

**Non-fluorescent optical schemes for single molecule detection, imaging,
sensing and spectroscopy**

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Abstract

The detection and study of single molecules with light has for decades relied on fluorescence-based detection. The development of other optical forms of single-molecule interrogation would, however, greatly expand the range of addressable molecules beyond those species that are highly emissive and photostable, and offer new applications beyond molecular biophysics and imaging. Here, we review the range of recent developments in optical label-free detection and imaging schemes that offer single molecule sensitivity, with an emphasis on plasmonic-enhanced and scattering approaches. We highlight the advantages and challenges facing these emerging methodologies and briefly outline their potential future applications.

Introduction

Spectroscopy is one of the dominant tools for characterising, understanding and thus eventually controlling the properties of optically active molecules and materials. Yet, the interaction between electromagnetic radiation and matter, irrespective of the investigated transitions is generally weak due to the large mismatch between the wavelength of light and the size of the molecules, atoms and electrons whose excitation and motion contains information about molecular properties. Most spectroscopic investigations therefore rely on the generation of a signal by a large ensemble of molecules that is strong enough to be measurable. This approach is useful because it enables the measurement of interest by raising the signal above the noise, but also somewhat unsatisfactory because it requires significant amounts of sample and, most importantly, only provides ensemble-averaged information. In addition, certain experiments cannot be performed at the ensemble-level, such as those requiring single photon sources¹ or investigations into the mechanism by which molecular motors move.² A more fundamental motivation was indirectly set by James Maxwell,³ who stated in 1873: “Molecular science ... is one of those branches of study which deal with things invisible and imperceptible by our senses, and which cannot be subjected to direct experiment”. Today, such experiments are not only possible but also routine across multiple disciplines. For instance, single electrons are used to perform nuclear magnetic resonance on single molecules,^{4,5} single atoms are trapped and manipulated⁶ and single emitters have become ubiquitous in a variety of applications in the life sciences^{7,8} and quantum information science.⁹

The origin of single molecule optics and microscopy traces back to the seminal experiments by Moerner and Kador, who detected individual dye molecules embedded in a crystalline matrix at cryogenic temperatures by absorption.¹⁰ This work was quickly followed by another low-temperature experiment by Orrit and coworkers, but in this case relying on fluorescence rather than absorption detection.¹¹ The far superior signal-to-noise ratio of the latter experiment laid the foundation for the dominance of fluorescence microscopy over all other optical studies at the single molecule level over the next 25 years. In both cases the low temperature, achieved by cooling the system to or near liquid Helium temperatures, and the immobilisation of the molecules within a solid matrix, converted the broad room temperature absorption spectrum to effectively a single narrow feature, which enhanced the interaction with light by increasing the absorption

cross-section by almost six orders of magnitude.

To understand the origin of this experimental approach, it is instructive to consider the basics of room temperature light-matter interactions at the single molecule level, in particular the ratio between the absorption cross-section (σ) and excitation area (A): σ/A . At room temperature, the interaction strength is dominated by the mismatch between the diffraction limited focussing area of visible light and the effective size of the molecule interacting with the incident photons. As an example, the absorption cross-section for a dye molecule is on the order of $\sigma = 5 \times 10^{-16} \text{ cm}^2$, while a high numerical aperture objective focuses light to an area on the order of $A = 5 \times 10^{-10} \text{ cm}^2$. As a result, $\sigma/A = 1 \times 10^{-6}$, which translates to the absorption of at best one photon in 10^6 incident photons by the molecule of interest. Thus, in a traditional absorption experiment, where light transmission is compared in the presence and absence of the substance of interest, the differential signal is only on the order of 1 in 10^6 , making it extremely difficult to detect. In cryogenic conditions under an illumination wavelength, λ , and for the correct host-guest system, the absorption cross-section can approach λ^2/π , which in turn results in σ/A reaching unity and therefore much larger interaction strengths.¹²

This consequences of the room-temperature mismatch between σ and A , at least from a detection perspective, become dramatically less important once the detection mechanism switches from photon absorption to fluorescence emission. The shift of the emitted photons to a lower energy compared to the excitation light converts a high-background measurement into a zero-background one, at least in the absence of any other species with similar absorption and emission properties.

The direct comparison between fluorescence and extinction, defined as the sum of scattering and absorption, leads to the immediate conclusion that extinction detection at the single molecule is unnecessarily complex compared to fluorescence. Although the signal-to-noise ratio can be comparable in an idealised setting, detecting a parts-per-million variation on top of a large background is experimentally much more challenging. Commercial Laser sources rarely achieve intensity fluctuations lower than 0.1% root mean square, detectors suffer from dark counts and read noise, and sample drift and background scattering affect the detected light intensities. As a result, such direct extinction measurements rarely achieve the required sensitivity levels except in highly optimised environments.¹³

Nevertheless, there are many reasons why extinction detection is worth pursuing. For instance, species that absorb or scatter light efficiently are not subject to the main

drawbacks of fluorescence microscopy such as photobleaching, photoblinking or limited excited state lifetimes, all of which restrict photon flux and duration. Extinction detection opens single-molecule investigations to a vast number of molecules that interact with light, but do not fluoresce very efficiently, such as small organic molecules or most proteins. Beyond these purely photophysical and photochemical arguments, there are numerous other disadvantages associated with the addition of a label in general. Labelling may perturb the system under study, as efficient light absorbing and emitting molecules are frequently larger than the molecule they are attached to. Take for example lipids, where dye molecules are known to alter lipid partitioning and distribution within a membrane,^{14,15} or larger sized scattering labels, which can induce variations in the mobility of lipids.^{7,8,16} Similarly, processes involving assembly or disassembly on the nanoscale, such as amyloid formation are difficult to monitor with true single-molecule resolution due to the necessity of distinguishing bound molecules from those at high concentration in solution.

As a result, there have been numerous attempts to image and study single molecules without relying on fluorescence-based detection schemes. To keep the scope and length of this progress report manageable, we focus on techniques that use light to excite and detect single molecules. Nevertheless there are examples where either the excitation or detection is non-optical.^{17,18} We emphasise that the goal of these developments is not to replace the hugely successful single-molecule fluorescence approach, especially in the life sciences, but rather to broaden the scope of single-molecule techniques towards applications that are fundamentally inaccessible to fluorescence-based methodologies.

All non-fluorescent single-molecule detection techniques discussed in this review aim to improve the detectability of extinction signatures either by increasing the signal generated by a single molecule or by reducing or optimising the measurement of the background. In general, they can be split into three major categories depending on the conditions of how single molecule detection is achieved: resonance-enhanced, plasmonically-enhanced and non-resonant. For resonant detection, the molecules of interest must possess an optical transition matching the excitation source; for plasmonic enhanced detection, no restriction on the type of molecule is imposed but a nano-plasmonic structure is required, and non-resonant detection, which only relies on the read-out of the extinction signature of the analyte molecule. Strictly speaking, only the last two categories qualify as truly label-free, defined in this review as the detection of

single molecules in the absence of any modifications and applicable to any (bio)molecule.

Resonant detection

Given the weak interaction of single molecules with light, means must be found to improve the signal to noise ratio of the detected signal. Resonant detection achieves this by only using molecules with a strong optical transition at the illumination wavelength, such as a ground to excited state electronic absorption. This optical transition increases the polarisability of the system at the incident wavelength, which in turn amplifies the extinction signature from a single molecule. In most cases, however, the use of molecules with a strong optical transition alone is insufficient to achieve single-molecule sensitivity; instead the single molecule signature must either be amplified either by increasing the light-matter interaction strength or significantly suppressing the background noise.

The conceptually most intuitive approach to improve single molecule signatures involves changing the σ/A ratio, i.e. the relative magnitudes of the area of the incident light beam and the size of the molecule of interest. Diffraction places firm boundaries on the minimal achievable focus size, A , which leaves changing the effective size of the molecule, σ , as the only viable alternative. This is the approach that enabled the first detection of single molecules reported by Moerner and Kador more than 25 years ago by cooling the molecules to liquid Helium temperatures, making σ/A approach unity. While the original work struggled with the then available optics and detectors, more recent low-temperature studies have demonstrated extinction of a laser beam by almost 10% with a single molecule, a phenomenon with potential applications in optical switching at the single molecule level.¹² Despite ample potential applications in quantum information science, for example, this effect is limited to a small number of dye molecules in very specific conditions including ultralow temperatures and crystalline matrices and is therefore not broadly applicable. Furthermore, the presence of crystalline matrices and long acquisition times on the order of seconds make dynamic investigations unfeasible.

Although the first experimental study reporting single-molecule sensitivity relied on absorption detection at cryogenic conditions,¹⁰ at room temperature the large mismatch between the interaction of light and single molecule ($\sigma/A = 1 \times 10^{-6}$) and the vastly superior background suppression achievable with fluorescence detection largely

discouraged further development in this direction. Some progress in fluorescence-free detection at room temperature conditions was made by implementing interferometric techniques,¹⁹ but it was only recently that three different approaches achieved single-molecule sensitivity almost simultaneously.²⁰

The conceptually simplest approach of the three involves a microscopic version of a standard absorption spectrometer.^{13,21} Light transmission is measured as a sample containing single molecules is translated through the tight focus of a microscope objective. The use of a strong light absorber, such as a molecular dye, optimises the interaction between the light and the molecule to the point where the difference in transmitted light amounts to several parts-per-million in the presence vs. the absence of the molecule in the focus. The second approach²² also probes an electronic transition, in this case stimulated emission, rather than absorption, leading to signals of similar magnitudes, but employing high-frequency modulation for noise suppression and two light sources for excitation and stimulated emission. A similar experimental approach is used in the third technique known as photothermal detection of single molecules,²³ except that here the electronic transition of the molecules is not probed directly, but rather through the dissipation of heat by the molecule to the environment. This is recorded in an interferometric fashion through the resulting local change in refractive index and induced phase shift on a probe beam.

All three techniques have their respective advantages and disadvantages. The first requires a highly uniform refractive index environment to distinguish the molecule from the background, but enables single-molecule detection with very low incident powers and theoretically provides the highest achievable signal-to-noise for a given amount of incident light as it probes the strongest, electronic absorption, transition. The other two techniques have the advantage that they enable modulation of the optical signal, which especially at high frequencies strongly suppresses laser and electronic noise, thus leading to the desired single-molecule sensitivity. In this way, the molecule of interest can be identified even in the presence of a large number of background scatterers. On the downside, these techniques effectively require driving the molecule into saturation to maximise the detectable signal, increasing the occurrence of adverse photochemical effects.

These experiments were critical in that they changed the prevalent impression that optical single-molecule detection was only possible either through the use of signal amplifiers or through the established fluorescence route. Their widespread adoption,

however, has remained limited largely for three reasons. Firstly, all techniques require a significant effort in noise suppression due to the still very small signals that are observable. Secondly, they rely on the presence of a strong optical resonance. Thirdly, the samples are embedded in a non-aqueous matrix (crystalline, polymer or glycerol) and the typical imaging rate was on the timescale of seconds. Although this expands the scope of single-molecule detection beyond fluorescence, most biological species cannot be studied under these conditions.

Plasmonically-enhanced detection

To expand single-molecule sensitivity to, at least in principle, all molecules, a handful of conceptually different techniques have exploited the ability of plasmonic structures to dramatically increase the interaction between light and matter, and at the same time amplify the extinction signatures from individual analytes. These techniques can be categorised into surface-enhanced Raman scattering (SERS), nano-plasmonic spectral and optical transmission detection, and hybrid plasmonic/whispering gallery mode detection.

Surface-enhanced Raman scattering

Surface-enhanced effects have been most prominent in Raman scattering,^{24,25} which is an inherently inefficient inelastic light scattering phenomenon. With off-resonant Raman cross-sections on the order of 10^{-30} cm² or less, detection of Stokes-shifted Raman scattering becomes effectively impossible in the presence of even the most weakly autofluorescent background. From this perspective, strong, near-field enhancement of the electric field at nanoscopic sites on the surfaces of plasmonic nanoparticles is helpful in three ways: (i) it increases the field strength and thereby the scattered photon flux, (ii) it acts on a significantly subdiffraction-limited volume thereby reducing the amount of non-resonant background, and (iii) it drastically decreases the interaction area (Fig. 1a). The combination of all three effects enables the observation of Raman spectra from single molecules (Fig. 1b).²⁶ One major advantage of this approach is that Raman spectra provide chemically specific information about the species of interest. A disadvantage is the difficulty associated with controlled fabrication of the nanoscale features that allow for the highest levels of enhancement as well as controlled placement of the molecule of interest in their vicinity.

Figure 1. Surface-enhanced Raman spectroscopy (SERS). **a**, Schematic of plasmonic hotspot amplification of single-molecule light-matter interactions. **b**, SERS spectra of rhodamine 6G using a dumbbell structure.²⁷ **c**, Principle of tip-enhanced Raman spectroscopy (TERS). **d**, Single-molecule TERS spectra of rhodamine 6G.²⁸

Recently, controlled combination of gold nanoparticles and placement of single molecules in the gap between the resulting dimers has been combined with non-linear implementations of Raman spectroscopy.²⁹ Using coherent anti-Stokes Raman scattering, the authors reported vibrational motion directly at the single molecule level. Some of the downsides of SERS, such as the difficulty of controlled placement of the molecules of interest in the plasmonic hotspot can be partially avoided using tip-enhanced Raman spectroscopy, TERS, (Fig. 1c).³⁰ Here, molecules are bound to a surface, over which a nanoscopic probe is scanned, leading to strongly enhanced scattering when the two come into close proximity allowing for improved control in the enhancement conditions (Fig. 1d).^{31,32} At room-temperature, the typical optical resolution achieved with TERS is on the order of 3-15 nm; however sub-molecular optical resolution allowing the chemical- and internal structure-imaging of single molecule was recently reported using a scanning tunnelling microscope at low temperature and pressure.³³

The main challenge facing SERS technology at the single-molecule level remains the controlled fabrication of hotspots and the correct placement of the molecules of interest in their vicinity. Although the latter is somewhat alleviated by scanning probe approaches, these experiments are more complex to set up and implement than a simple inverted microscope. Tip fabrication remains an art, especially when very high spatial resolution is necessary. Alternative approaches could benefit from the remarkable improvements in DNA origami technology over the past decade allowing for geometric control on the molecular scale²⁷ or by taking advantage of non-linear phenomena such as multi-photon absorption used for light-assisted molecular immobilisation.³⁴

Nanoplasmonic spectral detection

In SERS, the primary role of the nanoparticle is to enhance the local electric field in the vicinity of the molecule to improve the light-matter interaction so as to generate a detectable signal for weak scatterers. Alternatively, one can envision recording an optical signature of the nanoparticle and measuring any effects the binding or detachment of a single molecule on or near its surface has. Plasmonic nanoparticles exhibit specific resonances whose spectral appearance and location is tightly coupled to their immediate refractive index environment. As the nanoparticle size decreases and, ideally approaches that of individual proteins, binding of a single molecule to the nanoparticle surface results in a change of the overall refractive index environment (Fig. 2a). This refractive index change in turn will shift the plasmon resonance, ultimately enabling the detection of individual binding events. The experimental challenge from a pure sensitivity perspective involves the detection and spectral characterisation of the smallest possible nanoparticles, as these exhibit the largest spectral shifts upon single-molecule binding.

This challenge has been met by two, complementary approaches. In the first, total internal reflection microscopy is used to illuminate single nanoparticle sensors and the scattered light is extracted before being recorded by a spectrometer (Fig. 2b). The availability of high-power white light lasers enables high signal-to-noise ratios in determining the precise position of the resonance and thereby leads to single-protein sensitivity.³⁵ Alternatively, photothermal detection has been used, which has the advantage of *a priori* higher background suppression since the signal can be specifically modulated leading to the detection of smaller nanoparticles and thus higher sensitivity (Fig. 2c).³⁶ Rather than recording the entire spectrum, the photothermal approach uses a combination of heating and read-out lasers to probe shifts in the plasmon resonance position through the heating of the surrounding medium. Ultimately, the photothermal technique indirectly measures the position of the resonance by probing a spectral region where the resonance exhibits a steep slope. There, changes in the overall resonance position lead to significant modifications of the resonance condition, which is eventually read-out through changes of refractive index through heating.

Figure 2. Spectral nanoplasmonic detection of single molecules. a, Concept behind plasmonic sensing of single-molecule binding events. Attachment of a single molecule changes the local refractive index and thereby the plasmon resonance

frequency of a gold nanorod. **b,c** Single-protein detection by direct nanorod spectroscopy³⁵ and photothermal microscopy.³⁶

The preferential use – due to much higher achievable sensitivities caused by electric field hotspots – of nanorods means that binding at different sites leads to large variations in the detected spectral shift, making a clear calibration between signal magnitude and molecular weight of the analyte difficult. This has been alleviated partially by tip-specific functionalization,³⁶ yet the corresponding step-size histograms remain broad. In addition, multiplexing of the detection modality is difficult and both photothermal and spectral implementations have been limited to investigating one particle at a time. Only recently has sensor multiplexing been achieved by measuring the change in normalised scattering intensity in darkfield images, thus significantly improving throughput and opening the possibility to study single-molecule interactions.³⁷ The sensitivity level demonstrated in this work was enabled by the use of superluminescent diodes and tuning the amount of incident light and exposure time of each acquired image. The achievable sensitivity levels need to be explored further, although there are indications that spectral diffusion may pose a fundamental barrier.³⁶

Nanoplasmonic detection by optical transmission

By changing the geometry of the plasmonic structure from a nanorod to a dual nanohole and recording optical transmission rather than the plasmonic response, single-molecule detection has recently been reported using a double nanhole slit (DNS) (Fig. 3a). In this technique, the plasmonic structure optically traps a binding partner of the analyte of interest and the dynamics of the individual analyte molecules interacting with the optically trapped binding partner are registered as changes in optical transmission.³⁸ Rather than relying on the limited ability of far field optics to focus the incident light, this arrangement optimises the light-matter interaction and leads to a significant difference in light transmission in the presence and absence of a molecule in a nanoscopic slit (Fig. 3b,c). A second advantage is the intrinsic ability of the plasmonic structure to trap the molecule of interest without any additional assistance. The approach has been reported to be sensitive beyond pure detection events, such as substrate binding, denaturing and most recently, even the presence of low-frequency Raman transitions.³⁹ The latter is particularly exciting as it promises to provide a molecule-specific signature beyond the

size of the molecule as otherwise only available in SERS. To which degree this approach can be used as a sensor is too early to judge. In its current implementation it relies on diffusion to deliver the molecule of interest to a single trapped and much larger binding partner, with multiple binding sites that are specific to the analyte, which heavily depends on both the concentration of the analyte and the presence of other species. At the same time, there are ample prospect for future developments in terms of both sensitivity and multiplexing.⁴⁰

Figure 3. Double nanohole slit single-molecule detection. **a**, Trapping of a single molecule in a double nanohole slit (DNS). **b**, Transmitted light intensity changes during individual trapping events. **c**, Schematic of DNS trapping sensing with vibrational sensitivity.⁴⁰

Hybrid microcavity-plasmonic detection

The final approach that takes advantage of plasmonic enhancement is based on a technique that relies on the concept of multiple interactions of a single photon with the same molecule. As a result, even if the interaction between the incident light and the species of interest is very weak due to the inherently small cross-sections of single molecules, it can be amplified by repeating the interaction many times. This concept has been used by microcavity-based detection schemes for some time,⁴¹ but recently achieved robust single-molecule sensitivity through the combination with plasmonic enhancement.^{42,43}

Experimentally, light is coupled into a microresonator where it performs many roundtrips leading to a very narrow linewidth transmission enabled by the high quality factor of the resonator (Fig. 4a). In the case of silica resonators, binding of protein molecules to the surface effectively results in an increase of the resonator radius and thereby a phase shift, which is measured as a change in the resonance wavelength of the microcavity system (Fig. 4b). Despite early reports of single-molecule sensitivity,⁴⁴ the widespread implementation based on microcavity detection via whispering gallery modes, however, has suffered from the two main requirements to achieve high sensitivity levels for single-molecule detection: high quality factors and small mode volumes.

Nevertheless, a recent study has shown that incorporation of nanoplasmonic structures to microcavities provide up to a three order of magnitude improvement in the sensitivity levels. In this work the interaction kinetics of single nucleic acids were monitored with a time resolution of 20 ms (Fig. 4c), and the exceptional sensitivity was demonstrated by the detection of single molecules with a molecular weight of just 1 kDa.⁴³

Figure 4. Cavity-enhanced detection. **a** Hybrid microcavity-plasmonic whispering gallery mode approach to single molecule detection including a dielectric sphere, a gold nanorod, a tunable laser light source and appropriate detection.⁴³ **b**, Analyte binding detected by a shift in resonance frequency.⁴¹ **c**, Whispering gallery mode⁴³ detection of single-molecule binding events.

Advantages of using microcavities/nanoplasmonic hybrids include the potential for parallel detection and in principle extremely high sensitivity levels. At the same time, there are some drawbacks: the recorded signal magnitude does not strictly scale with molecular properties such as the molecular weight, but also depends on the location where the molecule binds on the sensor, which is difficult to control as for plasmonic sensors. In addition, no spatial information is available, which can be highly advantageous for facile multiplexing.

Non-resonant label-free detection

In all previously discussed techniques, the molecule requires a strong optical transition or an external amplifier to make it detectable. The question thus arises to which degree single molecules can be detected and studied without the addition of plasmonic or external structures. Re-examining the basic aspects of single-molecule detection approaches outlined in the introduction, the biggest drawback is the large mismatch between the molecular size and the diffraction limit of visible light. A single protein molecule is nevertheless only about a factor of 50 times smaller than a diffraction-limited beam, leading to a non-negligible interaction between the two. Such a molecule will therefore scatter a small percentage of photons from an incident beam, and that percentage will remain constant for a non-resonant interaction, irrespective of the incident power. The difficulty in detecting a single molecule thus is only one of

controlling or measuring the background scattering precisely. Even if this background is much larger than the single-molecule signature, it can be subtracted and removed if it can be measured precisely and does not change on the timescale of the measurement. Ultimately, this description is one of any sensor, which relies on the difference between a measurement in the presence and absence of the analyte. The simplicity of fluorescence detection is in that it is performed on top of a very low, almost negligible background, while scattering or extinction detection takes place on top of a large background.

Furthermore, one often-overlooked benefit of non-resonant optical microscopy based on extinction detection is the ability to visualise structural dynamics on the nanoscale. For example, different condensed matter phases exhibit different refractive indices and therefore scatter light to varying degrees, which can be imaged with sufficient sensitivity. Highly sensitive imaging of refractive index variations, an alternative perspective on label-free single molecule imaging, could be applied to study lipid and membrane associated processes such as vesicle fusion⁴⁵ or nanoscopic phase separation.⁴⁶ Similarly, the ability to image single proteins without labels may enable dynamic studies of biological self-assembly phenomena such as amyloidosis⁴⁷ or microtubule dynamics.⁴⁸

Interferometric scattering microscopy⁴⁹⁻⁵¹ combines the above principles, iSCAT, (Fig. 5a). Shining light on the sample and detecting the light returning towards the illumination path reduces the background over which the single-molecule signal needs to be measured. The resulting image contains numerous contributions from the surface, many of which are orders of magnitude larger than a single protein signal, such as actin filaments in a standard myosin 5 landing assay (Fig. 5b). Subtraction of this constant signal then reveals only mobile scatterers, for example single myosin 5 molecules binding and translocating along the actin filaments immobilised on the coverslip surface (Fig. 5c).⁵² The same concept has been used more recently to demonstrate single-protein sensitivity down to the 60 kDa level (Fig. 5d).⁵³

Figure 5. Single-molecule detection and imaging via interferometric scattering microscopy. **a**, Schematic of interferometric scattering microscopy (iSCAT). **b**, iSCAT image of unlabelled actin filaments bound to a microscope coverslide.⁵² **c**, Individual myosin 5 molecules binding to and walking along actin in the presence of ATP.⁵² Note the 30-fold reduction in the intensity scale. Scale bars: 1 μm . **d**, Label-free detection down to the 60 kDa level with iSCAT.⁵³

Apart from the experimental simplicity, one advantage of this approach is that it is compatible with most of the technologies developed for surface plasmon resonance sensing, i.e. detection of binding/unbinding events to specifically functionalised surfaces. The substrate becomes even more simplistic as it only consists of a glass coverslide, rather than a gold surface. The sensitivity is higher, with current limitations set largely by the availability of optimised detectors rather than the approach itself. The signal magnitude scales linearly with molecular weight and is independent of the binding site. Furthermore this approach opens the possibility to study nanoscale phase transitions currently inaccessible to any other technique. In fact, it appears that the main challenges of iSCAT as a biosensor are no longer in the optics, but rather in the design and specificity of the sensor.⁵³ A drawback of iSCAT is that little information about the molecule can be extracted beyond the molecular weight and that any scatterer produces a signal leading to potentially high backgrounds when residual scattering cannot be controlled.

Conclusion and Outlook

Optical investigations at the single-molecule level without relying on fluorescence detection have made remarkable progress over the last decade. In particular, the levels of sensitivity achieved in the past few years were until recently thought to be categorically unachievable, not too dissimilar to the beliefs that were changed by the seminal works of Moerner and Orrit 25 years ago. Single-molecule detection is for the first time entering the realm of no longer relying on resonant interactions between the incident light and the species of interest, and thus rapidly opening up new avenues of research and applications. Experiments that have relied on single-molecule fluorescence detection and grappled with its limitations can now be addressed in novel ways with applications in both biosensing and in studies of structural transitions.

One important aspect regarding the applicability of these techniques is the achievable time-resolution. In terms of the state-of-the-art, all existing techniques reported time-resolutions on the tens of ms – s timescale.^{35-37,39,43,52} All techniques, in principle, provide routes to higher detection speeds, but invariably require (i) more incident light and (ii) near shot noise-limited detection. Even for non-resonant interactions the incident photon flux will at some point cause damage or become difficult to detect with sufficient precision. At this stage, however, it appears that the more critical

challenges that need to be addressed first are: simplification of the experimental setup, and improving the robustness of the detection assays; whereas higher time-resolution should be driven by specific applications. Achieving high time-resolution in an idealised assay does not necessarily provide relevant information as to the capabilities of a particular technique as a single molecule sensor in a realistic setting. Here, the limits of sensitivity and achievable time resolution need to be explored further to make a quantitative comparison amongst the existing non-fluorescent single-molecule techniques.

For future success and growth of the field, lessons need to be learned from the development of activation-based super-resolution fluorescence imaging, which was considerably propelled by its simple experimental implementation and thus widespread adoption with respect to single molecule switching. Here, decades of single-molecule biophysics research ensured that the technological challenges were largely addressed upon arrival of the technique itself, a scenario that is currently non-existent for any, bar the simplest approaches discussed here.

One of the strengths of the field is the wide range of potential future applications and its multidisciplinary character. Nanoplasmonic detection enables large-scale multiplexing and will benefit immensely from the implementation of nanofabrication and microfluidic approaches. Trapping without relying on fluorescence emission may lead to prolonged investigations of single molecules, their activities and structural dynamics. Label-free biosensing is of enormous importance for studying, understanding and tuning protein-protein and protein-substrate interactions. In this context, surface-based biosensing technology provides potentially higher sensitivity levels and higher detection speeds. Finally, not having to rely on fluorescence labelling may enable precise characterisation of cell-to-cell variations with single-molecule pull-down assays.⁵⁴

Many of the technological challenges have been met or are likely to become routine over the next few years given the leaps achieved in this decade. The next important step in this field will be to apply them and demonstrate the potential of label-free single-molecule techniques and begin to challenge the currently justified dominant role of single-molecule fluorescence microscopy.

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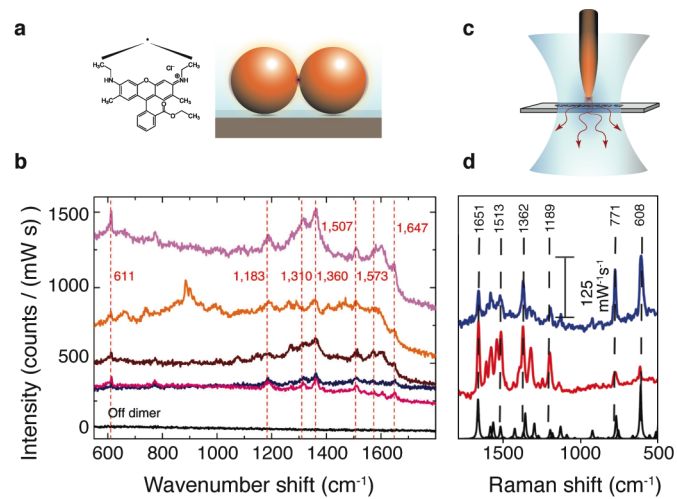


Figure 1

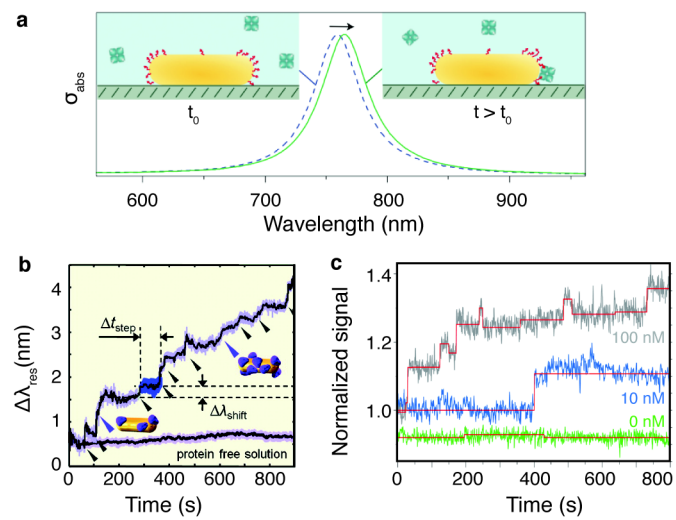


Figure 2

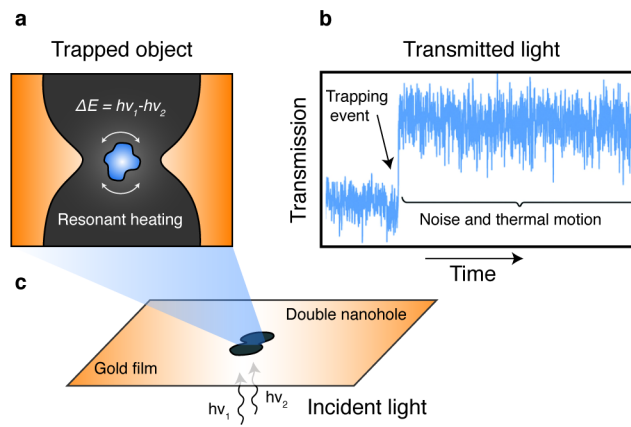


Figure 3

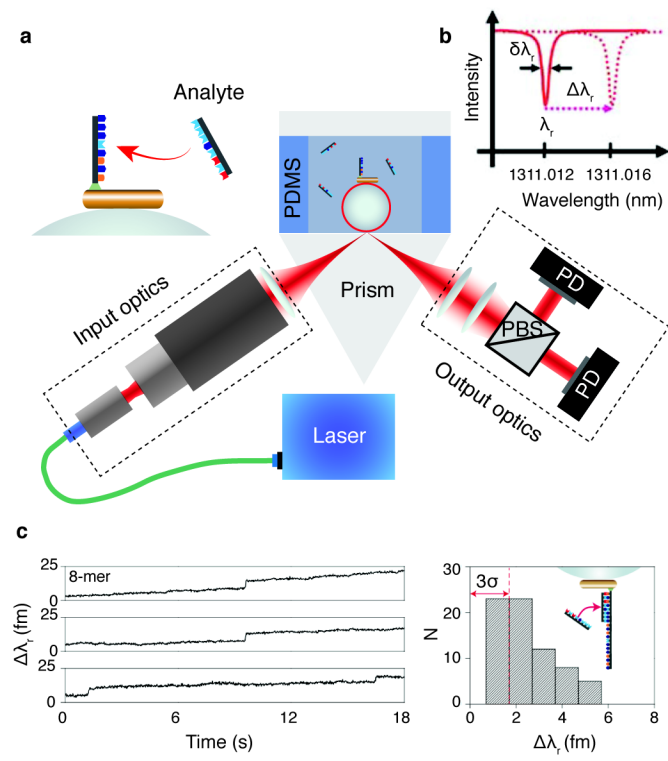


Figure 4

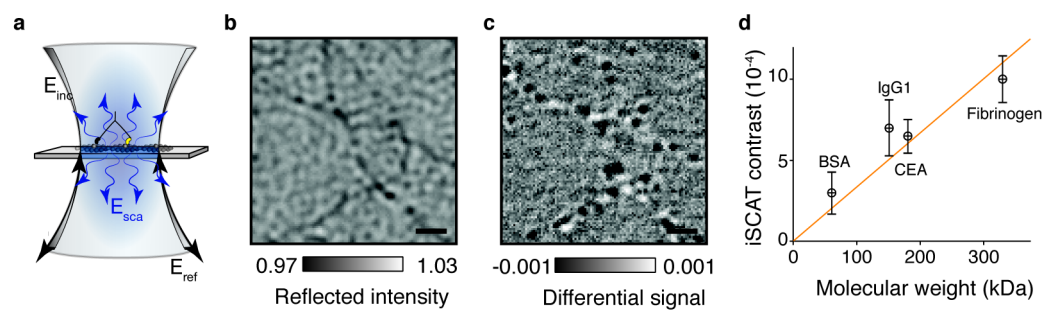


Figure 5