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Single-Molecule Sensors: Challenges and Opportunities for Quantitative Analysis

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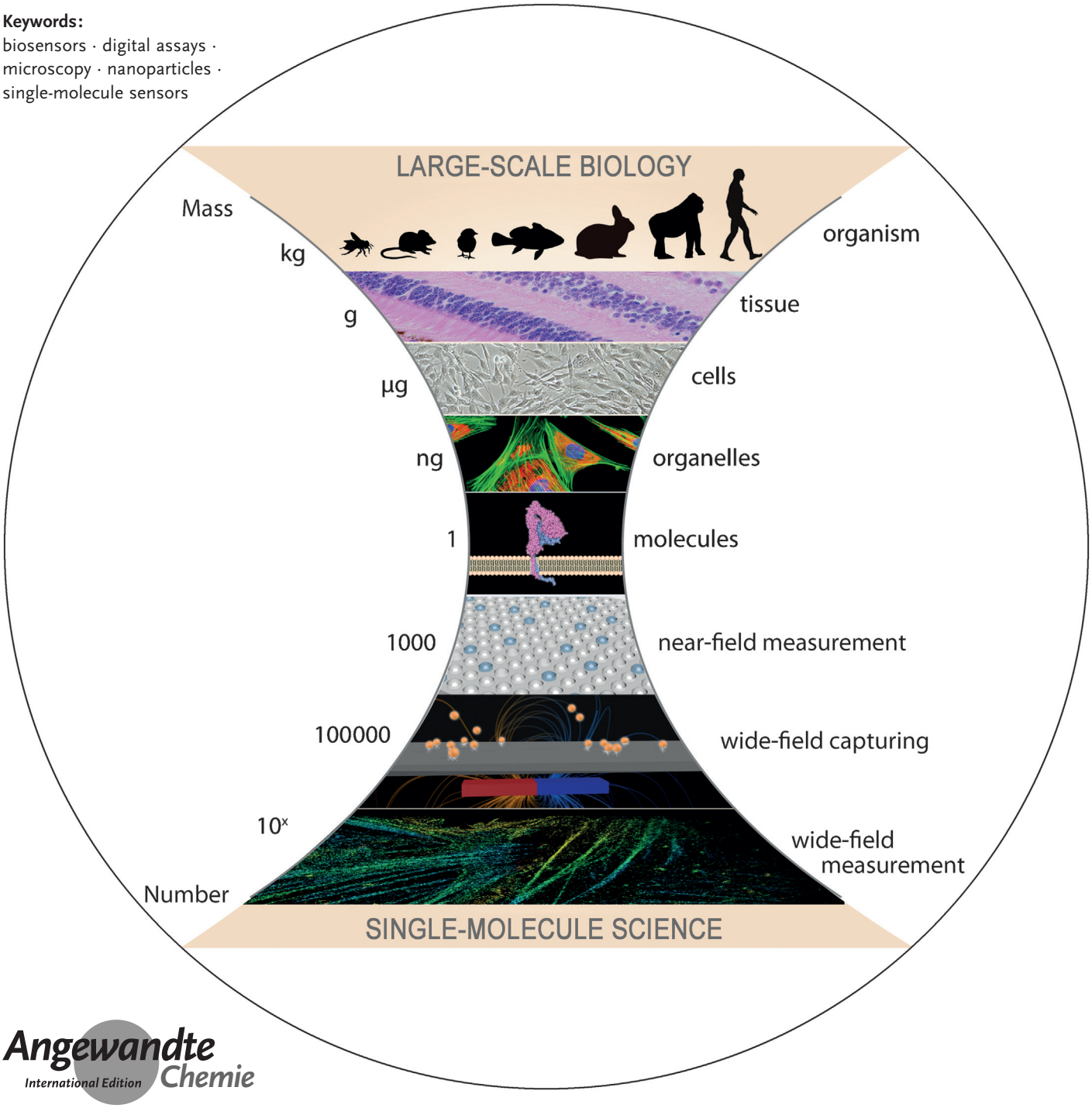

Measurement science has been converging to smaller and smaller samples, such that it is now possible to detect single molecules. This Review focuses on the next generation of analytical tools that combine single-molecule detection with the ability to measure many single molecules simultaneously and/or process larger and more complex samples. Such single-molecule sensors constitute a new type of quantitative analytical tool, as they perform analysis by molecular counting and thus potentially capture the heterogeneity of the sample. This Review outlines the advantages and potential of these new, quantitative single-molecule sensors, the measurement challenges in making single-molecule devices suitable for analysis, the inspiration biology provides for overcoming these challenges, and some of the solutions currently being explored.

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1. Introduction

The intertwined nature of advances in measurement and advances in knowledge is no better exemplified than in microscopy and biology. Leeuwenhoek's application of the microscope to biology in the 17th Century was the starting point of modern biology and triggered technical developments in optics that culminated in the invention of super-resolution fluorescence microscopy in our time.[1] Microscopy, however, is not the only analytical tool that is trying to resolve and detect smaller and smaller amounts of material. Electrochemical methods, mass spectrometry, microfluidics, and spectroscopy have all undergone similar developments in terms of reduction in the sample size, albeit in a faster timeframe. Thus, historically speaking, analytical methods have converged to the single-cell and the single-molecule level (Figure 1). Compared to ensemble measurements (comprising thousands or millions of entities), single-molecule and single-cell measurements provide far richer information because they have the capability of revealing heterogeneities and stochastic processes within biological systems.[2] A vitally important aspect of this richness in analytical information is the ability to detect and quantify rare, aberrant species which would be lost in the noise of an analytical device that presented information from an ensemble measurement. However, the measurement of single entities in complex systems brings its own challenges, both from a technical point of view as well as for data interpretation. Can a biological system be defined or classified as "healthy" or "diseased" by measuring only a few components? How can we ensure that the detected outlier in a single-entity assay is biological relevant? Will it ever be possible to design smart sensors that tell us whether a mutation or altered molecule is the cause of, or response to, a disease? Ultimately, we need to readjust our thinking of how individual components relate to the overall system response for sensor technologies and also for biological systems. Indeed, there are many analogies between single biological building blocks/analytes and system/sensor responses, which will be discussed at the end of this Review.

The first step in turning the detection of a single molecule into a means to perform quantitative analysis is to massively increase the number of detection events (Figure 1). Rather than detecting one molecule in one cell, for example, the onus is on measuring all, or most, of the copy numbers of that molecule in a cell, or identifying the same molecule in many cells or analyzing many different molecules in the same cell. This means that single-molecule sensing does not automatically mean small samples or fewer samples. In fact, single-molecule sensing may quickly encounter a similar "big data" problem as has arisen in systems biology.[3] The motivation to face these challenges comes both from molecular biology, where one wants to gain new molecular and mechanistic insights, and from the diagnostics perspective, where one wants better-performing analytical devices.

From the analytical perspective, it is prudent to ask why we would want to complicate things by formulating strategies for quantifying many single-molecule events. There are several answers to this question. The obvious answer is that by being able to detect single molecules we can develop sensors with the ultimate detection limit and where the signal to noise ratio does not decrease with decreasing concentrations of the analyte. A further answer comes from the depth of analytical information that can be achieved. Typically, we want sensors to respond to only one type of species and, thus, classify all interactions of other species with the sensor as

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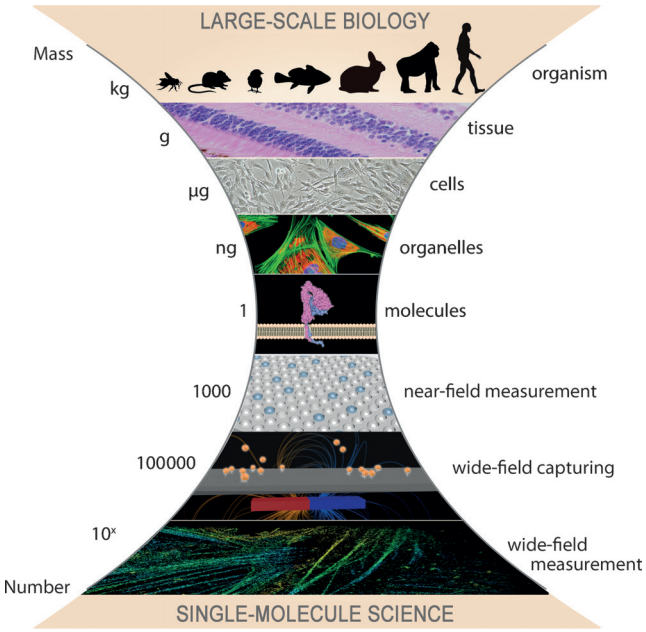

Figure 1. The development of measurement techniques from whole bodies to single cells and single molecules. To detect smaller and smaller entities, the sample size has drastically reduced. Measurement science now sits at the cusp where single-molecule measurements emerge into quantitative science, thus diverging back to larger sizes to capture many individual molecules. This will result in the convergence of measurement science and molecular biology. The diagram outlines strategies arising from confining single-molecule measurements to many individual wells (near-field measurement), to capturing the molecules in a bulk sample before the measurement takes place in a near-field configuration (wide-field capturing), to future wide-field measurements where many single-molecule measurements are monitored simultaneously.

undesirable or nonspecific. However, single-molecule sensors that detect *how* a molecule interacts with a surface, for example, do not need this classification, because the target analyte will have unique interaction properties. This shifts the emphasis away from single-molecule detection (with digital outputs) towards the quantification of single-molecule interactions (with more nuanced outputs richer in information). Single-molecule sensors that could measure molecular interactions across a wide spectrum of interaction properties, could also measure rare events, such as aberrant behavior. With traditional ensemble measurements, where only the mean response is measured, such rare events are lost within the noise. In single-molecule science, the distribution of the interaction properties is, in fact, more informative than the value of the mean. Finally, the sensing of many single molecules also means that the number of analyte molecules could be counted directly, which would simplify, or even completely remove, the need for calibration.

As we outline in this Review, developing single-molecule sensors for quantitative analysis is not simple, nor does the solution necessarily involve conventional sensing techniques. This is because most current sensing devices have two overriding features in common that make them unsuitable for single-molecule sensors. The first is that most sensors perform ensemble measurements, which means there is a single readout that is the mean of many single-molecule reactions or interactions. Secondly, conventional sensors aim for high selectivity and/or high affinity, because it is these attributes that give the sensor the possibility to only respond to one species even when exposed to the complex mixture of a biological sample.

This Review outlines the challenges in single-molecule sensing to emphasize why such measurements are so different to ensemble measurements and how sensing design needs to be adapted. The Review then covers strategies currently being employed to develop single-molecule sensors for quantitative analysis, outlining their strengths and weaknesses. It then looks at emerging strategies and finishes by drawing on biological examples of quantitative single-molecule sensing. Importantly, this Review does not seek to give a comprehensive overview of all the reported methods and technologies capable of detecting a single molecule, but rather seeks to present selected examples that demonstrate conceptual advances that allow the development of better quantitative sensors of single molecules. Furthermore, it is important to differentiate the quantitative single-molecules sensors discussed herein from the much more mature, and extensively reviewed[2b,4] field of single-molecule measurement that seeks to understand the properties of single molecules or single-molecule binding pairs.

2. The Challenges in Developing Quantitative Single-Molecule Sensors

The entry into the world of individual molecules has arisen from the incredible advances in measurement science. Optical trapping of individual kinesin molecules in 1993

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provided Svoboda et al. the first insights into the biophysics of molecular motors.[5] Other methods soon followed, such as force measurements with atomic force microscopy for protein–ligand binding[6] and DNA–DNA duplexes,[7] single-molecule fluorescence measurements of the turnover of individual ATP molecules by a single myosin molecule,[8] and the translocation of DNA through α-hemolysin nanopores.[9] Single-molecule measurements have progressed at an astonishing rate ever since. We are now at the point where the subtleties of enzymatic reactions can be monitored at the single-molecule level to reveal mechanistic information that would not otherwise be possible. For example, optical tweezer experiments of the interaction of enzymes (NS3 helicases) with RNA have revealed kinetic effects such as pauses, reversing of the enzyme reaction, and rewinding of unwound portions of the double-stranded RNA.[10] Similarly, single-molecule studies have been used to identify new potential antibiotics by screening molecules that block ion transport through single ion channels embedded within a lipid bilayer.[11]

These examples of single-molecule studies have three overriding aspects in common. Firstly, the methods can only detect a single or a few molecules at a time, which limits the number of interacting species in the sample that can be explored. Secondly, these methods typically require purified components and ex vivo assembly of the structure under investigation. Thirdly, to achieve single-molecule detection, the vast majority of approaches reduce the measurement space to very small volumes or areas. As a consequence, single-molecule measurements have thus far been a "near-field" science.

The key advantage of single-molecule approaches is their ability to detect heterogeneity in a population of molecules. As the interaction of biomolecules is typically stochastic, ensemble average measurements cannot tease out the subtleties of dynamic behavior, even if all the reactions were initiated at the same time. For example, Xie and co-workers explored Michaelis–Menten enzyme kinetics at a single-molecule level.[12] Although this classical enzyme mechanism still holds at the single-molecule level, the enzyme turnover was characterized by clusters of events separated by periods of low activity—fluctuations that could not be observed at the ensemble level.

An implication of stochastic behavior is that a population of molecules must be explored at the single-molecule level to understand the range of behavior that makes up the average ensemble measurement. This implies either serial measurement of many single molecules or massively parallel single-molecule measurements. Single-molecule electron-transfer measurements using a scanning tunneling microscope[13] have shown that temporal separation of many single-molecule measurements is viable for static systems when the measurement is rapid. However, for biomolecular interactions, the near-field approaches that are used in single-molecule fluorescence microscopy, and other biophysical techniques, have some significant limitations.

Reducing the measurement volume to a few femtoliters, as frequently done with near-field single-molecule measurements, places two limits on the range of biomolecular interactions that can be investigated. These are a concentration limit and a thermodynamic limit, as elegantly discussed by Tinnefeld and co-workers in the context of single-molecule fluorescence measurements. These limitations are equally true for all methods that use a near-field approach.[2a] The concentration limit arises from near-field single-molecule measurements requiring only one molecule in the detection volume at any one time. If the volume is 1 fL, this correlates to a concentration of about 2 nM in the detection volume. Measuring solutions of higher concentration will result in a high probability of more than one molecule being in the detection volume and an unreliable readout. Lowering the concentration, however, means the measurement takes inordinately long (Figure 2). For quantification in biological media, this lower concentration limit is above what one would normally want to detect. In a typical analysis in serum, the required concentrations are usually in the range of 10^{-16}–10^{-12} M but can be even lower. Rissin et al.[14] explained this with an example of a 1 mm^3 tumor of a million cells secreting 5000 proteins into a circulating blood stream with a volume of 5 L, which results in a concentration of about 2 fM. With single-molecule measurement techniques, it would take almost 10 minutes between single-molecule recordings at a concentration of 1 fM, assuming a typical protein diffusion coefficient of 1×10^{-7} $cm^2 s^{-1}$.

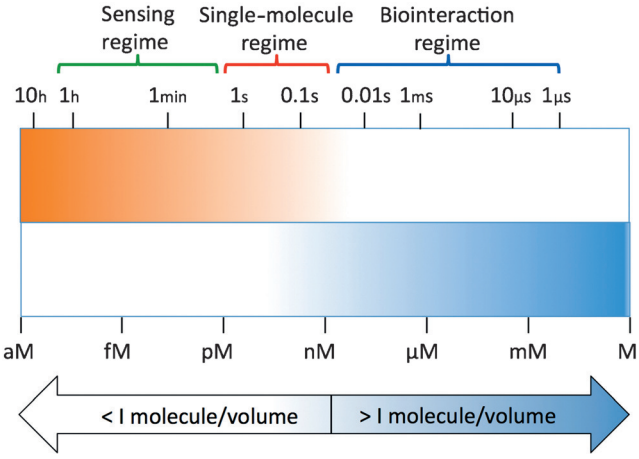

Figure 2. The different concentration regimes in which sensing, single molecules, and biological interactions typically operate. The single-molecule regime refers to a classical single-molecule experiment where measurements are performed by reducing the measurement volume (1 fL) so that there is only a single molecule or less in that volume. This equates to a concentration of 2 nM. At higher concentrations, where interactions between biological molecules typically occur in living systems, there will be more than one molecule per measurement volume and, hence, such concentration ranges are not amenable to single-molecule measurements. On the other hand, typical sensing concentrations are significantly lower than this regime. The limitation of the sensing region is that the time between measurements can be very long. This not only results in complications with regard to measurement time but also because the lifetime of the biological interacting complex on a single-molecule level may be shorter than the time between measurements. Assumptions are made that the measurement volume is 1 fL and the diffusion coefficient of a typically biomolecule is 1×10^{-7} $cm\,s^{-1}$.

The long timescale between measurements described above is exacerbated when quantitative analysis is required. This is because the samples taken for analysis must be representative of the bulk population. At low concentrations, sampling statistics are determined not by a Gaussian distribution but by a Poisson distribution. This distribution is used to calculate the probability of rare events occurring in a large number of trials. For the example above with a 1 fM concentration, a volume of over 50 nL must be processed to achieve 95% confidence that the analysis reflects the sample. With 10 minutes between single-molecule recordings, it would take at least 6 hours for all the molecules in the sample to pass through the measurement volume. As we will discuss in Section 4.1, this limitation explains why the nanopore sensors that can detect single molecules passing through a pore typically operate in the nanomolar to micromolar range.[9b] As a consequence, these pores are more frequently used for DNA sequencing,[15] where identification rather than quantification is the goal.

A consequence of "scaling down" to the single-molecule level is that the kinetics of biomolecular interactions can no longer be ignored; this is the thermodynamic limitation. The static viewpoint of biological binding events that arises from ensemble measurements may need to be rethought as we start to appreciate the dynamics of the binding processes. High-affinity interactions typically have dissociation constants in the micromolar to nanomolar range. This means that in enzyme-linked immunosorbent assays (ELISA), for example, the reversibility of the binding equilibrium is not detected, as the change in the number of binding events at any given time is too small to be recorded using conventional transducers. However, with single-molecule measurements—or measurement methods close to single-molecule resolution—the reversibility of affinity reactions becomes visible within the measurement.[16] This is important because the lifetime of the complex is linked to the dissociation equilibrium constant and association rate constant. Hence, the measurement method must be sufficiently rapid to detect the complex. One can expect the lifetime of a complex with a typical dissociation constant in the micromolar range to be about 1 second, when the diffusion-limited association constant is around $5 \times 10^{-5}\,\text{M}^{-1}\,\text{s}^{-1}$ (Ref. [2a]), and upwards of 3 minutes for high-affinity antibodies with a dissociation constant in the nM range.[17] As can be seen, with 10 minutes between measurements, such lifetimes can deleteriously influence the quality of analytical information obtained.

By considering the challenges of single-molecule measurements in terms of these concentration and thermodynamics limits, new opportunities arise in measurement design to bias equilibriums and drive binding reactions, for example, through compartmentalization and immobilization.

3. Learning from Nature To Address Challenges in Single-Molecule Sensing

A cell can sense and respond to the cues in its environment and adapt its morphology, behavior, and fate accordingly. Indeed, there are examples in biology where a single molecule can influence the overall outcome. A single peptide antigen, for example, can trigger the T-cell receptor and initiate an activation program that facilities an immune response.[18] To understand how this is possible, the T cell not only needs to detect and identify unique peptides at exceedingly low concentrations but also make a fate-determining decision: Is a given peptide sequence a benign peptide or is it an immunogenic peptide? In principle, there are two strategies for the design of sensors to meet this measurement challenge. Firstly, we could assemble a large peptide database that contains peptide sequences of all known pathogens, cancer as well as host proteins, and compare the detected peptide sequence to the database. Secondly, we could learn from T cells that utilize variations in the binding properties between the peptide antigen and the T-cell receptor for peptide detection and classification.

The binding affinities for the T-cell receptor with its cognate peptide ligands are relatively low ($K_d = 1$–$100\,\mu\text{M}$) and binding affinity does not necessarily correspond to T-cell responses.[19] Hence, the astonishing discrimination power of T cells between benign peptides and immunogenic peptides cannot solely be derived from the specificity of a single receptor–ligand interaction. Indeed, to overcome the low affinities, cells use tricks such as confining the interacting species into small volumes. This alters the apparent interaction kinetics, which are read out by the cell.[20] For example, the T-cell receptor is clustered into nanosized domains on the cell surface, which not only increases the concentration of receptors in that space but also restricts the mobility of each receptor.[21] Thus, it is possible that one ligand can serially bind to the same or neighboring receptors.[20,22] This is an example where many single-molecule interactions may compensate for the low affinity of one such interaction.

Other cellular structures that confine molecules and thus alter the "detection" rate are vesicles, two-dimensional membranes, or one-dimensional fibers. The dimensionality of these objects profoundly influences the reaction kinetics between molecules confined in these spaces. Here, sensor geometries could learn from biological confinement strategies. Whereas cells use confinement into small volumes to overcome the concentration limitation, other elements such as pores and receptors predominately operate on a single-molecule basis and can act as "gatekeepers" to these compartments. Such a selective confinement into a small volume would greatly simplify single-molecule measurements.

Another cellular strategy is to combine compartmentalization with multiplexing. Many proteins, particularly signaling molecules, have multiple interaction partners.[23] Hence, biological systems are not only heterogeneous in terms of interactions and reactions involving the same molecules, but also with respect to the interaction or reaction partners. A molecule that can interact with different partners under different circumstances is, biologically speaking, more valuable (and thus may have been evolutionally selected) than a molecule that can only bind one other partner.[23] The diversity in binding partners is, however, only functionally useful if diverse interactions can be communicated. It is far more likely that properties of the binding events are

communicated by signaling networks, rather than the identity of the binding partner. For example, which antigenic peptide is bound to the T-cell receptor is ultimately not important for the T cell to elicit an immune response. Thus, a common signaling mechanism exists that communicates the lifetime of the ligand–receptor interaction, and it is this binding property that is ultimately used for ligand discrimination. This is the biologically important message, while the actual sequence of the antigenic peptides is irrelevant. There is an important lesson here for single-molecule sensors: Reducing the specificity of the biorecognition species but increasing the amount of information obtained per single-molecule binding event (i.e. quantification of the complex lifetime at the expense of identification of the binding partner) can open the door for multiplexed sensing that yields valuable insight into complex systems. Sensors that quantify single-molecule binding events would have the same reliability for rare binding events as they have for common ones. Thus, the characteristics that define a good ensemble sensor (e.g. specificity, high-affinity binding) are not necessarily the same as for single-molecule sensors.

4. Emerging Approaches for Single-Molecule Sensors

What are the solutions to overcoming these limitations and allow single-molecule measurements to be performed with sufficient speed and statistical accuracy? We classify the emerging strategies into 1) fast near-field measurements, 2) massively parallel near-field measurements, 3) wide-field sampling/near-field detection measurements, and 4) wide-field measurements. The last three classes of emerging strategy are depicted in the bottom three panels of Figure 1.

4.1. Rapid Near-Field Measurements

Perhaps the first single-molecule measurement strategies to be used for quantitative analysis were the nanopore strategies pioneered by Bayley and co-workers[9b,24] for the detection of metal ions and organic molecules. These sensors operated by employing modified α-hemolysin nanopores embedded in a lipid bilayer that separated two sides of a solution such that species could only pass through the pore. This design is remarkably similar to pores and channels in cellular membranes. Electrodes placed on each side of the pore allowed the conductivity across the membrane to be measured. The conductivity decreases as a species translocates through the pore and partially blocks it (Figure 3). Selectivity is achieved by the analyte of interest having an affinity for the modified pore. Hence, the analyte is recognized by a resistive pulse and each resistive pulse is associated with a single species passing through the pore. The number of pulses, thus, reflects the amount of the species passing through the pores. In the early studies, detection limits on the order of nM for ions[24] and μM for organic species[25] were determined. Since those early days, there has been an enormous amount of research into using both protein-based and solid-state nanopores for sensing, with a strong focus being on detecting and sequencing DNA. With DNA sequencing, the key issue was

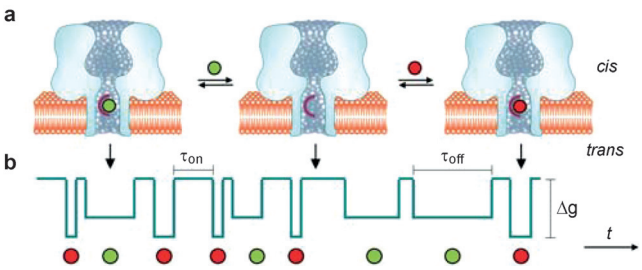

Figure 3. A single-molecule nanopore sensor. a) Reversible binding of different single analyte molecules (represented by red and green circles) with a receptor within the nanopore. b) The magnitude of the associated resistance pulses (indicated as a change in conductivity Δg) reflects the nature of the analyte, thus allowing differentiation between different analytes. Reproduced from Ref. [28] with permission from The Royal Society of Chemistry. Copyright 2010.

that the DNA molecules moved too fast through the nanopore for readout of the device, making the speed of molecules coming to the pore irrelevant.[26] The interested reader is referred to some of the many reviews[25,27] on this topic. Nanopores serve as an excellent example of how detection limits with single-molecule sensors can be decreased by increasing the rate at which the analyte of interest reaches the near-field measurement volume.

In a nanopore experiment, the time between single molecules encountering the pore (the encounter time) is governed by the same time rules as described above for a fluorescence experiment (Section 2), that is about 10 minutes between resistive pulses for a protein in a 1 fM solution. However, nanopores are not purely diffusive systems. The measurement is conducted with an applied electric field between the two electrodes. Hence, if the analyte of interest is charged, then migration of the analyte to the nanopore can greatly increase the encounter time with the pore for a given concentration. Höfler and Gyurcsányi[29] have addressed this issue theoretically by using a random walk model, and shown that the encounter time decreases as the electric field increases, with an up to two orders of magnitude decrease in encounter time and a concomitant decrease in detection limit. Despite the electric field decreasing the encounter time with a pore, the fact that measurements need to be performed in an electrolyte solution does mean that the electric field does not penetrate far from the pore. Thus, in reality, the mass transport of an analyte molecule far from the nanopore is typically by diffusion, with migration in electric fields becoming important only when the analyte comes close to the pore.

Freedman et al.[30] elegantly outlined the effect of the electrolyte on the influence of the electric field in bringing species to the nanopore. They suggest that if the capture radius of the pore as a result of the electric field is 3 μm, there would only be 34 molecules in the capture volume with a 1 nM solution. If the solution concentration was sub-picomolar, then there would only be 0.03 molecules in the capture volume and, hence, the sensor could only be operated with inordinately long measurement times again. Meller and co-workers[31] have addressed this issue using salt concentration

gradients. They showed that rather than have 1M KCl on each side of the pore, reducing the concentration to 0.2M on the side containing the DNA (the *cis* side) and maintaining 1M on the *trans* side led to the creation of an ionic gradient that emanated from the pore into the *cis* side. The consequence was a 30-fold increase in the rate of detection for a 3.8 pM DNA solution. A steep concentration gradient of 0.2M on the *cis* side and 4M on the *trans* side resulted in a resistive pulse approximately every second compared to several seconds between resistive pulses with 1M KCl on each side.

Building on the ideas of Meller and co-workers[31] for extending the volume of influence of the electric field from the nanopore, Freedman et al.[30] employed the alternating current technique of dielectrophoretic trapping. By using a metallic coating on a nanopipette for the measurement of DNA by resistive pulses, it is shown that the peak-to-peak voltage influenced the capture rate and, hence, the capture volume. A doubling of the peak-to-peak voltage from 10 V to 20 V resulted in a 10-fold increase in the capture rate. By using this approach, Freedman et al.[30] showed they could detect 5 fM DNA with 315 events per minute—quite a dramatic improvement compared with the absence of the dielectrophoretic trapping.

An alternative approach to using electrophoretic approaches to increase the capture volume is to use the tried and trusted approach of magnetic capture. Thus far, such strategies have been used to preconcentrate the analyte[32] prior to the nanopore measurement instead of using magnetic nanoparticles to bring the analyte to the pores.

The limitation of this general strategy of rapid near-field measurement volume is that it is restricted to measurement methods where the analyte translocates through the near-field measurement volume. If the single-molecule technique requires the molecules to reside in the measurement volume, then alternatives are required.

4.2. Massively Parallel Near-Field Measurements

Massively parallel near-field measurements of many single-molecule events in parallel can be performed in many small isolated sample wells. This is exactly what was achieved by Walt and co-workers using optical fiber bundle technology.[33] The ends of the fiber bundle are etched such that each fiber defines a well of 46 fL. A 1 mm bundle has 24000 of these wells, with each well spatially segregated from all the other wells. The first application of this technology was a single-enzyme assay, where the low concentrations of the enzyme β-galactosidase resulted in wells with either 1 or 0 enzyme molecules, whose activity was monitored with a fluorescent substrate.[34] Hence, the key principle of this single-molecule assay format is the confinement of the to-be-analyzed species into small volumes such that the concentration of the fluorescent substrate per well is sufficiently high to measure. An interesting variant of this experiment was recently published, where the enzyme was immobilized onto paramagnetic beads such that droplets containing the beads could be magnetically directed for loading of the wells.[35] This technology enabled single nucleic acid molecules to be amplified.[36] Another variant is the so-called slip-chip method developed by Ismagilov and co-workers.[37] The slip chip is a microfluidic device where a column of fluid is mechanically moved over nL-sized wells, where a reaction is allowed to proceed, with each well containing as little as a single molecule. In this way, single-molecule DNA amplification was demonstrated.[38]

There are many variants of this type of experiment for the monitoring of enzyme kinetics, as has been recently reviewed.[39] Small volumes have also been used for monitoring protein–protein binding in a zero-mode waveguide.[40] A particularly exciting development in this field is the development by Bohn and co-workers of arrays of zero-mode waveguides for spectroelectrochemical investigation of a single flavin mononucleotide (FMN) imaged in a wide-field format.[41] FMN is a redox enzyme cofactor, and closely related electrochemically and optically to the ubiquitous enzyme cofactor flavin adenine dinucleotide. Importantly, when excited, the oxidized form is fluorescent but the reduced form is less fluorescent. A feature of the zero-mode waveguides are that they have a gold optical cladding layer (Figure 4). This means only the oxidized FMN molecules that reach the bottom of the zero-mode waveguide will be stimulated. The gold cladding also serves to oxidize and reduce the FMN in each zero-mode waveguide. The volume of the zero-mode waveguides was estimated to be 200 zL, such that concentrations below 10 μM are expected to have an occupancy per waveguide of 1 or 0 FMN molecules. Thus, by alternating the potential from when the FMN is in its reduced form to an oxidizing potential results in the FMN fluorescence being observed in the zero-mode waveguides containing FMN molecules. This study opens the door to spectroelectrochemical investigation of single enzyme molecules and, further afield, to bioassays being performed. The advantages of this approach is that it is electrochemical, which is highly compatible with sensing, that the readout is in a wide-field format—so the responses are simply counted optically—and the species freely diffuse within each zero-mode waveguide.

An important aspect of the techniques described in this section are that they have the potential to be a digital assay,[42] since counting the number of wells with activity provides quantification. Poisson statistics must be used to determine the concentration from digital counting.[34] In the case where the concentration is sufficiently low so that observing anything other than a 0 or 1 event is highly improbable, the relationship between the percentage of "active" wells and bulk enzyme concentration approximates to linear. The limitation of this technique is that even though up to 24000 single molecules could be detected, in the case of the Rissin and Walt study,[34] this still represents a very small sample size in quantification terms (ca. 1 nL). This means that rare target species, where large samples are required, are not compatible with this approach. It should also be noted that this assay format compartmentalized the biology under investigation prior to analysis, which may preclude the measurement of interactions between molecules within a sample.

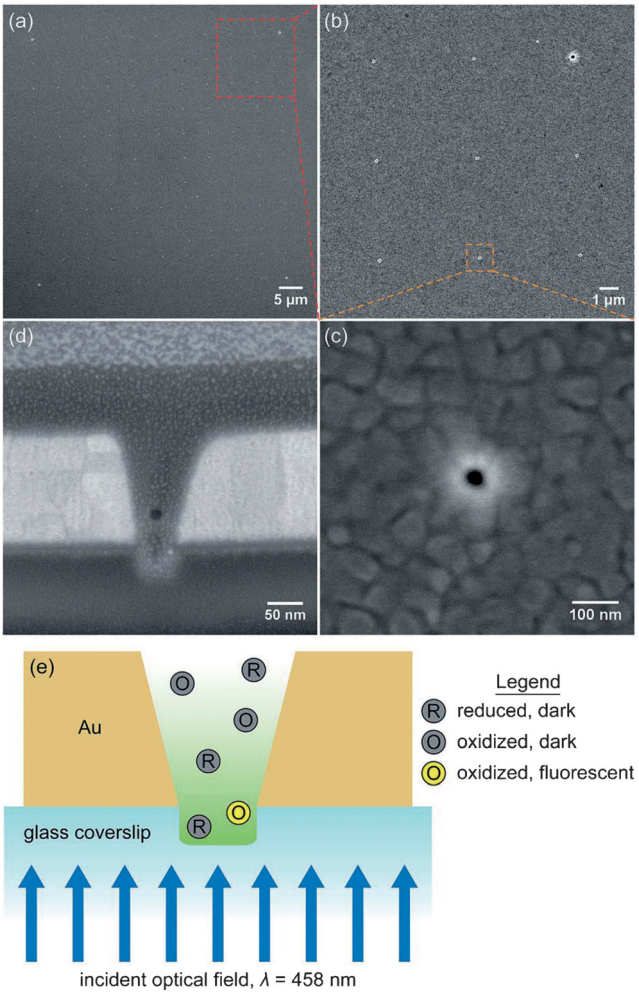

Figure 4. Scanning electron micrographs of the zero-mode waveguides used by Bohn and co-workers.[41] a) Top view of an 11×11 array of waveguides; enlarged views of b) nine waveguides and c) a single zero-mode waveguide; d) cross-sectional scanning electron micrograph of a single zero-mode waveguide; and e) schematic representation of the spectroelectrochemical setup of a single waveguide, where the gold serves as both electrodes for oxidizing and reducing FMN and as an optical cladding such that only oxidized FMN in the bottom section can be excited to fluoresce. Reproduced from Ref. [41] with permission from The Royal Society of Chemistry. Copyright 2015.

4.3. Wide-Field Sampling/Near-Field Detection

Extending the idea of massively parallel near-field measurement further, Rissin et al.[14] used the same principle to develop a digital single-molecule ELISA, which allows large sample volumes, and hence low analyte concentrations, to be analyzed (Figure 5). This is achieved by using antibody-modified magnetic beads that capture the analyte in solution, followed by binding of a secondary antibody with biotin labels to which streptavidin-β-galactosidase conjugate is attached. Only then are the magnetic nanoparticles loaded into the wells for single-molecule quantification of the number of "active" wells. Hence, confinement is only employed during the digital readout. We refer to this assay format as wide-field sampling/near-field detection measurements. The detection limits achieved are exquisite: for biotin–streptavidin it was 350 zM, which is equivalent to 10–20 enzyme labels in a 100 μL sample.[14] This technique has been termed single-molecule analysis or SiMoA, and became the basis of the company Quanterix. The Walt research group has demonstrated the power of this technique for a wide range of assays such as the cancer biomarker prostate-specific antigen,[43] the toxin ricin,[44] cytokines,[45] dengue fever,[46] and bacterial genomic DNA.[47] The range of different analytes exemplifies the generic nature of the method and, importantly, in all cases the detection limits are exquisitely low and in the sub-femtomolar range.

In assay formats with particles as sampling agents, both the number of beads and the number of biomolecules per bead is critical. As discussed above, at the single-molecule level the biomolecular complex is reversible and, hence, the lifetime of the complex is determined by the affinity constant. With particle assays, however, the presence of many antibodies on the bead (ca. 80000 in the case of Rissin et al.[14]) biases the equilibrium to the complex. That is, in a 100 μL sample with 200000 beads, the concentration of the capturing molecule is about 0.3 nM and a high percentage of the target species will be captured (ca. 70%). Lowering the antibody concentration on the beads lowers the capture efficiency. Furthermore, in the digital ELISA assay by Rissin et al.,[14] the number of beads must be sufficiently high to ensure that sufficient beads are captured in the 50000 wells, but too many beads lowers the ratio of target species to capturing agent. The implication is that many beads that have captured the target molecule may not end up in one of the wells and some analytical information will be lost. This is because only 20000–30000 of the 200000 beads are captured.

An important aspect of digital readouts relates to calibration. Calibration matches the instrument response to the amount or activity of the analyte in the sample. Calibration is always a challenge because standard solutions used in calibration should match the sample, but rarely do. Hence, calibration-free strategies of analysis are advantageous. In principle, analytical methods that can count the amount of material, such as is possible in gravimetry (where mass is measured) or coulometry[48] (where charge is counted) do not require calibration. Parallel single-molecule measurements with digital readouts can potentially be calibration-free.

Numerous other strategies are emerging for wide-field sampling/near-field measurements that employ other transduction methods. One strategy that allows wide-field sampling/near-field measurement, but which eliminates the compartmentalization of the sample during both capture and measurement is to exploit the optical properties of individual nanoparticles immobilized on a surface. There are a number of emerging assay formats that are approaching single-molecule sensitivity and have the potential for massive parallelization and continuous recording from many single nanoparticles. Many employ surface-bound nanoparticles that are plasmonic. For example, we developed arrays of plasmonic nanoparticles on gold surfaces for surface-enhanced Raman spectroscopy (SERS), where the SERS hotspots were reversibly opened for analyte capture and closed again for

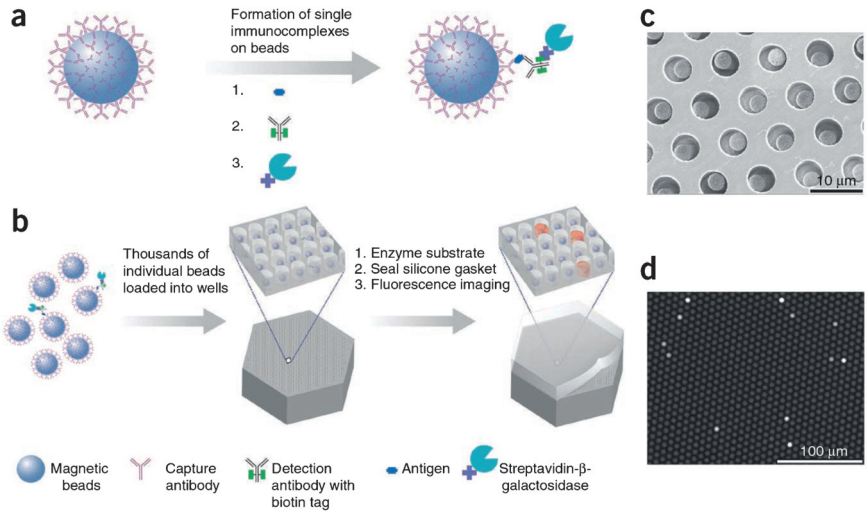

Figure 5. Digital ELISA by Rissin et al., which uses a) magnetic beads modified with antibodies to capture the analyte of interest followed by the binding of a secondary antibody which is labeled with an enzyme. b) The beads capture the analyte and are then brought to an array of femtoliter-sized wells, where the enzyme substrate is added. Color is only observed in the wells where a bead is located that has captured the analyte. c) Scanning electron microscopy image of the femtoliter wells, some with captured beads. d) Fluorescence image of a section of the array of wells showing bright spots where signal was generated by the enzyme attached to each bead with analyte. Reproduced with permission from Macmillan Publishers Ltd. Nature Biotechnology.[14] Copyright 2010.

measurement using thermoresponsive polymers.[49] Similar to several other SERS methods, this technique was shown to be capable of detecting single molecules, and by interrogating individual particles many single-molecule events could be detected. Mayer et al.[50] demonstrated a single-molecule immunoassay by using a dark-field microscope and surface-bound gold bipyramids modified with antibodies. The antibodies captured a target protein in solution, and dissociation of a single antibody from the pyramid surface caused a discernable shift in the plasmonic signature of the nanoparticles. Similarly, Sannomiya et al.[51] developed a single-molecule DNA-hybridization assay by using DNA-modified gold nanoparticles. This approach employed a label to obtain the optical response. DNA hybridization allowed a second smaller gold nanoparticle to bind to the main particle, thereby causing a change in the plasmonic signature of the surface-bound particle. In this example, the spectra of nine nanoparticles were monitored sequentially to acquire information on the DNA hybridization. However, more recently, Zijlstra et al.[52] and Ament et al.[53] have shown single-protein binding to gold nanorods in a label-free approach. None of these examples have been made massively parallel yet. However, a nonbiological application of the plasmonic imaging of many single nanoparticles to understand electrocatalysis demonstrates the potential for wide-field microscopy approaches.[54]

Approaches based on plasmonic nanoparticles on surfaces that allow wide-field measurement are emerging. An interesting example is the study by Käll and co-workers,[55] where biotinylated nanoparticles arrays on a surface are imaged using a dark-field condenser, which uses a liquid-crystal tunable filter that scans through the spectral region at 1 nm increments, prior to a CCD camera. In this way, restacking the images allows the spectrum of each particle to be monitored. The power of the approach has been demonstrated using horseradish peroxidase–streptavidin bioconjugates. The enzyme binds to the nanoparticles, with the enzyme reaction of a single or a few enzymes causing a precipitate to form on the nanoparticles with a concomitant spectral shift. Another example returns to the idea of nanopores. The reason single, or a few, nanopores are typically used is because, with resistive measurements, if there are too many pores then a single resistive pulse does not change the resistance enough to give a discernable digital response. In essence, this means the single-molecule capability is lost as a large number of pores gives an analogue signal. Wallace and co-workers[56] have recently come up with an ingenious solution to this problem by using a biomimetic strategy. Rather than using conductivity to record ion flux through the nanopores, they employed the calcium signaling fluorophore Fluo-8. Analogous to the resistance approach, the fluorescence arising from calcium flowing through the pore is high until a sequence of DNA passes through the pore, whereupon there is a drop in fluorescence. Thus, translocations through the pores are characterized by localized decreases in the fluorescence signals. A plasmonic equivalent of this type of measurement seems possible. Oh and co-workers[57] have shown that the dielectrophoretic capture trapping approach described in Section 4.1 can be used to measure low levels of analyte driven through plasmonic nanohole arrays, as the spacing between the holes is greater than the diffraction limit.

4.4. Wide-Field Measurements

The approaches described at the end of the last section are beginning to converge to fully wide-field measurements. Optical microscopy affords wide-field measurements and is frequently employed to detect surface-confined reactions on particles. The principle is also used in single-molecule pull-down assays to characterize and quantify the copy numbers of protein complexes in cells (Figure 6).[58] Here, an antibody-modified glass surface is used as the capturing method, and immunofluorescence and total internal reflectance fluorescence (TIRF) is used as the readout. The temporal separation between capture and measurement, however, restricts the scope of the method, as binding associations must be stable for minutes or hours, thereby restricting the method by the kinetic limitation discussed in Section 2. Lee et al.[59] extended the single-molecule co-immunoprecipitation assay to real time, such that protein–protein interactions with lifetimes of a few hundred milliseconds could be observed in crude cell

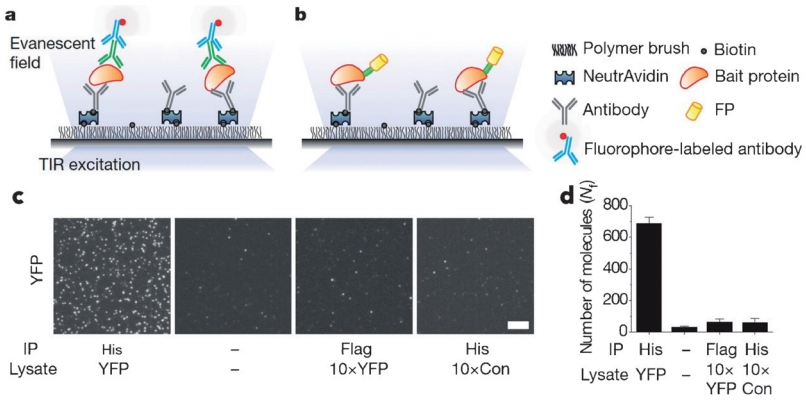

Figure 6. Single-molecule pull-down assays where an antibody-modified surface is used to capture a bait protein, which is either a) visualized using a secondary fluorescently labeled antibody or b) was genetically fused to a fluorescent protein (FP). The visualization is performed using TIRF microscopy, where the captured labels must be spaced further apart than the diffraction limit. c,d) TIRF images and quantification of yellow fluorescent protein (YFP) pulled down from cells expressing His_6-YFP and negative control surfaces (–), tag (Flag), and cells (Con). Adapted with permission from Macmillan Publishers Ltd. Nature.[58] Copyright 2011.

extracts. Gambin et al. inverted the format and used fluorescence fluctuations in a confocal detection volume, instead of surface immobilization and TIRF microscopy, to analyze the stoichiometry of protein complexes in lyzed cells.[60] Although these methods can even detect transient single-binding events, the strategies typically require cell transfection and low levels of protein expression.

Single-molecule localization microscopy (SMLM, also known as photoactivation localization microscopy (PALM),[61] (direct) stochastic optical reconstruction microscopy ((d)STORM),[62] ground-state depletion (GSD) microscopy)[63] may become the tool of choice for wide-field detection. This super-resolution fluorescence microscopy approach uses temporal separation of fluorescent events to identify single molecules in highly dense samples and in a wide-field format. So far, single-molecule localization microscopy has been mainly used as an imaging tool in cell biology.[64] The photoswitching and -activation of the fluorophores introduces a temporal resolution limit, and counting molecules, even in fixed samples, requires careful calibration.[65] Hence, the barrier to quantification lies in differentiating single-molecule events from noise. From a technical perspective, one must take into account the detection probability of the signal. This issue is particularly acute for SMLM, where photoactivation/-switching of fluorophores can destroy the molecule before its signal is recorded. A recent study estimated that the detection probability of commonly used fluorescent proteins in SMLM was about 50–70 %.[66] However, there is also the biological challenge of identifying rare events. For example, in a recombinant system, it was showed that activation of the small GTPase Ras by the exchange factor Son of Sevenless (SOS) was entirely driven by rare, but long-lived, high-activity states that contributed relatively little to the mean catalytic rate.[67] In this example, the majority of the exchange reaction may be regarded as noise. Thus, detection reliability becomes an issue, particularly in diagnosis where rare events are being searched for.

An intriguing, nonfluorescent variant of wide-field measurement comes from the characterization of dopants in graphene by Raman spectroscopy.[68] Strano and co-workers[69] have exploited the sensitivity of graphene to small changes in dopants to image localized changes in dopant levels. In effect, they suggest a graphene sheet is an independently addressable array of optical sensors. They used the method to image pH values with sub-20 nm spatial resolution, and demonstrated the ability to determine localized pH changes in a cell on the graphene surface as a measure of the cells metabolism.

At this point in time, we are of the opinion that massively parallel near-field-strategies are well-suited for quantification, while wide-field strategies are better suited for biological experiments where one can engineer the sample to some extent to suit the assay format. However, wide-field microscopy has also been used to quantify the hybridization kinetics of DNA duplexes on a surface and count the number of fluorescently labeled target strands that actually bind to the surface down to 1 pM.[70] The restriction here, however, is ensuring that there is temporal separation of the target sequences that contain the fluorophores, that binding to the surface occurs, or that the density is low, so as to avoid spectral overlap.

5. Summary and Outlook

The transition from single-molecule detectors to single-molecule sensors that are suitable for quantitative analysis is just beginning. Establishing such techniques is both analytically and biologically important. From an analytical perspective, quantitative single-molecule sensors could provide sensors with the ultimate resolution of a single molecule, and the ability to count molecules could provide calibration-free sensors. However, beyond the capability of current sensing strategies, single-molecule sensors could have the ability to go beyond the digital counting of molecules to being able to parameterize single-molecule interaction events themselves. Such a capability to quantify single-molecule interactions leads us to the biological imperative for quantitative single-molecule sensors. Biological systems are characterized by heterogeneities, both within the reaction/interactions kinetics of the same species and the reaction/interaction partners. Ultimately, there is no alternative to understanding biological systems than to map these heterogeneities, and only single-molecule sensors can measure heterogeneities on the molecular scale.

The challenges for developing quantitative single-molecule sensors are essentially twofold. The first is getting enough signal from a single molecule. This is a challenge that has been successfully solved in the last 20 years by the amazing advances in single-molecule detection and monitoring schemes. Without trivializing the difficulties in performing

such measurements, single-molecule detection methods can nowadays almost be regarded as routine, such has been the success of this emerging field of research. Predominantly, sufficient signal is achieved by confining the measurement volume to the nanoscale. The second challenge is measuring many single-molecule events to provide enough information for quantitative analysis. The confinement of measurement space to very small volumes turns this challenge into one of mass transport—getting enough single molecules into the measurement volume over a viable timescale.

Incredible advances have been made since the first single-molecule sensors for quantitative analysis little more than 10 years ago, and we are now at the point where companies develop single-molecule sensors for quantitative analysis. In this Review, we have classified the published approaches for quantitative single molecule sensors that rise to the mass-transport challenge in four ways. These are:

1) bringing the molecules to the measurement volume more rapidly for rapid near-field measurements,
2) making the near-field measurement volumes massively parallel,
3) collecting the single molecules from the solution space in conjunction with massively parallel near-field measurement volumes,
4) performing wide-field measurements at a single-molecule level.

The first three of these approaches are becoming well-established, with several different strategies reported within each classification. It is evident from Section 3 that the idea of confinement into small volumes or onto two-dimensional surfaces and one-dimensional fibers are well-utilized. The other common theme in the single-molecule sensing ideas thus far developed is that they are predominantly end-point assays. That is, the individual molecules are brought into the measurement volumes and then a measurement is made. A biological strategy that still requires translation to sensors is discrimination based on different timescales and other binding properties of the interactions of different molecules with a common receptor. In only a few cases, such as some nanopore methods,[24b] are the characteristics of the interaction of the single species used in the identification of the species. This is effectively an open, unexplored area for future investigations.

The fourth strategy, where single molecules on a surface are monitored in a wide-field format, has also hardly been explored. This last category is similar to a traditional sensor, in that many molecules will interact with the surface to give a signal. The important distinction though is that, whereas signals from a traditional sensor are an average of the interactions of many molecules with a surface, a single-molecule sensor will yield a signal from each individual molecule. Obtaining information from each individual molecule as it interacts with a surface is one obvious pathway to develop single-molecule sensors that respond to the second challenge from nature: quantifying single-molecule interactions and recognizing heterogeneities in binding properties and binding partners. This will take single-molecule sensors beyond providing digital information and will completely change the current way of thinking about sensing. With traditional sensors, because a single piece of information is obtained, the approach has been to make the sensing interfaces highly specific for the target species of interest. However, quantifying single-molecule interactions shifts the approach to sensing interfaces that are moderately selective for a range of species. Being able to quantify single-molecule interactions has immense benefits, from simply distinguishing specific from nonspecific interactions to giving us the tools to better understand the importance of single-molecule interactions in biology.

Quantifying single-molecule interactions, however, will create a massive amount of data that is intrinsically heterogeneous. We must now start to think about how we describe and quantify heterogeneities. Standard statistical tools, such as means and standard deviations, are simply inadequate. Thankfully, new methods are being developed such as Conditional-Density Resampled Estimate of Mutual Information (DREMI) to quantify the strengths of the underlying complex relationships between parameters within noisy data.[71]

Finally, the reliability of a single-molecule sensor requires test samples that may not yet exist. For example, a device that claims to count the absolute number of molecules of a given species in a sample requires a standard with a known number of molecules. Notwithstanding all the challenges, analytical scientists and biologists working together could make for exciting times ahead, as we create the opportunity of entering the single-molecule world for a truly bottom-up perspective of life itself.

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