GE PRIZE-WINNING ESSAY

A New Approach to Fluorescence Microscopy

Precise localization of switchable fluorescent molecules facilitates nanoscale biological imaging.

Mark Bates

GE Healthcare and *Science* are pleased to present the prize-winning essay by Mark Bates, a regional winner from North America, who is the Grand Prize winner of the GE & *Science* Prize for Young Life Scientists.

The fluorescence microscope is a powerful tool for the study of molecular and cell biology, enabling researchers to peer into cells and living organisms to understand their spatial organization. Methods for tagging specific biomolecules with fluorescent labels, such as green fluorescent protein (GFP), provide a toolbox for the observation of protein organization, interactions, and dynamics (*1*). This enables the visualization of a host of biological phenomena, such as enzymatic processes, the development of cells and organisms, and the structural dynamics of individual biomolecules.

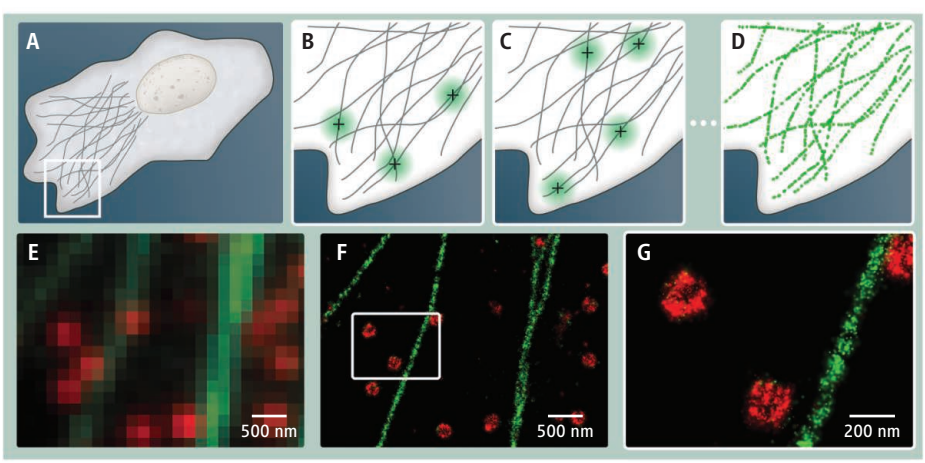
Despite its benefits, a significant drawback of fluorescence microscopy is its spatial resolution. Beyond a certain magnification, the fine structure of a specimen is obscured when viewed through the optical microscope. As described by Abbe in the late 19th century, the diffraction of light imposes a resolution limit, which makes it impossible to resolve spatial features smaller than about 250 nm using visible light. This leaves much of the architecture of cells and their inner workings inaccessible to study. The electron microscope allows researchers to obtain images with higher resolution, but in this case the sample preparation is not compatible with live cell imaging, and it is difficult to achieve the molecular specificity and multicolor capability offered by fluorescent labeling.

My graduate thesis focused on the development of an imaging modality that would combine the advantages of both electron microscopy and fluorescence imaging. In the past several years, a number of innovative approaches have been conceived to capture fluorescence images with nanometer-scale spatial resolution (*2*, *3*). These techniques take advantage of nonlinearities inherent in molecular fluorescence, such as saturation or depletion of the excited state, to obtain optical resolutions an order of magnitude beyond the classical diffraction limit. We invented a new approach that is based on single-molecule detection and localization, using a standard fluorescence microscope and readily available fluorescent labels, for wide applicability to the life sciences.

Early in my doctoral research, I made a surprising observation about a commonly used red fluorophore, Cy5: Its fluorescence emission can be switched on and off using pulses of light. We studied this "optical switching" effect and found that during each excitation, Cy5 emits thousands of photons before going dark. A brief pulse of ultraviolet light will then efficiently reactivate the molecule to its fluorescent state, and this process can be repeated for hundreds of cycles (*4*, *5*). We realized that the switchable fluorescence exhibited by Cy5 is a strongly nonlinear process, and we reasoned that this nonlinearity could be used to overcome the diffraction limit of resolution.

Inspired by this idea, we found a novel method to obtain sub–diffraction limit fluorescence images (see the figure, parts A to D). If a biological sample is labeled with photoswitchable fluorophores, the experiment can be controlled so that only one fluorophore, or a sparse subset of fluorophores, are in the fluorescent state at any given time. The image of each isolated fluorophore appears as a diffraction-limited spot several hundred nanometers in size. By measuring the centroid of this spot, the position of the fluorophore can be determined with a high degree of precision, often on the nanometer scale (*6*, *7*). When the active fluorophores have been switched off, a new subset is stochastically activated by a pulse of activation light, and the process is repeated. When a sufficient number of fluorophore positions have been obtained, a plot of their coordinates reconstructs an image of the sample. The spatial resolution of the resulting image is not diffraction-limited. Rather, it is limited only by the precision of each fluorophore localization and the number of localizations present. We called this method stochastic optical reconstruction microscopy, or STORM (*8*). This concept for imaging was simultaneously developed by several groups, and it is also known as PALM and fPALM (*9*, *10*).

Multicolor fluorescence imaging enables the study of the relative organization and interactions of cellular components, and we developed a strategy for multicolor STORM using Cy5 as the photoswitchable fluorophore. We found that when Cy5 is paired



STORM imaging. (**A** to **D**) A cell labeled with switchable fluorophores (A) is imaged by activating only a subset of fluorophores at a time (B and C). The locations of each fluorophore are determined computationally (crosses) and a plot of their coordinates yields a super-resolution image which is not limited by diffraction (D) (*17*). A comparison of conventional epifluorescence imaging (**E**) and the corresponding STORM image (**F**) of clathrin-coated pits (red) and microtubules (green). The boxed region in (F) is magnified in (**G**).

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with a second fluorophore (such as Cy2 or Cy3), its activation wavelength is strongly influenced by the spectral characteristics of its neighbor (*11*). We took advantage of this property to selectively activate specific populations of dye pairs, enabling us to capture images of multiple molecular targets within the same sample. Using antibodies labeled with either Cy3 and Cy5, or Cy2 and Cy5, we imaged microtubules and clathrin-coated pits in cultured mammalian cells (see the figure, parts E to G). In comparison with the conventional fluorescence image, the STORM image reveals previously unseen detail due to its high spatial resolution (~25 nm) and clearly resolves the rounded structure of clathrin pits only 150 to 200 nm in diameter (*11*).

Many cellular structures have a complex three-dimensional (3D) shape, requiring a 3D imaging method for study. Taking advantage of a technique for 3D particle localization, we used astigmatic imaging to generate 3D STORM images with 25-nm lateral resolution and 50-nm axial resolution. With this strategy, we were able to obtain images of the full spherical structure of clathrin-coated pits (*12*).

Although imaging techniques based on this concept are relatively recent, they have already been used to understand mechanisms in biology. Greenfield *et al*. (*13*) have used these techniques to develop a model of how chemotaxis receptors in *Escherichia coli* organize in growing cells. Biteen *et al.* (*14*) have visualized the nanoscale structure of MreB in live *Caulobacter crescentus*, taking advantage of the photoswitchable fluorescence of enhanced yellow fluorescent protein (EYFP). Also, Hess *et al*. (*15*) have obtained high-resolution images and dynamic information from influenza hemagglutinin, a clustered membrane protein, to differentiate between membrane organization models in fibroblast cells.

The development of sub–diffraction limit fluorescence microscopy has created new possibilities for the observation of biological processes, and a new assay for the organization and composition of biomolecular complexes. With continued advances in fluorescent labels and labeling methods *(16)*, it will be exciting to see how these techniques are applied to bring about insights into life at the nanometer scale.

References and Notes

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17. Figure panels A to D are adapted from *Current Opinion in Chemical Biology*, vol. 12, "Super-resolution microscopy by nanoscale localization of photo-switchable fluorescent probes," M. Bates, B. Huang, and X. Zhuang, pages 505–514, Copyright 2008, with permission from Elsevier.

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2010 Grand Prize Winner

Mark Bates was born in Toronto, Canada. He received a B.Sc. degree in engineering physics from Queen's University and an M.Sc. degree in physics from McGill University. He conducted his doctoral research at Harvard University, working under the guidance of Xiaowei Zhuang, where he studied the properties of photoswitchable fluorescent molecules and applied these results to develop a new method for high-resolution optical imaging. Dr. Bates is now a postdoctoral fellow in the laboratory of Stefan Hell in Göttingen, Germany, where he is applying super-resolution fluorescence microscopy to study prokaryotic cell biology.

Regional Winners

Europe: **Ataman Sendoel** for his essay "Is Death Without Oxygen as Sweet as Apoptosis?" Dr. Sendoel was born in Zurich, Switzerland. He studied medicine at the Universities of Zurich and Lausanne. After finishing medical school, he joined the M.D.-Ph.D. program of the University of Zurich. He conducted his Ph.D. work in the laboratory of Michael Hengartner, where he studied mechanisms of controlling programmed cell death in *Caenorhabditis elegans*. Dr. Sendoel is currently a postdoctoral fellow and continues to work on hypoxia responses in *C. elegans*.

Japan: **Sakiko Honjoh** for her essay "Is Aging Necessary?" Dr. Honjoh was born in Yokohama, Japan. Inspired by a high-school biology teacher, she decided to major in molecular biology and entered Kyoto University. Continuing on this track, Dr. Honjoh completed her Ph.D. in the laboratory of Eisuke Nishida at the Graduate School of Biostudies, Kyoto University, working on the signal transduction networks that regulate life span. She is continuing her work in the same lab, still trying to elucidate the molecular changes that occur during aging.

All Other Countries: **Melissa Fullwood** for her essay "Genome-Wide Chromatin Loops Regulate Transcription." Dr. Fullwood, born and raised in Singapore, graduated from Stanford University in 2005 and completed her Ph.D. in 2009 at the Genome Institute of Singapore under the auspices of the National University of Singapore where she was supervised by Yijun Ruan. In 2009, she was selected for the inaugural L'Oreal for Women in Science National Fellowships in Singapore. She is currently a Lee Kuan Yew Post-Doctoral Fellow at the Duke-NUS Graduate Medical School Singapore under the supervision of Shirish Shenolikar.

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