Spectroscopy of single molecules provides detailed data on microenvironments and dynamic state changes within materials.

Single-Molecule Magic

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Single-molecule spectroscopy (SMS) has become a powerful technique for exploring the individual nanoscale behavior of molecules in complex local environments. Because a single molecule is only a few nanometers in size, it is able to sense its local environment on a very small scale; thus, its optical properties are often influenced by its immediate surroundings.

For a heterogeneous condensed phase system (for example, crystals, polymers, and biomolecular systems), a wide variety of local environments exist. A single molecule localized within such a nano-environment is exposed to a unique set of conditions and can be considered to “march to its own drummer.” In a conventional optical measurement, we observe many molecules at once and record the ensemble average of an optical property. In the case of single-molecule techniques, however, we can observe the full distribution of values for the optical property. SMS thus offers the capability to either study heterogeneity in a system or to measure time-dependent state changes molecule by molecule; both data types are obscured with conventional ensemble averaging.
The Singles Scene

To probe a single molecule, we use a light beam (typically a laser) to pump a strongly allowed optical transition of the molecule of interest, sensing this absorption through the detection of fluorescence emission. Consider an energy-level diagram for a typical single-molecule probe (see figure 1). Here the molecule is excited from the singlet ground state ($S_0$) to some vibrational level of the electronic excited state. The molecule quickly relaxes to the lowest-energy first excited state ($S_1$) through a nonradiative process. From $S_1$, the molecule can return to $S_0$ through the emission of a photon with a probability defined as the fluorescence quantum yield of the molecule. The molecule may also undergo intersystem crossing from $S_1$ into a triplet state ($T_1$) from which it can then return to the ground state at rate $k_T$. Efficient single-molecule fluorophores should have high fluorescence quantum yields (near unity) and low rates of intersystem crossing and short triplet lifetimes. A large absorption cross section is also desirable.

We generally guarantee that only one molecule is in resonance with the excitation light by using dilution and tightly focused excitation. In figure 1, only one molecule is in the focal volume of the excitation light, with the result that fluorescence emission stems from that molecule only. Several techniques allow us to visualize the emission from a single molecule, including confocal, total internal reflection, near-field optical scanning, and wide-field epifluorescence microscopy (see figure 2). 1 Wide-field epifluorescence imaging allows us to collect the emission from several single molecules within the excitation volume, so long as they are sufficiently separated in space. Because this type of imaging is a wide-field technique, the diffraction limit defines the spatial resolution to be approximately $\lambda/(2NA)$, where $NA$ is the numerical aperture of the objective.

A successful single-molecule experiment must provide a signal-to-noise ratio (SNR) for the single-molecule signal that is greater than unity for a reasonable averaging time (on the order of microseconds to seconds). The full expression for calculating the SNR depends on both the experimental parameters (for example, excitation power, laser spot size, and integration time) and the properties of the single-molecule emitter (fluorescence quantum yield, absorption cross section, saturation intensity, photobleaching). We can achieve the required SNR by careful selection of the emitting molecule and the transparent host, in addition to ultrasensitive detection methods. Considerations that are critical for the selection of the host include high optical quality to minimize Rayleigh scattering and minimization of the volume probed to reduce Raman scattering from the host matrix.

Obtaining the largest possible signal from the molecule requires a combination of factors, including a large absorption cross section, high fluorescence quantum yield, and a weak bottleneck into dark states such as triplet states. Molecules should also demonstrate high photostability and provide a large number of fluorescent photons before irreversibly photobleaching. At room temperature, these requirements have previously been fulfilled by fluorescent labels based on laser dyes such as rhodamines, cyanines, oxazines, and so on, or by derivatives of rigid polynuclear aromatic hydrocarbons such as terrylene or perylene.

New and Improved

Recent work in the Moerner laboratory has focused on a new class of fluorophores that meet the strict demands for single-molecule imaging and offer additional interesting properties such as large ground-state dipole moments, moderate hyperpolarizability, and sensitivity to the local environment. This last property is of particular interest because of the number of complex systems with dynamic (time-dependent) heterogeneity that could benefit from study at the single-molecule level. The molecules within the class all contain an amine

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**Figure 1** Energy level scheme for a single-molecule fluorophore (left) shows the ground singlet state ($S_0$), the first excited singlet ($S_1$), and the lowest triplet state or other intermediate state ($T_1$). Pump photons at energy $h\nu$ (blue arrow) excite an upper vibrational level of the dipole-allowed singlet-singlet transition, followed by rapid relaxation to $S_1$ (black arrow). Dotted arrows indicate fluorescence emission. The intersystem crossing rate ($k_{ISC}$) and triplet decay rate ($k_T$) are shown with gray arrows. Precise system control allows us to pump a single resonant molecule.

**Figure 2** In wide-field epillumination microscopy, a dichroic beamsplitter (D) directs excitation light into a high-numerical-aperture microscope objective (O). A lens (L) focuses this light at the back focal plane of the objective to allow collimation at the sample. Sample emission is collected by the objective, where it passes through scatter rejection filters (F) before capture by CCD camera.
donor and a dicyanodihydrofuran (DCDHF) acceptor linked by a conjugated unit such as benzene, thiophene, alkene, or styrene; the molecules are named the DCDHF fluorophores after the acceptor unit. An example of one of the DCDHF molecules called DCDHF-6 is shown in figure 1. The absorption and emission maxima of these molecules extend over much of the range of visible wavelengths.

We can image individual DCDHF-6 molecules in a polymethyl methacrylate (PMMA) film (see figure 3). We captured the image using wide-field epi-illumination with excitation provided by the 488-nm line of an argon-ion laser; each peak corresponds to a single molecule of DCDHF-6. Figure 3 shows the high SNR and signal-to-background data achievable with a good single-molecule fluorophore in an optically clear polymer film. In this polymer environment, DCDHF-6 has a fluorescence quantum yield of 0.92 and, on average, emits more than $10^6$ total photons before irreversibly photobleaching; moreover, the molecule is stable against “blinking” or flickering in emission, with approximately 85% of the molecules showing no blinking behavior on the 100-ms time scale of the measurement.\(^2\)

The fluorescence quantum yields of the DCDHF dyes in toluene solutions were surprisingly low for such strong single-molecule emitters, however. In the case of DCDHF-6 in toluene, the fluorescence quantum yield was almost an order of magnitude below that of the polymer film. This result suggests that there is an environmentally sensitive path through which the molecule can return nonradiatively to the ground state. Quantum mechanical calculations of the electronic structure of this system suggest the presence of two excited-state minima—one radiative and the other nonradiative—accessed through different intramolecular twists in the excited state.\(^3\)

The twist leading to nonradiative decay has an environmentally dependent energy barrier, resulting in a DCDHF fluorescence quantum yield that varies with local environment.

We can probe this dependence on local environment at the single-molecule level through the excited-state lifetime ($\tau_F$), which is proportional to the fluorescence quantum yield.\(^4\) Using a time-correlated single-photon counting (TCSPC) method to record delay times between excitation by a pulsed laser source and emission from a single molecule, we can measure the excited-state lifetime of single DCDHF-6 molecules in different hosts (see figure 4). The figure also shows histograms of single-molecule lifetimes of DCDHF-6 in two different polymer hosts: PMMA and a (butyl methacrylate)/(iso-butyl methacrylate) copolymer. Each distribution is fit with a Gaussian function, revealing that the average value of $\tau_F$ is longer in the PMMA than the copolymer, which is consistent with bulk experiments. The distribution of lifetimes is broader in the copolymer, however, suggesting that a greater number of microscopic local environments exist in this matrix.

With the discovery and development of new single-molecule fluorophores comes the ability to design and implement new single-molecule experiments. Molecules that can sense changes in the local environment are of particular value due to the dynamic heterogeneity intrinsic to many biological and polymer systems. In addition to environmental sensitivity, DCDHF molecules offer synthetic flexibility, allowing us to optimize the properties of the fluorophores for specific applications. DCDHF fluorophores should thus be useful for a wide variety of single-molecule experiments, with each molecule acting as a nanoscale sensor marching to the beat of a different drummer.\(^6\)

**Figure 3** In a wide-field epifluorescence image of DCDHF-6 in a PMMA film, each peak corresponds to a single molecule of DCDHF-6. Acquisition time was 100 ms for a 4.8 $\mu$m x 4.8 $\mu$m sample area excited with 0.4 kW cm$^{-2}$ from an argon ion laser. The color bar shows the emission intensity scale (z-axis) in arbitrary units.

**Figure 4** Histogram of single molecule fluorescence lifetimes of DCDHF-6 in the copolymer (red) and PMMA (blue) fit to Gaussian curves show larger $\tau_F$ for the PMMA but a broader distribution of microscopic local environments in the copolymer. Inset shows representative TCSPC data (fit with a single exponential) for a single DCDHF-6 molecule in PMMA, which tells us the excited-state lifetime.

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**References**


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