Invited Review

Meeting Report: SMART Timing—Principles of Single Molecule Techniques Course at the University of Michigan 2014

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ABSTRACT:

Four days after the announcement of the 2014 Nobel Prize in Chemistry for "the development of super-resolved fluorescence microscopy" based on single molecule detection, the Single Molecule Analysis in Real-Time (SMART) Center at the University of Michigan hosted a "Principles of Single Molecule Techniques 2014" course. Through a combination of plenary lectures and an Open House at the SMART Center, the course took a snapshot of a technology with an especially broad and rapidly expanding range of applications in the biomedical and materials sciences. Highlighting the continued rapid emergence of technical and scientific advances, the course underscored just how brightly the future of the single molecule field shines. © 2014 Wiley Periodicals, Inc. Biopolymers 103: 296–302, 2015.

Keywords: *superresolution imaging; single particle tracking; single molecule fluorescence; fluorescence correlation spectroscopy; meeting summary*

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Sophisticated microscopes have emerged over the last two to three decades that can visualize single molecules within virtually any complex mixture (Figure 1) based on either their optical absorption or fluorescence, and mechanically manipulate and detect them through the use of magnetic tweezers, optical tweezers, and atomic force microscopes.[1–3] From a May 2006 symposium entitled "At the Single Molecule Frontier: Integration in Biology and Nanotechnology", which gathered several thought leaders for 2 days at the University of Michigan, emerged the idea that instrument- and training-focused centers of expertise were needed to enable the broader integration of single molecule microscopy into biology and nanotechnology.[4] Eight years later, after a successful 3.5-year, $1.7Mio NSF Major Research Instrumentation grant funded from federal stimulus moneys and a year of negotiations over how to support it beyond the grant's lifetime, the University of Michigan's Single Molecule Analysis in Real-Time (SMART) Center embodies this call for action. Housed in dedicated space in Biophysics, the SMART Center has grown into a unique open-access facility—with currently ~90 trainees and users from the University of Michigan and across the nation—that is on a mission to bring basic scientists, engineers, and clinical researchers together to apply single molecule tools to the most relevant questions in medicine and nanotechnology (http://singlemolecule.lsa.umich.edu). As an

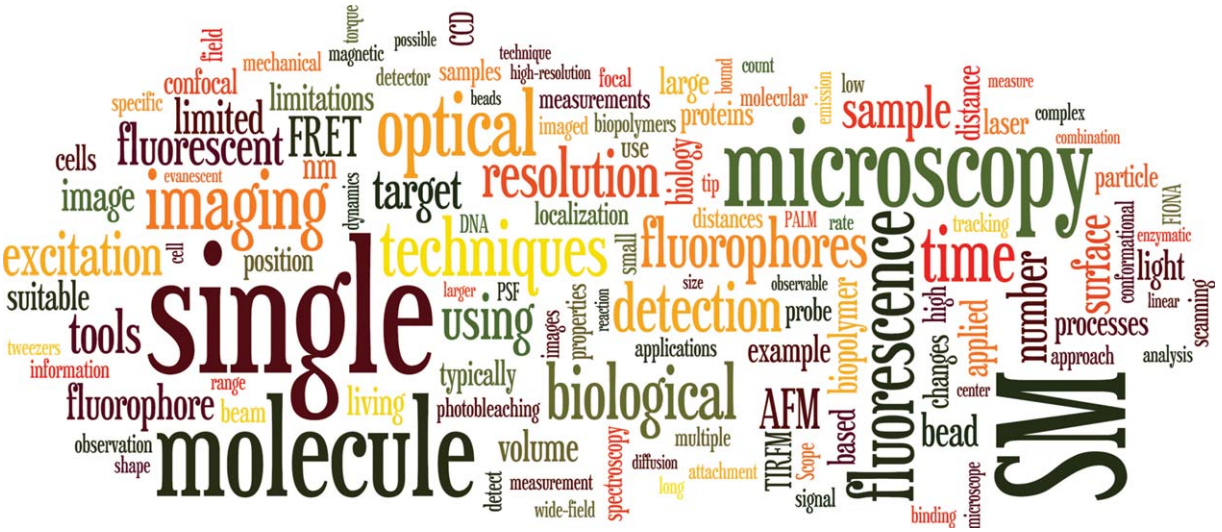

FIGURE 1 Word cloud of the single molecule field, generated using Wordle™.

outreach activity to further broaden its user base and foster discussions about current capabilities and applications of single molecule techniques, the SMART Center on October 13 and 14, 2014, organized the "Single Molecule Techniques Workshop 2014", bringing together 12 speakers, including five from outside the University of Michigan, for a full day of exciting plenary lectures, followed by a day-long hands-on introduction to single molecule tools through a SMART Center Open House. The date was set much in advance, but turned out to follow just 4 days after the announcement of the Nobel Prize for Chemistry 2014 "for the development of super-resolved fluorescence microscopy" based on single molecule detection. The three awardees—Eric Betzig, Stefan W. Hell and William E. Moerner—represent a cross-section of the exciting developments in this rising field over the past quarter century, and their accomplishments and those of many others were reflected in the lectures that took a snapshot of the current standing of the field, which is summarized in the following.

Sua Myong (University of Illinois at Urbana-Champaign, Bioengineering) kicked off the workshop by discussing several single molecule fluorescence techniques that her group employs to illuminate the mechanism of RNA interference (RNAi)—an evolutionarily conserved, cellular innate immunity pathway that uses small interfering RNAs (siRNAs) to mediate sequence-specific degradation of undesired virus and transposon derived RNAs.[5,6] In addition to its roles as a fundamental genome defense mechanism and antiviral immune response,[7,8] RNAi has been developed into a valuable gene-silencing tool with profound applications in functional genomics and therapeutics.[9] In an effort to improve gene silencing potency with minimal off-target effects, Myong and colleagues studied various stages of the RNAi pathway. Using protein induced fluorescence enhancement,[10] single-molecule pulldown (SiMPull),[11,12] as well as two- and three-color single molecule fluorescence resonance energy transfer (smFRET; Figure 2),[13] they found that TRBP, an RNA binding co-factor of Dicer involved in the processing of long double-stranded RNAs (dsRNAs) into siRNAs, binds and slides along its RNA substrate *in vitro*.[14] They additionally found that sliding efficiency was dependent on both the length and the structure of the RNA, and on a linker region within the protein. SiMPull-FRET[12] further revealed that the processing of long dsRNA by Dicer (i.e., "dicing") was more efficient when the substrates contained occasional bulges and when TRBP sliding was observed. Using SiMPull of Ago-Dicer-TRBP ternary complexes containing doubly labeled siRNAs, Myong and colleagues confirmed previous findings that one strand within the ds-siRNA is preferentially retained to guide the RNA induced silencing complex (RISC) to cleave a target RNA. To study the final step in the RNAi pathway, cleavage of a target RNA (or "slicing") was probed in cells using single molecule fluorescence *in situ* hybridization (smFISH). Within 12 hours of cellular siRNA delivery, a significant fraction of messenger RNAs (mRNAs) were found to be cleaved. Slicing efficiency was strongly correlated with that of dicing, while 3′ UU or TT overhangs increased silencing efficiency compared to blunt ended RNAs. Overall, this talk highlighted the wide array of single molecule fluorescence spectroscopy tools currently available to mechanistically dissect biologically relevant cellular pathways.

On the other end of the spectrum of single molecule tools (Figure 2), Julie Biteen (University of Michigan, Chemistry) began her discussion with a brief overview of super-resolved single particle tracking in live bacterial cells.[15] Briefly, when

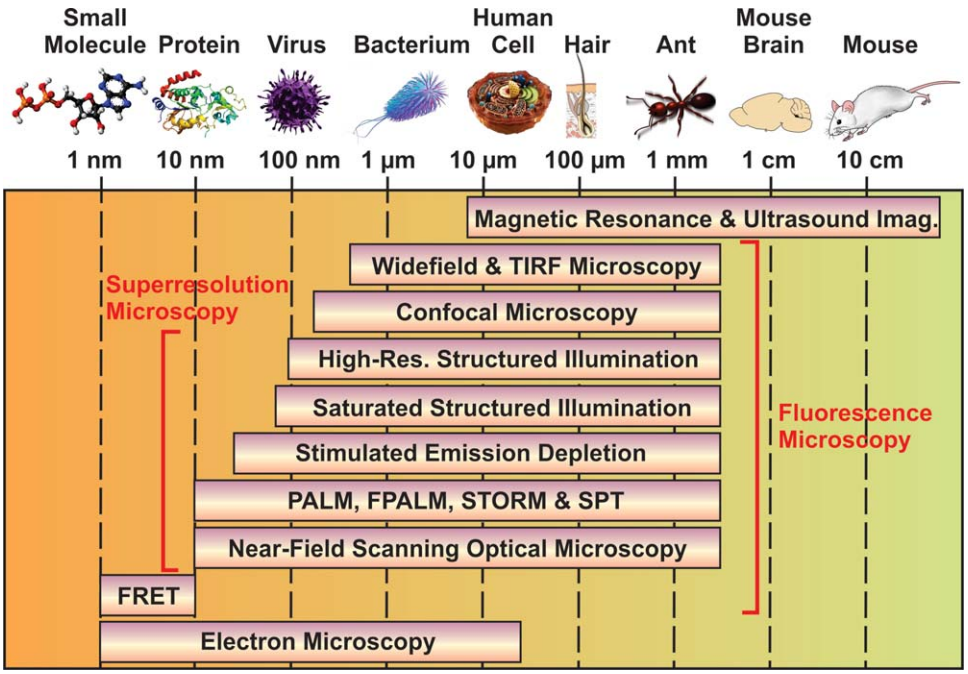

FIGURE 2 Resolution range of modern microscopy and imaging techniques.

detected in a microscope, the fluorescence emanating from a sub-diffraction (low-nanometer) sized particle generates an Airy disc diffraction pattern called the point spread function (PSF). The center of this hundreds of nanometer large signal peak encodes the exact location of the particle, which can be pinpointed to tens of nanometer precision by fitting the PSF, typically recorded on a pixelated CCD camera chip, with a 2D Gaussian profile approximation. Through a time series of images, the location of the particle while diffusing within a bacterial cell, for example, can be tracked in time. A mean squared displacement can be calculated from changes in particle location over time and used to calculate a diffusion coefficient.[15] For some molecules, or large particles, distinct movement characteristics are observed. Through the addition or removal of various components or stimuli, one can observe a shift in the population of particles within a class of diffusion behaviors and coefficients. Using these techniques, Biteen and colleagues are able to overcome the diffraction limits associated with traditional microscopy techniques and use single molecule imaging to study living bacterial cells with super-resolution (resolving particles with a distance < 40 nm).[15]

As an example, the Biteen group has studied the movement characteristics of TcpP, a protein of the cholera-toxin producing *Vibrio cholera* virulence cascade, and its interactions with ToxR and the *toxT* promoter.[16] To visualize the single TcpP protein molecules in an overexpressing cell, they labeled and ectopically expressed the gene with a photoactivatable mCherry protein. Without further perturbation, they detected three distinct populations of diffusion coefficients for the labeled TcpP protein: fast, slow, and immobile. They hypothesized that the two slowly diffusing populations reflect the association of TcpP and its interacting partner ToxR with the *toxT* promoter in the genomic DNA. Unexpectedly, knockout of the ToxR protein and/or removal of the *toxT* promoter resulted in the redistribution of a significant fraction of the fast moving particles into the slowly diffusing and immobile populations. Further data analysis suggested that ToxR acts as a "broom" to clear the DNA, scans for the *toxT* promotor, and recruits TcpP to activate transcription.[16] Biteen then also gave a summary of several other directions of her recent work, including plasmon-enhanced fluorescent probes, fluorophore incorporation into proteins via unnatural amino acids, CRISPR/Cas9 technologies for site-specific fluorescent labeling of genomic sequences,[17] 3D imaging for more accurate diffusion coefficient calculations, and new correlation schemes to track extremely fast particles.[18]

Joong Hwan Bahng (University of Michigan, Biomedical Engineering, Nick Kotov's group) was unfortunately unable to present due to sudden illness, but provided a manuscript showcasing work on how the surface of microscale particles can be physically engineered to enable solubility in phobic solvents without the use of chemical surfactant camouflage.[19] Such unconventional dispersion breaks the "similarity rule" by imparting an interfacial nanoscale topography on so-called "hedgehog particles"

with a layer of stiff ZnO nanospikes normal to the surface. Using confocal microscopy of single particles in the SMART Center and theoretical calculations, Bahng and colleagues revealed several underlying physical properties affecting the enhanced stability. They concluded that factors contributing to increased hedgehog particle stability include the limited contact area and increased minimum interaction distance due to surface corrugation that together drastically reduce the attractive potential. Entrapped air pockets and the solvent auto-ionization on their interface provide stronger electrostatic repulsive potential in the case of aqueous dispersion of hydrophobic hedgehog particles, whereas low ionic strength provides long-range electrostatic repulsion in the case of hydrophilic hedgehog particles in non-polar organic media. Bahng's work thus yielded a deeper understanding of interparticle and particle-solvent interactions. In the future, these findings may provide a simple and robust method for preparing colloids for nanotechnological applications.[19]

After a coffee break, Brent Krueger (Hope College, Chemistry) began his introduction to FRET by observing that the acronym would better be referred to as fluorescence-detected or Förster resonance energy transfer to clarify the physical mechanism as, contrary to common misconception, no fluorescent photon transfer occurs as part of the resonance energy transfer. Krueger highlighted the assumptions underlying the application of the Förster equation in its popular biological applications and discussed sources of potential error specifically in the ideal dipole approximation (IDA).[20,21] For example, most users of smFRET assume that all spectral parameters are the same for all donor/acceptor probe pairs in an ensemble of molecules, largely disregarding environmentally induced fluctuations. Similarly, it is typically assumed that donor and acceptor have an isotropic spatial orientation such that an average relative orientation factor, κ^2, of 2/3 applies; however, this value may not be accurate since when simulating the orientation of dyes coupled to a biopolymer, an average error of $\sim$10% from the IDA is observed, with an error as large as $\sim$20% for some of the most commonly used dyes, such as Cy3 and Cy5.[21] Krueger closed his talk suggesting a possible solution for this dilemma, which is a Markov chain approach that calculates the probability of all possible events (excitation of donor or acceptor, static and dynamic quenching, energy transfer etc.) over a molecular dynamics simulation trajectory with fluorophores attached to a biopolymer, then correlates these predictions with an experimental smFRET trajectory of the molecule.[21]

Next, Kristen Verhey (University of Michigan, Cell and Developmental Biology) spoke about her work on kinesin motor function in mammalian cells. The kinesin protein family is one of several classes of motor proteins in eukaryotic cells responsible for the intracellular trafficking of cellular cargo.[22] Kinesins use the hydrolysis of ATP to drive a walking motion towards the positive end of microtubules and often do so through attachment of multiple kinesin motors to each cargo. How different kinesins attached to the same cargo interact with each other to achieve directed motion through a crowded cellular environment is not well understood.

Of the 14 families of kinesin motor proteins, Verhey focused on kinesin-3, known as the "marathon motor" capable of transporting at a high rate over very long distances, and kinesin-1, a much slower motor with low processivity.[23] Using single molecule total internal reflection fluorescence (TIRF) microscopy in the SMART Center, Verhey and colleagues investigated the effects of both the fast (kinesin-3) and slow (kinesin-1) motor attached to the same single cargo. To this end, the two kinesin motors were co-expressed with a scaffold protein capable of tethering them together and serving as a cargo mimic.[23] Surprisingly, *in vitro* experiments revealed that a majority of cargo complexes move along microtubules at a slow pace, characteristic of kinesin-1, with some cargoes undergoing speed changes to the fast pace of kinesin-3. This would appear to indicate that the presence of one motor does not interfere with the function of the other and that the two proteins work independently, resulting in the slow motor dominating. By contrast, the fast motor dominates on certain subpopulations of microtubules in cells.[23] The question now under investigation is whether 'road marks' are present that allow kinesin-1 and kinesin-3 to discriminate different populations or regions of microtubules.

Sethuramasundaram Pitchiaya (University of Michigan, Chemistry, Nils Walter's group), presented an overview of how intracellular Single-molecule High-Resolution Localization and Counting (iSHiRLoC)[6,24,25] has been successfully used to examine biological pathways involving non-coding RNAs (ncRNAs) in mammalian cells, in particular microRNAs (miRNAs) and DNA-damage response RNAs (DDRNAs). A key challenge in the study of ncRNAs is understanding their intracellular trafficking to find specific binding partners. For example, the maturation pathways and regulatory functions of miRNAs involve a number of processes in both the nucleus and the cytoplasm. Using two-color iSHiRLoC, Pitchiaya and colleagues characterized the distribution and movement of individual miRNAs in the cell, and found that only a small fraction localizes to processing bodies (P-bodies, membraneless cytoplasmic foci enriched in RNA-processing enzymes) and the extent of colocalization evolved temporally: miRNAs assembled quickly with P-bodies, followed by a slow and gradual release. This work demonstrates how iSHiRLoC can be used to access spatiotemporal information about individual

molecular species and their interaction partners in the complex environment of the cell.

Pitchiaya's second example focused on DNA-damage response RNAs (DDRNAs), a relatively new entry in the field of ncRNAs.[26,27] He highlighted the broad applicability of iSHiRLoC by showing that DDRNAs appear to accumulate at DNA double-strand breaks before known damage-response proteins, in a sequence-specific manner that requires active transcription.

After lunch, Yale Goldman (University of Pennsylvania, Physiology, and Pennsylvania Muscle Institute) described the structural dynamics of several motor proteins using single molecule techniques. Using a high-speed polarized TIRF (polTIRF) setup, his group characterized the hand-over-hand movements of myosin V as it traverses an actin filament.[28] Incorporation of fluorophore probes into the myosin V lever arms allowed Goldman and colleagues to determine tilting angles of the lever arms during myosin V stepping, demonstrating an example for the broad applicability of the polTIRF tool. In a separate study, a single-molecule FRET setup was used to characterize the translocation of elongation factors EF-G and EF-Tu during bacterial protein biosynthesis.[29,30] Domain rotations of the elongation factor were examined to determine how the ribosome utilizes metabolic energy to ensure the fidelity of tRNA selection and adherence to the correct reading frame. Goldman and colleagues found that EF-G changes conformation while bound to the ribosome and thus revealed the coupling of ribosome and cofactor conformational dynamics during bacterial protein translation.

Sivaraj Sivaramakrishnan (University of Michigan, Cell and Developmental Biology) presented on how the interaction between different myosin motors modulates their collective motion. Inside cells, multiple classes of myosins act collectively in carrying cargo, yet the rules governing their emergent behaviors are not well understood. Using DNA origami scaffolds, assemblies of multiple myosin V and/or myosin VI motors—which have antagonistic directionalities—were generated to investigate their emergent movement on an actin network from keratocytes.[31] Using equipment in the SMART Center, Sivaramakrishnan and colleagues observed that homogeneous multi-myosin assemblies move larger distances but at lower average speed than single-myosin assemblies. Multi-myosin V assemblies showed skewed (non-linear) movement, whereas multi-myosin VI assemblies had more linear trajectories. In contrast, heterogeneous multi-myosin assemblies containing both myosin V and VI showed unidirectional motion and traveled equally well towards the cell periphery and cell center. Using a combination of stochastic simulations and experiments, the observed differences in skewness of movement of the homogeneous assemblies were attributed to differences in the flexural rigidity of the myosin lever arms relative to thermal fluctuations.[31] Consequently, it is possible to alter the trajectory shape of multi-myosin motors by swapping the lever arms of the two myosin classes. This work therefore showed how the collective motion of myosin assemblies can be predictively engineered, paving the way for the knowledge-based custom design of molecular motor assemblies.[31]

Joerg Bewersdorf (Yale University, Cell Biology) described the basic principles of super-resolution fluorescence microscopy techniques including fluorescence photoactivation localization microscopy, stochastic optical reconstruction microscopy, and stimulated emission depletion, further highlighting their relative capabilities and significant technical challenges.[32] Although diffraction-unlimited resolution is achieved, the typical spatial resolution of these techniques is 25–50 nm. In addition, implementation in live cells remains a challenge due to the slow speed of image acquisition. To circumvent the latter problem, Bewersdorf described collaborative work in which he and his colleagues successfully improved the spatial-temporal resolution using a technique called single-molecule switching nanoscopy (SMSN) that was applied to super-resolution imaging of both live and fixed cells.[33] Augmenting a new, faster camera, termed scientific complementary metal-oxide semiconductor, of reasonable quantum yield (~72%) with software-based corrections for pixel-dependent readout noise, SMSN can localize single molecules with a precision of up to ~10 nm and super-resolved imaging speeds of up to 32 reconstructed images per second.[33] This development may offer high-throughput imaging of a wide range of biological samples, including dynamic live cells.

After another short break, Sarah Veatch (University of Michigan, Biophysics) emphasized that many protein-protein interactions are transient and new methodologies are needed to resolve such brief events. Veatch then reported on her development of a time-resolved cross-correlation (TRXC) analysis technique for super-resolution localization to examine immune cell signaling in live cells. Upon binding to antigen, B-cell receptors (BCRs) interact with a complex network of proteins within the membrane that regulates receptor signaling.[34] By fluorescently tagging BCRs and Lyn kinase, one of the downstream regulatory proteins, the Veatch group was able to utilize TRXC to monitor the dynamics between these two proteins. Prior to antigen binding, movements of the two proteins are uncorrelated; they undergo Brownian diffusion of different step sizes, whereas after stimulation by antigen binding the BCR becomes highly correlated with itself as well as Lyn kinase. It was also possible to determine the dissociation rate constant of Lyn to ~1 second. The TRXC approach may be broadly applicable to many other biological systems.

Moving on to another form of correlation analysis, Keith Berland (Emory University, Physics) discussed the strengths as well as limitations of fluorescence correlation spectroscopy (FCS), presenting a new method that overcomes many of its limitations.[35] Berland discussed the applications of FCS to intracellular dynamics, including ongoing studies of amyloid fiber assembly. He and his colleagues found that improvements to FCS were required to address some of the questions raised by this biological system, such as how aggregates of amyloid-β form. He then presented some of the limitations of FCS, focusing on the difficulty of choosing the proper diffusion model with which to fit and interpret the experimental data. Standard FCS data can often be fit relatively well by a variety of models, such as one-, two- or three-species models or anomalous diffusion. However, overlapping and/or small subpopulations are difficult to resolve.

Berland then asked how these problems of limited resolution and model determination can be overcome, and proposed the solution of new contrast parameters combined with global analysis.[35] New contrast parameters are additional observables such as molecular brightness, color, fluorescence lifetime, anisotropy or FRET, which can be measured independently of FCS. Berland proposed lifetime and FCS as a particularly powerful combination of contrast parameters. One measures FCS and lifetime data on the same sample and performs a global analysis, fitting both datasets to a common set of parameters. Lifetime data depend on the fluorescence lifetime of each species, whereas FCS depends on the diffusion constant of each species, and both depend on the concentration and brightness of each molecular species involved. Berland then tested this combination technique, termed τFCS,[35] on a mixture of rhodamine 6G and rhodamine B, which have nearly identical diffusion coefficients but different lifetimes. He found that by applying a global fit of the τFCS data with a two-species model, accurate lifetimes, diffusion coefficients and relative concentrations of the two dyes could be recovered across a wide range of relative concentrations. Standard FCS would have been unable to differentiate the two dyes as they have the same diffusion coefficient. Berland concluded that τFCS is just one possible implementation of a broad strategy that combines multi-modal fluorescence acquisition with global analysis, further improving the power to resolve sample composition in complex environments.

Finishing up an exciting day of science, Beniamino Barbieri (president of ISS, Champaign, IL) reviewed the background and capabilities of fluorescence fluctuation spectroscopy (FFS) modalities (e.g., FCS, photon counting histograms) and time-resolved methods [e.g., fluorescence lifetime imaging (FLIM), time-resolved FRET].[36] These modalities enable quantitative interrogation of molecular dynamics within the cell, and thus complement high-resolution imaging techniques, which can provide detailed structural but limited molecular information. By examining temporal fluctuations in fluorescent signals, FFS modalities provide real-time information about fluorescent probe concentration, diffusion coefficient and brightness. From these parameters, one may draw inferences about dynamic events such as binding kinetics or changes in local concentration or mobility. For example, FCS has been used to examine assembly of paxillin in focal adhesions, finding that paxillin assembles as monomers, but disassembles as small aggregates with variable dynamics based on the location of the adhesion relative to the cell perimeter.[37] Barbieri then briefly discussed practical considerations in the implementation of modern FFS/FLIM, and recent advances in the algorithms and hardware to make these techniques faster, more sensitive, and more accessible.[36] An instrument to perform such measurements, the ALBA fluorescence fluctuation microscope from ISS, is available for broad use in the SMART Center.

In summary, the workshop showcased the leaps and bounds with which single molecule tools have advanced, especially over the past decade.[2,3] Bringing basic physical scientists and engineers together with biomedical and materials scientists through activities such as the SMART Center is starting to pay off. Further nurturing these synergies will likely lead to the ability to ask and answer many new scientific questions, often from entirely new angles, so one Nobel Prize may be just the beginning.

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