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Highlights from Faraday Discussion 184: Single-Molecule Microscopy and Spectroscopy, London, UK, September 2015

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The 2015 Faraday Discussion on single-molecule microscopy and spectroscopy brought together leading scientists involved in various topics of single-molecule research. It attracted almost a hundred delegates from a broad spectrum of backgrounds and experience levels – from experimentalists to theoreticians, from biologists to materials scientists, from masters students to Nobel Prize Laureates. The meeting was merely a reflection of how big of an impact the ability to detect individual molecules has had on science over the past quarter of a century. In the following we give an overview of the topics covered during this meeting and briefly highlight the content of each presentation.

On September 14, 2015, around 90 delegates from 5 continents arrived in London to attend the Faraday Discussion meeting dedicated to single-molecule microscopy and spectroscopy. The city welcomed them with overcast skies and drizzle, in conformity with the English meteorological stereotype. The conference took place in the library of the London residence of the Royal Society of Chemistry (RSC) in the beautiful, Palladian-style Burlington House.

Faraday Discussions go back over 100 years, with the first gathering taking place in London in 1907. The meetings have gained recognition quickly and the format has grown from initially just 1 annual meeting to 9 meetings per year in 2015.

The present Faraday Discussion was the 184th meeting and the first one specifically dedicated to single molecule (SM) research. The award of the 2014 Nobel Prize in Chemistry to scientists that have made major contributions to the development of single molecule research and super-resolution microscopy (S. Hell, E. Betzig and W. E. Moerner) was undoubtedly a nice bonus and definitely enhanced the impact of the meeting.

Since the first detection of a single molecule 26 years ago, the field has expanded markedly. The first ground-breaking work by W. E. Moerner and M. Orrit made scientists realize that the efficient detection of individual molecules at cryogenic conditions is possible.[1,2]

Once the detection of single molecules at room temperature had been reported by E. Betzig in 1993,[3] a large spectrum of experiments became feasible, causing the field to expand tremendously. Soon, the detection of SMs and other isolated quantum emitters (such as quantum dots and impurity centres in solid crystals) was utilized in physics, biology, chemistry and materials sciences. In particular, the detection of individual molecules through fluorescence became an everyday tool in laboratories across the world.

The variety of research topics involving individual emitters was readily reflected in the wide range of sessions in this Faraday Discussion. They were loosely assigned to four different themes: [1] quantum optics and plasmonics; [2] probes and sensors for molecular biophysics; [3] super-resolution and imaging of soft and biological matter; and [4] nonlinear optics and coherence in biophysics. These themes were divided into a total of 9 sessions, which focussed on particular aspects of single-molecule research. In the following we give a brief overview of the presentations.

After having finished their lunch, the attendees were welcomed by the chair of the scientific committee, M. Orrit (Leiden University, The Netherlands), who delivered the opening remarks and explained the particular format of the meeting (Fig. 1). The discussions were preceded by a 45 minute opening lecture given by W. E. Moerner (Stanford University, USA) and followed by an equally long perspective lecture by E. Betzig (Howard Hughes Medical Institute, USA). The concluding remarks were offered by N. van Hulst (ICFO, Spain). Prior to the meeting, all the remaining speakers had submitted a paper. During their session they were given 5 minutes each to briefly summarize the main findings of their paper, followed by an open discussion, with 25 minutes allotted for public and live peer-review of each paper contributed.

Opening lecture

W. E. Moerner (Stanford University, USA) gave the introductory lecture entitled

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Fig. 1 Michel Orrit opening the Single-Molecule Microscopy and Spectroscopy Faraday Discussion (credit: the Royal Society of Chemistry and John Rogers).

"Single molecule spectroscopy and imaging over the decades" (DOI: 10.1039/C5FD00149H). He gave a historical overview of the milestones of single molecule detection, starting with how people came to believe that it is actually possible to detect single molecules. Once it was realized that the optical absorption of a bulk sample has a reproducible fine-structure, the pursuit to detect a single molecule began.[1] Shortly after, M. Orrit started measuring molecules through their fluorescence with a much higher signal-to-noise ratio, which lead the majority of the field to use this approach.[2] In this new regime, unexpected effects like spectral jumps and blinking were first observed. These effects are studied and used as probes of the local nanoenvironment to this day.[4–6] The extension to room temperature through near-field imaging techniques boosted the field even further because many imaging experiments on biological samples were made possible.[3] This coincided with the introduction of a broad range of other new techniques for room temperature experiments like confocal[7] and widefield[8,9] imaging.

The development of super-resolution techniques circumvented the restriction of the optical diffraction limit. By now, a number of super-resolution techniques (STED, STORM, PALM *etc.*) are used to identify molecules and localize them with nanometric precision. As there seem to be at least as many slightly different techniques as acronyms, W. E. Moerner suggested the use of the general acronym SMACM (Single-Molecule Active-Control Microscopy). Many new things have been possible thanks to super-resolution such as learning the actin fine-structure, the observation of Huntington aggregates using mutant proteins and neuron axon dynamics, to name just a few.

The lecture was concluded with an overview of the current projects pursued by the Moerner group. Standard super-resolution microscopy techniques are typically two-dimensional. By changing the point spread function (PSF) of the microscope, 3D images can be realized. Various masks such as double helices, corkscrews, bisected or other, complex masks have already been utilized for this purpose. In this context, W. E. Moerner also emphasized the difference between *accuracy* and *precision*.

Finally, he explained the application of anti-Brownian electrokinetic (ABEL) traps for measuring the diffusivity and mobility of single molecules in a solution. With such a trap, the observation of single dissociation events such as DNA-binding and unbinding has been recently demonstrated.

Session 1: Super-resolution techniques

Super-resolution techniques are continuously being developed and extended at a high pace. One of the big dreams of biologists and biochemists is the ability to record super-resolution images with high temporal resolution. A method that takes a step towards achieving this goal is the parallelization of the STED approach. In the opening session B. Lounis (University of Bordeaux, France) presented their lattice-STED microscopy approach (DOI: 10.1039/C5FD00092K) (see Fig. 2). In particular, he compared the resolution of various optical lattice configurations and their theoretical simulations and concluded that polarization information can be retrieved even at the single molecule level.

Even though a range of super-resolution approaches is already available, there is still a strong drive to develop new techniques. One such approach that is still in the making is based on fluorescence resonance energy transfer (FRET) to increase the resolution in scanning near-field optical microscopy (SNOM). Thanks to its highly nonlinear distance dependence, FRET offers a much-improved resolution compared to traditional SNOM, without loss in sensitivity. S. K. Sekatskii (EPFL, Switzerland) presented their efforts towards the development of single molecule FRET SNOM (DOI: 10.1039/C5FD00097A). Here nitrogen-vacancy (NV) centers were chosen due to their superior photostability. He discussed the challenges in achieving their goal, as up to now all of their attempts have failed. This has been ascribed to the difficulty in finding NV centers very close to the crystal's surface and to the fact that the NV centers likely become increasingly unstable the closer they are to the crystal's surface.

Session 2: Biophysics with nanostructures

After the lightning poster presentation, and an afternoon tea and poster session, the second discussion of the day took place. This session focused on utilizing nanostructures for the detection of individual, biologically relevant analytes. As the first contribution by A. Peñaherrera (Polytechnic Army School, Ecuador) had been cancelled (DOI: 10.1039/C5FD00106D), X. Shi (East China University of Science and Technology, China) opened this session by discussing an integrated system for the simultaneous detection of synchronized optical and electronic signals from a single molecule passing through a solid-state metal-coated nanopore (DOI: 10.1039/C5FD00060B). This label-free approach is based on the detection of the change in the nanopores' scattered light under dark field illumination in the presence of a passing particle, combined with the simultaneous detection of the electrical signal.

P. W. Bohn (University of Notre Dame, USA) also presented work based on the simultaneous detection of the optical and electrical signals, but using zero-mode waveguide (ZMW) structures and fluorescence detection (DOI: 10.1039/C5FD00072F). In their work they addressed the electroluminescence behavior of freely diffusing single flavin molecules under both static and active potential control, which enabled the discrimination between the oxidized and reduced state and thus directly probed the electron transfer dynamics on a single molecule level. The extremely small volume and efficient trapping of the optical fields in the waveguides make this a nice platform for electrochemical measurements in various systems.

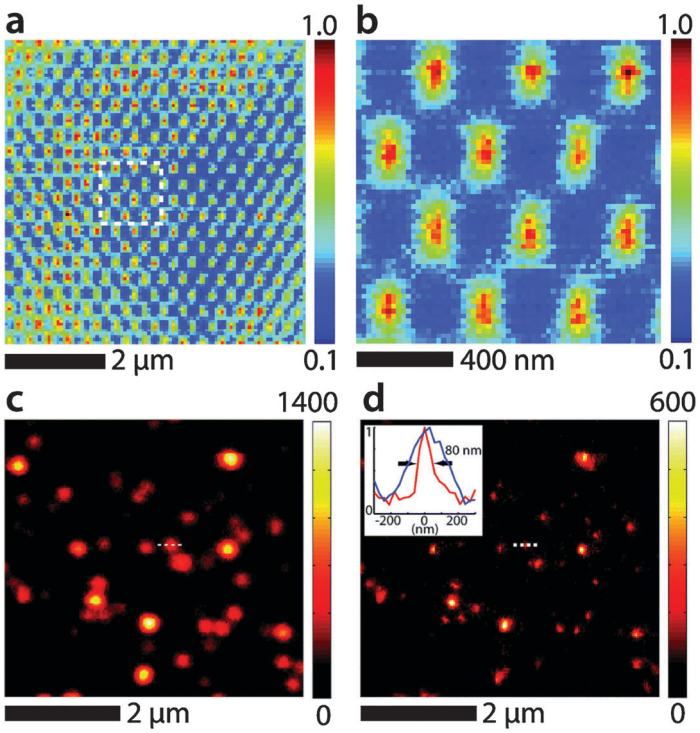

Fig. 2 (a) Experimental depletion pattern of the three-beam lattice-STED microscope with (b) a zoom of the central area. In (c) and (d), images without and with the depletion optical lattice of the same sample region containing 35 nm fluorescent nano-diamonds are compared (reproduced from DOI: 10.1039/C5FD00092K with permission from the Royal Society of Chemistry).

Session 3: Fluorescence energy transfer

The third session of the conference was dedicated to fluorescence resonance energy transfer (FRET) between individual emitters. Owing to its strongly nonlinear distance dependence, FRET has been used extensively in structural biology as a molecular ruler reporting on the distances between the energy exchanging sites. The first talk was given by J. Michaelis (Ulm University, Germany), who presented a new data analysis algorithm to gain structural information using FRET-based nano-positioning systems (NPS) (DOI: 10.1039/C5FD00110B). With this method, the analysis time was reduced by two orders of magnitude compared to pre-existing algorithms while getting results with the same quality. The effect of various model assumptions on the extracted relative distances and their respective uncertainties were discussed.

These theoretical studies were complemented by the work of V. Birkedal (Aarhus University, Denmark), who presented a new procedure to determine single molecule FRET efficiencies that are independent of the instruments used (DOI: 10.1039/C5FD00100E). In their work they evaluated various corrections arising from direct acceptor excitation and detection channel cross-talk, as well as detection efficiencies for donors and acceptors in order to obtain the most accurate FRET efficiencies between the donor–acceptor pairs as a function of the base-pair distance on a DNA strand.

Session 4: Quantum optics

The next morning began with a session in which quantum phenomena were at the center of attention. The application of these phenomena is an important aspect of single molecule research in order to advance state-of-the-art measurement techniques even further. In the first talk of this session, J. Wrachtrup (University of Stuttgart, Germany) showed how to achieve single proton sensitivity with nuclear magnetic resonance (NMR) spectroscopy (DOI: 10.1039/C5FD00116A). To this end, NV centers were used as magnetic field sensors whose two levels within the triplet subspace are resonantly coupled to a two-level ancillary nuclear spin. This feat beats the relaxation rate of the sensor and enables the detection of weakly coupled distant spins with high contrast as shown in Fig. 3.

Entanglement can help probe the transition from the quantum to the classical regime, which might also be interesting for quantum computing applications. The presentation of T. Farrow (University of Oxford, UK) dealt with the theoretical framework for the quantum entanglement of complex molecules to explore under what conditions they entangle, if at all (DOI: 10.1039/C5FD00101C). A generic experimental scheme was proposed to entangle two molecules through the interference of their fluorescence spectra.

The third presenter of this session, R. Hanson (Delft University of Technology, The Netherlands) was unfortunately not able to present at the meeting (DOI: 10.1039/C5FD00113G), allowing more time to discuss the other two papers.

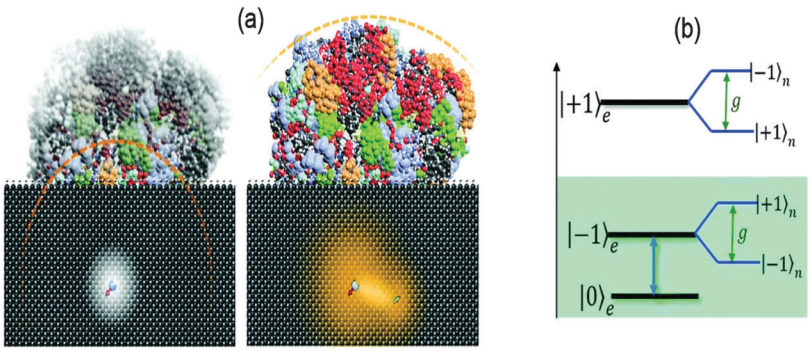

Fig. 3 (a) Schematic of the enlargement of the detection volume through indirect sensing. (b) The energy levels of the system where two levels of the sensor are resonantly coupled to a two-level ancillary spin (reproduced from DOI: 10.1039/C5FD00116A with permission from the Royal Society of Chemistry).

Session 5: Molecular spectroscopy

The molecular spectroscopy session took place after the morning tea. Right from the beginning of the field, a large variety of spectroscopic techniques at the single molecule level have been developed. This session showed that there is still space for innovation despite the abundance of pre-existing methods. L. Novotny (ETH Zürich, Switzerland) explained how the local environment in nanocarbon systems can be studied using tip-enhanced Raman scattering (TERS) to understand defects, strain and doping levels (DOI: 10.1039/C5FD00050E). It was shown that defects in carbon nanotubes lead to additional bands in the Raman spectrum. When combining this technique with photoluminescence or electroluminescence microscopy, a more complete picture of the investigated sample can be drawn.

It is important not to forget about the inhomogeneity of single molecule properties when developing new techniques. The next presenter, L. Piatkowski (ICFO, Spain) remarked that in the case of broad spectral distributions of single molecules it is impossible to probe the entire molecular distribution by just a single narrowband excitation wavelength (DOI: 10.1039/C5FD00107B). He advocated the use of several excitation wavelengths in this case and finished his talk by presenting a broadband excitation technique to measure single molecule high-resolution fluorescence excitation spectra at room temperature.

One of the biggest challenges of single molecule spectroscopy under ambient conditions is the limited photostability of the system under investigation. T. Cordes (University of Groningen, The Netherlands) proposed the covalent linkage of organic fluorophores to photostabilizers to efficiently release the triplet excitations (DOI: 10.1039/C5FD00114E). Thanks to this intramolecular ''self-healing'' the bleaching rate can be reduced by 2 orders of magnitude. Examples of this improvement are shown in Fig. 4.

Session 6: Low-temperature spectroscopy

After lunch, the attention shifted from room temperature experiments to cryogenic conditions. The variability of single molecule spectra is typically explained by differences in the nano-environment. To date, the exact parameters that lead to spectral heterogeneity are not always clear. In his talk, Y. G. Vainer (Russian Academy of Sciences, Russia) presented the distribution of spectral line widths of single tetra-*tert*-butylterrylene (TBT) molecules as a function of embedding diffusion depth in a thin film of amorphous polymer (DOI: 10.1039/C5FD00055F). He showed that the molecules exhibited increasingly faster and more complex spectral dynamics the closer to the surface they were located. Only at a distance of at least 20 nm from the surface, the bulk properties were recovered.

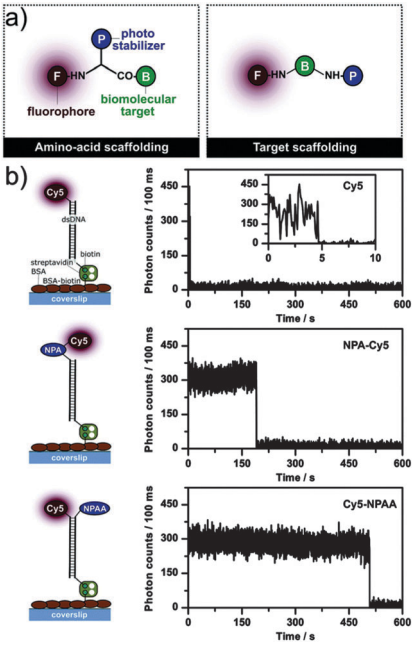

Fig. 4 (a) Different chemical techniques to scaffold photostabilizer–dye conjugates. (b) Different experimental realizations to study Cy5 derivatives together with the representative time traces where the inset in the first time trace is a zoom in of the first 10 seconds of the scan (reproduced from DOI: 10.1039/C5FD00114E with permission from the Royal Society of Chemistry).

The concept that the local environment affects the molecule cannot only be studied, but also utilized. S. Faez (Utrecht University, The Netherlands) proposed the use of designed organic molecules as nanoprobes of the electric field (DOI: 10.1039/C5FD00065C). To this end, they wanted to make use of molecules that exhibit large Stark shifts while keeping narrow zero-phonon lines. Some measurements on the most promising candidate showed that the host matrix was not suitable for narrow and bright zero phonon lines, so other design strategies might have to be employed.

Alterations in the local environment not only change the behaviour of the molecule itself, but also affect other parameters like the refractive index due to local field effects. M. G. Gladush (Institute for Spectroscopy of the Russian Academy of Sciences, Russia) demonstrated that one can determine the local refractive index and its fluctuations through its dependence on the radiative lifetime of single molecule probes embedded in a solid (DOI: 10.1039/C5FD00086F). At cryogenic temperatures, the radiative lifetime can be determined by the lifetime-limited spectral width of the zero phonon lines and it was found that fluctuations in the refractive index are more pronounced in disordered than in ordered media.

Session 7: Plasmonics

The plasmonics session was scheduled after the afternoon tea break. Plasmonic nanostructures are widely used in various applications. These nanosized systems exhibit novel properties compared to the bulk that are not always fully understood and are challenging to probe.

P. Borri (Cardiff University School of Biosciences, UK) presented a technique to characterize the optical extinction cross-section of individual gold nanoparticles quickly and in parallel using wide-field imaging (DOI: 10.1039/C5FD00079C). Using polarization-dependent excitation, it is possible to deduce the geometrical aspect ratio of the particles and discriminate single particles from dimers and larger aggregates. Finally, transient resonant four-wave mixing was performed on the same particles, which proved promising for localized surface plasmon resonance measurements.

An important property of plasmonic nanostructures for single molecule applications is the local electric field enhancement in close proximity to a plasmonic nanostructure. However, it is often difficult to place the molecules at the position of maximum field enhancement. In his talk, A. J. Meixner (University of Tübingen, Germany) explained that high particle positioning precision can be achieved when placing quantum dots at the tip of a plasmonic nanocone (DOI: 10.1039/

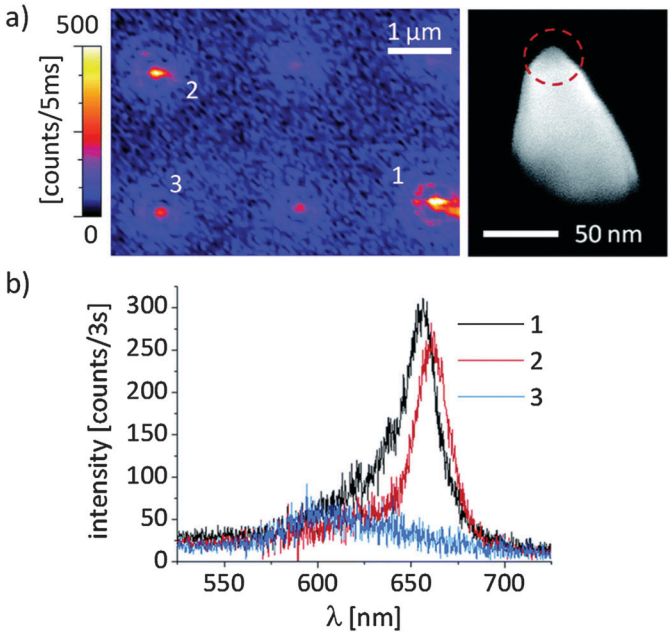

Fig. 5 (a) Confocal image of the nanocone–quantum dot hybrid structure (left) and a SEM image of it (right). The spectra of the cones labelled 1, 2 and 3 in the confocal image are given in (b) where 3 is a cone without a quantum dot (reproduced from DOI: 10.1039/C5FD00074B with permission from the Royal Society of Chemistry).

C5FD00074B). For this hybrid system, an increase in the quantum dot photoluminescence intensity and a significantly shorter lifetime, as well as a spectral shift of the emission spectrum were observed. This is demonstrated in Fig. 5.

The positive effects of plasmonic field enhancement are exploited in a wide range of techniques and applications. It can, for example, be combined with Raman scattering to perform surface enhanced Raman spectroscopy (SERS). In his contribution, P. Z. El-Khoury (Pacific Northwest National Laboratory, USA) talked about determining the molecular orientation with respect to the local electric field using SERS (DOI: 10.1039/C5FD00036J). When moving to the single molecule regime the ensemble averaging is removed so that the molecular orientation can be deduced from the spectral signature of the molecule due to the tensorial nature of the Raman scattering process.

RSC Fellowship, poster session reception and conference dinner

This Faraday Discussion was very special as it hosted two out of three of the 2014 Nobel Prize Laureates in chemistry: William E. Moerner and Eric Betzig. Both of them actively participated in the discussions, and they additionally gave the opening and closing lectures, respectively. For their remarkable contributions to the field of single molecule research they were awarded Honorary Royal Society of Chemistry Fellowships, presented by the president of the Royal Society of Chemistry, Professor Dominic Tildesley (see Fig. 6a).

Following this event the delegates moved to the Science Room, where the poster session took place. There was plenty of time to discuss in detail the 29 posters in a relaxed manner, while enjoying some snacks and drinks.

The evening was concluded with a celebrative conference dinner, which took place in the home of the Royal Society in Carlton House Terrace. After the dinner, the Faraday Discussions Poster Prize was awarded to M. Lee (Stanford University, USA) for his poster on "3D single-molecule super-resolution fluorescence microscopy with point spread function engineering". However, the most anticipated dinner event was another Faraday Discussion tradition, namely the Loving Cup Ceremony. During this event the attendees pass each other an 18th century silver cup, from which they sip port wine and toast to the long term employees of the Faraday Society, "in piam memoriam of G. S. Marlow and Angela and Tony Fish" (Fig. 6b).

Session 8: Tracking and manipulating

On Wednesday morning everybody gathered in the Library again for the last two sessions of this Faraday Discussion meeting. Various methods are used to study the nanoscale local dynamics of biological processes with high spatial or temporal resolution. However, for a high spatial resolution one typically has to sacrifice acquisition speed and *vice versa*. H. Yang (Princeton University, USA) presented a thorough study of the parameters that limit the acquisition speed in super-resolution microscopy (DOI: 10.1039/C5FD00090D). To this end, new detection and feedback methods that are fast and sensitive are needed in which one should concentrate on the object of interest rather than scan a large area. He concluded that at the moment, the real limiting factor is the speed of the 3D piezoelectric stages.

In order to understand a complex system, it is often easier to study a simpler system first. Artificial self-propelled swimmers are for example developed to study the mechanisms of thermophoresis and thermoosmosis – processes which happen far from equilibrium. F. Cichos (University of Leipzig, Germany) explained to the audience how they methodically changed experimental parameters such as particle size or input power in order to understand which role each parameter plays on the motion of their self-thermophoretic swimmers (DOI: 10.1039/C5FD00111K). These findings were accompanied by numerical calculations. They find that the experiments and theory are in good agreement and were able to find the parameters that lead to maximum efficiency in real systems.

Session 9: Living cells

After morning tea, this last session of the Faraday Discussion had its focus on the measurements of living cells. The ability to localize and track single molecules in living cells is key to understanding biological systems on the nanoscale. Consequently, it enables one to address many of the open questions about nuclear dynamics and molecular organization within cells.

Fig. 6 (a) Royal Society of Chemistry president Dominic Tildesley awards Honorary Royal Society of Chemistry Fellowships to Eric Betzig and W. E. Moerner (credit: the Royal Society of Chemistry and John Rogers). (b) The loving cup. (c) Poster session in the Science Room.

It is, for example, not fully understood how DNA binding proteins (DBPs) find their target sequence through non-specific binding. M. Dahan (Université Pierre et Marie Curie, France) and his team followed DBPs in 3D and were able to discriminate between 1D sliding along the DNA and 3D diffusion (DOI: 10.1039/C5FD00112A). The retrieved distribution of binding times provided vital information about the differences between the genomes, and its organization and nuclear environment in mammalian and bacterial cells.

Many cellular processes are facilitated by proteins and regulated through protein concentration. M. C. Leake (University of York, UK) presented a new experimental protocol to estimate protein concentration in different parts of living cells (DOI: 10.1039/C5FD00077G). They studied the dependence of Mig1 concentration in the cell as a function of controlled changes of the glucose concentration outside the cell with ms time resolution. Since this technique studies cells individually it is sensitive to more subtle local concentration variations than ensemble measurements.

E. coli cells replicate their DNA during cell division. The binding protein SeqA helps with the regulation of chromosome replication. Using photo-activated localized spectroscopy (PALM), J. T. Mika (KU Leuven, Belgium) has followed the localization of SeqA throughout the entire cell cycle (DOI: 10.1039/C5FD00058K). The application of super-resolution microscopy resulted in much improved sensitivity, which uncovered that SeqA is sometimes localized outside the foci structures and even in the cell membrane. They found that the amount of SeqA showed a broad distribution between the cells and had no correlation to the cell cycle stage.

Perspective lecture

In the perspective lecture, E. Betzig (Howard Hughes Medical Institute, USA) discussed the challenges and trade-offs in super-resolution fluorescence microscopy. In this context, he focused on the meaning of resolution, which in his opinion should reflect the resolution one can achieve in crowded samples rather than on isolated single molecules.

He started his lecture by talking about the Nyquist criterion, which states that the sampling interval must be at least twice as fine as the desired resolution. With some examples he clearly demonstrated that the Nyquist criterion, while necessary, is often not sufficient and that a labelling density that is at least 10 times higher is required to see the structure of interest with high fidelity. At the same time, he warned that a high labelling density may lead to overexpression artefacts and be disruptive to physiological environments. E. Betzig concluded this part of his talk by demonstrating that for localization microscopy, labelling directly on the target with high specificity is far more important for the resolution than the brightness of the labels.

He continued with the comparison between fluorescence proteins, which have a short linker length and 100% specificity with antibodies, and dyes, which have a longer linker length and only 80% specificity. Here, he reminded the audience that averaging over several linecuts of the same feature is not a faithful measure of resolution since it is only possible in the case of isolated features, illustrating again that resolution is a very slippery metric. One can thus only claim lower-bounds of the resolution due to its dependence on properties such as the sample density.

Using a combination of Points Accumulation for Imaging in Nanoscale Topography (PAINT) and photoactivated localization microscopy (PALM) one obtains a combined global and protein-specific contrast. Still, with electron microscopy progressing rapidly towards protein specificity, the end of super-resolution for structural biology would be near if it were not the case that live imaging is impossible with electron microscopy. Optical super-resolution could therefore still be the path forward for the study of dynamical processes even though several challenges have to be overcome to reach this goal. Currently, one of the main issues is that live cell imaging requires many photons for each frame, leading to fast photobleaching.

Betzig continued with an intermezzo about practical resolution limits and their dependence on noise. He demonstrated how photobleaching, phototoxicity, and sample motion all influence the signal-to-noise ratio and thus the image resolution. Therefore, resolution is not just one number for each microscope, but depends highly on the signal-to-noise ratio. Deconvolution, when done with care, can help in making a more representative image since it compensates for the filtering properties of the microscope. However, since it amplifies the noise alongside the signal it cannot improve the resolution. As a bottom line, the signal-to-noise ratio and hence the practical resolution is sample dependent.

With all the above in mind, structured illumination microscopy (SIM) was introduced. When combined with total internal reflection fluorescence (TIRF), it becomes a powerful tool for live cell imaging because it requires a much lower photon flux and has a faster acquisition time than other techniques while it also provides a very good resolution, especially for dense samples. Consequently, according to Betzig, the nearest future for super-resolution microscopy lies in the TIRF-SIM approach.

Concluding remarks

The concluding remarks were presented by N. F. van Hulst (ICFO, Barcelona) (DOI: 10.1039/C5FD00147A). He recapitulated the meeting by giving a brief overview of all the topics covered during the Discussion. Importantly, he pointed out that in spite of a broad range of topics covered in the meeting, it did not exhaust the full potential. Some of the emerging topics have not been represented like those that include new single molecule detection schemes based on scattering (iSCAT), or the detection of individual objects through electrical read-out or photothermal contrast. Other topics omitted during the meeting were ultrafast coherent control, detection volume confinement in complex, concentrated media, nano-objects possessing magnetic and non-dipolar transitions as well as new families of quantum emitters based on rare earth ions and perovskites. All of this suggests that we should see a number of interesting experiments in the coming years which perhaps will stimulate the single molecule community to propose a follow-up of this Faraday Discussion. Van Hulst ended wondering and asking the audience whether there is a milestone in sight that could match for instance the first single molecule detection at room temperature; a single experiment that would lift the field of single molecules to yet another level of sophistication. With that in mind and plenty to think about, the audience said their goodbyes and dispersed to continue their daily adventures with science.

The 2015 Faraday Discussion on single-molecule microscopy and spectroscopy has been a vibrant and stimulating meeting. That the meeting was successful was obvious during the discussion sessions of the meeting, through the abundance of questions and remarks, which often forced the sessions' chairs to conclude the discussions prematurely due to time restrictions.

The meeting gave a great opportunity not only to look back on how the field of single molecule microscopy and spectroscopy has developed over the past 26 years but more importantly also to present the latest advances and to discuss future plans.

It is indeed hard to resist the impression that the field should not be considered a single, concise field anymore. In each session the researchers addressed different aspects, defined explicit boundaries to be broken, limits to be beaten and goals to be achieved. Can we expect another major breakthrough that would revolutionize the field? Only time will show.

After having one last chance to discuss the talks with the other delegates over lunch, everybody dispersed into an overcast and rainy London.

Acknowledgements

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