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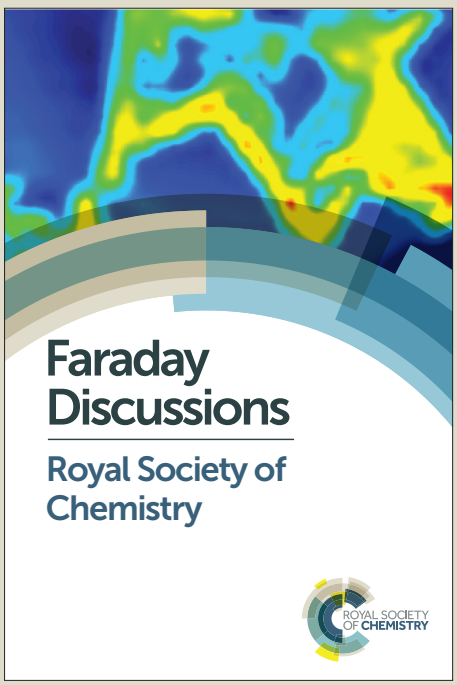

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Single Molecule Microscopy and Spectroscopy: Concluding remarks

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Chemistry is all about molecules: control, synthesis, interaction and reaction of molecules. All too easily on a blackboard one draws molecules, their structure and dynamics, to create an insightful picture. The dream would be to see those molecules in reality. This is exactly what "Single Molecule Detection" provides: a look at molecules in action at ambient conditions; a breakthrough technology in chemistry, physics and biology. Within the realms of the Royal society of Chemistry the Faraday Discussion on "Single Molecule Microscopy and Spectroscopy" has been a very appropriate topic for presentation, deliberation and debate.

Undoubtedly the Faraday Discussions have a splendid reputation in stimulating scientific debates along the traditions set by Michael Faraday. Interestingly, back in the 1830's, Faraday himself pursued an experiment that led to the idea that atoms in a compound were joined by an electrical component. He placed two opposite electrodes in a solution of water containing a dissolved compound, and observed that one of the elements of the compound accumulated on one electrode, while the other was deposited on the opposite electrode. Though Faraday was deeply opposed to atomism, he had to recognize that electrical forces were responsible for the joining of atoms. Probably a direct view on the atoms or molecules in his experiment would have convinced him. As such Michael Faraday might have liked the gathering at the Burlington house in September 2015. Surely with the questioning eyes of his bust on the 1st floor corridor the non-believer Michael Faraday has incited each passer-by to enter into discussion and search for deeper answers at the level of single molecules.

In these concluding remarks, highlights of the presented papers and discussions are summarized, complemented by a conclusion on future perspective.

Fig.1. The Royal Society of Chemistry at the Burlington House in London. The entrance, the stained glass window on the stairway towards the first floor corridor where one finds the bronze bust representing Michael Faraday, protagonist of the early-day lively scientific discussions which have inspired the "Faraday Discussions".

Introduction

The detection of atoms and molecules has long been restricted to the gas phase at ultrahigh vacuum conditions. Yet in 1989, now over 25 years ago, the first detection of the absorption of a single molecule in the condensed phase was realized by the group of W. E. Moerner, in a highly engineered frequency and Stark modulated scheme [1]. Soon after, in 1990, Michel Orrit and his team also observed the absorption of a single molecule by detecting the background-free fluorescence [2]. The early experiments by Moerner and Orrit all took place at cryogenic conditions, with narrow lines and large absorption cross-sections. In fact the molecules could be separated spectrally even without microscopic selection. In 1993 Eric Betzig entered into the world of "single molecule detection" using near-field fluorescence microscopy to image the first individual molecules at room temperature [3]. His near field approach offered a tiny sub-wavelength excitation volume, which was advantageous to discriminate the individual molecules against their surroundings. Soon it was shown that also with regular confocal microscopy, and even wide field microscopy and photon counting CCDs, single molecules can readily be traced. Through these early pioneering actions Moerner, Orrit and Betzig have laid the grounds for a highly active research field, "single molecule detection", which has led to so many discoveries and applications. Today single molecule fluorescence detection has evolved to a new frontier in science with high impact in a wide range of disciplines, such as material research, analytical chemistry, and the biological sciences. Single-molecule fluorescence is particularly a subject of intense interest for biological imaging, through the labeling of biomolecules such as proteins and nucleotides to study enzymatic function which cannot easily be studied on the bulk scale. The motion and action of several types of motor proteins (myosin/actin) is being followed at the single protein level. More and more we are getting closer to following the action of single molecules directly in living cells.

Many of these topics were discussed at the Faraday Discussions. Asking around in the Burlington house corridors some said it was quite late to have Faraday Discussions on single molecule detection, after 25 years. Others found the meeting very timely as the field is very active and rapidly spreading out in many techniques and applications in both chemistry and biology. Again others pointed out the crucial role of single molecule detection in the current developments of nanoplasmonics and super-resolution "nanoscopy".

Indeed, it is exactly the capacity to detect and image single molecules at room temperature that triggered the original ideas towards "super-resolution" optical imaging. Essentially the position of a single isolated molecule can be determined with very high accuracy, limited only by the amount of detected signal: ideally if N photons are detected Poison statistics tells us that the location precision improves with $\sqrt{N}$. It was Eric Betzig who in 1994 stated that if each molecule could be discriminated by "some unique optical feature" a map of their positions could be reconstructed, allowing "molecular resolution imaging" [4]. In 2006 Betzig introduced time dependent photoactivation as a discriminator: with an optical stimulus a tiny fraction of molecules is activated, imaged until photo-bleaching, and the exact positions determined. By successive iteration of activation-mapping-bleaching the position map gets filled: Photo-Activation Localization Microscopy (PALM) with nanometric localization accuracy [5]. STORM is an alternative approach, switching molecules temporarily without bleaching [6]. PALM and STORM are commonly termed super-resolution microscopies, yet it should be noted that the diffraction limit is not broken: it is the combination of a well-defined point spread function and high signal which yields super localisation, all along the rules of diffraction and photon statistics.

Interestingly Stefan Hell [7] also in 1994 demonstrated that a fluorescent sample can be switched off by depleting the excited state through stimulated emission, where the reduction of fluorescence shows a highly non-linear response on the depletion power. This non-linearity now is the key to gain spatial accuracy, allowing to leave a narrow non-depleted spot at the center of an excitation profile. Thus Stefan Hell made STED, Stimulated Emission Depletion microscopy, to work as a super-resolution method [8]. Again STED obeys perfectly the diffraction limit, but it is the non-linear response of the fluorescent molecule which squeezes the effective spatial imaging response to far below the diffraction limit. It is important to notice that STED does not need detection of single molecules.

The 2014 Nobel Prize in Chemistry was awarded jointly to William E. Moerner, Eric Betzig and Stefan W. Hell, for "the development of super-resolved fluorescence microscopy". Extraordinarily at these Faraday Discussions, chaired by Michel Orrit, both William E. Moerner and Eric Betzig were presented. All three pioneers, Orrit, Moerner and Betzig, played a crucial role in the early development and first demonstrations of single molecule detection, thus bringing great standing to the meeting at the Royal Society.

To capture new developments the Faraday Discussion did focus on 4 discussion themes:

- *Super-resolution and Imaging of Soft and Biological Matter*
  The revolution of super-resolution imaging and several nonlinear optical imaging modalities, to be discussed in the frame of single molecules and single objects.
- *Quantum optics and Plasmonics*
  Single molecules combined with plasmonics opens new routes for enhancement of both excitation and emission, with connections to quantum optics.
- *Probes and Sensors for Molecular Biophysics*
  Plasmonic structures as bright and stable labels, as rulers to probe dynamics, as antennas to efficiently extract information from the nanoscale; all particularly attractive in biophysical applications.
- *Nonlinear optics and Coherence in Biophysics*
  Tailored light pulses opens the way to manipulate quantum states of single molecules, and to explore coherent effects in biological processes.

Here let's revisit and discuss particular highlights of the various themed sessions.

Super-resolution and Imaging of Soft and Biological Matter

The introductory lecture was presented by WE Moerner of Stanford University, on single-molecule active control microscopy (SMACM). He gave a superb introduction into the history and current highlights of single molecule detection in general, and into super-resolution microscopy particularly, thus nicely kicking-off the topic. Beyond a wealth of high-resolution images of new biological applications, such as labelled free amines on living bacterial surfaces, he expressed the continuing need for new robust photoswitchable molecules to pursue the technology further. Next he presented various solutions to bring super-resolution microscopy from 2D towards 3D. Building on his previously developed double helix illumination he now showed the use of saddle point masks and azimuthal polarisation detection. It should be noted that his student Maurice Lee received the conference poster award for a poster on that topic. Finally WE Moerner made a fine distinction between localization accuracy of light spots and determination of real positions of molecules. Due to field dependent aberrations positions can easily be off by more than 20 nm and up to 100 nm. Using arrays of sub-diffraction limited nanoholes, filled with fluorophores, he presented corrections of absolute positions to better than 25 nm, thus improving substantially the 3D imaging and tracking. He presented an application of the aberration correction in tracking diffusing molecules simultaneously confined in an ABEL-trap.

The complementary technique to SMACM (PALM and STORM) is STED, not requiring photo-switchability, yet an engineered two colour Gaussian/donut excitation. As such STED is a single point method, which requires serial point-by-point scanning and relatively slow imaging. Use of an array of illumination spots is a solution put forward by the Hell group. The array of donut spots for state depletion needs only to be scanned to cover the distance between adjacent donuts, which enhances frame rate. Yet the single point detection needs to be replaced by a CCD camera; one needs to avoid cross-talk between the various donuts; and polarisation becomes critical. Brahim Lounis from University Bordeaux [9] pushed the parallel “lattice” STED to high frame/rate, using four incident beams to create a 2D interference pattern of dips for state depletion, together with a rapid CMOS CCD camera. Mutual polarization of the four beams appeared critical to avoid long depletion lines. He presented STED images with 75 nm resolution and 80ms frame time.

Scanning probe microscopy and near field optical microscopy, are the predecessors to the current far-field super-resolution microscopies. Sergey Sekatskii from EPFLausanne [10] presented a challenging idea to use a FRET (Fluorescence Resonance Energy Transfer) pair for imaging at molecular resolution: scanning the acceptor molecule on a local probe versus the donor molecules in the sample. To avoid premature bleaching of the probe he designed a single diamond NV centre at the end of the scanning probe. He presented a scanning probe, with force control and nice high resolution force microscopy images of ssDNA, yet the optical FRET contrast so far did not yield results.

The use of FRET at the level of single donor and acceptor is generally very powerful. The sixth power distance dependence allows distance determination at sub-nanometer scale and has been used extensively to monitor protein dynamics and

folding. Jens Michaelis of Ulm University [11] presented an approach to retrieve quantitative structural information from a single protein using single-molecule FRET. Labelling a protein at many different sites with FRET pairs and measuring all FRET responses for each labelling case he developed a nano-positioning system (NPS). Interpretation of the data appeared very challenging, also due to length of linkers and mobility of the donor/acceptor. As such he could determine the most credible volume for each position and thus work his way towards the full protein structure. Clearly an interesting approach, making progress and hopefully delivering a first protein structure one day.

Connected to the single molecule FRET probing, Victoria Birkedal of Aarhus University [12] showed methods to retrieve quantitative single molecule FRET efficiencies for use in TIRF microscopy. A particular problem in FRET experiments is the cross-talk, in bleed-through of donor when detecting acceptor and in direct excitation of acceptor. She showed practical approaches and checks for correction and thus achieve quantitative FRET.

High resolution microscopy technology was being pushed firmly by many presenters at the Faraday Discussions. H Yang of Princeton University [13] argued that the sequential localization in PALM/STORM is a severe limit on the speed and thus it is better to localize only the object of study and follow that fast. He presented a localization microscopy with 3D feedback of a single molecule, acting as a fast 3D particle tracking system with nanometric resolution. The 3D speed barrier was not anymore the detected signal level, rather the piezo-electric feedback of the various high frequency position controls. Particle tracking was also shown by Frank Chicos of Chemnitz University [14]. Instead of fluorescence he detected directly the scattering of metal-coated polymer particles of different sizes, from microns to nanometer scale. The particles were Janus particles, with a metal hemisphere, which absorb the incident laser power and convert the energy into heat, thus acting as self-propelled photophoretic swimmers. Tracking the particles, the local heating and speed appeared to depend strongly on particle size.

A powerful alternative for efficient detection of single molecules, particularly in conditions with a certain background, is the nanoscale reduction of the detection volume, very analogous to the original near field work of Eric Betzig in 1993. A more practical approach is the detection in a limited volume such as “zero-mode” waveguides and Nano pores, with characteristic dimensions far below 100 nm. X. Shi, of East China University [15], presented fabrication of Nano pores for simultaneous local optical and electrical detection. Yet presented pores were still quite large, ~100 nm compared to the biomolecular dimensions. Paul Bohn, of University of Notre Dame [16], presented ultra-small zero-dimensional nanophotonic structures to detect freely diffusing single Flavin mononucleotide and detect both spectral and electrochemical response. Interestingly he could control the local oxidation/reduction conditions, making the nucleotides to go bright or dim and reveal bimodal distributions at certain pH. Definitely these nanovolume approaches are simple, sensitive and versatile, allowing relatively high background concentrations up to biologically relevant concentration levels of micromolar. Their scalability is very promising for applications in biosensing arrays.

Besides pushing the technology also the applications of super-resolution microscopy are rapidly advancing towards nanoscale imaging into living cells. Maxim Dahan of University 6 in Paris [17], Mark Leake of University of York [18] and Jacek Mika of Leuven University [19] applied localization microscopy to show (de)attachment of protein binding, local distributions of protein concentrations, quantification and stoichiometry of proteins all on single cells and bacteria.

The last word on super-resolution was for Eric Betzig, presenting a perspective lecture. In his typical persuasive self-critical style Eric Betzig made clear that he had moved on beyond the topic that had earned him the Nobel Prize in 2014. No word on his single molecule near field work in his past. Some hard words on STED, especially critical as to the high power levels needed for the depletion. Next he scrutinized the resolution limits of localization microscopy: within the Nyquist criterion the claimed 10-20 nm accuracy is not realistic; label-linker-length can shift actual positions; local index variations create aberrations; the labelling density has limits. Altogether he reasoned that ~50 nm accuracy was a more realistic number for super-resolution microscopy. Based on that he advertised structured illumination microscopy (SIM), which also reaches resolution below 100 nm, yet does not need photoswitchable labels and allows much faster frame rates. He presented a dashing amount of high resolution images of living cells using SIM. At the same time he advocated light-sheet microscopy to address the 3rd dimension. Combining light-sheet excitation and structured illumination Eric Betzig presented a wide variety of detailed full 3D images of cells.

Quantum optics and plasmonics

The detection of a single molecule implies the detection of single photon emission with fundamentally non-classical photon statistics, also at room temperature. Together with the observation of discrete quantum jumps and discrete blinking the detection of single photon emitters is tightly connected to the field of quantum optics. Most quantum optical experiments on single emitters are carried out at cryogenic conditions as they require pure states with very narrow lines. Yet also at room temperature there is room for certain quantized experiments.

Jörg Wrachtrup, of Stuttgart University [20], presented advances in characterizing very weakly coupled spins, using diamond NV centres. Diamond NV centres are relatively pure quantum systems with microsecond coherence time even at room temperature, and stable luminescence. Most importantly the NV system has an electronic spin which can be used for local field sensing up to about 20 nm range. Moreover effects on nuclear spin can be felt up to 2 nm. The idea of Wrachtrup is to use the NV centre as a local spin sensor for a protein (or other biomolecule) in proximity, ideally towards a single molecular NMR sensors. For nuclear spin sensing he proposes to couple the electron and nuclear spin such that the nuclear spin range can be enhanced towards 10-15 nm. He presented results on the specific sensing of local isotopes deposited on the diamond surface, an encouraging step towards the projected biological applications.

Pushing the quantum optics even further T. Farrow of Oxford University [21] proposed to witness directly quantum effects in complex molecules, specifically based on a theoretical protocol for entangling two organic molecules by interfering

their fluorescence spectra. Authors are working on a set-up to achieve spectral overlap of both molecules at cryogenic conditions to next explore their entanglement predictions.

In the last decade the fields of nanoscale optics, plasmonics and optical antennas have found a direct connection to the detection of individual nanoparticles and molecules. Nanoplasmonic particles display highly localized fields with strong mode density; ideal for nanofocussing and enhancement of excitation and emission rates. Thus, when engineered well, the plasmonic particles can be of great advantage to enhance the single molecule response and control the emission characteristics. Enhancement of fluorescence and Raman response are well-known examples. Several works in this direction were present at the Faraday Discussion.

Lukas Novotny of ETH Zurich [22] gave a nice display on the enhanced Raman response of carbon-nanotubes and graphene in the proximity of a sharp metallic tip. Super-resolution to 10 nm details was resolved while at each position the full Raman spectrum was retrieved, showing effects of strain, defects, and doping. It should be noted that the Raman response of nanocarbon materials is particularly strong, orders of magnitude above the response of organic molecules. Paola Borri, of Cardiff University [23], presented transient resonant four-wave mixing on individual metallic nanoparticles, where the phase controlled experiment allowed to retrieve the spectral phase development for both single and coupled plasmonic particles. Fredy Meixner, of Tubingen University [24], showed the enhancement of single quantum dot luminescence when coupled to plasmonic nanocones. Lifetime experiments revealed that both excitation, radiative and non-radiative rates were enhanced. Controlling the excitation polarization he could choose to enhance the top or bottom side of the cone. Finally P.Z. El-Khoury [25] showed the enhanced Raman scattering from aromatic dithiols electrosprayed into plasmonic nanojunctions. Clearly in all these examples the control of the near field interaction between a metallic particles and single emitters is of large benefit to enhance the detection sensitivity of a single unit.

Probes and Sensors for Molecular Biophysics

Since the early days of single molecule detection it is clear that single molecules are ideal probes of their local nano-environment. Spectral shifts, blinking dynamics, lifetime variations, and polarization changes are all direct observables of fluctuations in the local conformation, chemistry, fields, etc. In this context, Lukasz Piatkowski, of ICFO in Barcelona [26], showed a multicolour excitation approach, to reveal large spectral shifts in emission spectra for molecules embedded in PMMA, even up to almost 200 nm. Such dramatic shifts hamper detection of all molecules and require changing excitation wavelength and detection bands. As a result one can easily be biased by failing to detect a substantial percentage of the molecules. As a solution he presented first broadband Fourier transform excitation spectroscopy of single molecules, which seems promising to capture the full variation in excitation spectra. Also Y.G. Vainer of the Russian Academy [27] presented spectral changes of dyes in a host matrix; yet this time at cryogenic temperature, where the linewidth can be lifetime limited. In practise he showed the spectral linewidths would change as function of the nanometric diffusion depths in

the polymer, thus revealing subsurface layer dynamics of polymers with nanometer resolution. Finally M. G. Gladush, also of Russian Academy [28], showed how single molecule spectro-microscopy can even be used for sub-wavelength refractometry.

Discrete photodissociation is the most limiting effect when using single molecule, both for probing local properties and in super-resolution applications. Enhancing the total number of emitted photons is permanent wish for the whole community. One of the main routes of photodissociation is through the triplet state. Thorben Cordes, of Groningen University [29], presented an effective solution towards photostabilization by quenching of the triplet-state. Attaching a triplet quencher to the Cy5 dye he proved intramolecular healing and bleach rate reduction with even 2 orders of magnitude. The enhanced photon number is impressive and surely will prove important in application for both imaging and sensing.

Sanli Faez of Utrecht University [30] proposed an original idea to use single molecules for the local sensing of electric fields at the nanoscale. To make an aromatic molecule more field-sensitive he identified 3-fluoroterrylene with a strongly different LUMO and HOMO. Based on first estimates sufficient Stark shift in the ZPL could be expected in a polymer host with external field applied. Synthesis and experiments are in progress.

Nonlinear optics and Coherence in Biophysics

During the meeting the non-linear response of nanoparticles and coherences in the emission of single molecules and NV-centres were touched in discussions. Yet as a whole the topic appeared rather underrepresented compared to the dominance of super-resolution contributions.

Topics beyond the discussion

The collective set of papers presented at the Faraday Discussion reflects the wide range of advances in the field in recent years. The field is gradually coming of age and particularly the growing diversity of the applications should be noted. And yet the field is constantly looking for new directions. Let's reflect on some very recent developments.

Detection beyond fluorescence: The vast majority of successes in the imaging and spectroscopy of individual molecules at room temperature rely on fluorescence. Yet most relevant molecules do not fluoresce or only weakly. Thus the field has been limited to efficient fluorophores, often used as biolabels. As such alternative contrast methods have always been high on the agenda. Direct detection of a molecule in absorption has been shown, yet remains very challenging [31]. Reduction of the excitation volume towards dimensions of the molecular cross-section is needed for effective absorption detection; a way to go is nano-focusing using plasmonic particles. Besides absorption also scattering is a valid alternative. Through clever interferometric detection schemes scattering detection is now reaching the level of individual proteins [32]. Raman spectroscopy is a valid alternative to fluorescence detection, as also presented during the discussion. Yet enhancements of 10^{10} are needed to compete with the spontaneous emission, making single molecule Raman

still challenging, and moreover harder to interpret than enhanced spectroscopy. Stimulated emission is again another way to go; effective stimulated emission imaging has been shown [33], yet the single molecule limit is still challenging. The photothermal detection of nanoparticles has reached limits of particles only a few nanometers, and the photothermal approach even allowed to detect a single molecule in absorption [34, 35]. Electrical read-out of single molecules in tunnel junctions has been shown, but needs scaling up to ambient conditions for applications.

Ultrafast and coherent control: ultrafast femtosecond and picosecond spectroscopy is an important topic in spectroscopy and physical chemistry as it reveals the dynamics on the molecular time scale. Single molecule detection based on fluorescence detection only yields photons and typically with nanosecond lifetime. As a result the single molecule detection is fundamentally too slow for ultrafast spectroscopy. Yet the stimulated excitation is instantaneous and can be used to encode fs effects, while still detecting the fluorescence response. Through pump-probe and coherent control schemes indeed ultrafast signatures of single molecules have been observed [36]. The route is open to detect coherences, fs-ps energy transfer, charge transfer, etc. Recently the vibration of a single molecule was reported using surface enhanced single molecule CARS [37].

New single emitters: The detection of single entities started with atoms and molecules and has extended to single quantum dots and single color centers, such as NV-centers in diamond. New emitters are fanatically pursued. Detection of single lanthanides has been reported [38], while systems such as perovskites are explored. Beyond the conventional emitters based on a transition dipole moment, one could also attempt to detect the weaker non-dipolar transitions or even magnetic transitions. Single molecules are detected in the very weak field linear regime; yet even at the few photon level, operating at the nanoscale, a nonlinear single molecule response could be expected provided one couples efficiently to the molecule. Finally all users of super-resolution microscopy are constantly craving for new bright and stable photoswitchable molecules, preferentially small with short linkers to allow higher labelling density.

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