25 years since the development of single-molecule fluorescence microscopy

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[About my research]

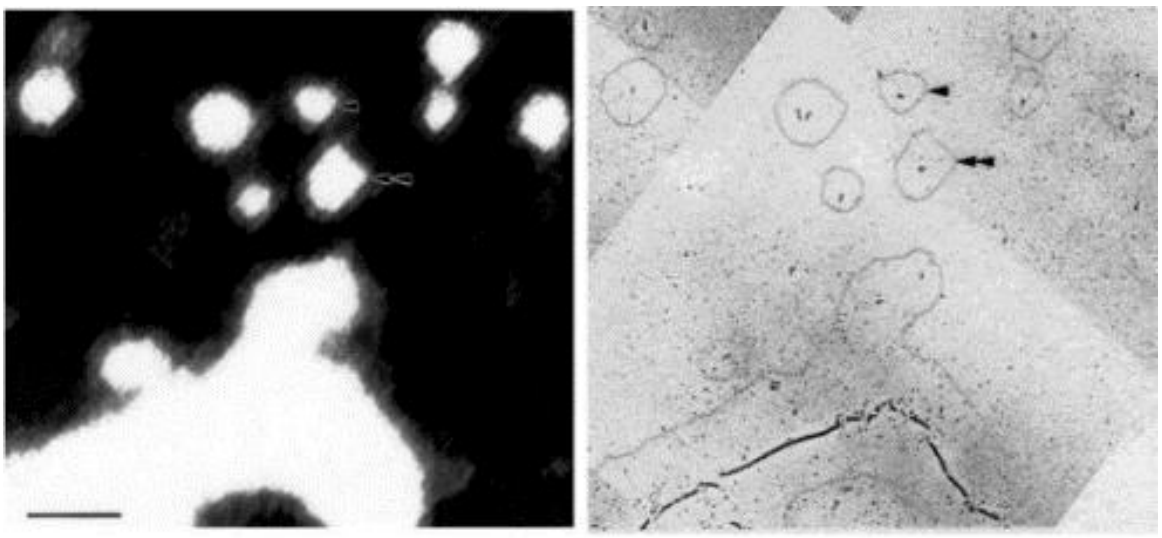
The research field called “single-molecule biology” is well-established. One of the technologies that support this field is "single-molecule imaging," which allows us to observe directly at a single molecule.

In 1992, YANAGIDA Biomotron of the Research Development Corporation of Japan (now the Japan Science and Technology Agency) started, and I participated as one of the researchers. The project consisted of three groups, and I was assigned to the first group led by Dr. Takashi Funatsu (currently a professor at the Graduate School of Pharmaceutical Sciences, The University of Tokyo). First, I discussed the research theme with Dr. Funatsu. Both Dr. Funatsu and I have studied about the molecular mechanism of muscle contraction. Here, in order to understand the molecular mechanism, we wanted to directly observe the behavior of individual molecules. Therefore, we named our group's research theme as "direct observation of a single molecule.”

To observe single biomolecules working in an aqueous solution under the optical microscope, they need to be marked. We decided to use the most typical fluorescent dye molecule as the marker. Then, we focused on the development of the fluorescence microscope that could observe a single fluorescent dye molecule. In the modified microscope, background light was reduced as much as possible. We began to get positive results that we might see single fluorescent dye molecules. However, we could not be confident enough. When the project leader, Dr. Toshio Yanagida (currently a specially-appointed professor at the Graduate School of Frontier Biosciences, Osaka University), asked us, "Do you really see single molecules?" we could only reply as, "I think, yes..." Then, the third group leader, Dr. Hideo Higuchi (currently a professor at the Graduate School of Science, The University of Tokyo), suggested us to compare the image obtained under the fluorescence microscope with that by the electron microscope. I thought, "such an experiment is too difficult to do." But, Dr. Funatsu and I finally agreed that we had no choice but to do it.

We labeled myosin molecules, muscle protein that we were familiar with by their characteristic shape, with fluorescent dye Cy3. First, we observed the sample from one end to the other under our improved fluorescence microscope to capture fluorescent images on video. A small amount of fluorescently labeled actin filament was present as a marker for later comparison with electron microscopy images. Then, the samples were dried in a vacuum, shadowed by low-angle rotational deposition, observed by electron microscopy, and photographed, and all images

were developed. The electron micrographs were laid out on a table and compared to the fluorescence image, and we searched for the exact locations. It was not easy to find it, and I repeated the experiment day after day. One day, I realized that if I flip it over, the shape of the fluorescence image of the actin filament and the electron microscope image might match. As I continued to observe it that way, I became more confident. I will never forget the excitement I felt at that moment. In fact, I had made a mistake in counting "negatives" and "positives" by one in the process of microscopic observation, sample preparation, photography, and printing. So I observed the fluorescence microscope image and the electron microscope image as mirrored images. Now the position of the spot in the fluorescence image and the position of the myosin molecule in the electron microscope image was the same (see figure below). This demonstrated that we had observed a single molecule of fluorescent dye.



Images of myosin molecules labeled with fluorescent dye Cy3 and actin filaments labeled with rhodamine-phalloidin attached a mica thin film and observed under the fluorescence microscope that has been modified for single-molecule imaging (left). An electron microscope image (right) of the same field of view that has been shadowed by low-angle rotational deposition. The black line-like structures, disconnected in places, in the lower part of the image are actin filaments. About half of the myosin molecules in the sample are labeled with one molecule of Cy3 and one quarter with two molecules of Cy3. The remaining approximately one-fourth is unlabeled. Judging from the brightness of the fluorescent spots, one arrowhead indicates a myosin molecule labeled with one molecule of Cy3 and two arrowheads with two molecules of Cy3. The scale bar is 1 μm.

Later, total internal reflection illumination, an illumination method that dramatically reduces

background light, was incorporated into fluorescence microscopes. And we can stably observe single molecules of fluorescent dyes for long periods. Over the past quarter-century, various key technologies necessary for single-molecule imaging have been dramatically improved. These are the development of high-sensitivity cameras with reduced noise, lasers as light sources, and improved performance of mirrors and filters used in the optical system. Today, fluorescence microscopy systems incorporating objective-type total internal reflection illumination are commercially available from leading microscope manufacturers, allowing anyone to perform single-molecule imaging experiments. The super-resolution microscope that won the Nobel Prize in Chemistry in 2014 was also based on single-molecule fluorescence imaging technology.

We have since then used single-molecule fluorescence microscopy not only to study muscle proteins, but also to study various motor proteins responsible for material transport in cells, RNA polymerase, proteins involved in DNA repair, and other proteins involved in gene expression, maintenance, and regulation. Various single-molecule imaging methods, other than single-molecule fluorescence imaging and manipulating techniques, are continuously developed worldwide. Imaging all molecules within a single cell have become a research goal for the near future. We expect that single-molecule observation will reveal sophisticated functions of protein molecules and behaviors of biomolecules in the cell.

Reference

"Single-Molecule Biology," edited by Yoshie Harada and Shinichi Ishiwata, published by Kagaku-Dojin Publishing Company, INC. October 10, 2014.