields. Cryogenic single-molecule spectroscopy enables the study of spatial and temporal heterogeneities on a nanometer length scale. Aside from the spectroscopy of NV centers in diamond, which expanded as a whole new independent field,[84] many quantum-optical experiments have been performed on the narrow lifetime-limited optical zero-phonon lines of single molecules under cryogenic conditions. One of their first applications has been as sources of single

TABLE I. Some photo-physical properties of various photoluminescent systems. The values indicated are coarse estimates meant for qualitative discussion purposes only. Low (room) T: low (room) temperature.

System of interest	Size	Quantum yield	Absorption cross section	Spectral line-width	Photostability	Luminescence lifetime
Organic fluorescent dyes (at low T)	$\lesssim$1 nm	$\lesssim$1	10^{-10} cm^2 [47]	~0.2 μeV[48]	Minutes to days	~4 ns[49]
Organic fluorescent dyes (at room T)	$\lesssim$1 nm	0.1–0.95[50]	10^{-16} cm^2 [51]	~0.2 eV[52]	Seconds to minutes	~4 ns[53,50]
Semiconducting quantum dots (at low T)	5–50 nm	Up to 1.0[54]	~10^{-14} cm^2 [55]	~300 μeV[56]	Several days[56]	~200 ns[57]
Semiconducting quantum dots (at room T)	5–50 nm	Up to 1.0[54]	~10^{-15} cm^2 [58]	~50 meV[59]	Several hours	~20 ns[58]
NV center (at low T)	0.3 nm	0.7–0.8		~0.4 μeV[60]	Hours to days	~10 ns[61]
NV center (at room T)	0.3 nm[62]	0.7–0.8[63]	5 × 10^{-17} cm^2 [62]		Hours to days	10–25 ns[64]
Perovskite nanocrystals (at low T)	50–500 nm			~170 μeV[65]		~200 ps[65]
Perovskites nanocrystals (at room T)	~10 nm[65]	0.5[65]–0.85[36]		~90 meV[65]	Minutes to hours[66]	
Auto-fluorescent proteins	$\lesssim$5 nm	0.2–0.8[67]	$\lesssim 10^{-16}$ cm^2 [67]	~0.1 eV	Seconds to minutes[68]	~4 ns[69]
Metallic nanoparticles	5–100 nm	10^{-6}–10^{-7} [70]	10^{-13}–10^{-9} cm^2 [70]	~0.1 eV	Years	$\lesssim$1 ps
Conjugated polymers	5–10 nm	~0.1[71]	10^{-15}–10^{-14} cm^2 [71]	~0.1 eV	Seconds to minutes	$\lesssim$300 ps[72]
Carbon dots	2–25 nm[73]	0.01–0.9[73]		~0.1 eV	Several hours[73]	~10 ns[73]
Defects in van der Waals materials (hBN, MoS_2)	<1 nm	0.01–0.95[74]		~50 meV[74]		~10 ns[74]
Carbon nanotubes	40–1000 nm	0.001–0.01[75]	~10^{-17} cm^2/C atom[75]	~20 meV[75]	Minutes to hours[75]	~200 ps[75]

photons.[85] Several groups demonstrated many quantum and nonlinear optics experiments[86] with cryogenic single molecules. A spectacular example is the Hong–Ou–Mandel two-photon interference (see an example in the top panel of Fig. 2), where two photons from two different molecules were brought together on a beam splitter, leading to a dip in the coincidence rate from two detectors in the reflection and transmission arms.[87,88] Single molecules can now be detected without fluorescence, through extinction of a strongly focused excitation beam. Although this extinction is of the order of a few percent and more difficult to detect than fluorescence, its advantage is the coherent optical signature of the molecule it provides. Recently, a single molecule was coupled to a micro-cavity, which enhanced the purity of the molecular emission by suppressing vibronic sidebands.[89] Entangled superpositions could be obtained through coherent coupling of two single molecules.[90,91] Such entangled states could be used for information treatment and storage and make single-molecule emitters potential contenders as qubits for quantum information.[92] Beyond their uses in quantum optics, the narrow lines of single molecules are sensitive oscillators that provide first-hand information about their environments, such as the local electric field,[93] the motion of laser-induced charge carriers,[48] or local strain and stresses by acoustic waves or macroscopic deformations.[94] Detailed knowledge of the dynamics and structure of the nano-environment leads to a more detailed understanding of condensed matter. An interesting application of cryogenic conditions discussed below is the exploration of the 3D structure of a protein with Angstrom resolution using the so-called COLD (Cryogenic Optical Localization in 3D) method.[95]

D. Chemistry at SM level and complex heterogeneous systems

Photochemical reactions take place from an excited state, in closed-shell molecules most often from the metastable triplet state, which has longer lifetime than singlet states.[51,96] It is often very hard to identify the intermediate states in single-molecule studies because fluorescence is usually lost as soon as the initial molecule reacts. A few exceptions have been identified, in particular in the case of photo-oxidation of aromatic molecules.[96,97] Reversible transitions between a fluorescent and a dark state under (photo)-chemical reactions give rise to fluorescence blinking, i.e., sudden changes of fluorescence brightness as a function of time until the molecule bleaches and fluorescence is lost irretrievably. For example, Zondervan *et al.*[51] reported the blinking behavior of Rhodamine 6G in polyvinyl alcohol polymer by intermolecular charge transfer mechanism, which created the dark state. The role of charge transfer in the blinking of dyes in aqueous solutions started to be better appreciated, which led to strategies to minimize or control blinking by playing on chemical conditions. Often, the strategy amounts to reducing the triplet lifetime by introducing triplet quenchers[98] or by introducing electron donors or acceptors, which assist charge transfer to restore the initial fluorescent ground state.[99] These strategies became particularly important and useful in the context of dSTORM superresolution schemes, where the conditions are tuned to obtain short bright times and long dark times. Molecules can then be activated, in turn, and super-localized. Charge transfer processes such as photo-induced interfacial electron transfer have been studied using single-molecule fluorescence spectroscopy. The blinking of the

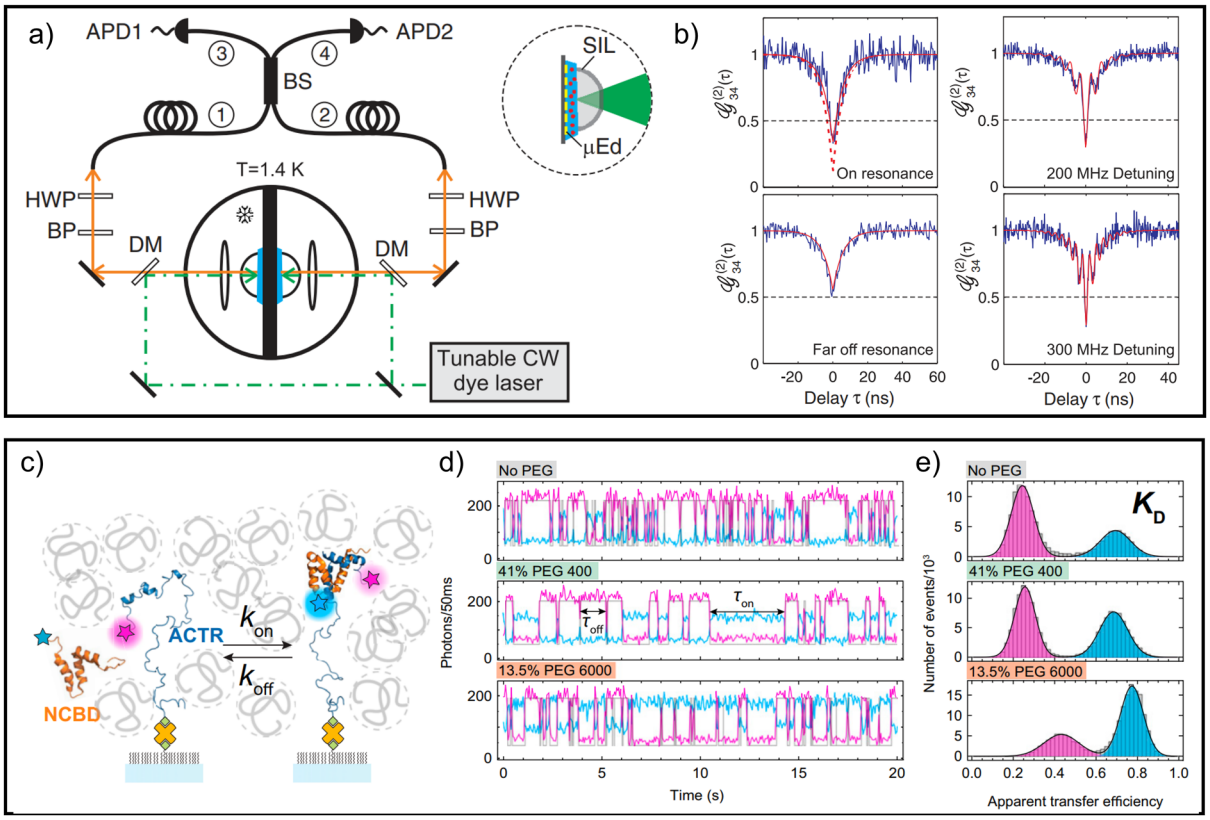

FIG. 2. Top panel: quantum interference of two indistinguishable photons emitted by two remote single organic molecules. (a) Schematic representation of the experimental setup. DM: dichroic mirror, BP: bandpass filter, HWP: half-wave plate, BS: beam splitter, APD: avalanche photodiode, SIL: solid immersion lens, and μED: gold electrodes. (b) Intensity cross correlations of two photons under four different conditions: (i) on resonance, i.e., with the same zero-phonon line (ZPL) frequency for two molecules; (ii) off resonance, i.e., the ZPL of one molecule is detuned by about 5 GHz; and (iii) and (iv) near resonance, i.e., two molecules are detuned by 200 and 300 MHz, respectively. The red curves represent the calculations. Reproduced with permission from Lettow *et al.*, Phys. Rev. Lett. **104**, 123605 (2010). Copyright 2010 American Physical Society. Bottom panel: single-molecule experiment probing the interaction of intrinsically disordered protein molecules in a crowded environment. (c) Schematic representation of the molecular interaction of acceptor-labeled NCBD protein (orange) binding to the surface-immobilized donor-labeled ACTR protein (blue) in the presence of polymeric crowders (gray). (d) Single-molecule FRET timetraces (donor signal: magenta; acceptor signal: light blue) at different poly(ethylene) glycol (PEG) concentrations. The most likely states are shown in gray. (e) Histograms of apparent FRET efficiency at different PEG concentrations. The histograms can be used to calculate the equilibrium dissociation constant, k_D. Reprinted with permission from Zosel *et al.*, Proc. Natl. Acad. Sci. U. S. A. **117**, 13480 (2020). Copyright 2020 Author(s), licensed under a Creative Commons Attribution (CC-BY-NC-ND) license.[144]

fluorescence signal between bright and dark states allows us to study static and dynamical heterogeneity of these processes.[100,101] The electrochemical redox reaction of a redox-sensitive dye molecule, methylene blue, was studied at the single-molecule level via fluorescence blinking.[102] The weak fluorescence of methylene blue was enhanced by the plasmonic near field of a gold nanorod. The mid-point potential of individual molecules was determined through their redox-induced blinking behavior according to the Nernst equation. The mid-point potentials of individual methylene blue molecules are broadly distributed by local interactions with the surface. Such differences are averaged out in ensemble experiments. Another similar example is the change in fluorescence lifetime and brightness upon oxidation or reduction of a metal center in an azurin protein, demonstrated by Pradhan *et al.*[103] The long timetraces of individual electron transfer processes demonstrate signatures of dynamical heterogeneity at the single protein level. The dynamical heterogeneity, i.e., the temporal changes of the electron transfer rates, is assigned to conformational changes of the single protein although its size is comparatively small, only 14 kDa.

Single-molecule studies of complex and heterogeneous systems offer a unique insight into differences from site to site and the influence of defects. This is particularly appealing in studies of heterogeneous chemical reactions and in material science. Super-resolution fluorescence microscopy can be used to study complex heterogeneous polymeric systems and chemical reactions.[104] Single-molecule methods were applied to studies of spatial and dynamical heterogeneity in glass-forming materials, such as polymers[105–107] and supercooled liquids.[108–110] Glass-forming materials show a non-exponential relaxation close to the glass-transition temperature.

Single-molecule experiments demonstrate that this non-exponential behavior arises from spatial heterogeneity, i.e., a spatial distribution of exponential relaxation times, and to a lesser extent from dynamical heterogeneity, i.e., temporal changes of relaxation times at each particular location. Many catalysts present surface sites with very variable activity. Catalytic sites can be imaged by fluorogenic reactions in zeolites[111,112] or in metal nanoparticles.[113,114] For example, the catalytic activity of individual gold nanoparticles shows heterogeneity correlated with size so that the binding affinity to the substrate decreases, whereas the binding affinity to the product increases with decreasing size.[114] Diffusion in porous materials also displays large heterogeneity, reflecting the complex connectivity of the channels confining the molecular motion.[115]

Proteins are complex biomolecules that present various conformations affecting their properties, often to a large extent. Examples are the conformational fluctuations of a protein by means of a fluorescent flavin quenched by electron transfer to a tyrosine residue,[116] the catalytic activity of single lipase molecules,[117] or the redox cycles of the copper protein azurin.[103]

E. Biophysics and cell biochemistry

Biological systems, as a consequence of their high degree of organization, are highly heterogeneous in space and time. Single-molecule optical microscopy and superresolution are therefore first-rate tools to comprehend the spatial and time-dependent aspects of their organization. Methods and progress have been extensively reviewed,[118–121] and we just give a few highlights here. Several of the superresolution methods, distinguished by the 2014 Nobel Prize in Chemistry, were invented in the early 2000s. As discussed in Sec. II C, most of the superresolution methods, such as PALM, STORM, dSTORM, and PAINT, rely on single-molecule observations. Remarkable discoveries have arisen from the large gain in detail and correlation brought by superresolution studies of cells such as bacteria[122] and neurons.[123] Single-molecule observations give an unprecedented view of dynamical processes in such complex systems,[121] where ensemble observations are impossible due to the lacking synchronization mechanisms. Good examples are the elucidation of the stepping behavior of molecular motors thanks to fluorescence and optical tweezers[124,125] or the visualization of interactions between proteins and DNA.[126,127] Single-molecule studies notably helped understand kinesin transport over microtubules, which follows a hand-over-hand mechanism,[124] and the molecular mechanism of RAD51 nucleoprotein filament disassembly.[125]

Structural information about distances and/or angles in biochemical systems is often obtained through single-molecule Förster resonance energy transfer (FRET) measurements. Förster resonance energy transfer (FRET; see an example in Fig. 2, bottom) probes the rate of energy transfer between a donor and an acceptor and is widely used as a molecular ruler to measure distances between a donor and an acceptor and their variations.[120,128] It was used to probe protein folding[129,130] by changes of donor–acceptor distances, the structure of chromatin,[131] and the dynamics of nucleosomes or the changes of an electronic state of an acceptor, such as the copper center of azurin, which becomes an energy acceptor in its oxidized state.

Fluorescence correlation spectroscopy (FCS)[132,133] accumulates statistics from many events corresponding to the crossing of the confocal volume by many individual molecules. Although these many molecular events are often integrated and averaged in a given integration time, cross correlation can also provide true single-molecule information when individual bursts are analyzed. FCS is currently used to study mobility and dynamics of complex heterogeneous biological systems. Joined with multi-dimensional fluorescence measurements, such as lifetime, FRET, and cross correlations,[134] it may provide enough resolution of different species in a mixture to assign individual bursts to a given type of molecule. Alternatively, the focus may be spatially scanned or moved to track single molecules,[135] virus particles,[136] or carbon nanotubes.[137]

The spectacular improvement in optical resolution makes it possible to contemplate correlative optical and electron imaging. Cryo-electron microscopy is compatible with single-molecule fluorescence in frozen samples, where molecules are physically trapped. Such a correlation would be very attractive as complement to TEM and to the images and structures provided by cryo-electron microscopy, preferably *in situ* on the same specimens. However, standard super-resolution schemes have been developed and perfected for room temperature and do not work properly at cryogenic conditions. The diffusion and photochemical processes at the core of the STORM or PAINT superresolution schemes, for example, are dramatically slowed down or impeded at low temperatures so that new super-resolution techniques must be developed. Yet, several demonstrations have already appeared on protein molecules[95,138] and assemblies and even on whole bacteria[139] and highlight the tantalizing promise of these methods. Another interesting prospect for biomolecular studies at low or cryogenic temperatures is that of microsecond thermal cycles, inspired by simulated annealing in molecular dynamics. Switching a single molecule between a low temperature where the process of interest is frozen and a high temperature where its activation is possible would make it possible to resolve even very complex heterogeneous dynamics into sequences of steps. The crucial idea thereby is that the whole immediate environment of a single molecule, down to a few tens of nm, can be cycled in extremely short times, well below a microsecond, and the cycles can be repeated many times. Such short cooling times cannot be reached for larger ensembles. First experiments in this direction have demonstrated the soundness of the idea.[140–143] Yuan *et al.*[141,142] tracked the conformational dynamics of a single polyproline oligopeptide on the timescale of few minutes, showing several transitions between bright and dark states of the donor. These transitions were assigned to interactions between the dye and proline residues, correlated with conformational changes of the dye link.

F. Nanoparticles

Nanoparticles are small pieces of a bulk material with sizes ranging from a few nm to a fraction of a micrometer. They can be made out of different solid materials, metals, semiconductors, organics, etc. Their physical and chemical properties can differ strongly from those of the bulk material and in some cases can be varied through size and shape. Indeed, nanoparticles have higher surface-to-volume ratios compared to bulk materials. In the case of semiconductor and metal nanoparticles, their strongly

delocalized electrons are sensitive to the boundary conditions created by surfaces. For good metals, plasmonic interactions are exquisitely sensitive to the size and shape of the nanoparticle. Nanoparticles are subject to intense research because of their fundamental interest, of their potential applications in fields such as catalysis or magnetism, or of their impact in environmental science.[145] Some of them, usually semiconductors or insulators doped with emitters such as organic molecules or rare-earth ions, are strongly fluorescent or, more generally, photoluminescent. Metals usually have very low luminescence quantum yields because the absence of an energy gap in their excited-state spectrum leads to a fast relaxation of charge carriers toward the Fermi level. Despite their much weaker photoluminescence quantum yields, metals are usually so highly resistant to photo-damage that their photoluminescence not only is observable, but also allows one to study them at the single-nanoparticle level.[146,147] As nanoparticles exist in a bewildering variety of sizes, shapes, facet development, impurities, and defects, single-particle observations are the only practical way to eliminate heterogeneity because it is impossible to prepare ensembles of identical nanoparticles. Single nanoparticles give insights into the distributions of their parameters in the ensemble and into the influence of variations of these parameters on their properties.

Here, we focus on nanoparticles made out of noble and highly conductive metals, whose optical properties are particularly interesting because their collective electronic oscillations, usually called localized surface plasmons, enhance the interaction of these particles with light. This enhancement takes place not only inside the nanoparticle but also in its immediate vicinity in the so-called near field, which extends a few nm up to some 20 nm around the particle. Near-field effects are particularly pronounced near tips and sharp edges. The enhanced interaction of metal nanoparticles with light can be observed in absorption, emission, Raman scattering, or nonlinear optics. The case of gold is particularly important because of its low optical dissipation in the red and near-infrared spectral range. Thanks to its chemical inertness, gold is interesting in complex chemical environments where other plasmonic metals, such as silver, copper, or even aluminum, would degrade. The optical enhancement by metal nanoparticles themselves has been used for harmonic generation,[148] two-photon-excited photoluminescence,[44] or one-photon-excited photoluminescence.[45] Time-dependent properties of individual gold nanoparticles in pump–probe spectroscopy showing coherent acoustic oscillations[149,150] allow us to determine the mechanical properties of each nanoparticle.

Owing to strong near fields in their vicinity, nanoparticles can be seen as light concentrators or as components of optical micro-, nano-, or even pico-cavities.[151–154] Although these open cavities are very leaky (their quality factor rarely exceed a few tens), they present extremely small mode volumes. Such high confinement of photons leads to very high Purcell factors and to enhanced interactions with all kinds of nano-objects from molecules to other nanoparticles. Plasmonic enhancement makes single-molecule fluorescence more readily observable,[155] for molecules with high[156] and for low[157] quantum yields. Thanks to plasmonic nanoparticles, single-molecule detection can be generalized to dyes with poor emission properties, provided that their transition dipole moments are not too small.

Plasmonic cavities can be assembled with sub-nanometer positioning accuracy in scanning probe microscopes, in effect combining optics with near-field probes, often STM. Single-molecule emission can be excited through electroluminescence at the STM tip[158] or by photoluminescence,[159] and surface-enhanced Raman scattering is obtained with a well-defined geometry.[160] The plasmonic properties are involved in both excitation and emission enhancement, which makes even very weak Raman scattering processes observable.[161]

G. Non-fluorescent imaging methods

The power of fluorescence-based methods lies in their weak background, as fluorescence is a comparatively rare property in nature, and their easy use. However, fluorescence entails several limitations: it requires fluorophores, a very restricted kind of rigid, absorbing molecules that often must be either attached chemically to the sample or doped into it and, therefore, may alter its properties. Even when fluorophores are natural molecules, such as autofluorescent proteins, their use in un-natural conditions may bring undesired side effects. Fluorophores, presenting long-lived excited states, are prone to photochemistry. Examples of photochemically induced processes are blinking, which involves excursions to transient dark states, or bleaching, when this transition to a dark state is irreversible, leading to a non-fluorescent product. A further limitation of fluorescence is its comparatively long lifetime, on the order of a few nanoseconds, which limits the intensity of single-molecule fluorescence signals and thereby the signal-to-noise ratio. These limitations of fluorescence make non-fluorescence-based optical methods very attractive for single-molecule studies. The two main optical properties that have been used so far for that purpose are absorption and scattering.

Absorption-based methods rely on the optical detection of a change of state consecutive to optical absorption. The absorption and detection steps are usually performed at two different wavelengths, which can then be easily separated spectrally, as done in fluorescence. Several methods have been developed, which all are nonlinear techniques of the pump–probe or $\chi^{(3)}$ type.[162] As any absorbing object will give rise to a signal, independently of any possible emission of fluorescence, these methods are more general than fluorescence, although they are still about 1000 times less sensitive when applied to fluorescent molecules. The most common absorption-based method is photothermal imaging,[6] which makes use of the thermal lens caused by dissipation of optical energy by the object to be detected. The optical response of the excited molecule can also be modified by excitation, leading to transient absorption, stimulated emission, ground-state depletion, and coherent Raman scattering signals.[23] Optical pump–probe signals can be isolated in many different ways depending on the sample studied. For single molecules and point-like absorbers, spatial signatures are difficult to use, but time-resolved methods work well, either with short laser pulses or with fast modulation of at least one of the beams. In photothermal microscopy, a modulated heating laser causes a synchronous change of the scattered probe signal due to modifications of the probe propagation in the vicinity of the heated object by a refractive-index profile known as the thermal lens. Modulation at high frequencies, 100 kHz–10 MHz, restricts the spatial extent of the time-dependent thermal lens and enables

imaging with high spatial resolution. A sensitive lock-in detection enables shot-noise-limited detection of the thermal lenses created by individual absorbers. Other types of photothermal detection use whispering-gallery modes[163] or nanomechanical devices together with heating.[164,165] The sharp resonance of a whispering-gallery mode microresonator or of a silicon nitride mechanical drum resonator is highly sensitive to changes of local refractive index and/or mechanical properties due to local heating and thus provides high photothermal sensitivities. Photothermal detection has been applied to a variety of objects, including single gold nanoparticles,[6] semiconductor quantum dots,[166] carbon nanotubes,[167] or conjugated polymers.[71] Single-dye-molecule absorption by photothermal imaging[7] was reported in 2010 (see Fig. 3, left panel), simultaneously with direct extinction[168,169] and with ground-state depletion.[170] Correlative photothermal and fluorescence imaging of single nanoparticles or molecules enables a direct determination of the luminescence quantum yield at the single-molecule level, as shown in the example of Fig. 3 (right panel). Photothermal microscopy has further been adapted to the detection and quantitative measurement of circular dichroism (CD) of single chiral nanoparticles. This method called photothermal circular dichroism (PT CD) microscopy[171,172] relies on the true absorption of individual particles rather than on their extinction. In contrast to intensity-modulated photothermal microscopy, PT CD microscopy uses modulations between left and right circularly polarized lights and measures differential absorption of chiral nanoobjects. Thus, PT CD microscopy images single chiral nanoobjects with a sign correlated with the handedness of the object. The recent development of mid-IR photothermal microscopy[173,174] enables the combination of spatial resolution with high spectral resolution through vibrational imaging with chemical specificity. Although mid-IR photothermal microscopy uses mid-IR as heating beam, the spatial resolution is mostly determined by the visible probe beam and thus improves the diffraction-limited spatial resolution by an order of magnitude compared to traditional IR spectroscopy. Wide-field photothermal microscopy,[175–177] although less sensitive than the standard confocal version, makes much faster imaging possible. Very recently, a fluorescence-detected photothermal method[178,179] may open broad applications in biology.

The second kind of fluorescence-free optical detection is through pure scattering and does not require any optical absorption by the sample. A simple index contrast with the surrounding medium suffices to give rise to scattering, and the challenge is then to distinguish the weak interference signal arising from scattering from background and noise. As discussed above, dark-field scattering's intensity decays as the square of the scatterer's volume and the method is not well-adapted to very small particles. Indeed, background from all kinds of defects or other small scatterers dominates the signal of the particle of interest. Bright-field scattering, on the other hand, consists in observing changes of scattered intensity in the presence of background. As this method involves an interference between two fields, it has been called iSCAT. Because background is part of the detected signal, iSCAT requires very careful subtraction of background intensity. This can be done in different ways, most often by imaging in space, on images from a pixelated detector array,[9,10,180] and/or in time by comparing the signals at different times when scatterers have been entering or leaving the diffraction-limited detection volume.[13,181] The iSCAT signal provides the scattering strength, nearly directly related to the mass of an individual protein molecule (see examples in Fig. 4, right panel). The change of the scattering spectrum of a plasmonic particle, for example, a single gold nanorod, can also act as a detector for small objects, down to single protein molecules, in the near field close to the nanorod tips,[182,183] as illustrated in the left panel of Fig. 4. Other

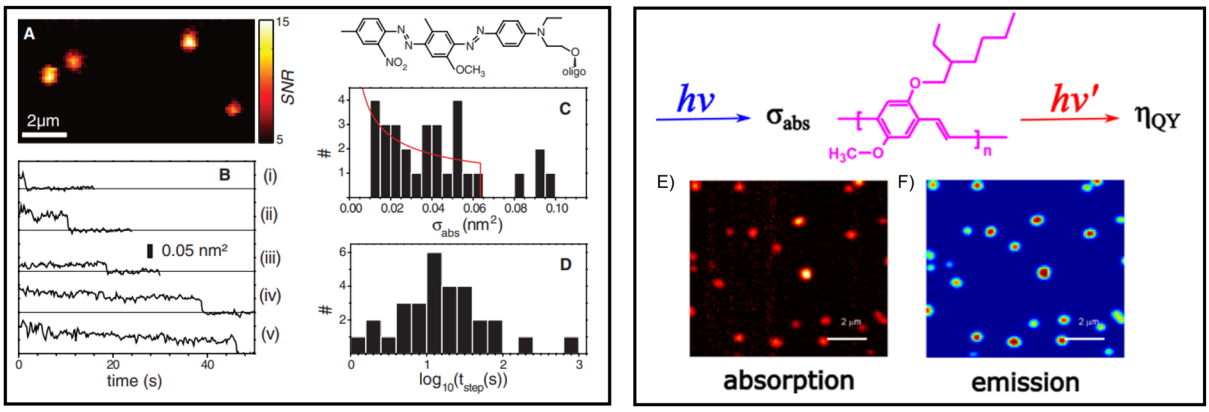

FIG. 3. Left panel: photothermal imaging of single organic dye molecules at room temperature. (a) Photothermal image of four black-hole quencher (BHQ) constructs (BHQ1-10T-BHQ1), each construct consisting of two BHQ molecules. (b) Photothermal timetraces of single BHQ1-10T-BHQ1 molecules showing single-step photobleaching in an oxygenated environment. (c) Histogram of absorption cross sections from 30 single-step photobleaching events. The average cross section is 0.04 nm^2. The red line is expected for an isotropic distribution of the transition dipole moments of single chromophores. (d) Histogram of single-step photobleaching events. The average survival time is 49 s. Reproduced with permission from Gaiduk *et al.*, Science **330**, 353 (2010). Copyright 2010 American Association for the Advancement of Science. Right panel: simultaneous absorption and fluorescence measurements of single conjugated polymer molecules. In the top, a schematic representation of the experimental approach for the determination of the quantum yield of a single MEHPPV conjugated polymer. Simultaneous (e) photothermal and (f) fluorescence images of single MEHPPV molecules. Scale bar 2 μm. Reprinted with permission from Hou *et al.*, Nano Lett. **17**, 1575 (2017). Copyright 2017 Author(s), licensed under a Creative Commons Attribution (CC-BY-NC-ND) license.

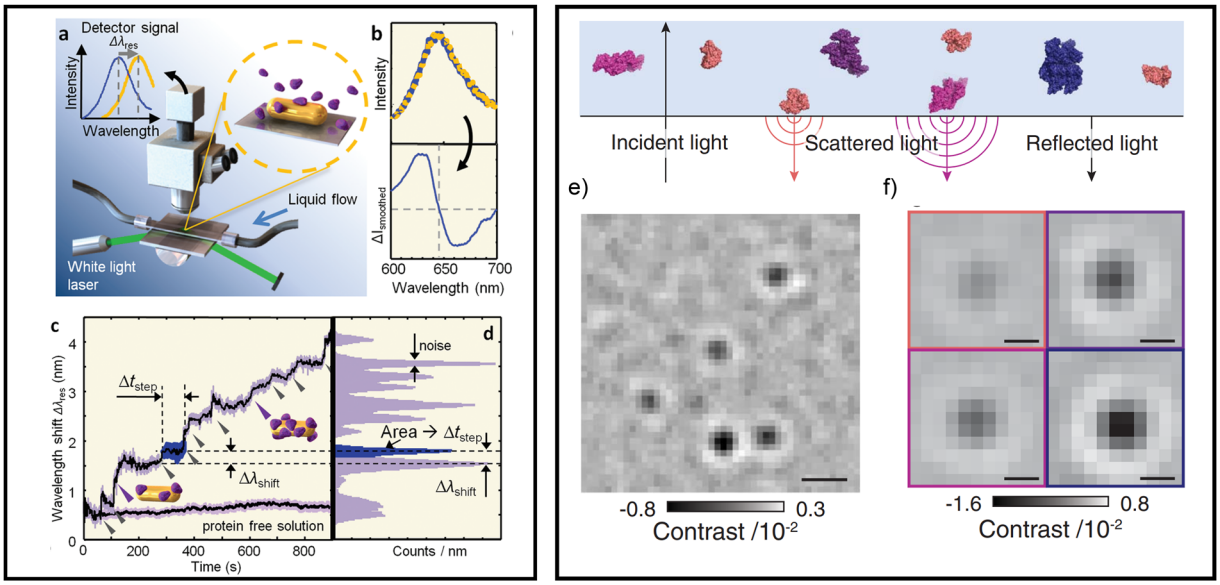

FIG. 4. Left panel: detection of a single protein molecule's attachment to a single gold nanorod. (a) Schematic diagram of the microscope with an enlarged view of the flow cell. Inset: schematic representation of the plasmonic wavelength shift. (b) The measured plasmonic spectra of a single gold nanorod before and after a single protein's attachment (upper panel) and difference between those two spectra (bottom panel). (c) The plasmonic resonance wavelength shift of a single gold nanorod during single protein attachment events and in protein-free solution. (d) Histogram of the time trace shown in (c), showing distinct peaks for each single binding event. Reproduced with permission from Ament *et al.*, Nano Lett. **12**, 1092 (2012). Copyright 2012 American Chemical Society. Right panel: The top part shows the experimental approach. Oligomers of protein molecules in different colors are immobilized near an interface with a different refractive index. The incident light gets scattered by the molecules and reflected by the interface. (e) Interferometric scattering (iSCAT) image of BSA protein molecules. Scale bar: 500 nm. (f) Images of different BSA oligomers: monomer, dimer, trimer, and tetramer. Scale bar: 200 nm. Reproduced with permission from Young *et al.*, Science **360**, 423 (2018). Copyright 2018 American Association for the Advancement of Science.

variants of the iSCAT technique can involve polarization-sensitive opto-plasmonic detection.[184,185]

H. Control of diffusion and trapping

Because of the mismatch between molecular sizes and the light wavelength, the interactions of single molecules and light are rather weak so that detecting them requires fairly long integration times. An obvious manner to confine molecules is to immobilize them in a solid, either a crystal or a glassy matrix, such as a polymer. However, as the natural environment of most biomolecules is fluid, dedicated methods are needed to keep the molecules in their native fluid environment, while extending the optical observation times. Chemical tethering to a solid surface is possible, but involves several tedious chemical preparation steps and requires controls that the molecule's properties are not altered by the tether. Several tether-free confinement methods have been demonstrated in recent years. Proteins and other biomolecules can be corralled in small bilayer-bounded droplets called liposomes or vesicles.[186] In this method, water-soluble biomolecules interact only with the hydrophilic side of the phospholipid bilayer, which is a rather innocuous interaction-free interface to most molecules. Other tether-free confinement methods exploit passive potentials or active feedback to lengthen the dwell time of the molecule in the detection volume.[187–190] The anti-Brownian electrokinetic (ABEL) trap developed in Moerner's lab exploits optical information on the molecule's location, initially its fluorescence[191,192] and more recently its scattering iSCAT.[193] Fast feedback voltages and currents applied through electrodes compensate in nearly real time the Brownian displacement by electrokinetic flows of the buffer solution, which keep the molecule in the illuminated area. Electrostatic traps rely on surface charges on properly treated substrates to create trapping potentials in small three-dimensional spaces, where molecules with a given charge can be confined for long times.[194] Plasmonic holes (or zero-mode waveguides) and nanopores can simultaneously confine molecules and enhance optical fields in these small regions to improve single-molecule sensitivity. Various methods such as optical,[195,196] magnetic,[197] or acoustic[198] tweezers can be combined with fluorescence to reach the same goal.

III. BEST HOPES FOR THE FUTURE

In this section, we propose some ideas for new single-molecule developments in the future, starting with projects actively pursued in the authors' group.

A. Cryogenic SMS

The sharp optical resonances of single molecules at cryogenic temperatures are sensitive detectors, which have been used to

explore the reorganization degrees of freedom of atoms or groups of atoms, known as two-level systems, in disordered solids. Coherent and incoherent charge transfer in solids is usually much faster (ps to s) than the reorganization of atoms, even in tunneling systems at low temperatures (microseconds to days and longer). However, in suitable structures, single charge carriers can be trapped for long times. Single molecules could monitor the charge state of small metallic islands in insulating environments. Such experiments have already been carried out at room temperature, where fluorescence can probe the charge state of a molecule,[102,103] considered as a sub-nm capacitor for single charges. Coulomb blockade occurs for $k_BT < e^2/2C$, where e is the electron charge, C is the capacitance, k_B is Boltzmann's constant, and T is the temperature. A single molecule as a capacitor produces Coulomb blockade at room temperature. A metallic island some tens of nm in diameter, however, requires much lower temperatures to perform as a single-charge box. Cryogenic temperatures make it possible to reach the single-charge regime with 50 nm metal islands while guaranteeing the sharpness of single-molecule lines. Under such conditions, the molecular line could monitor the island charge through the electrostatic field it generates in its vicinity. In previous work, relaxation processes of a complex charge distribution were visualized as drifts of the single-molecule lines,[199,200] but the interpretation of these observations was difficult owing to the simultaneous motion of many charges. A single-charge box or transistor would be much better defined and cause clear frequency shifts of a single molecule placed in its vicinity. First steps in this direction were set in Moradi's Ph.D. thesis.[201]

A single molecule can perform as an optical transistor in different ways, as demonstrated in Ref. 202. An attractive prospect is to use a molecule's metastable triplet state as a toggle to switch its interaction with the excitation laser and, thereby, its fluorescence, on and off.[203] The triplet state itself could be excited optically from the ground singlet with a high-intensity auxiliary beam. Due to the difficulty in locating the very weak singlet-to-triplet transitions in organic molecules, this has not been possible yet. Should such a transition be found, all kinds of double-resonance experiments would become possible, similar to those carried out on single NV centers,[204] thanks to the additional degrees of freedom of the electronic triplet spin and of nuclear spins.

Because up to hundreds of single molecules in a diffraction-limited volume can be separated spectrally at low temperatures,[205] the near field of a plasmonic structure could be sampled at different points by several immobile molecules. One would thus obtain a better view of excitation and emission enhancements by the nanostructure's near field.[206] The very short lifetimes due to quenching by the metal and to radiative enhancement could be measured directly at low temperature in the spectral domain through the broadening of zero-phonon lines. However, apart from the first steps reported in Ref. 206, previous experiments on molecules near the tips of plasmonic nanorods did not provide the expected sharp single-molecule lines yet. Reasons for this failure may be sought in the spectral diffusion induced by many defects in the lattice structure induced by the proximity of the metal or in the low volume of the near field and the low probability to find molecules at the right distance, not too close to avoid quenching, but close enough to feel significant enhancement.[207] Further experimental attempts are required to answer this question.

B. Plasmonics

Plasmonic enhancement becomes particularly efficient for higher-order nonlinear optical processes if more than one interaction step can be enhanced by the same plasmonic structure. Two-photon-excited luminescence can be enhanced by gold nanorods when the emitter is placed in the vicinity of a tip. For example, CdSe quantum dots were efficiently excited with two red photons at 780 nm,[208] although the emitted wavelength, around 650 nm, did not match any resonance of the rod. Higher enhancement factors could be obtained with doubly resonant structures, which would enhance both excitation and emission steps. Such structures could help excite the very weak electronic transitions of single lanthanide ions or complexes and improve the efficiency and emission rate of luminescence photons. These rates are in the 0.1–10 kHz range for isolated single ions, i.e., too low for efficient optical detection.

Another intriguing prospect is the plasmonic sensing of non-absorbing nanoparticles and (bio-)molecules. Such particles cause a change of the index of refraction in the environment of a plasmonic structure, for example a gold nanorod, which slightly modifies its plasmon resonance. The particle's presence in the near field causes a slight change of the absorption[182] or of the scattering[184] of a probe beam by the plasmonic structure.[185] Detecting a number of characteristics from such transients could provide enough information to identify the diffusing or transiently binding analytes.

C. Photothermal microscopy

Circular dichroism (CD) spectra are routinely recorded to analyze protein structures and interactions. Because the chiral electromagnetic interactions responsible for circular dichroism are very weak, standard CD spectrometers require solutions with large ensembles of molecules. Single-molecule or single-nanoparticle CD would provide structural information free from ensemble averaging, but is exceedingly difficult. A few years ago,[171] we started exploring photothermal CD detection on single metal nanoparticles. Each individual gold nanoparticle, albeit quasi-spherical on average, displays specific morphological and surface defects giving rise to a significant CD signal, which varies in sign and magnitude from particle to particle. In particular, magnetic nanoparticles provide a CD signal through the polar magneto-optical Kerr effect (MOKE). Due to the polar MOKE effect, the strength and sign of the CD signal scale with the projection of the magnetic moment along the propagation direction of the photothermal heating light. Thus, PT CD is a probe of the magnetic properties of individual nanoparticles. A fascinating prospect is to monitor the magnetic state of individual nanoparticles by means of photothermal contrast, including their vortex states, superparamagnetic states, and their temperature-, light- or microwave-activated magnetization reversal.[209]

Super-resolution microscopy is mostly applied under fluorescence modalities, but could be generalized to photothermal contrast, for example, with PAINT or DNA-PAINT and molecules or other objects tagged with absorbing gold nanoparticles. Superlocalization of many transient binding events would then reveal the underlying pattern of binding sites.

Vibrational CD is much weaker than optical CD in the UV, but provides information of a different nature on chemical bonds in the biomolecules. Combining mid-IR photothermal microscopy with photothermal CD microscopy would help study the conformations of biomolecules with a higher spatial resolution than with traditional vibrational CD spectroscopy. The fluorescence-based and wide-field modalities of photothermal detection could also help in this context.

D. Broader outlook

An exciting prospect of cryogenic single-molecule experiments is their use for quantum optics. A major disadvantage of organic molecules as emitters is their broad fluorescence spectra due to the spread of fluorescence photons over their many intramolecular vibrations and matrix phonons. Coupling the molecule to a cavity tuned to resonance with the zero-phonon line can narrow down the emission to a Fourier-limited line, largely suppressing emission on the vibrational modes.[210] Coherent coupling to resonant light opens the path to state manipulations, photon–photon interactions, and all-optical switching circuits. A strong advantage of molecules is their control through chemical synthesis, which makes it possible to arrange desired nuclei spatially, in ways allowing for controlled hyperfine interactions. Quantum computation with NMR on a large ensemble of specially designed molecules has been demonstrated 20 years ago.[211] Electronic spin degrees of freedom, either through excitation of the triplet state of a closed-shell molecule or in the ground and excited electronic states of an open-shell molecule, would make the control and manipulation of the nuclear spins of a single molecule possible. These nuclear spins, in turn, could be placed at desired positions by chemical synthesis.

Studies of frozen biomolecules under cryogenic conditions enable correlations between cryo-electron microscopy and superresolution optical images.[138,139] Controlling dark states and blinking dynamics to obtain superresolution poses original photophysics questions. The ability to freeze-quench single molecules and assemblies thereof by temperature cycles opens detailed studies of their potential energy landscapes, dynamics, and kinetic pathways. The first feasibility studies[140,141] must now give way to convincing demonstrations to illustrate the potential of the method for single-molecule studies of intermediates, reaction pathways, or folding. Long-standing conundrums, such as allostery or the alternative mechanisms of conformational selection vs induced fit, could thus be revisited in each particular case.

The ideal near-field optical source is a bright dipole emitter placed on a movable tip. This old dream was first attempted in a heroic experiment by Michaelis *et al.*[212] Ideally, such an emitter should be monochromatic and Fourier-limited. Could a single molecule at low temperature serve as such an emitter? As cryogenic plasmonic experiments have shown, it is difficult to keep a lifetime-limited narrow line for a molecule close to a surface. Comparatively, narrow lines were obtained in UHV and close to an ultraclean surface,[213] but applying such a source to near-field imaging of a real sample appears very challenging. A similar problem arises for NV centers in nanocrystals, where the proximity of surface states induces disorder and dynamical fluctuations, reminding us of Pauli's remark that "if bulk was God's invention, the surface was invented by the devil."

The coupling of single emitters to plasmonic cavities leads to superemitters with broader but still lifetime-limited lines. These small optically active systems would be very useful for photonic applications and for quantum optics either under cryogenic conditions or outside the cryostat.[214] In further steps, such interacting single molecules or emitters could be brought into interaction, creating entangled superpositions, as proposed in an early publication.[90]

IV. CONCLUSION

After an explosive beginning in the 1990s, single-molecule optics has reached a more steady rate of growth. It has turned into a standard method in cell bio-physics because of its ultimate resolution and of the advantages of optics vs more invasive electron microscopy, scanning-tip microscopy, and mass spectrometry. Single-molecule chemistry is a unique tool for basic bio-chemistry, in particular, for proteins because their large complexity is a stepping stone before the full complexity of cells. The complex potential energy landscapes of these biomolecules require dedicated methods to explore their potential energy surfaces, the pathways, and the intermediates involved in their complex reactions. For a deeper understanding of this complexity, complete removal of ensemble averaging is indispensable. Only single-molecule methods offer this ability. The single-molecule methods pioneered in bio-science have started to be generalized to heterogeneous catalysis, porous materials, soft matter, and, generally, (soft) material science, where the potential for new applications is huge.

For the future quantum Internet, communication hubs are required between information-storing qubits and flying qubits, in practice photons. Single small emitters in condensed matter, such as molecules, and also color centers and (self-assembled) quantum dots are all strong contenders in this endeavor and present specific advantages compared to ions and atoms in the gas phase. A unique asset of molecules is their control and flexibility under chemical synthesis, although interactions with the matrix may perturb a molecule's properties beyond recognition. Techniques must be developed to imbed these molecules into strong matrices that do not perturb the guest molecule too much. Inclusion of the molecules in a hexagonal boron nitride multilayer[215] is an original and attractive development. After more than 30 years of constant re-invention, the field of single-molecule experiments having become an indispensable tool for the study of living and complex systems will continue to expand in unexpected new directions.

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AUTHOR DECLARATIONS

Conflict of Interest

The authors have no conflicts to disclose.

DATA AVAILABILITY

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

REFERENCES

[1]W. E. Moerner and W. E, "Single-molecule spectroscopy, imaging, and photocontrol: Foundations for super-resolution microscopy (Nobel Lecture)," Angew. Chem., Int. Ed. **54**(28), 8067–8093 (2015).

[2]E. Betzig, "Single molecules, cells, and super-resolution optics (Nobel Lecture)," Angew. Chem., Int. Ed. **54**(28), 8034–8053 (2015).

[3]S. W. Hell, "Nanoscopy with focused light (Nobel Lecture)," Angew. Chem., Int. Ed. **54**(28), 8054–8066 (2015).

[4]W. E. Moerner and L. Kador, "Optical detection and spectroscopy of single molecules in a solid," Phys. Rev. Lett. **62**(21), 2535–2538 (1989).

[5]M. Orrit and J. Bernard, "Single pentacene molecules detected by fluorescence excitation in a *p*-terphenyl crystal," Phys. Rev. Lett. **65**(21), 2716–2719 (1990).

[6]D. Boyer, P. Tamarat, A. Maali, B. Lounis, and M. Orrit, "Photothermal imaging of nanometer-sized metal particles among scatterers," Science **297**(5584), 1160–1163 (2002).

[7]A. Gaiduk, M. Yorulmaz, P. V. Ruijgrok, and M. Orrit, "Room-temperature detection of a single molecule's absorption by photothermal contrast," Science **330**(6002), 353–356 (2010).

[8]F. V. Ignatovich and L. Novotny, "Real-time and background-free detection of nanoscale particles," Phys. Rev. Lett. **96**(1), 013901 (2006).

[9]K. Lindfors, T. Kalkbrenner, P. Stoller, and V. Sandoghdar, "Detection and spectroscopy of gold nanoparticles using supercontinuum white light confocal microscopy," Phys. Rev. Lett. **93**(3), 037401 (2004).

[10]V. Jacobsen, P. Stoller, C. Brunner, V. Vogel, and V. Sandoghdar, "Interferometric optical detection and tracking of very small gold nanoparticles at a water-glass interface," Opt. Express **14**(1), 405–414 (2006).

[11]J. Ortega Arroyo, J. Andrecka, K. M. Spillane, N. Billington, Y. Takagi, J. R. Sellers, and P. Kukura, "Label-free, all-optical detection, imaging, and tracking of a single protein," Nano Lett. **14**(4), 2065–2070 (2014).

[12]M. Piliarik and V. Sandoghdar, "Direct optical sensing of single unlabelled proteins and super-resolution imaging of their binding sites," Nat. Commun. **5**(1), 4495 (2014).

[13]G. Young, N. Hundt, D. Cole, A. Fineberg, J. Andrecka, A. Tyler, A. Olerinyova, A. Ansari, E. G. Marklund, M. P. Collier, S. A. Chandler, O. Tkachenko, J. Allen, M. Crispin, N. Billington, Y. Takagi, J. R. Sellers, C. Eichmann, P. Selenko, L. Frey, R. Riek, M. R. Galpin, W. B. Struwe, J. L. P. Benesch, and P. Kukura, "Quantitative mass imaging of single biological macromolecules," Science **360**(6387), 423–427 (2018).

[14]R. W. Taylor, R. G. Mahmoodabadi, V. Rauschenberger, A. Giessl, A. Schambony, and V. Sandoghdar, "Interferometric scattering microscopy reveals microsecond nanoscopic protein motion on a live cell membrane," Nat. Photonics **13**(7), 480–487 (2019).

[15]P. Tamarat, A. Maali, B. Lounis, and M. Orrit, "Ten years of single-molecule spectroscopy," J. Phys. Chem. A **104**(1), 1–16 (2000).

[16]A. Hoffmann and M. T. Woodside, "Signal-pair correlation analysis of single-molecule trajectories," Angew. Chem., Int. Ed. **50**(52), 12643–12646 (2011).

[17]M. Orrit, "Single-molecule spectroscopy: The road ahead," J. Chem. Phys. **117**(24), 10938–10946 (2002).

[18]C. F. Bohren and D. R. Huffman, *Absorption and Scattering of Light by Small Particles* (Wiley-VCH, Weinheim, 2004).

[19]H. Siedentopf and R. Zsigmondy, "Uber sichtbarmachung und größenbestimmung ultramikoskopischer teilchen, mit besonderer anwendung auf goldrubingläser," Ann. Phys. **315**(1), 1–39 (1902).

[20]C. Sönnichsen, S. Geier, N. E. Hecker, G. von Plessen, J. Feldmann, H. Ditlbacher, B. Lamprecht, J. R. Krenn, F. R. Aussenegg, V. Z.-H. Chan, J. P. Spatz, and M. Möller, "Spectroscopy of single metallic nanoparticles using total internal reflection microscopy," Appl. Phys. Lett. **77**(19), 2949–2951 (2000).

[21]R. W. Taylor and V. Sandoghdar, "Interferometric scattering microscopy: Seeing single nanoparticles and molecules via Rayleigh scattering," Nano Lett. **19**(8), 4827–4835 (2019).

[22]S. Adhikari, P. Spaeth, A. Kar, M. D. Baaske, S. Khatua, and M. Orrit, "Photothermal microscopy: Imaging the optical absorption of single nanoparticles and single molecules," ACS Nano **14**(12), 16414–16445 (2020).

[23]*Coherent Raman Scattering Microscopy*, edited by J.-X. Cheng and X. S. Xie (CRC Press, Boca Raton, 2012).

[24]R. Strack, "Organic dyes for live imaging," Nat. Methods **18**(1), 30 (2021).

[25]F. Hu, C. Zeng, R. Long, Y. Miao, L. Wei, Q. Xu, and W. Min, "Supermultiplexed optical imaging and barcoding with engineered polyynes," Nat. Methods **15**(3), 194–200 (2018).

[26]J. C. Bolinger, M. C. Traub, J. Brazard, T. Adachi, P. F. Barbara, and D. A. Vanden Bout, "Conformation and energy transfer in single conjugated polymers," Acc. Chem. Res. **45**(11), 1992–2001 (2012).

[27]H. Lin, S. R. Tabaei, D. Thomsson, O. Mirzov, P.-O. Larsson, and I. G. Scheblykin, "Fluorescence blinking, exciton dynamics, and energy transfer domains in single conjugated polymer chains," J. Am. Chem. Soc. **130**(22), 7042–7051 (2008).

[28]F. A. Feist, M. F. Zickler, and T. Basché, "Origin of the red sites and energy transfer rates in single MEH-PPV chains at low temperature," ChemPhysChem **12**(8), 1499–1508 (2011).

[29]Y. Ebihara and M. Vacha, "Relating conformation and photophysics in single MEH-PPV chains," J. Phys. Chem. B **112**(40), 12575–12578 (2008).

[30]Z. Chen, S. Berciaud, C. Nuckolls, T. F. Heinz, and L. E. Brus, "Energy transfer from individual semiconductor nanocrystals to graphene," ACS Nano **4**(5), 2964–2968 (2010).

[31]I. Chung, K. T. Shimizu, and M. G. Bawendi, "Room temperature measurements of the 3D orientation of single CdSe quantum dots using polarization microscopy," Proc. Natl. Acad. Sci. U. S. A. **100**(2), 405–408 (2003).

[32]P. P. Jha and P. Guyot-Sionnest, "Electrochemical switching of the photoluminescence of single quantum dots," J. Phys. Chem. C **114**(49), 21138–21141 (2010).

[33]B. Ji, E. Giovanelli, B. Habert, P. Spinicelli, M. Nasilowski, X. Xu, N. Lequeux, J.-P. Hugonin, F. Marquier, J.-J. Greffet, and B. Dubertret, "Non-blinking quantum dot with a plasmonic nanoshell resonator," Nat. Nanotechnol. **10**(2), 170–175 (2015).

[34]M. J. Fernée, P. Tamarat, and B. Lounis, "Spectroscopy of single nanocrystals," Chem. Soc. Rev. **43**(4), 1311–1337 (2014).

[35]M. Fu, P. Tamarat, J.-B. Trebbia, M. I. Bodnarchuk, M. V. Kovalenko, J. Even, and B. Lounis, "Unraveling exciton–phonon coupling in individual $FAPbI_3$ nanocrystals emitting near-infrared single photons," Nat. Commun. **9**(1), 3318 (2018).

[36]P. Tamarat, M. I. Bodnarchuk, J.-B. Trebbia, R. Erni, M. V. Kovalenko, J. Even, and B. Lounis, "The ground exciton state of formamidinium lead bromide perovskite nanocrystals is a singlet dark state," Nat. Mater. **18**(7), 717–724 (2019).

[37]H. Yuan, E. Debroye, G. Caliandro, K. P. F. Janssen, J. van Loon, C. E. A. Kirschhock, J. A. Martens, J. Hofkens, and M. B. J. Roeffaers, "Photoluminescence blinking of single-crystal methylammonium lead iodide perovskite nanorods induced by surface traps," ACS Omega **1**(1), 148–159 (2016).

[38]H. Utzat, W. Sun, A. E. K. Kaplan, F. Krieg, M. Ginterseder, B. Spokoyny, N. D. Klein, K. E. Shulenberger, C. F. Perkinson, M. V. Kovalenko, and M. G. Bawendi, "Coherent single-photon emission from colloidal lead halide perovskite quantum dots," Science **363**(6431), 1068–1072 (2019).

[39]P. Neumann, R. Kolesov, V. Jacques, J. Beck, J. Tisler, A. Batalov, L. Rogers, N. B. Manson, G. Balasubramanian, F. Jelezko, and J. Wrachtrup, "Excited-state spectroscopy of single NV defects in diamond using optically detected magnetic resonance," New J. Phys. **11**(1), 013017 (2009).

[40]F. Dolde, H. Fedder, M. W. Doherty, T. Nöbauer, F. Rempp, G. Balasubramanian, T. Wolf, F. Reinhard, L. C. L. Hollenberg, F. Jelezko, and J. Wrachtrup, "Electric-field sensing using single diamond spins," Nat. Phys. **7**(6), 459–463 (2011).

[41]G. Balasubramanian, I. Y. Chan, R. Kolesov, M. Al-Hmoud, J. Tisler, C. Shin, C. Kim, A. Wojcik, P. R. Hemmer, A. Krueger, T. Hanke, A. Leitenstorfer, R. Bratschitsch, F. Jelezko, and J. Wrachtrup, "Nanoscale imaging magnetometry with diamond spins under ambient conditions," Nature **455**(7213), 648–651 (2008).

[42]G. de Lange, D. Ristè, V. V. Dobrovitski, and R. Hanson, "Single-spin magnetometry with multipulse sensing sequences," Phys. Rev. Lett. **106**(8), 080802 (2011).

[43]T. H. Taminiau, J. J. T. Wagenaar, T. van der Sar, F. Jelezko, V. V. Dobrovitski, and R. Hanson, "Detection and control of individual nuclear spins using a weakly coupled electron spin," Phys. Rev. Lett. **109**(13), 137602 (2012).

[44]P. Zijlstra, J. W. M. Chon, and M. Gu, "Five-dimensional optical recording mediated by surface plasmons in gold nanorods," Nature **459**(7245), 410–413 (2009).
[45]A. Carattino, M. Caldarola, and M. Orrit, "Gold nanoparticles as absolute nanothermometers," Nano Lett. **18**(2), 874–880 (2018).
[46]C. J. Reckmeier, J. Schneider, A. S. Susha, and A. L. Rogach, "Luminescent colloidal carbon dots: Optical properties and effects of doping (invited)," Opt. Express **24**(2), A312–A340 (2016).
[47]B. Kozankiewicz and M. Orrit, "Single-molecule photophysics, from cryogenic to ambient conditions," Chem. Soc. Rev. **43**(4), 1029–1043 (2014).
[48]M. Colautti, F. S. Piccioli, Z. Ristanović, P. Lombardi, A. Moradi, S. Adhikari, I. Deperasinska, B. Kozankiewicz, M. Orrit, and C. Toninelli, "Laser-induced frequency tuning of fourier-limited single-molecule emitters," ACS Nano **14**(10), 13584–13592 (2020).
[49]R. Smit, Z. Ristanović, B. Kozankiewicz, and M. Orrit, "Reverse intersystem crossing of single deuterated perylene molecules in a dibenzothiophene matrix," ChemPhysChem **23**, e202100890 (2022).
[50]P.-H. Chung, C. Tregidgo, and K. Suhling, "Determining a fluorophore's transition dipole moment from fluorescence lifetime measurements in solvents of varying refractive index," Methods Appl. Fluoresc. **4**(4), 045001 (2016).
[51]R. Zondervan, F. Kulzer, S. B. Orlinskii, and M. Orrit, "Photoblinking of rhodamine 6G in poly(vinyl alcohol): Radical dark state formed through the triplet," J. Phys. Chem. A **107**(35), 6770–6776 (2003).
[52]R. C. Schofield, K. D. Major, S. Grandi, S. Boissier, E. A. Hinds, and A. S. Clark, "Efficient excitation of dye molecules for single photon generation," J. Phys. Commun. **2**(11), 115027 (2018).
[53]J. Maillard, K. Klehs, C. Rumble, E. Vauthey, M. Heilemann, and A. Fürstenberg, "Universal quenching of common fluorescent probes by water and alcohols," Chem. Sci. **12**(4), 1352–1362 (2021).
[54]M. Nasilowski, P. Spinicelli, G. Patriarche, and B. Dubertret, "Gradient CdSe/CdS quantum dots with room temperature biexciton unity quantum yield," Nano Lett. **15**(6), 3953–3958 (2015).
[55]T. Matsumoto, M. Ohtsu, K. Matsuda, T. Saiki, H. Saito, and K. Nishi, "Low-temperature near-field nonlinear absorption spectroscopy of InGaAs single quantum dots," Appl. Phys. Lett. **75**(21), 3246–3248 (1999).
[56]L. Biadala, Y. Louyer, P. Tamarat, and B. Lounis, "Direct observation of the two lowest exciton zero-phonon lines in single CdSe/ZnS nanocrystals," Phys. Rev. Lett. **103**(3), 037404 (2009).
[57]O. Labeau, P. Tamarat, and B. Lounis, "Temperature dependence of the luminescence lifetime of single CdSe/ZnS quantum dots," Phys. Rev. Lett. **90**(25), 257404 (2003).
[58]B. Lounis, H. A. Bechtel, D. Gerion, P. Alivisatos, and W. E. Moerner, "Photon antibunching in single CdSe/ZnS quantum dot fluorescence," Chem. Phys. Lett. **329**(5), 399–404 (2000).
[59]S. Berciaud, L. Cognet, and B. Lounis, "Photothermal absorption spectroscopy of individual semiconductor nanocrystals," Nano Lett. **5**(11), 2160–2163 (2005).
[60]C. Santori, P. E. Barclay, K.-M. C. Fu, R. G. Beausoleil, S. Spillane, and M. Fisch, "Nanophotonics for quantum optics using nitrogen-vacancy centers in diamond," Nanotechnology **21**(27), 274008 (2010).
[61]A. Dräbenstedt, L. Fleury, C. Tietz, F. Jelezko, S. Kilin, A. Nizovtzev, and J. Wrachtrup, "Low-temperature microscopy and spectroscopy on single defect centers in diamond," Phys. Rev. B **60**(16), 11503–11508 (1999).
[62]T. Plakhotnik and H. Aman, "NV-centers in nanodiamonds: How good they are," Diamond Relat. Mater. **82**, 87–95 (2018).
[63]I. P. Radko, M. Boll, N. M. Israelsen, N. Raatz, J. Meijer, F. Jelezko, U. L. Andersen, and A. Huck, "Determining the internal quantum efficiency of shallow-implanted nitrogen-vacancy defects in bulk diamond," Opt. Express **24**(24), 27715–27725 (2016).
[64]J. Tisler, G. Balasubramanian, B. Naydenov, R. Kolesov, B. Grotz, R. Reuter, J.-P. Boudou, P. A. Curmi, M. Sennour, A. Thorel, M. Börsch, K. Aulenbacher, R. Erdmann, P. R. Hemmer, F. Jelezko, and J. Wrachtrup, "Fluorescence and spin properties of defects in single digit nanodiamonds," ACS Nano **3**(7), 1959–1965 (2009).
[65]M. Fu, P. Tamarat, H. Huang, J. Even, A. L. Rogach, and B. Lounis, "Neutral and charged exciton fine structure in single lead halide perovskite nanocrystals revealed by magneto-optical spectroscopy," Nano Lett. **17**(5), 2895–2901 (2017).
[66]K. Xing, S. Cao, X. Yuan, R. Zeng, H. Li, B. Zou, and J. Zhao, "Thermal and photo stability of all inorganic lead halide perovskite nanocrystals," Phys. Chem. Chem. Phys. **23**(32), 17113–17128 (2021).
[67]R. Y. Tsien, "The green fluorescent protein," Annu. Rev. Biochem. **67**(1), 509–544 (1998).
[68]N. C. Shaner, R. E. Campbell, P. A. Steinbach, B. N. G. Giepmans, A. E. Palmer, and R. Y. Tsien, "Improved monomeric red, orange and yellow fluorescent proteins derived from *Discosoma* sp. red fluorescent protein," Nat. Biotechnol. **22**(12), 1567–1572 (2004).
[69]L. Albertazzi, D. Arosio, L. Marchetti, F. Ricci, and F. Beltram, "Quantitative FRET analysis with the E0GFP-MCherry fluorescent protein pair," Photochem. Photobiol. **85**(1), 287–297 (2009).
[70]A. Gaiduk, M. Yorulmaz, and M. Orrit, "Correlated absorption and photoluminescence of single gold nanoparticles," ChemPhysChem **12**(8), 1536–1541 (2011).
[71]L. Hou, S. Adhikari, Y. Tian, I. G. Scheblykin, and M. Orrit, "Absorption and quantum yield of single conjugated polymer poly[2-methoxy-5-(2-ethylhexyloxy)-1,4-phenylenevinylene] (MEH-PPV) molecules," Nano Lett. **17**(3), 1575–1581 (2017).
[72]M. Vacha and S. Habuchi, "Conformation and physics of polymer chains: A single-molecule perspective," NPG Asia Mater. **2**(4), 134–142 (2010).
[73]K. Hola, Y. Zhang, Y. Wang, E. P. Giannelis, R. Zboril, and A. L. Rogach, "Carbon dots—Emerging light emitters for bioimaging, cancer therapy and optoelectronics," Nano Today **9**(5), 590–603 (2014).
[74]M. Amani, D.-H. Lien, D. Kiriya, J. Xiao, A. Azcatl, J. Noh, S. R. Madhvapathy, R. Addou, K. C. Santosh, M. Dubey, K. Cho, R. M. Wallace, S.-C. Lee, J.-H. He, J. W. Ager, X. Zhang, E. Yablonovitch, and A. Javey, "Near-unity photoluminescence quantum yield in MoS_2," Science **350**(6264), 1065–1068 (2015).
[75]S. Berciaud, L. Cognet, and B. Lounis, "Luminescence decay and the absorption cross section of individual single-walled carbon nanotubes," Phys. Rev. Lett. **101**(7), 077402 (2008).
[76]S. W. Hell and J. Wichmann, "Breaking the diffraction resolution limit by stimulated emission: Stimulated-emission-depletion fluorescence microscopy," Opt. Lett. **19**(11), 780–782 (1994).
[77]E. Betzig, G. H. Patterson, R. Sougrat, O. W. Lindwasser, S. Olenych, J. S. Bonifacino, M. W. Davidson, J. Lippincott-Schwartz, and H. F. Hess, "Imaging intracellular fluorescent proteins at nanometer resolution," Science **313**(5793), 1642–1645 (2006).
[78]M. J. Rust, M. Bates, and X. Zhuang, "Sub-diffraction-limit imaging by stochastic optical reconstruction microscopy (STORM)," Nat. Methods **3**(10), 793–796 (2006).
[79]S. T. Hess, T. P. K. Girirajan, and M. D. Mason, "Ultra-high resolution imaging by fluorescence photoactivation localization microscopy," Biophys. J. **91**(11), 4258–4272 (2006).
[80]M. Heilemann, S. van de Linde, M. Schüttpelz, R. Kasper, B. Seefeldt, A. Mukherjee, P. Tinnefeld, and M. Sauer, "Subdiffraction-resolution fluorescence imaging with conventional fluorescent probes," Angew. Chem., Int. Ed. **47**(33), 6172–6176 (2008).
[81]A. Sharonov and R. M. Hochstrasser, "Wide-field subdiffraction imaging by accumulated binding of diffusing probes," Proc. Natl. Acad. Sci. U. S. A. **103**(50), 18911–18916 (2006).
[82]G. Giannone, E. Hosy, F. Levet, A. Constals, K. Schulze, A. I. Sobolevsky, M. P. Rosconi, E. Gouaux, R. Tampé, D. Choquet, and L. Cognet, "Dynamic super-resolution imaging of endogenous proteins on living cells at ultra-high density," Biophys. J. **99**(4), 1303–1310 (2010).
[83]R. Jungmann, C. Steinhauer, M. Scheible, A. Kuzyk, P. Tinnefeld, and F. C. Simmel, "Single-molecule kinetics and super-resolution microscopy by fluorescence imaging of transient binding on DNA origami," Nano Lett. **10**(11), 4756–4761 (2010).
[84]D. D. Awschalom, R. Hanson, J. Wrachtrup, and B. B. Zhou, "Quantum technologies with optically interfaced solid-state spins," Nat. Photonics **12**(9), 516–527 (2018).

[85]B. Lounis and M. Orrit, "Single-photon sources," Rep. Prog. Phys. **68**(5), 1129–1179 (2005).
[86]A. Maser, B. Gmeiner, T. Utikal, S. Götzinger, and V. Sandoghdar, "Few-photon coherent nonlinear optics with a single molecule," Nat. Photonics **10**(7), 450–453 (2016).
[87]R. Lettow, Y. L. A. Rezus, A. Renn, G. Zumofen, E. Ikonen, S. Götzinger, and V. Sandoghdar, "Quantum interference of tunably indistinguishable photons from remote organic molecules," Phys. Rev. Lett. **104**(12), 123605 (2010).
[88]J.-B. Trebbia, P. Tamarat, and B. Lounis, "Indistinguishable near-infrared single photons from an individual organic molecule," Phys. Rev. A **82**(6), 063803 (2010).
[89]D. Wang, H. Kelkar, D. Martin-Cano, D. Rattenbacher, A. Shkarin, T. Utikal, S. Götzinger, and V. Sandoghdar, "Turning a molecule into a coherent two-level quantum system," Nat. Phys. **15**(5), 483–489 (2019).
[90]C. Hettich, C. Schmitt, J. Zitzmann, S. Kühn, I. Gerhardt, and V. Sandoghdar, "Nanometer resolution and coherent optical dipole coupling of two individual molecules," Science **298**(5592), 385–389 (2002).
[91]J.-B. Trebbia, J.-B. Trebbia, R. Baby, R. Baby, P. Tamarat, P. Tamarat, B. Lounis, and B. Lounis, "3D optical nanoscopy with excited state saturation at liquid helium temperatures," Opt. Express **27**(16), 23486–23496 (2019).
[92]C. Toninelli, I. Gerhardt, A. S. Clark, A. Reserbat-Plantey, S. Götzinger, Z. Ristanović, M. Colautti, P. Lombardi, K. D. Major, I. Deperasińska, W. H. Pernice, F. H. L. Koppens, B. Kozankiewicz, A. Gourdon, V. Sandoghdar, and M. Orrit, "Single organic molecules for photonic quantum technologies," Nat. Mater. **20**, 1615–1628 (2021).
[93]A. Moradi, Z. Ristanović, M. Orrit, I. Deperasińska, and B. Kozankiewicz, "Matrix-induced linear Stark effect of single dibenzoterrylene molecules in 2,3-dibromonaphthalene crystal," ChemPhysChem **20**(1), 55–61 (2019).
[94]Y. Tian, P. Navarro, and M. Orrit, "Single molecule as a local acoustic detector for mechanical oscillators," Phys. Rev. Lett. **113**(13), 135505 (2014).
[95]S. Weisenburger, D. Boening, B. Schomburg, K. Giller, S. Becker, C. Griesinger, and V. Sandoghdar, "Cryogenic optical localization provides 3D protein structure data with Angstrom resolution," Nat. Methods **14**(2), 141–144 (2017).
[96]R. Zondervan, F. Kulzer, M. A. Kol'chenk, and M. Orrit, "Photobleaching of rhodamine 6G in poly(vinyl alcohol) at the ensemble and single-molecule levels," J. Phys. Chem. A **108**(10), 1657–1665 (2004).
[97]T. Christ, F. Kulzer, P. Bordat, and T. Basché, "Watching the photo-oxidation of a single aromatic hydrocarbon molecule," Angew. Chem., Int. Ed. **40**(22), 4192–4195 (2001).
[98]T. Cordes, J. Vogelsang, and P. Tinnefeld, "On the mechanism of trolox as antiblinking and antibleaching reagent," J. Am. Chem. Soc. **131**(14), 5018–5019 (2009).
[99]J. Vogelsang, R. Kasper, C. Steinhauer, B. Person, M. Heilemann, M. Sauer, and P. Tinnefeld, "A reducing and oxidizing system minimizes photobleaching and blinking of fluorescent dyes," Angew. Chem., Int. Ed. **47**(29), 5465–5469 (2008).
[100]M. W. Holman, R. Liu, and D. M. Adams, "Single-molecule spectroscopy of interfacial electron transfer," J. Am. Chem. Soc. **125**(41), 12649–12654 (2003).
[101]Y. Wang, X. Wang, S. K. Ghosh, and H. P. Lu, "Probing single-molecule interfacial electron transfer dynamics of porphyrin on TiO_2 nanoparticles," J. Am. Chem. Soc. **131**(4), 1479–1487 (2009).
[102]W. Zhang, M. Caldarola, B. Pradhan, and M. Orrit, "Gold nanorod enhanced fluorescence enables single-molecule electrochemistry of methylene blue," Angew. Chem. **129**(13), 3620–3623 (2017).
[103]B. Pradhan, C. Engelhard, S. Van Mulken, X. Miao, G. W. Canters, and M. Orrit, "Single electron transfer events and dynamical heterogeneity in the small protein azurin from *Pseudomonas aeruginosa*," Chem. Sci. **11**(3), 763–771 (2020).
[104]D. Wöll and C. Flors, "Super-resolution fluorescence imaging for materials science," Small Methods **1**(10), 1700191 (2017).
[105]R. A. L. Vallée, M. Cotlet, J. Hofkens, F. C. De Schryver, and K. Müllen, "Spatially heterogeneous dynamics in polymer glasses at room temperature probed by single molecule lifetime fluctuations," Macromolecules **36**(20), 7752–7758 (2003).
[106]B. M. I. Flier, M. C. Baier, J. Huber, K. Müllen, S. Mecking, A. Zumbusch, and D. Wöll, "Heterogeneous diffusion in thin polymer films as observed by high-temperature single-molecule fluorescence microscopy," J. Am. Chem. Soc. **134**(1), 480–488 (2012).
[107]S. Adhikari, M. Selmke, and F. Cichos, "Temperature dependent single molecule rotational dynamics in PMA," Phys. Chem. Chem. Phys. **13**(5), 1849–1856 (2011).
[108]L. A. Deschenes and D. A. Vanden Bout, "Heterogeneous dynamics and domains in supercooled *o*-terphenyl: A single molecule study," J. Phys. Chem. B **106**(44), 11438–11445 (2002).
[109]R. Zondervan, T. Xia, H. van der Meer, C. Storm, F. Kulzer, W. van Saarloos, and M. Orrit, "Soft glassy rheology of supercooled molecular liquids," Proc. Natl. Acad. Sci. U. S. A. **105**(13), 4993–4998 (2008).
[110]S. A. Mackowiak, T. K. Herman, and L. J. Kaufman, "Spatial and temporal heterogeneity in supercooled glycerol: Evidence from wide field single molecule imaging," J. Chem. Phys. **131**(24), 244513 (2009).
[111]Z. Ristanović, M. M. Kerssens, A. V. Kubarev, F. C. Hendriks, P. Dedecker, J. Hofkens, M. B. J. Roeffaers, and B. M. Weckhuysen, "High-resolution single-molecule fluorescence imaging of zeolite aggregates within real-life fluid catalytic cracking particles," Angew. Chem. **127**(6), 1856–1860 (2015).
[112]Z. Ristanović, J. P. Hofmann, G. De Cremer, A. V. Kubarev, M. Rohnke, F. Meirer, J. Hofkens, M. B. J. Roeffaers, and B. M. Weckhuysen, "Quantitative 3D fluorescence imaging of single catalytic turnovers reveals spatiotemporal gradients in reactivity of zeolite H-ZSM-5 crystals upon steaming," J. Am. Chem. Soc. **137**(20), 6559–6568 (2015).
[113]W. Xu, J. S. Kong, Y.-T. E. Yeh, and P. Chen, "Single-molecule nanocatalysis reveals heterogeneous reaction pathways and catalytic dynamics," Nat. Mater. **7**(12), 992–996 (2008).
[114]X. Zhou, W. Xu, G. Liu, D. Panda, and P. Chen, "Size-dependent catalytic activity and dynamics of gold nanoparticles at the single-molecule level," J. Am. Chem. Soc. **132**(1), 138–146 (2010).
[115]J. Kirstein, B. Platschek, C. Jung, R. Brown, T. Bein, and C. Bräuchle, "Exploration of nanostructured channel systems with single-molecule probes," Nat. Mater. **6**(4), 303–310 (2007).
[116]H. Yang, G. Luo, P. Karnchanaphanurach, T.-M. Louie, I. Rech, S. Cova, L. Xun, and X. S. Xie, "Protein conformational dynamics probed by single-molecule electron transfer," Science **302**(5643), 262–266 (2003).
[117]O. Flomenbom, K. Velonia, D. Loos, S. Masuo, M. Cotlet, Y. Engelborghs, J. Hofkens, A. E. Rowan, R. J. M. Nolte, M. Van der Auweraer, F. C. de Schryver, and J. Klafter, "Stretched exponential decay and correlations in the catalytic activity of fluctuating single lipase molecules," Proc. Natl. Acad. Sci. U. S. A. **102**(7), 2368–2372 (2005).
[118]A. A. Deniz, S. Mukhopadhyay, and E. A. Lemke, "Single-molecule biophysics: At the interface of biology, physics and chemistry," J. R. Soc., Interface **5**(18), 15–45 (2008).
[119]H. Miller, Z. Zhou, J. Shepherd, A. J. M. Wollman, and M. C. Leake, "Single-molecule techniques in biophysics: A review of the progress in methods and applications," Rep. Prog. Phys. **81**(2), 024601 (2017).
[120]E. Lerner, T. Cordes, A. Ingargiola, Y. Alhadid, S. Chung, X. Michalet, and S. Weiss, "Toward dynamic structural biology: Two decades of single-molecule förster resonance energy transfer," Science **359**(6373), eaan1133 (2018).
[121]J. Elf and I. Barkefors, "Single-molecule kinetics in living cells," Annu. Rev. Biochem. **88**(1), 635–659 (2019).
[122]A. Gahlmann and W. E. Moerner, "Exploring bacterial cell biology with single-molecule tracking and super-resolution imaging," Nat. Rev. Microbiol. **12**(1), 9–22 (2014).
[123]Y. M. Sigal, R. Zhou, and X. Zhuang, "Visualizing and discovering cellular structures with super-resolution microscopy," Science, **361**, 880 (2018).
[124]A. Yildiz, M. Tomishige, R. D. Vale, and P. R. Selvin, "Kinesin walks hand-over-hand," Science **303**, 676 (2004).
[125]J. van Mameren, M. Modesti, R. Kanaar, C. Wyman, E. J. G. Peterman, and G. J. L. Wuite, "Counting RAD51 proteins disassembling from nucleoprotein filaments under tension," Nature **457**(7230), 745–748 (2009).
[126]J. S. Lewis, L. M. Spenkelink, S. Jergic, E. A. Wood, E. Monachino, N. P. Horan, K. E. Duderstadt, M. M. Cox, A. Robinson, N. E. Dixon, and A. M. van

Oijen, "Single-molecule visualization of fast polymerase turnover in the bacterial replisome," eLife **6**, e23932 (2017).
[127]S. A. Stratmann and A. M. van Oijen, "DNA replication at the single-molecule level," Chem. Soc. Rev. **43**(4), 1201–1220 (2014).
[128]R. Roy, S. Hohng, and T. Ha, "A practical guide to single-molecule FRET," Nat. Methods **5**(6), 507–516 (2008).
[129]B. Schuler and W. A. Eaton, "Protein folding studied by single-molecule FRET," Curr. Opin. Struct. Biol. **18**(1), 16–26 (2008).
[130]B. Schuler, A. Soranno, H. Hofmann, and D. Nettels, "Single-molecule FRET spectroscopy and the polymer physics of unfolded and intrinsically disordered proteins," Annu. Rev. Biophys. **45**(1), 207–231 (2016).
[131]R. Buning, W. Koopmans, H. Neumann, J. Chin, and J. van Noort, "Regulation of nucleosome conformational dynamics by post-translational histone modifications studied with single-pair FRET," Biophys. J. **98**(3), 475a (2010).
[132]E. Haustein and P. Schwille, "Fluorescence correlation spectroscopy: Novel variations of an established technique," Annu. Rev. Biophys. Biomol. Struct. **36**(1), 151–169 (2007).
[133]M. A. Digman and E. Gratton, "Lessons in fluctuation correlation spectroscopy," Annu. Rev. Phys. Chem. **62**(1), 645–668 (2011).
[134]E. Sisamakis, A. Valeri, S. Kalinin, P. J. Rothwell, and C. A. M. Seidel, "Accurate single-molecule FRET studies using multiparameter fluorescence detection," Methods Enzymol. **475**, 455–514 (2010).
[135]S. Semrau, A. Pezzarossa, and T. Schmidt, "Microsecond single-molecule tracking (μsSMT)," Biophys. J. **100**(4), L19–L21 (2011).
[136]K. Welsher and H. Yang, "Multi-resolution 3D visualization of the early stages of cellular uptake of peptide-coated nanoparticles," Nat. Nanotechnol. **9**(3), 198–203 (2014).
[137]A. G. Godin, J. A. Varela, Z. Gao, N. Danné, J. P. Dupuis, B. Lounis, L. Groc, and L. Cognet, "Single-nanotube tracking reveals the nanoscale organization of the extracellular space in the live brain," Nat. Nanotechnol. **12**(3), 238–243 (2017).
[138]H. Mazal, F.-F. Wieser, and V. Sandoghdar, "Deciphering a hexameric protein complex with Angstrom optical resolution," bioRxiv (2021).
[139]P. D. Dahlberg, S. Saurabh, A. M. Sartor, J. Wang, P. G. Mitchell, W. Chiu, L. Shapiro, and W. E. Moerner, "Cryogenic single-molecule fluorescence annotations for electron tomography reveal *in situ* organization of key proteins in *Caulobacter*," Proc. Natl. Acad. Sci. U. S. A. **117**(25), 13937–13944 (2020).
[140]R. Zondervan, F. Kulzer, H. van der Meer, J. A. J. M. Disselhorst, and M. Orrit, "Laser-driven microsecond temperature cycles analyzed by fluorescence polarization microscopy," Biophys. J. **90**(8), 2958–2969 (2006).
[141]H. Yuan, T. Xia, B. Schuler, and M. Orrit, "Temperature-cycle single-molecule FRET microscopy on polyprolines," Phys. Chem. Chem. Phys. **13**(5), 1762–1769 (2011).
[142]H. Yuan, A. Gaiduk, J. R. Siekierzycka, S. Fujiyoshi, M. Matsushita, D. Nettels, B. Schuler, C. A. M. Seidel, and M. Orrit, "Temperature-cycle microscopy reveals single-molecule conformational heterogeneity," Phys. Chem. Chem. Phys. **17**(9), 6532–6544 (2015).
[143]E. D. Holmstrom, N. F. Dupuis, and D. J. Nesbitt, "Pulsed IR heating studies of single-molecule DNA duplex dissociation kinetics and thermodynamics," Biophys. J. **106**(1), 220–231 (2014).
[144]F. Zosel, A. Soranno, K. J. Buholzer, D. Nettels, and B. Schuler, "Depletion interactions modulate the binding between disordered proteins in crowded environments," Proc. Natl. Acad. Sci. U. S. A. **117**(24), 13480–13489 (2020).
[145]C. de Mello Donegá, *Nanoparticles: Workhorses of Nanoscience* (Springer Verlag, Heidelberg, 2014).
[146]A. Tcherniak, S. Dominguez-Medina, W.-S. Chang, P. Swanglap, L. S. Slaughter, C. F. Landes, and S. Link, "One-photon plasmon luminescence and its application to correlation spectroscopy as a probe for rotational and translational dynamics of gold nanorods," J. Phys. Chem. C **115**(32), 15938–15949 (2011).
[147]M. Yorulmaz, S. Khatua, P. Zijlstra, A. Gaiduk, and M. Orrit, "Luminescence quantum yield of single gold nanorods," Nano Lett. **12**(8), 4385–4391 (2012).
[148]M. Lippitz, M. A. van Dijk, and M. Orrit, "Third-harmonic generation from single gold nanoparticles," Nano Lett. **5**(4), 799–802 (2005).
[149]M. A. van Dijk, M. Lippitz, and M. Orrit, "Detection of acoustic oscillations of single gold nanospheres by time-resolved interferometry," Phys. Rev. Lett. **95**(26), 267406 (2005).
[150]P. Zijlstra, A. L. Tchebotareva, J. W. M. Chon, M. Gu, and M. Orrit, "Acoustic oscillations and elastic moduli of single gold nanorods," Nano Lett. **8**(10), 3493–3497 (2008).
[151]T. H. Taminiau, F. D. Stefani, and N. F. van Hulst, "Optical nanorod antennas modeled as cavities for dipolar emitters: Evolution of sub- and super-radiant modes," Nano Lett. **11**(3), 1020–1024 (2011).
[152]H. M. Doeleman, C. D. Dieleman, C. Mennes, B. Ehrler, and A. F. Koenderink, "Observation of cooperative Purcell enhancements in antenna–cavity hybrids," ACS Nano **14**(9), 12027–12036 (2020).
[153]R. Chikkaraddy, B. de Nijs, F. Benz, S. J. Barrow, O. A. Scherman, E. Rosta, A. Demetriadou, P. Fox, O. Hess, and J. J. Baumberg, "Single-molecule strong coupling at room temperature in plasmonic nanocavities," Nature **535**(7610), 127–130 (2016).
[154]F. Benz, M. K. Schmidt, A. Dreismann, R. Chikkaraddy, Y. Zhang, A. Demetriadou, C. Carnegie, H. Ohadi, B. de Nijs, R. Esteban, J. Aizpurua, and J. J. Baumberg, "Single-molecule optomechanics in 'picocavities,'" Science **354**, 726 (2016).
[155]S. Khatua, P. M. R. Paulo, H. Yuan, A. Gupta, P. Zijlstra, and M. Orrit, "Resonant plasmonic enhancement of single-molecule fluorescence by individual gold nanorods," ACS Nano **8**(5), 4440–4449 (2014).
[156]B. Pradhan, S. Khatua, A. Gupta, T. Aartsma, G. Canters, and M. Orrit, "Gold-nanorod-enhanced fluorescence correlation spectroscopy of fluorophores with high quantum yield in lipid bilayers," J. Phys. Chem. C **120**(45), 25996–26003 (2016).
[157]X. Lu, G. Ye, D. Punj, R. C. Chiechi, and M. Orrit, "Quantum yield limits for the detection of single-molecule fluorescence enhancement by a gold nanorod," ACS Photonics **7**(9), 2498–2505 (2020).
[158]G. Reecht, F. Scheurer, V. Speisser, Y. J. Dappe, F. Mathevet, and G. Schull, "Electroluminescence of a polythiophene molecular wire suspended between a metallic surface and the tip of a scanning tunneling microscope," Phys. Rev. Lett. **112**(4), 047403 (2014).
[159]H. Imada, M. Imai-Imada, K. Miwa, H. Yamane, T. Iwasa, Y. Tanaka, N. Toriumi, K. Kimura, N. Yokoshi, A. Muranaka, M. Uchiyama, T. Taketsugu, Y. K. Kato, H. Ishihara, and Y. Kim, "Single-molecule laser nanospectroscopy with micro–electron volt energy resolution," Science **373**, 95 (2021).
[160]R. Zhang, Y. Zhang, Z. C. Dong, S. Jiang, C. Zhang, L. G. Chen, L. Zhang, Y. Liao, J. Aizpurua, Y. Luo, J. L. Yang, and J. G. Hou, "Chemical mapping of a single molecule by plasmon-enhanced Raman scattering," Nature **498**(7452), 82–86 (2013).
[161]E. Bailo and V. Deckert, "Tip-enhanced Raman scattering," Chem. Soc. Rev. **37**(5), 921–930 (2008).
[162]Y. R. Shen, *The Principles of Nonlinear Optics* (Wiley, 2022).
[163]K. D. Heylman, N. Thakkar, E. H. Horak, S. C. Quillin, C. Cherqui, K. A. Knapper, D. J. Masiello, and R. H. Goldsmith, "Optical microresonators as single-particle absorption spectrometers," Nat. Photonics **10**(12), 788–795 (2016).
[164]M.-H. Chien, M. Brameshuber, B. K. Rossboth, G. J. Schütz, and S. Schmid, "Single-molecule optical absorption imaging by nanomechanical photothermal sensing," Proc. Natl. Acad. Sci. U. S. A. **115**(44), 11150–11155 (2018).
[165]T. Larsen, S. Schmid, L. G. Villanueva, and A. Boisen, "Photothermal analysis of individual nanoparticulate samples using micromechanical resonators," ACS Nano **7**(7), 6188–6193 (2013).
[166]S. Berciaud, L. Cognet, G. A. Blab, and B. Lounis, "Photothermal heterodyne imaging of individual nonfluorescent nanoclusters and nanocrystals," Phys. Rev. Lett. **93**(25), 257402 (2004).
[167]S. Berciaud, L. Cognet, P. Poulin, R. B. Weisman, and B. Lounis, "Absorption spectroscopy of individual single-walled carbon nanotubes," Nano Lett. **7**(5), 1203–1207 (2007).
[168]P. Kukura, M. Celebrano, A. Renn, and V. Sandoghdar, "Single-molecule sensitivity in optical absorption at room temperature," J. Phys. Chem. Lett. **1**(23), 3323–3327 (2010).
[169]M. Celebrano, P. Kukura, A. Renn, and V. Sandoghdar, "Single-molecule imaging by optical absorption," Nat. Photonics **5**(2), 95–98 (2011).

[170]S. Chong, W. Min, and X. S. Xie, "Ground-state depletion microscopy: Detection sensitivity of single-molecule optical absorption at room temperature," J. Phys. Chem. Lett. **1**(23), 3316–3322 (2010).
[171]P. Spaeth, S. Adhikari, L. Le, T. Jollans, S. Pud, W. Albrecht, T. Bauer, M. Caldarola, L. Kuipers, and M. Orrit, "Circular dichroism measurement of single metal nanoparticles using photothermal imaging," Nano Lett. **19**(12), 8934–8940 (2019).
[172]P. Spaeth, S. Adhikari, M. D. Baaske, S. Pud, J. Ton, and M. Orrit, "Photothermal circular dichroism of single nanoparticles rejecting linear dichroism by dual modulation," ACS Nano **15**(10), 16277–16285 (2021).
[173]D. Zhang, C. Li, C. Zhang, M. N. Slipchenko, G. Eakins, and J.-X. Cheng, "Depth-resolved mid-infrared photothermal imaging of living cells and organisms with submicrometer spatial resolution," Sci. Adv. **2**(9), e1600521 (2016).
[174]Y. Bai, J. Yin, and J.-X. Cheng, "Bond-selective imaging by optically sensing the mid-infrared photothermal effect," Sci. Adv. **7**(20), eabg1559 (2021).
[175]Y.-C. Huang, T.-H. Chen, J.-Y. Juo, S.-W. Chu, and C.-L. Hsieh, "Quantitative imaging of single light-absorbing nanoparticles by widefield interferometric photothermal microscopy," ACS Photonics **8**(2), 592–602 (2021).
[176]Y. Bai, D. Zhang, L. Lan, Y. Huang, K. Maize, A. Shakouri, and J.-X. Cheng, "Ultrafast chemical imaging by widefield photothermal sensing of infrared absorption," Sci. Adv. **5**(7), eaav7127 (2019).
[177]M. Liebel, F. V. A. Camargo, G. Cerullo, and N. F. van Hulst, "Ultrafast transient holographic microscopy," Nano Lett. **21**(4), 1666–1671 (2021).
[178]Y. Zhang, H. Zong, C. Zong, Y. Tan, M. Zhang, Y. Zhan, and J.-X. Cheng, "Fluorescence-detected mid-infrared photothermal microscopy," J. Am. Chem. Soc. **143**(30), 11490–11499 (2021).
[179]M. Li, A. Razumtcev, R. Yang, Y. Liu, J. Rong, A. C. Geiger, R. Blanchard, C. Pfluegl, L. S. Taylor, and G. J. Simpson, "Fluorescence-detected mid-infrared photothermal microscopy," J. Am. Chem. Soc. **143**(29), 10809–10815 (2021).
[180]D. Cole, G. Young, A. Weigel, A. Sebesta, and P. Kukura, "Label-free single-molecule imaging with numerical-aperture-shaped interferometric scattering microscopy," ACS Photonics **4**(2), 211–216 (2017).
[181]L. Priest, J. S. Peters, and P. Kukura, "Scattering-based light microscopy: From metal nanoparticles to single proteins," Chem. Rev. **121**(19), 11937–11970 (2021).
[182]P. Zijlstra, P. M. R. Paulo, and M. Orrit, "Optical detection of single non-absorbing molecules using the surface plasmon resonance of a gold nanorod," Nat. Nanotechnol. **7**(6), 379–382 (2012).
[183]I. Ament, J. Prasad, A. Henkel, S. Schmachtel, and C. Sönnichsen, "Single unlabeled protein detection on individual plasmonic nanoparticles," Nano Lett. **12**(2), 1092–1095 (2012).
[184]M. D. Baaske, P. S. Neu, and M. Orrit, "Label-free plasmonic detection of untethered nanometer-sized Brownian particles," ACS Nano **14**(10), 14212–14218 (2020).
[185]M. D. Baaske, N. Asgari, D. Punj, and M. Orrit, "Nanosecond time scale transient optoplasmonic detection of single proteins," Sci. Adv. **8**, eabl5576 (2022).
[186]E. Boukobza, A. Sonnenfeld, and G. Haran, "Immobilization in surface-tethered lipid vesicles as a new tool for single biomolecule spectroscopy," J. Phys. Chem. B **105**(48), 12165–12170 (2001).
[187]C. Limouse, J. C. Bell, C. J. Fuller, A. F. Straight, and H. Mabuchi, "Measurement of mesoscale conformational dynamics of freely diffusing molecules with tracking FCS," Biophys. J. **114**(7), 1539–1550 (2018).
[188]F. Cardarelli, L. Lanzano, and E. Gratton, "Capturing directed molecular motion in the nuclear pore complex of live cells," Proc. Natl. Acad. Sci. U. S. A. **109**(25), 9863–9868 (2012).
[189]K. Welsher and H. Yang, "Imaging the behavior of molecules in biological systems: Breaking the 3D speed barrier with 3D multi-resolution microscopy," Faraday Discuss. **184**, 359–379 (2015).
[190]S. Hou, J. Exell, and K. Welsher, "Real-time 3D single molecule tracking," Nat. Commun. **11**(1), 3607 (2020).
[191]A. E. Cohen and W. E. Moerner, "Method for trapping and manipulating nanoscale objects in solution," Appl. Phys. Lett. **86**(9), 093109 (2005).
[192]Q. Wang, R. H. Goldsmith, Y. Jiang, S. D. Bockenhauer, and W. E. Moerner, "Probing single biomolecules in solution using the anti-Brownian electrokinetic (ABEL) trap," Acc. Chem. Res. **45**(11), 1955–1964 (2012).
[193]A. H. Squires, A. A. Lavania, P. D. Dahlberg, and W. E. Moerner, "Interferometric scattering enables fluorescence-free electrokinetic trapping of single nanoparticles in free solution," Nano Lett. **19**(6), 4112–4117 (2019).
[194]M. Krishnan, N. Mojarad, P. Kukura, and V. Sandoghdar, "Geometry-induced electrostatic trapping of nanometric objects in a fluid," Nature **467**(7316), 692–695 (2010).
[195]M. A. van Dijk, L. C. Kapitein, J. van Mameren, C. F. Schmidt, and E. J. G. Peterman, "Combining optical trapping and single-molecule fluorescence spectroscopy: Enhanced photobleaching of fluorophores," J. Phys. Chem. B **108**(20), 6479–6484 (2004).
[196]A. Candelli, G. J. L. Wuite, and E. J. G. Peterman, "Combining optical trapping, fluorescence microscopy and micro-fluidics for single molecule studies of DNA–protein interactions," Phys. Chem. Chem. Phys. **13**(16), 7263–7272 (2011).
[197]J. Lipfert, J. W. J. Kerssemakers, T. Jager, and N. H. Dekker, "Magnetic torque tweezers: Measuring torsional stiffness in DNA and RecA-DNA filaments," Nat. Methods **7**(12), 977–980 (2010).
[198]G. Sitters, D. Kamsma, G. Thalhammer, M. Ritsch-Marte, E. J. G. Peterman, and G. J. L. Wuite, "Acoustic force spectroscopy," Nat. Methods **12**(1), 47–50 (2015).
[199]A. A. L. Nicolet, M. A. Kol'chenko, C. Hofmann, B. Kozankiewicz, and M. Orrit, "Nanoscale probing of charge transport in an organic field-effect transistor at cryogenic temperatures," Phys. Chem. Chem. Phys. **15**(12), 4415–4421 (2013).
[200]A. Shkarin, D. Rattenbacher, J. Renger, S. Hönl, T. Utikal, P. Seidler, S. Götzinger, and V. Sandoghdar, "Nanoscopic charge fluctuations in a gallium phosphide waveguide measured by single molecules," Phys. Rev. Lett. **126**(13), 133602 (2021).
[201]A. Moradi, "Towards optical detection of a single electron," Ph.D. thesis, Leiden University, 2021.
[202]J. Hwang, M. Pototschnig, R. Lettow, G. Zumofen, A. Renn, S. Götzinger, and V. Sandoghdar, "A single-molecule optical transistor," Nature **460**(7251), 76–80 (2009).
[203]M. Orrit, "Celebrating optical nanoscopy," Nat. Photonics **8**(12), 887–888 (2014).
[204]A. Gruber, A. Dräbenstedt, C. Tietz, L. Fleury, J. Wrachtrup, and C. von Borczyskowski, "Scanning confocal optical microscopy and magnetic resonance on single defect centers," Science **276**(5321), 2012–2014 (1997).
[205]A. V. Naumov, A. A. Gorshelev, Y. G. Vainer, L. Kador, and J. Köhler, "Far-field nanodiagnostics of solids with visible light by spectrally selective imaging," Angew. Chem., Int. Ed. **48**(51), 9747–9750 (2009).
[206]J. Zirkelbach, B. Gmeiner, J. Renger, P. Türschmann, T. Utikal, S. Götzinger, and V. Sandoghdar, "Partial cloaking of a gold particle by a single molecule," Phys. Rev. Lett. **125**(10), 103603 (2020).
[207]I. Gerhardt, B. Grotz, P. Siyushev, and J. Wrachtrup, "Coherent interaction of single molecules and plasmonic nanowires," Int. J. Mod. Phys. B **31**(24), 1740004 (2017).
[208]W. Zhang, M. Caldarola, X. Lu, and M. Orrit, "Plasmonic enhancement of two-photon-excited luminescence of single quantum dots by individual gold nanorods," ACS Photonics **5**(7), 2960–2968 (2018).
[209]A. Balan, P. M. Derlet, A. F. Rodríguez, J. Bansmann, R. Yanes, U. Nowak, A. Kleibert, and F. Nolting, "Direct observation of magnetic metastability in individual iron nanoparticles," Phys. Rev. Lett. **112**(10), 107201 (2014).
[210]D. Wang, H. Kelkar, D. Martin-Cano, T. Utikal, S. Götzinger, and V. Sandoghdar, "Coherent coupling of a single molecule to a scanning fabry-perot microcavity," Phys. Rev. X **7**(2), 021014 (2017).
[211]L. M. K. Vandersypen, M. Steffen, G. Breyta, C. S. Yannoni, M. H. Sherwood, and I. L. Chuang, "Experimental realization of Shor's quantum

factoring algorithm using nuclear magnetic resonance," Nature **414**(6866), 883–887 (2001).
[212]J. Michaelis, C. Hettich, J. Mlynek, and V. Sandoghdar, "Optical microscopy using a single-molecule light source," Nature **405**(6784), 325–328 (2000).
[213]M. C. Chong, N. Afshar-Imani, F. Scheurer, C. Cardoso, A. Ferretti, D. Prezzi, and G. Schull, "Bright electroluminescence from single graphene nanoribbon junctions," Nano Lett. **18**(1), 175–181 (2018).
[214]I. M. Palstra, H. M. Doeleman, and A. F. Koenderink, "Hybrid cavity-antenna systems for quantum optics outside the cryostat?," Nanophotonics **8**(9), 1513–1531 (2019).
[215]S. Han, C. Qin, Y. Song, S. Dong, Y. Lei, S. Wang, X. Su, A. Wei, X. Li, G. Zhang, R. Chen, J. Hu, L. Xiao, and S. Jia, "Photostable fluorescent molecules on layered hexagonal boron nitride: Ideal single-photon sources at room temperature," J. Chem. Phys. **155**(24), 244301 (2021).