

Photonics at the NIH

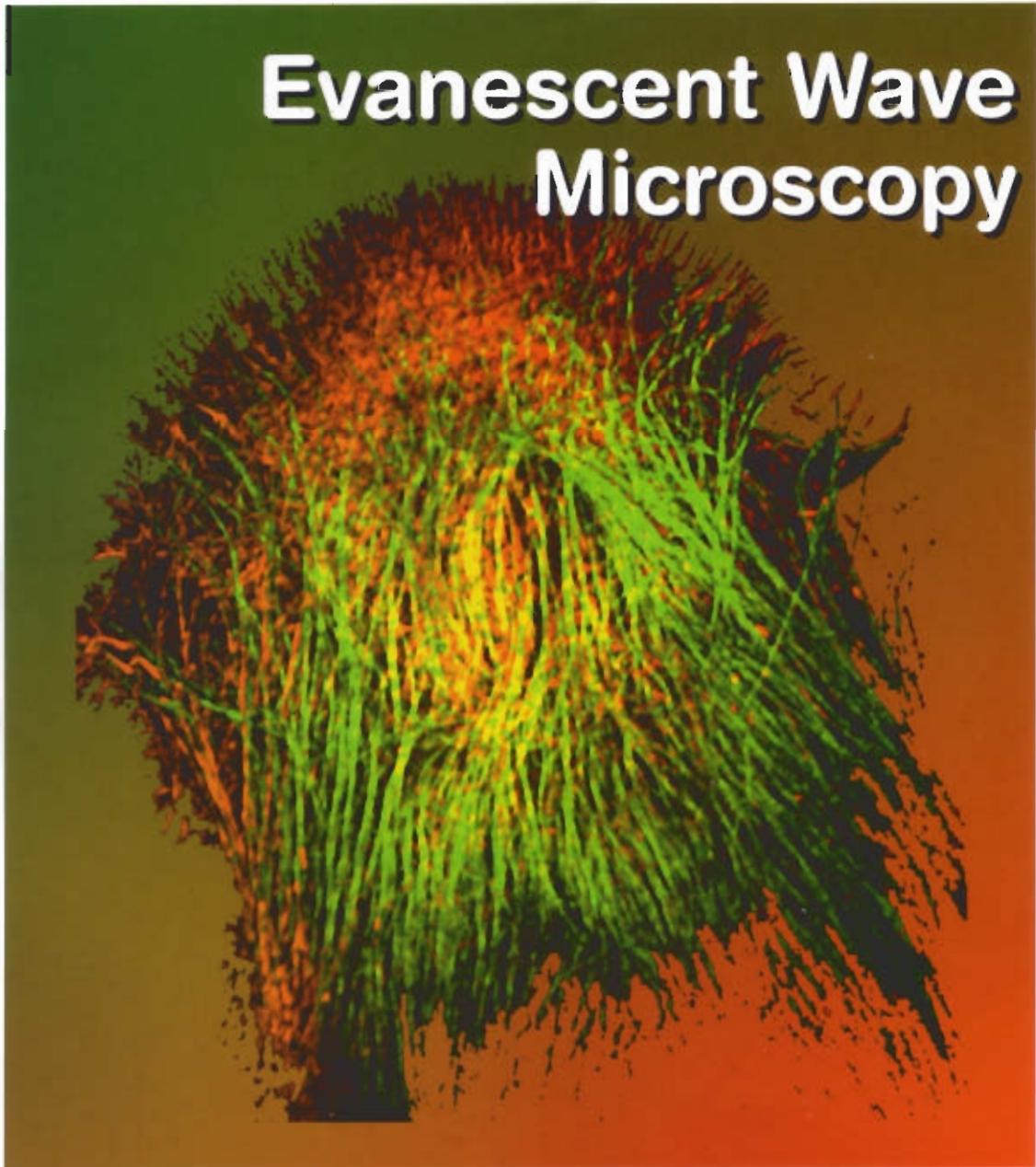
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Getting the local — and whole — picture

A mask with tiny holes aids in image correction

Sometimes to get the whole picture you first need to get the hole image. That's the case with a molecular colocalization detection technique recently developed and demonstrated by researchers from Nagoya University in Japan. The investigators used the data they derived from an array of micron-diameter holes to achieve simultaneous, dual-color single fluorescent molecule colocalization imaging.

The researchers tracked individual molecules and were able to determine the colocalization of two molecules in living tissue to an accuracy of 64 nm. The technique will enable the tracking of two-molecule processes in living cells, allowing scientists to obtain crucial information about cellular signaling events.

"All of the important reactions and cellular processes are carried out by molecular interactions, and it is, in fact, critical that the behaviors of two molecules are tracked simultaneously," said Akihiro Kusumi, a professor of biophysics at Kyoto University. At the time the research was conducted, his group was at Nagoya University. The team reported its findings in the April issue of *Biophysical Journal*.

Two fluorescent probes are colocalized if they're attached to molecules interacting with or bound to one another. Conventional fluorescence microscopy can image colocalization but requires several

tens of molecules to be present. Signaling events, on the other hand, typically involve only a few molecules and often are transient. What was needed to detect single molecules in living cells, and with high spatial precision, the researchers noted, was an imaging system capable of at least video frame rates.

Holey mask

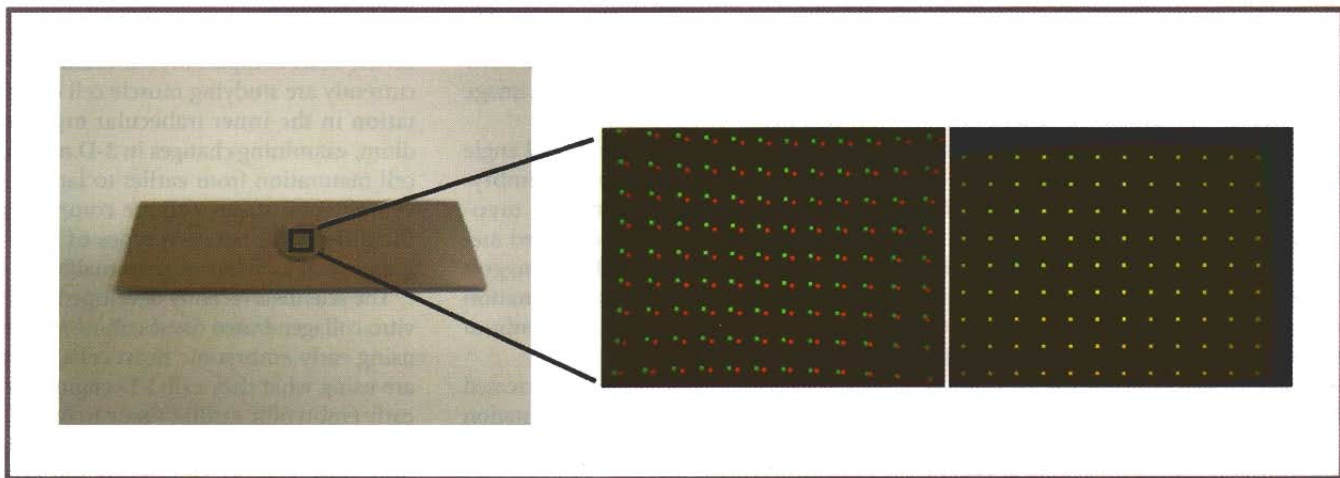
The researchers developed an imaging setup that involves two optical detection paths. In the instrument, an argon-ion laser at 488 nm and a HeNe laser at 594.1 nm illuminate the sample. These beams enter a modified Olympus microscope, and a series of dichroic mirrors, filters and other optical components collect the fluorescence emission and separate it into two components. The two channels travel separately and are both terminated at separate identical Hamamatsu cameras (either silicon-intensified-target or electron-bombardment CCD cameras). The researchers synchronize the two cameras frame by frame.

With the channels, the scientists can image two single fluorescent molecules individually. Colocalization, though, requires the images to be stacked with the greatest possible accuracy. To correct for differences between the optical paths and to achieve the best possible colocalization images, the investigators ordered a

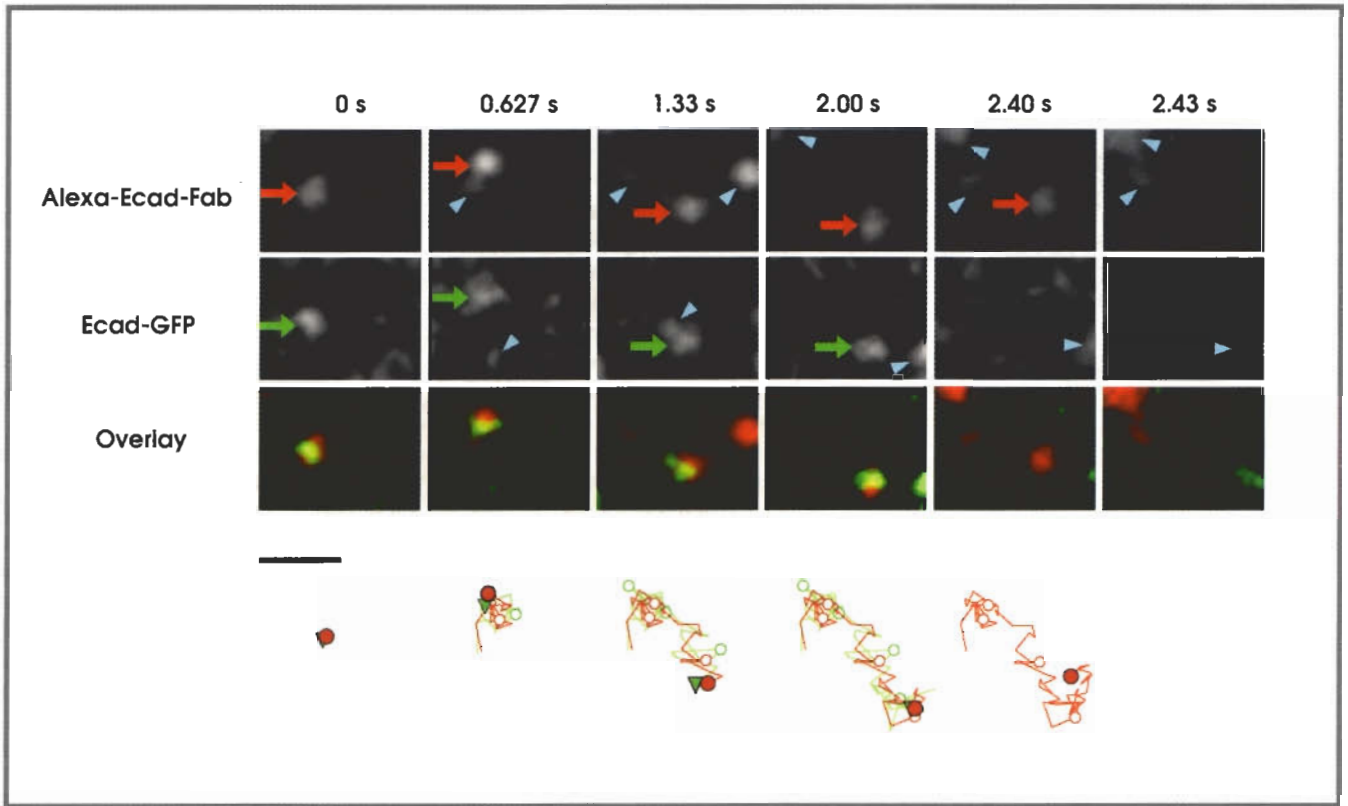
mask comprising an array of small holes made by Shibuya Kogaku of Wako, Japan. The mask cost several thousand dollars, and some of its features were at the limit of what the vendor could supply. "The size of the holes, a micron diameter, was the best they could do," Kusumi said.

The 1.0- μm holes on the mask are spaced 5.0 μm apart with a center spot accuracy of 0.3 μm . The researchers imaged the array through each of the channels, capturing and averaging 100 frames to find the center of each hole. From these images, they developed a correction table that adjusted the measured image to match the ideal. The table also allowed for correction between the holes. Kusumi noted that the design of the array and the size of the holes were key to accomplishing successful corrections, and, he added, there were just enough to make viable correction tables.

Working with stored images, the researchers applied these correction factors to their data. In effect, they superimposed one camera's image over the other. Using their system, they stacked images to an accuracy of about 13 nm. The accuracy of X and Y coordinates of a point is also a factor in determining the exact location of a fluorescent molecule. The instrument's superpositioning and localization uncertainties combined to create a colocalization accuracy of 64 nm.



Researchers used a chromium oxide mask (left) to spatially correct dual-color images captured on two detection channels (GFP and Alexa 633) of a microscope. Bright-field transmission microscope images of the array of holes in the mask were recorded on both channels and superimposed. The raw superimposed images (center) are compared with the same images after spatial correction (right). The yellow spots represent superimposed green and red spots.



The technique was used to detect colocalization of single molecules diffusing in the plasma membrane of living cells. Spatially corrected, superimposed video images are shown. A single molecule of E-cadherin (Ecad)-GFP (indicated by green arrows) was colocalized with the Fab fragment of a monoclonal antibody of Ecad (Alexa-Ecad-Fab) (indicated by red arrows). Other spots representing single molecules are shown by blue arrowheads. Beneath each column, the concurrent positions (green triangles and red circles) and the trajectories of these molecules are shown. (The scale bar is 1 μm for the images and 0.5 μm for the trajectories.)

However, there's a trade-off involved in the number. According to the scientists, **colocalized** molecules may be within **64 nm of each** other in only two out of **three images** captured by the system. Thus, the condition may be too strict and the criteria may need to be changed to ensure that colocalized molecules are detected.

"To increase the detectability to the level of 90 percent, you have to change the distance to 64 to 100 nm, depending on the observation conditions," Kusumi explained. He added that a larger cutoff for the distance increased the chance that two probes that just happened to be near each other would be mistakenly identified as being colocalized; therefore, proper experimental control is a must.

The researchers demonstrated and proved their instrument's capabilities in several ways. For example, they used the system to track the movement of proteins labeled with two probes. They matched

the single molecules in each detection channel to its corresponding signal in the other channel, which proved the instrument capable of handling what could be described as the ideal colocalization situation. They also used the device to detect colocalization in fixed cells, again with good results.

To demonstrate the instrument's potential in live cells, the investigators labeled the Fab fragment of a monoclonal antibody of E-cadherin — a transmembrane protein responsible for calcium-dependent cell-to-cell adhesion of epithelial tissues — with the fluorescent probe Alexa Fluor 633. They cultured live cells expressing E-cadherin-enhanced GFP molecules on the cell surface and captured images of these interacting with the Alexa-labeled Fab fragment of the antibody. They discarded images with intensities that indicated that more than one protein was involved and those with a measured GFP-Alexa 633 distance of >100

nm. They tracked the movement of the remaining candidate single molecules labeled with the two probes and were able to determine that they were indeed colocalized: The probes stayed within 100 nm of each other for a given candidate, and they exhibited similar diffusion coefficients.

Better microscope optics and higher-quality cameras could improve the instrument. Fluorescent probe characteristics also play a part in localization accuracy, with stronger signals resulting in greater precision.

Kusumi said that, although improvements in all three areas are needed, the current system could be put to use as is. He sees the instrument and its successors being used for "understanding how cellular nanosystems work at very fundamental levels and the development of better ways to develop new drugs and clinical testing methods." □

Hank Hogan