# The SMART ways of seeing

**Technique 1: Single-molecule imaging to precisely localize particles**

Single particle localization is one of the foundational single-molecule techniques where the position of individual fluorescently labeled particles (e.g. single proteins or RNA molecules) is precisely localized within a cell.

There is a theoretical limit to the resolution of any light microscopy due to the physics of diffraction. The theoretical best resolution is situated at about half the wavelength of the light being observed, typically somewhere around 250 nm. However, if scientists are interested in sparse single particles, they can use computational tricks (like fitting the point spread function), to find the particle's true location with much better precision, usually around 20 nm.

For each of these localized particles, one can measure their positions, and observe their states or behaviors. For example, one can see whether the particle is co-localized with other (differently colored) molecules and how many labeled subunits constitute the particle, what functional state it is in, etc. To measure diffusion patterns and quantify transient behaviors, one can also localize each particle frame-by-frame over time in live cells.

For example, Pitchiaya et al.[1] tracked the dynamic localization of individual RNAs (miRNA, mRNA, and lncRNA) in processing bodies (P-bodies), using either live-cell particle tracking or single-molecule hybridization in fixed-cells. From these data, these scientists were able to reveal where and when mRNA turnover occurs and how much a particular RNA is recruited to P-bodies for storage and decay.

*"RNA localization is central to gene regulation and cellular function. With the SMART Center's state-of-the-art instrumentation, especially the single-particle tracking and single-molecule imaging technology, I'm able to visualize intracellular RNAs at unprecedented spatiotemporal resolution and understand mechanisms of RNA(-based) regulation."*
*—Sethu Pitchiaya, Ph.D. Research Investigator, Pathology, Medical School*

*Pitchiaya working on the single particle tracking TIRF microscope (SPT), doing some single molecule FISH (fluorescence in situ hybridization).*

Single-molecule imaging overlaps with one of the families of super-resolution microscopy, the single-molecule localization microscopy (in particular, STochastic Optical Reconstruction Microscopy, STORM, and PhotoActivated Localization Microscopy, PALM). With these newest techniques, sparse sets of fluorescent molecules are imaged and localized, but a different set of molecules is observed at each frame, and this over thousands of frames, while the vast majority of molecules are held in a dark non-fluorescent state. Scientists then combine all the localized molecule positions into one final "pointillism" super-resolution image, which has an effective resolution around 20 nm. The SMART Center has recently begun offering STORM imaging, and has plans to expand this capability in the future, enabling more colors and faster imaging.

**Technique 2: Fluorescence fluctuation spectroscopy for diffusing molecules**

Fluorescence Fluctuation Spectroscopy (FFS) is a family of quasi-single molecule techniques that allows fluorescence-based measurements on single or small numbers of diffusing molecules. A laser is focused to a very small spot, and as molecules diffuse in and out of the laser focus, one gets a snapshot of the properties of each particle. FFS can measure several aspects of a particle: its size, how fast it

[1] Pitchiaya, S., Mourao, M.D.A., Jalihal, J., Xiao, L., Jiang, X., Chinnaiyan, A.M., Schnell, S. and Walter, N.G., Dynamic Recruitment of Single RNAs to Processing Bodies Depends on RNA Functionality (2019) *Molecular Cell* **74**, 521-533.

diffuses, how often it is co-localized with a binding partner, and its conformation (via Förster Resonance Energy Transfer, or FRET).

Importantly, these measurements are taken on diffusing molecules. Many single-molecule studies require the molecules to be attached to a solid substrate like a microscope slide; this is a very effective technique, but there is often a question about whether the proximity of the substrate is affecting the behavior of the target molecules. Measurements on diffusing particles avoid such caveats and are closer to actual physiological conditions. These measurements can even be taken inside living cells.

FFS allows to construct a picture of the population statistics that often provides new and complementary information compared to bulk assays. For example, FRET traditional technique may indicate that two probe locations are 5 nm apart, while single-molecule measurements will show that one population is, say, 7 nm apart while another one is 3 nm apart. These detailed data were looking like noise in the FRET bulk measurement.

With FFS, one can measure the dynamics or kinetics of molecular states and observe how often they transition. One can also record the relative likelihood of transitions between different states. FFS can monitor these interactions considerably faster than single-molecule imaging, with temporal resolution on the order of microseconds, allowing direct observation of short-lived transient states.

The SMART Center's FFS instrument is also time-resolved, which means that it measures the arrival time of each detected photon with picosecond resolution. This allows fluorescent lifetime to be measured alongside any other properties one might be interested in. This extra dimension of information can be very useful for discriminating between fluorophores and auto-fluorescent backgrounds. Fluorescent lifetime is also responsive to changes in the local chemical environment or processes like FRET or molecular rotation.

Dr. Hoff has also worked with the SMART Center's users to develop new techniques or analysis methods. For example, with Jens-Christian Meiners's group (U-M LSA, Biophysics and Physics), he has developed a method for improving FFS on slowly diffusing molecules, e.g., DNA inside crowded environments like bacterial cells.[2] Developing such technique is particularly challenging because slowly diffusing molecules may photobleach quickly compared to the timescale of their motion, which limits the usefulness of FFS under these conditions. Dr. Meiners's group demonstrated how to computationally correct for this photobleaching, allowing quantitative FFS information about dynamic DNA motion to be recovered.

*"The SMART Center has provided us with its modular time-tagged fluorescence correlation spectroscopy instrument as a platform to develop a novel way of looking at diffusion and fluctuations on the sub-cellular scale. Because the instrument is not a black box but allows us access to hardware and raw data, we were, with the competent help of the SMART Center staff, able to implement our own data processing methods and extract information that is otherwise unobtainable. We are using this method to study the dynamics of the bacterial chromosome with high temporal and spatial resolution to answer basic questions about the mechanics of genetic regulation."*
*—Jens-Christian Meiners, Professor of Physics and Biophysics, University of Michigan*

**Technique 3: Force spectroscopy to measure molecular mechanical forces**

A large number of molecular biological events have a mechanical component to their activity. Protein conformational changes, winding or unwinding DNA, or cell surface mechanoreceptors sensing their environment are all activities that involve molecules generating and/or measuring mechanical forces. The force exerted by a single molecule is, of course, very small.

At the SMART Center, we study these forces with two types of instruments: optical tweezers and atomic force microscopy (AFM). With these tools, one can measure or exert forces from around a piconewton up to nanonewtons. No one really has an intuitive understanding for forces at such small scales. For reference, a single kinesin motor protein that transports cargo in the cell can exert around 5pN as it takes 8 nm steps. This is roughly similar to the force exerted by a standard laser pointer when it is pointed at a wall.

[2] Hodges, C., Kafle, R.P., Hoff, J.D., Meiners, J-C. (2018) Fluorescence Correlation Spectroscopy with Photobleaching Correction in Slowly Diffusing Systems, *Journal of Fluorescence* **28**, 505-511.

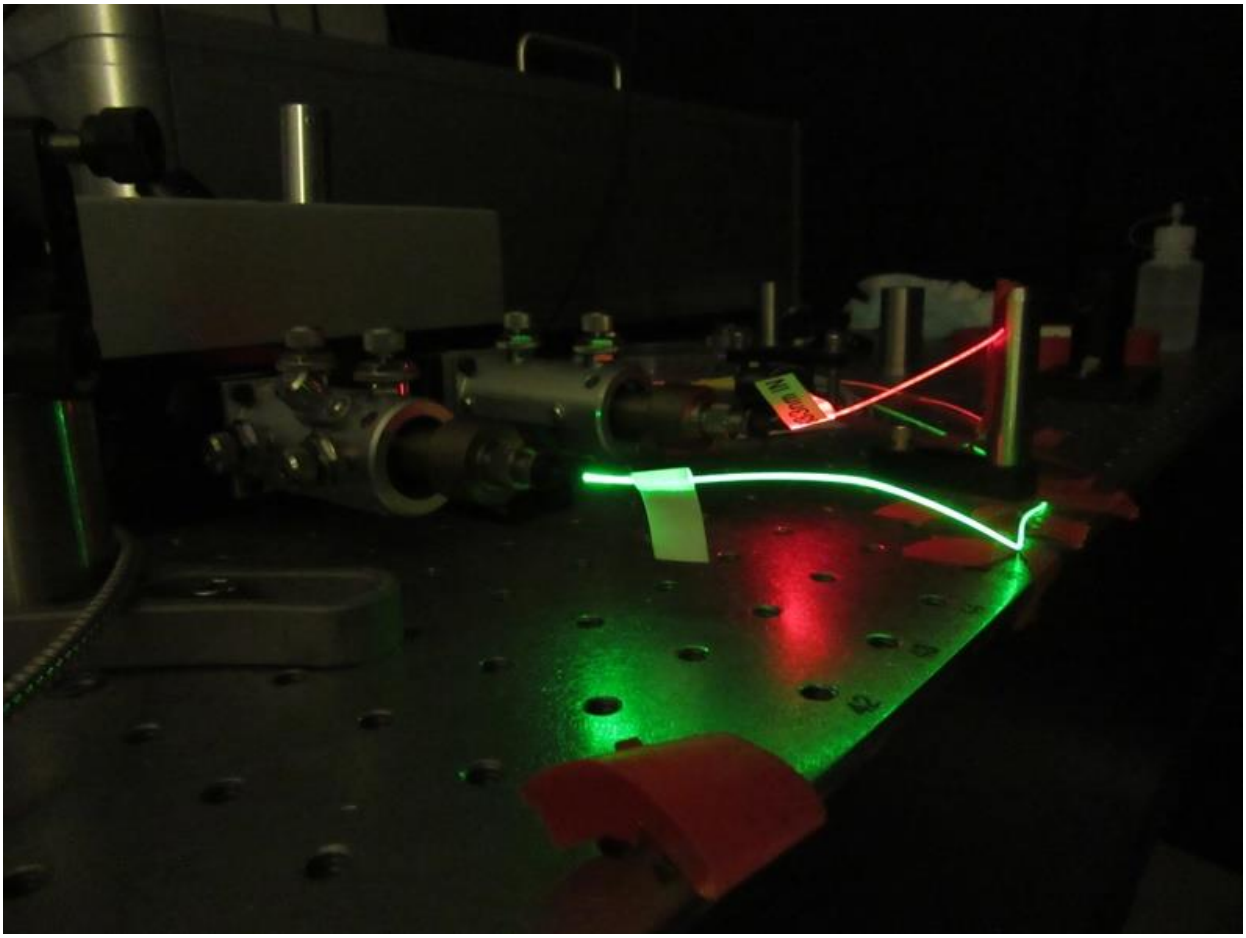
*No one really has an intuitive understanding for forces at such small scales.*

Researchers can use these high-precision instruments to measure the mechanical properties of biomolecules: How much force can they exert? How strongly do they bind to their partners? How stiff are they?

One of the SMART Center's optical tweezers incorporates single molecule fluorescent imaging.

By combining optical tweezers with FRET, we can simultaneously measure conformational changes or the positioning of binding partners (via FRET) while also measuring the mechanical forces generated by these events (via tweezers). Or vice versa: impose a mechanical force and monitor the structural changes via FRET.

The SMART Center's team has not yet applied this technique to RNA studies, but research groups at other universities have used it in investigations about RNA polymerase force production, and RNA unfolding forces and kinetics. We look forward to applying this technique to RNA related projects.



*Pulsed white light being filtered into whatever excitation wavelength we want (here, green or 532nm and red or 640nm) on the time-resolved confocal. The light from the two fibers hit the sample about 20 ns apart. This allows us to do pulsed interleaved excitation (PIE), where the sample is hit by two colors almost simultaneously, but the pulses of light are spaced just far enough apart that the two measurements are independent of each other.*