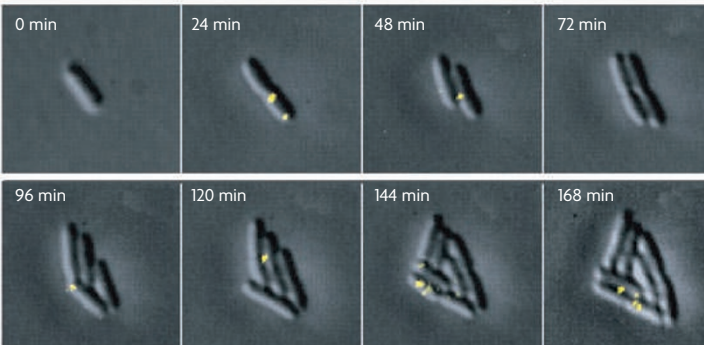
MILESTONE 17

Single molecules in the dark



Time-lapse images of *Escherichia coli* cells showing single-molecule imaging of the expression of single fluorescent fusion proteins. The fluorescent molecules are photobleached in the intervals between each image. Image is reproduced, with permission, from J. Yu *et al.* © (2006) American Association for the Advancement of Science.

The potential to visualize bright fluorescent molecules on a dark background using fluorescence microscopy lit up the field by providing molecular specificity and image contrast unparallelled by other dyes. Theory suggested that these properties should permit the detection and imaging of single molecules. A first glimpse of the possibilities came in 1976, when Thomas Hirshfeld used a fluorescence microscope to detect single molecules bound by dozens of fluorophores as they passed through a thin layer of illumination. But technical limitations hindered progress. Although the next two decades witnessed the detection of single fluorophores at low temperatures, or while transiting a highly focused laser beam (see MILESTONE 10), such methods were incapable of repeatedly imaging identified molecules in ambient environments.

In 1993, this situation changed markedly when Eric Betzig and Robert Chichester reported the first repetitive imaging of single fluorophores at room temperature with a new technique called near-field scanning optical microscopy (NSOM) that repeatedly scans an extremely small optical probe over a sample. This provided molecule-scale spatial localization and information on molecular orientation. The potential biological applications of single-molecule imaging captured the imaginations of microscopists and biologists alike, but because of its invasiveness and complexity NSOM proved largely unsuitable for complex biological samples.

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Although a number of people continued to try to improve NSOM — including its use for Förster (or fluorescence) resonance energy transfer (FRET) as a molecular ruler for single molecules — others turned to alternatives. In 1995, Toshio Yanagida and colleagues used an optimized implementation of total internal reflection fluorescence microscopy (TIRF) to image single myosin molecules labelled with one fluorophore and to detect the turnover of ATP labelled with a different fluorophore. Unlike previous methods using immobilized molecules, this work showed that TIRF could image many molecules in aqueous solution for periods of several seconds before bleaching occurred, and demonstrated the suitability of this method for true biological applications.

Although TIRF would come to dominate most single-molecule imaging applications, it was not the first technique to image the movement of single fluorophores. This first was claimed by conventional epifluorescence microscopy (see MILESTONE 4) with a highly sensitive charge-coupled device (CCD) camera. Hansgeorg Schindler and his co-workers adapted the method of Jeff Gelles *et al.* for single-particle tracking based on Gaussian-peak fitting to determine the positions of individual fluorophore-labelled lipids in a membrane with an accuracy of about 15 nanometres and to track their movements over time.

Up until this point, single-molecule fluorescence microscopy applications had an almost proof-of-principle feel to them, and for the most part novel biological insights were limited. But in 1998, Sunney Xie and colleagues used the intrinsic on–off fluorescence switching of flavin adenine dinucleotide in the active site of cholesterol oxidase to reveal that enzyme activity is influenced by a form of molecular memory residing in conformational changes in the protein. This behaviour was completely unanticipated from ensemble experiments and showed unequivocally that single-molecule microscopy could shed new light on seemingly well-characterized systems. Similar enzymatic-turnover assays developed by other groups eventually led to the sequencing of individual DNA molecules.

Methods continued to be developed and refined as highlighted by a number of groundbreaking studies on ribozymes, molecular motors and gene expression in single living cells as scientists pushed the limits of technology and imagination.

Daniel Evanko, Editor, Nature Methods

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