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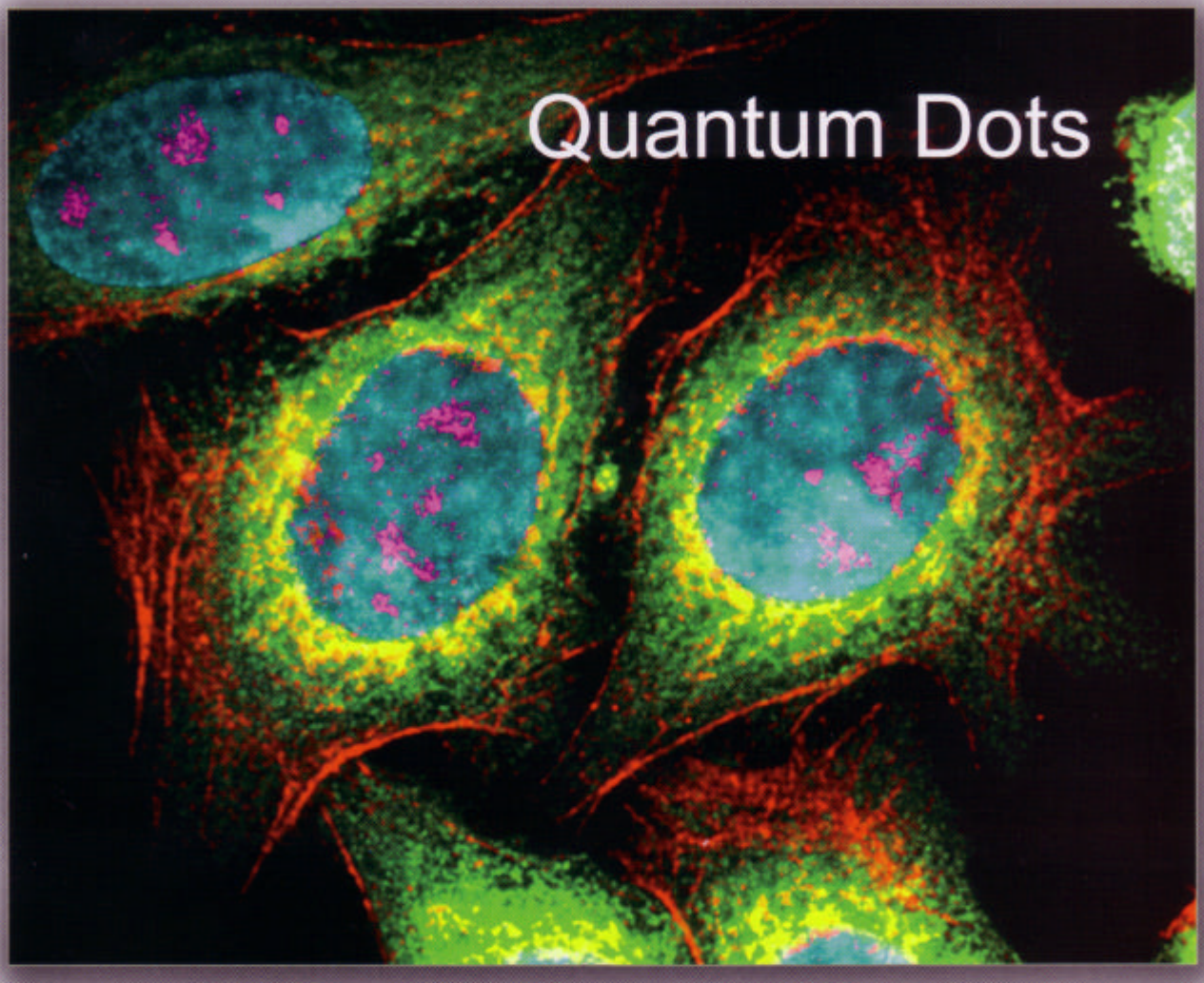


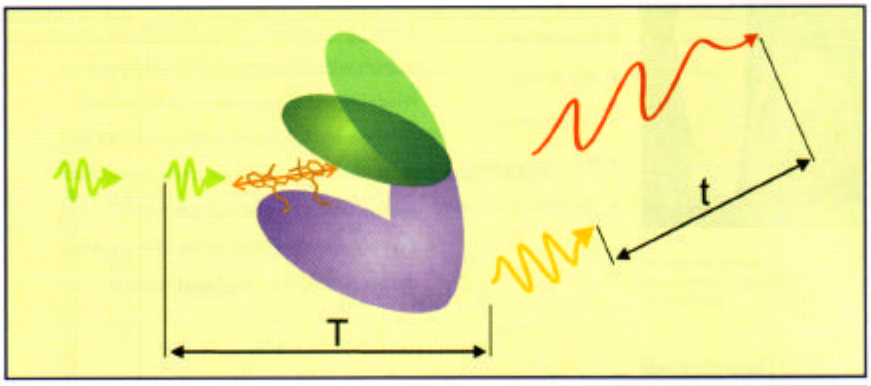
Spectroscopy Applications Grow

Single-molecule analytical method measures 14 parameters

Experiment shows how many photons are required for measurements

Pharmaceutical manufacturers, food inspectors, medical laboratories and even labs that test for biological or chemical weapons may benefit from study of single-molecule probes being conducted by researchers at the Swiss Federal Institute of Technology, Zürich. The research demonstrates that up to 14 parameters can be measured from a single fluorescent molecular probe and estimates the number of photons required to make the measurements. Each parameter, in turn, can provide several pieces of information about the molecule and its environment.

According to Michael Prummer, a member of the team and corresponding author of the research, which was published in the March 15 issue of *Analytical Chemistry*, single-molecule detection reaches the theoretical sensitivity limit for biological testing. In addition, it allows the probing of basic biology on the molecular scale. "By monitoring the behavior of single molecules with high time resolution," he explained, "uncorrelated dynamic processes [such as the stepwise rotation of ATP synthase rotary motors] become accessible to experiments." Conventional approaches tend to obscure these processes, he added.

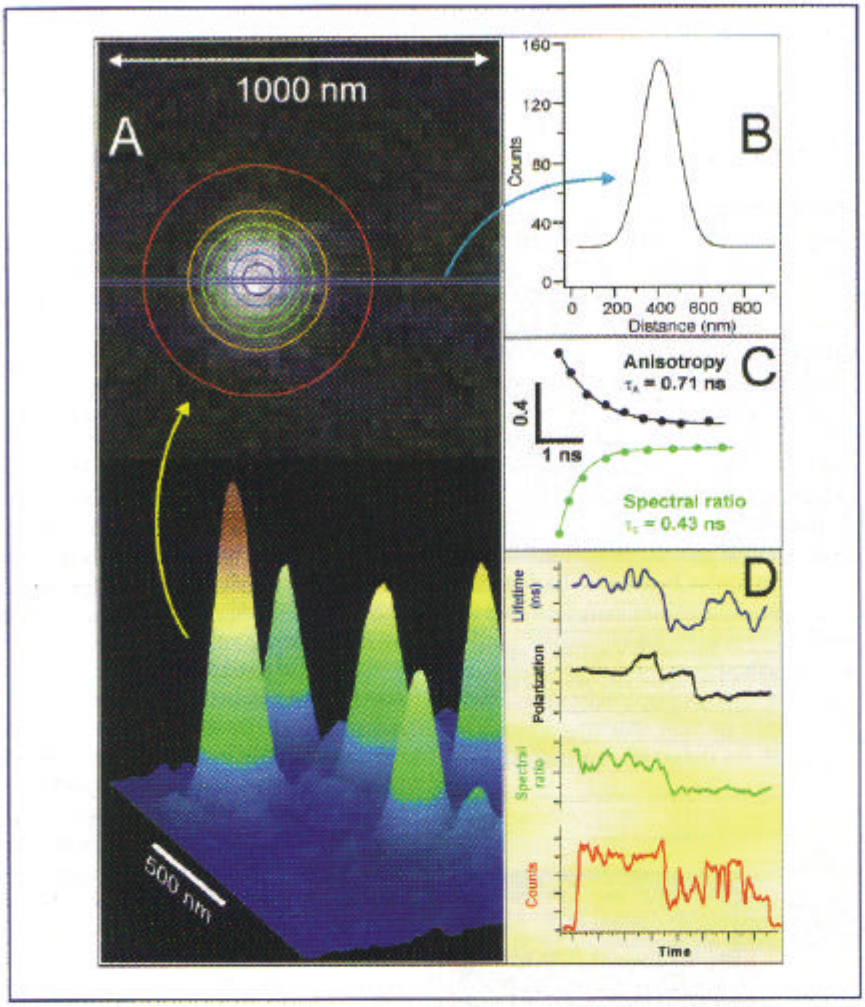


Multiparameter single-molecule spectroscopy uses a pulsed laser source (light green) to excite a fluorophore (orange) attached to a protein (blue-green), which is undergoing a conformational change. This change influences the fluorescence properties, which shows up in the photons that the fluorophore emits. Researchers can determine 14 parameters by recording several properties of the emitted photons (red, yellow) — the wavelength, the polarization, the time (T) after excitation, and the time (t) after the last photon. Images courtesy of Michael Prummer.

This research builds on previous work developing a sensitive optical setup that allowed the group to distinguish one dye molecule out of four species with an accuracy of 99.9 percent using only a "few hundred" photons.

The researchers built the microscope with the demands of single-molecule detection in mind. Based on a confocal microscope with annular illumination and time-correlated single-photon counting, it depends on one polarization and two spectral detection channels. The microscope achieves single-molecule sensitivity by using a high-numerical-aperture objective and single-photon-sensitive detectors with a high quantum yield (avalanche photodiodes).

For excitation, they used a pulsed Nd:YAG laser frequency-doubled to 532 nm and focused with a 1.4-NA oil immersion objective. The objective also collected the fluorescence, which passed through a dichroic mirror and a polarizing beamsplitter to the first avalanche photodiode, which detected vertical polarization. The horizontally polarized light was then split by another dichroic mirror, and the short- and long-wavelength



Researchers at the Swiss Federal Institute of Technology have demonstrated the ability to simultaneously measure 14 parameters of single-molecule fluorescence. They combined high-resolution confocal imaging (A) allowing for nanometer localization of dye molecules (B) with single-molecule fluorescence spectroscopy to yield, for instance, the anisotropy and the spectral ratio (C), and the trajectories of the fluorescence lifetime, the polarization and the intensity (D).

components passed to a second and third avalanche photodiode, respectively. The system recorded images by scanning a diffraction-limited spot across the sample, and recording each point. According to the published research, the system can determine the position of a fluorophore within 1 nm.

For their experiments, they chose the fluorescent dye DiI in a thin film made of the polymer PMMA, a combination commonly used as a model system for single-molecule detection. "The 20-nm-thin PMMA film protects the dye molecules, immobilizes them and provides a low-background environment," Prummer explained. He added that the DiI molecules have a high extinction coefficient and fluorescence quantum yield. They also are very stable in the PMMA.

The scientists also experimented with a polyaromatic dye and proteins labeled with Alexa. They measured 14 fluorescence parameters from the single probe, including:

- The X and Y position of the dye molecule, which allowed them to investigate the mobility of the molecules as well as the concentration heterogeneities, collocalization, active transport and others.
- The emission intensity, which provided information on absorption cross

section, quantum yield and the presence of quencher molecules in the vicinity.

- Two angles of the absorption dipole orientation, which revealed the three-dimensional orientation of the molecule, allowing them to follow conformational changes; specifically, how easily the molecule rotates. This parameter allowed them to estimate the local viscosity of the fluorophore's environment.
- The in-plane emission dipole orientation, which combined with the previous measurements to provide information on differences in the relative orientation of the absorption and emission dipoles, as well as on the activity of rotary motor molecules.
- The excited-state lifetime, which enabled the monitoring of energy transfer or quenching from other nearby molecules, Förster resonance energy transfer (FRET) and binding events.
- The intersystem crossing yield and the triplet lifetime, which allowed them to monitor the presence of triplet quencher molecules such as oxygen and iodine.
- The polarization anisotropy decay time and amplitude, which provided information about the rotational mobility of a dye molecule; specifically, how easily and how far the molecule rotates. These parameters facilitated the estimation of, for instance, the local viscosity of the fluorophore's environment.
- Measurement of the spectral relaxation time, the solvent shift and the steady-state emission wavelength, which allowed the group to identify different dye molecules with different emission spectra, and to monitor FRET as well as binding events and the dielectric properties of the molecules' environment.

Prummer said the method can be used with a wide variety of fluorophores. "They should be bright and photostable and should be excitable at [wavelengths longer than] 450 nm — because of background fluorescence. There are many commercial fluorophores that can be used as probes in our method — cyanine dyes, rhodamine derivatives and polyaromatic dyes. Single fluorescent protein constructs [such as GFP] can be detected, but because of their limited photostability, only about 10 parameters can be determined simultaneously."

When it comes to actually using single molecules as probes, photobleaching is a concern because enough photons must be detected to complete the test before the fluorophore photobleaches. To investigate this further, the researchers tested the number of photons required to get a reliable result for each parameter. Intensity is the easiest to determine, needing only 100 to 200 photons to be accurate to within 10 percent. Determining position accuracy and fluorescence lifetime requires about 500 photons each, and determining the intersystem crossing kinetics, analysis of on-off times and orientational imaging requires more. On the whole, they found that all 14 parameters could be obtained from about 10,000 photons. Increasing the number of photons would increase the accuracy.

They then determined which fluorophores could withstand the excitation required to measure all 14 parameters. They used the combination of DiI and PMMA to exemplify the class of dye-doped polymer films, and proteins labeled with Alexa immobilized in an aqueous medium. The polymer-bound dyes produced an average photon count of 10^5, and the proteins produced 20,000 photons. They determined that 80 percent of the polymer-bound dyes could provide all 14 parameters. The labeled proteins, however, proved more fragile, with only 32 percent able to provide all 14. Of this group, 92 percent allowed measurement of the first 10 parameters.

Prummer also said that, in some cases, they can measure additional parameters. "For example, the fluorescence quantum yield of cyanine dyes depends on their degree of cis-trans-isomerization. The isomerization and back-isomerization rates can be directly determined by analyzing the intensity fluctuations of a single fluorophore. These two parameters could be obtained with the same precision and the same number of photons required for the remaining 14."

Prummer said the group will continue using the multiparameter approach to study molecular interactions. "We want to monitor conformational changes in ATP-dependent enzymes by FRET between donor and acceptor on two different positions within the protein." □

Kevin Robinson