SnapShot: Single-Molecule Fluorescence

Cell

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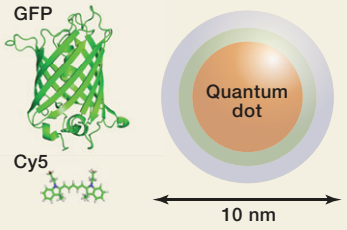
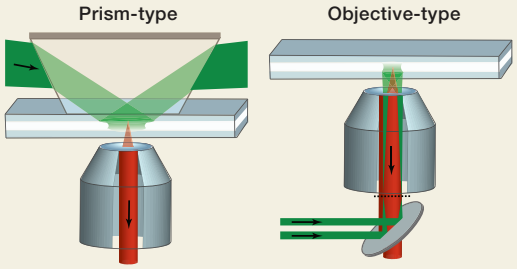
Fluorophores



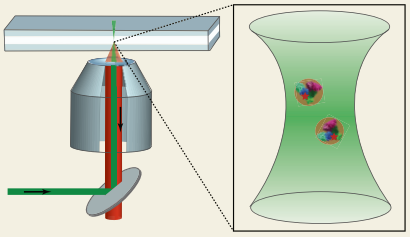
Common fluorescent agents include organic fluorophores (e.g., Cy5), fluorescent proteins (e.g., GFP), and semiconductor quantum dots each with different size, photobleaching, blinking, and labeling efficiency properties.

Total internal reflection fluorescence (TIRF)

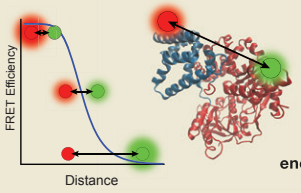
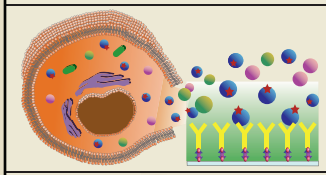
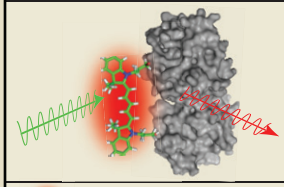
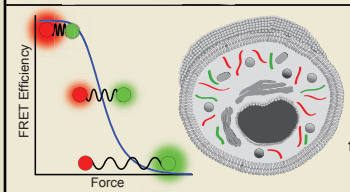
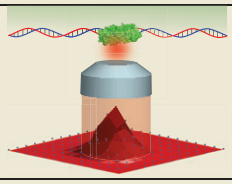
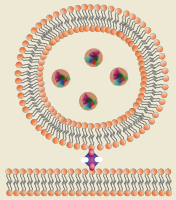
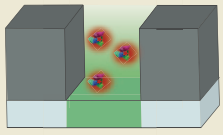


Wide-field detection with EMCCD camera enables parallel measurements. The temporal resolution is >ms, and the working concentration is limited to nM or less.

Confocal fluorescence



Confocal detection from an effective volume of ~10-15 l with an avalanche photodiode or photomultiplier tube. The temporal resolution is <ms, and the working concentration is limited to ~nM or less.

TECHNIQUE	DESCRIPTION	APPLICATION	COMMENTS
 **Single-molecule Förster resonance energy transfer (smFRET)** TIRF, confocal	Measure distance-dependent Förster resonance energy transfer (FRET) between a donor and an acceptor fluorophore attached to a single biomolecule or to two different biomolecules Three- and four-color FRET measure multiple distances	Dynamics of intra- and intermolecular motion Association and dissociation kinetics Confocal detection from an effective volume of ~10-15 l with an avalanche photodiodeor photomultiplier tube; the temporal resolution is <ms, and the working concentrationis limited to ~nM or less. Structure determination	Sensitive to ~1–10 nm distance changes Accuracy limited by background noise and fluorophore mobility Calibration of absolute distances is challenging
 **Single-molecule pull-down (SiMPuLL)** TIRF	Protein complexes from cell lysate are captured by immobilized antibodies and visualized using fluorophore-labeled antibodies or fluorescent protein (FP) tags	Stoichiometry, intermolecular interactions, and biochemical analysis of cellular protein complexes	Simple and more sensitive alternative to western blots Possibly applicable to single-cell analysis
 **Single-molecule fluorescence polarization (SMFP)** TIRF	Polarized evanescent wave generated by total internal reflection excites dipole moments of individual fluorophores, the orientation of which is measured by the polarization of the fluorescence emission	Rotational dynamics	Unique approach with ~ms temporal resolution to measure rotational conformational dynamics with ~10° resolution Requires complex instrumentation and rotationally constrained fluorophore attachment to protein
 **Single-molecule fluorescence force spectroscopy (SMFFS)** TIRF	In vivo FRET-based tension sensor using FPs genetically encoded in reporter protein; tension extends the protein, which results in a change in the FRET signal	In vivo measurement of mechanical forces (≤10 pN) on reporter protein	Calibrating the FRET vs. tension relationship requires a combined single-molecule manipulation and fluorescence instrument
 **Single-molecule fluorescence tracking (SMFT)** TIRF	Labeled proteins are tracked in one, two, or three dimensions with high spatial and temporal resolution	Translocation and diffusion of proteins on extended substrates or in solution	Tracking in the plane (x-y) relies on Gaussian fitting of the point-spread function; out-of-plane (z) motion is determined from changes in the intensity of the fluorophore in the TIRF evanescent field or from depth-dependent changes in the point-spread function
 **Nanovesicle trapping** TIRF, confocal	Labeled biomolecules are trapped inside lipid nanovesicles with ~50–100 nm diameter Effective detection volume is ~10^{-19} l	Biomolecular interactions (~μM or weaker)	Can be immobilized on surface, minimizes nonspecific interactions, and mimics the cellular environment Particularly suited for studying membrane-associated proteins Not suitable for studying extended substrates
 **Zero-mode waveguide (ZMW)** TIRF	Labeled molecules are excited inside metal-clad wells or lanes fabricated on silica substrate Excitation volume ~10^{-21} l reduces fluorescence background	Biomolecular interactions (~μM or weaker)	Easy exchange of solutions, suitable for studying extended substrates Potential changes in fluorophore properties and higher nonspecific binding due to the metal surface

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Choosing the Method

Single-molecule fluorescence has been widely used in the study of intra- and intermolecular dynamics in biological systems, in addition to binding and motion on extended substrates in vitro and in vivo. There are two complementary fluorescence techniques that differ in their excitation and detection modalities: total internal reflection fluorescence (TIRF) and confocal. TIRF is a wide-field technique in which an evanescent field is used to illuminate only the surface of the sample. TIRF is particularly suited for single-molecule tracking and is also suitable for measuring conformational dynamics, intermolecular interactions, and stoichiometry of immobilized protein complexes. Confocal florescence is a focal volume-limited technique suitable for measuring diffusion constants, association and dissociation kinetics, and conformational dynamics of freely diffusing biomolecules with faster timescales (< ms) compared to TIRF (> ms). Confocal detection can also be used to achieve higher time resolution and improved signal to noise in experiments with immobilized molecules, but this is a low-throughput approach. Variations and extensions of these two approaches have led to a wide array of techniques and associated acronyms.

Fluorescent Labeling

The first essential step is labeling the biomolecules of interest. The choice of fluorescent label depends on many factors, including photostability, brightness, size, environmental sensitivity, labeling efficiency, quantum yield, wavelength, linker size, and attachment chemistry. For Förster resonance energy transfer (FRET) measurements, the spectral overlap between the donor and acceptor dictates the characteristic length scale (r_0) and the range of separations that can be resolved (~$0.1r_0$ – $0.9r_0$).

Single-Molecule Fluorescence of Low-Affinity Interactions

Biomolecular interactions with ~μM or weaker affinities can be studied by employing nanovesicles or zero-mode waveguides (ZMW) to reduce the effective detection volume (<10^{-19} l) while increasing the local concentration (~μM). Advantages of nanovesicle trapping include (1) surface immobilization, (2) minimization of nonspecific interactions, (3) approximation of cellular environment, and (4) suitability for studying membrane-associated proteins. However, this technique is not suitable for studying extended substrates that exceed the vesicle diameter. Furthermore, it is difficult to effectuate buffer exchange inside of vesicles. Some of these drawbacks can be overcome using zero-mode waveguides that are metal-clad wells or lanes fabricated on a silica substrate. Effective detection volume can be as low as ~10^{-21} l, whereas the effective concentrations of labeled biomolecules can be in the mM range. Advantages of ZMWs include straightforward exchange of buffer and suitability for studying extended substrates. Disadvantages are the potential changes in fluorophore properties due to metal surface and higher nonspecific binding.

Analysis of Single-Molecule Data

Extracting meaningful information from single-molecule fluorescence data, which is typically noisy and can be quite complex, is challenging. There is an ever-growing array of analysis techniques ranging from simple histograms to sophisticated hidden Markov and Bayesian approaches, each with their attendant advantages and limitations. Independent of the choice of analysis methods, simulations are an invaluable tool to determine how the combined effect of the analysis procedures and noise distort the underlying phenomenon being investigated. Simulations and models can provide a rigorous test of the conclusions drawn from the data and the analysis techniques used to extract the pertinent quantities on which the conclusions are based.

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