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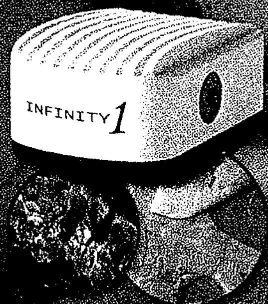

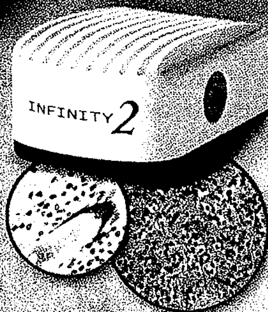

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addition and subsequent binding of cadmium ions prevented charge transfer from the molecule's amino group to the fluorophore — in this case, boradiazaindacene — and yielded a blueshift in the emission intensity peak to 597 nm. Continued addition of cadmium steadily increased the intensity at this wavelength and the ratio of 656- to 597-nm emission.

Further titration experiments demonstrated that, of common metal ions, only chromium (III) ions slightly enhanced the molecule's fluorescence. In competition experiments, neither zinc ions nor other metal ions increased the intensity ratio.

As a test of the sensor in living systems, the scientists incubated pheochromocytoma cells with the sensor. After excitation with green light from a mercury lamp with a filter block, they imaged the cells before and after cadmium addition with a Nikon fluorescence microscope. They repeated the procedure with dendritic cells but used a Leica confocal microscope in single- and double-channel modes with excitation from a Melles Griot HeNe laser at 543 nm. Universal Imaging Corp. (now Molecular Devices) software was used to analyze the data from the HeNe laser to obtain standard confocal and ratio fluorescence images.

For both cell types, cadmium exposure significantly increased fluorescence emission. Images of the dendritic cells, however, showed the change more clearly and also revealed an increase in the intensity ratio.

The team suggests that, based on these results, the fluorescent molecule could be used to selectively detect the heavy metal in living cells. □

Michael J. Lander

A fluorophore count reveals the right distribution

Method reveals the number of fluorescent molecules in a cluster

In an ideal world, fluorescent molecules used to label a biological sample always would be distinct from one another, making quantifying them a simple matter of counting. In the real world of fluorescence microscopy, however, fluorophores often congregate in clusters, or puncta, and determining how many of them are in puncta can be challenging.

Now researchers from the University of Washington in Seattle have developed and demonstrated an approach that reveals the number of single molecules present in a cluster once the relationship between single molecule and single puncta intensity distributions is known.

Daniel T. Chiu, professor of chemistry at the university, predicts that the technique could make fluorescence microscopy more quantitative by providing information on how many proteins, vesicles, organelles, DNAs, signaling complexes or other fluorescent units are in the puncta.

The scientists developed the method because other solutions to the problem of quantifying fluorophores had significant disadvantages. One potential approach is sequential single-molecule photobleaching, which, in theory, leads to a series of step decreases in fluorescence intensity as molecules are extinguished one by one. In practice, differences between molecules result in uneven steps, and several molecules sometimes photobleach at once, resulting in a sequence of fluorescence changes that cannot be tied to a definitive number of molecules.

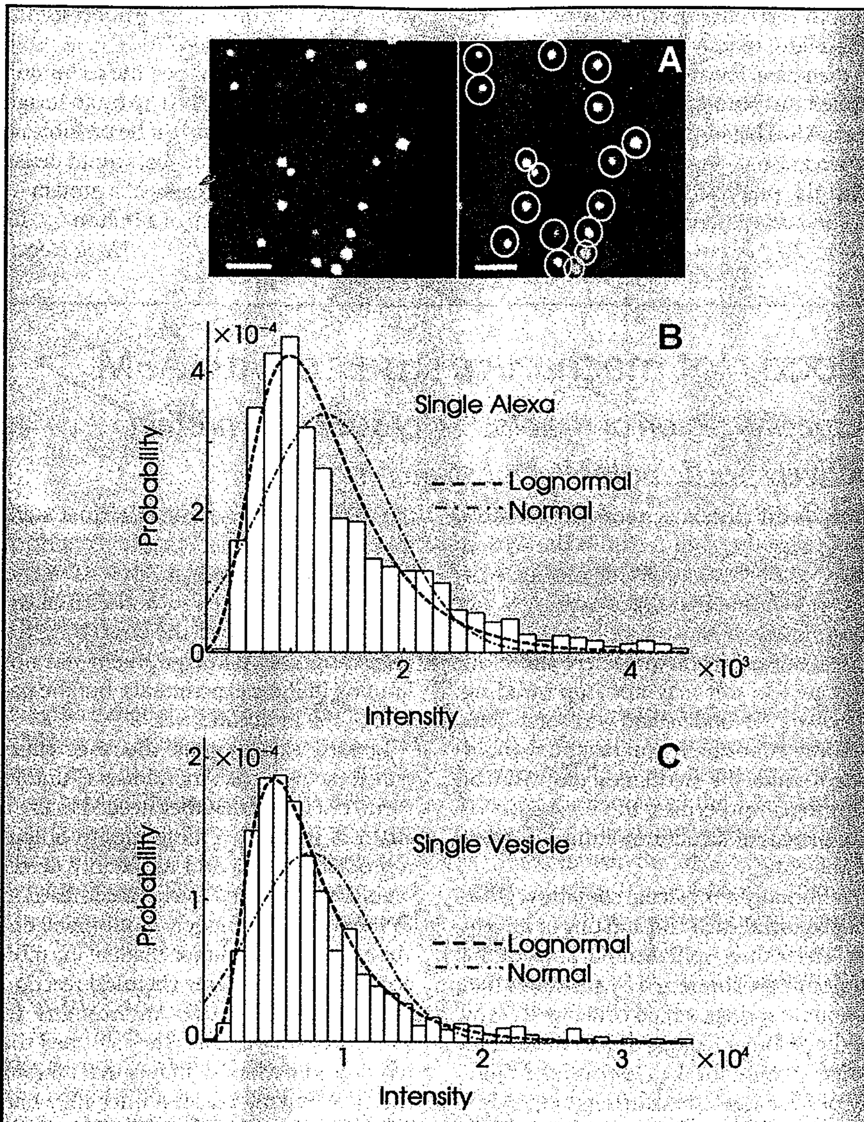
Such ambiguities, along with the tedious nature of sequential photobleaching, persuaded the researchers to try something else.

Another possible method involves simple intensity measurements. However, this works only when large numbers of fluorophores with substantially the same intensity are present in each punctum. In many situations, though, there are only a few tens of fluorescent molecules in a cell — too low a number for the method to work. Thus, the researchers decided that intensity measurements would not be suitable.

The group, therefore, came up with its own technique. According to statistical theory, the fluorescence intensity of puncta and that of single molecules

should be related, and the first should be predictable given the second. How the two are related affects the distribution of puncta intensities, and resolving that took the researchers years of work and a bit of luck. "We came up with this method purely by accident. Initially, we used the normal distribution," Chiu said.

If the puncta intensities resulted from adding together single largely independent fluorophore intensities, the fluorescence intensity of the clusters would follow a normal distribution. Instead, the intensity showed a lognormal distribution, which meant that it peaked at a lower value and more sharply than a normal



Knowing the fluorescence intensity distribution relationship of single molecules and particles can help in counting how many single molecules are in the particles. At top on the left is an image of single goat anti-mouse IgG antibodies (GAM) labeled with multiple Alexa Fluor 488; top right (A) shows each molecule circled automatically by the imaging software to define a region of interest. The plots below are intensity distributions of (B) single Alexa Fluor 488 carboxylic acid succinimidyl ester molecules, and (C) single synaptic vesicles tagged with anti-SV2 primary antibody and Alexa Fluor 488-labeled GAM secondary antibody. For (B, C), the dashed line is the best-fit lognormal distribution to the data, and the dash-dot line is the best-fit normal distribution to the data. The distribution of the intensity data is a better fit by a lognormal distribution in both cases. Reprinted with permission of Biophysical Journal.

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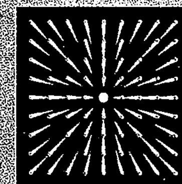

distribution. Thus, it seemed that the puncta intensity distribution was not simply the sum of independent fluorophores.

After thinking about these results, the researchers realized that the measurements could be accounted for if the intensity of each point in the region of interest encompassing a punctum were affected by its position in the image. In that case, the cluster intensity could be derived by scaling or multiplying single-fluorophore intensity. The result of what the researchers termed the multiplied distribution would be lognormal.

As for how this could happen, one possibility, Chiu noted, is that it arises from measurement artifacts. These could be the result of defocusing one point relative to another, of detector efficiency varying from one point as compared to another or because of a variety of other factors. Each of these issues might then be multiplied together, and the product would thereby dominate the intensity distribution.

Chiu recalled that it took years to come up with a conceptual framework to explain the observed lognormal distribution results, which, the group noted, others also had seen. He added that, if the source of this multiplied distribution is indeed many small effects occurring in the measurement process, it might be difficult to eradicate.

Whether intensities follow a random additive, a normal distribution or a multiplied distribution, lognormal distribution must be established for each case with care. In addition, Chiu noted another somewhat minor drawback to the technique. "It is computationally demanding in the fitting if there are many copies to be fitted, but with computers what they are nowadays, I think it can be handled just fine on a new desktop computer."

In a demonstration of the technique, the researchers reacted biocytin labeled with Alexa Fluor 488 with the protein avidin, making use of one of the strongest known affinities between biological molecules and also testing the technique on a heavily studied biological interaction.

They used a microfluidic chamber and total internal reflection fluorescence microscopy on a home-built system consisting of a Roper Scientific CCD camera, a Coherent 488-nm solid-state diode-pumped laser, and dichroic mirror and filter from Chroma. The researchers used total internal reflection microscopy because it allowed them to use a lower laser power than other techniques. They needed only 88 μW of power during the capture of each 300-ms image. The work is detailed in the April issue of *Biophysical Journal.*

They used avidin bound to a single biocytin for single-molecule calibrating intensities, capturing 800 intensity values. They did the same for avidin bound to multiple biocytins, saturating the solution to reach the stoichiometric ratio of one avidin to four biocytins. From the fit to their data, they concluded that the multiplied distribution led to 95 percent of the avidins having four biocytins, whereas 5 percent had three — good agreement with the predicted binding ratio of 1:4. In contrast, the random additive approach led to 20 percent puncta with ratios of 1:5 or more and 20 percent with nonphysical parameters, such as a negative number of biocytin per avidin.

The researchers are applying their technique to help understand synaptic function and are working to further refine the method. "We will continue to develop this technique and try different imaging modes, as we hope to make this method widely used for quantitative fluorescence microscopy," Chiu said.

He added that he hoped the technique would someday be picked up by an image analysis company. It might be possible to have a software module that would determine the number of copies of a protein in an image with the click of a button. □

Hank Hogan

Blood clot mechanics studied with AFM

A specific region of the blood clot structural protein actively participates in clot stretching

Blood clots are necessary to stop bleeding, but a clot in the wrong place can cause a heart attack or stroke. On one hand, very stiff clots are more likely to cause problems and potential death, but, on the other hand, clots that are too soft may not stop bleeding effectively. To understand why some clots become abnormally soft or stiff, it could help to study the mechanical properties of fibrinogen molecules, the precursors of the principal structural component of blood clots.

A fibrinogen molecule contains a globular region at each end and one in its center. These three spherical portions of the molecule are connected by structures that resemble springs, called coiled-coils. Any of the globular regions or the coiled-coils could account for the elasticity and rigidity of fibrinogen. Researchers at the University of Pennsylvania in Philadelphia recently used atomic force microscopy (AFM) to discover which of these structures governs the mechanical properties of fibrinogen.

To examine the mechanical properties of fibrinogen, they stretched the cross-linked fibrinogen molecules between an AFM probe and a mica surface. They chose this method because it can pull on large proteins with high enough forces and because it does not require complex chemistry to attach molecules to the probe or the surface — the molecules just stick there. The researchers employed silicon nitride cantilevers and a Veeco AFM and used software written in-house to calculate peak forces and the stretching dimensions. The AFM equipment was not limiting in these experiments, but the nature of the molecules was.

In early experiments, the researchers pulled on fibrinogen monomers to unfold them, but found that they needed to use a chain of fibrinogen molecules to obtain reproducible data and to reliably detect unfolding. They used an enzymatic reaction to cross-link fibrinogen monomers, enabling creation of a linear chain of the molecules. This is the same chemical reaction that naturally occurs in humans to strengthen blood clots. They stopped the reaction with a blood thinner that inhibits the cross-linking reaction once they had the desired length of molecular aggregates.

The forcible extension of the fibrinogen molecules resulted in a graph with many small peaks, a pattern that resembled sawteeth. To create that pattern, one structure in fibrinogen had to become 23 nm longer when unfolded. They verified their experimental data by performing Monte Carlo simulations, which produced nearly identical results and confirmed the measurements.

To determine which structure unfolded, the investigators compared the 23-nm