

may now be feasible with direct visualization. These studies will yield fundamental information on the order of events underlying fusion-based reprogramming and on the key properties that influence the phenotype of the resultant heterokaryon, such as cell-cycle or differentiation stage. An important engineering aspect that needs to be addressed is the capacity of the device to accommodate the wide range of cell sizes and types that researchers may wish to interrogate.

In the context of somatic cell reprogramming to a pluripotent cell state, the device may enable, either alone or combined with gene knockdown approaches, studies to identify new reprogramming factors. It is unlikely that the kinetics and molecular players that mediate reprogramming upon fusion between somatic cells and oocytes or embryonic stem cells are the same; quantitative comparisons of reprogramming efficiencies from successfully fused cell pairs may be informative.

Finally, this device, or its derivatives, may find use in the new area of lineage reprogramming—reprogramming of one

differentiated cell type to another³. In lineage reprogramming, transcription factors are introduced to try and switch one differentiated cell type directly to another. The success of this strategy depends on understanding which transcription factors may be effective for the generation of the target cell type. It may now be possible to explore fusion outcomes between relatively small numbers of purified stem cells and differentiated cells in a combinatorial manner, a capability that could accelerate the identification of candidate transcription factors for lineage reprogramming studies.

A particular strength of microfluidic systems, especially those that can be combined with visualization-based outcome analysis, is their ability to be multiplexed for the parallel analysis of multiple conditions; it will be fun to see how the strategy used by Skelly *et al.*¹ is put to use to generate new discoveries using cell fusion.

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Recently, new microscopy methods have successfully broken the diffraction limit. Localization microscopy methods combine photophysics and single-molecule imaging of PAFPs (Fig. 1) to improve resolution. The usual problem in light microscopy is that many molecules are visible at the same time, and their images are blurred together by diffraction, like coalesced raindrops on a windshield. Localization microscopy gets around diffraction by not looking at all the molecules at once. Instead, many small subsets of fluorescently labeled molecules within a sample are imaged separately, at low enough density that each molecule is distinct from the others.

The subsets are activated stochastically by illuminating the sample with light of a wavelength that switches the labeled molecules from an inactive (invisible) into an active (visible) state. Then, using a second wavelength of light, only the active molecules are excited and emit fluorescence, which is imaged by a high-sensitivity camera, and each visible molecule is then localized. Molecules remain visible for a short time and then photobleach under the high-intensity illumination. The cycle of activation, imaging, localization, and photobleaching is repeated many times to build up data on tens or hundreds of thousands of molecules. The key to improving the resolution is that fluorescent molecules can be localized with a precision of just a few nanometers, a significantly smaller value than the 200–250 nanometer limit imposed by diffraction. Once the positions of the molecules have been determined, the image can be obtained by plotting the positions of all localized molecules.

Three techniques introduced essentially simultaneously in 2006—fluorescence photoactivation localization microscopy (FPALM)³, photoactivated localization microscopy (PALM)⁴ and stochastic optical reconstruction microscopy (STORM)⁵—described the principles of localization microscopy and showed the first tantalizing applications. These technologies can now be used to image live cells⁶, multiple colors^{7,8}, three-dimensional samples^{9,10} and molecular orientations¹¹. Because localization microscopy can be carried out using standard fluorescence microscopes equipped with a high-sensitivity camera, many laboratories are beginning to try these powerful methods on their own biological systems.

However, the full potential of super-resolution microscopy is still limited by a shortage of suitable PAFPs. Several properties of these probes are crucial for successful localization

Red lights, camera, photoactivation!

Samuel T Hess

Two groups present new photoactivatable fluorescent proteins that will be useful for super-resolution fluorescence microscopy.

Super-resolution imaging methods are advancing rapidly and opening new windows into cellular structure. However, a shortage of genetically encoded photoactivatable and photoswitchable fluorescent probes (PAFPs) still limits some applications, in particular the imaging of multiple fluorescently labeled species. In this issue of *Nature Methods*, two groups present new photoactivatable fluorescent proteins that extend the range of available probes^{1,2}.

Subach *et al.*¹ screened many fluorescent proteins, identifying candidates with promise for two-color photoactivation-based imaging. McKinney *et al.*² succeeded in the daunting task of improving EosFP, one of the best existing photoswitchable fluorescent proteins. These new probes

will be crucial for advancing localization microscopy technology.

Light microscopes have led to great discoveries in biology. Unfortunately, their resolution is limited by diffraction of the light used to image the sample. As quantified by Ernst Abbe in the nineteenth century, the smallest features that can be imaged in a light microscope are a fraction of a wavelength in size, or about 200–250 nanometers for visible light using the best commercially available components. Of course, much of biology occurs on much smaller (molecular) scales. Electron microscopy, which can image on these smaller scales, cannot currently be used to image living specimens, motivating the need for improved technology.

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microscopy. PAFPs must be essentially invisible in at least one spectral region before they are activated. After activation, they must emit, within that same spectral region, a large enough number of photons (typically, tens of thousands) over a short enough time (typically, milliseconds) to be quickly imaged and identified as single molecules. Ideally, PAFPs should activate efficiently but only when illuminated by the activation wavelength. These demands drastically restrict the number of probes available for localization microscopy. Although there are many genetically encoded fluorescent protein variants available for standard fluorescence imaging, the number of genetically encoded PAFPs is relatively small. Of those that have efficient absorption and emission at convenient wavelengths, only a handful have good contrast between inactive and active forms, and are monomers when expressed as fusion proteins in cells.

Some of the existing PAFPs can certainly be used to image a single molecular species; however, many biological processes depend on how one species interacts with another, and thus live-cell multicolor imaging of genetically encoded PAFPs is highly desirable. STORM can be used to image multiple species but so far cannot be performed using genetically encoded probes and requires a low-oxygen (reducing) environment for imaging. The problem for PALM and FPALM imaging has been that the best genetically encoded PAFPs either activate from a dark state to a green-emitting state or switch from a green-emitting state to an orange-emitting state. Both of these options thus involve green-emitting species and are difficult to combine for two- or three-species imaging, in particular at high temporal resolution.

Reported in this issue of *Nature Methods*, an extensive mutagenesis screen by the Verkhusha and Lippincott-Schwartz labs now provides an exciting new option. Subach *et al.*¹ describe a photoactivatable version of monomeric Cherry (mCherry), called PAmCherry1, which is capable of switching from a dark state directly into a red-emitting state with high contrast and photostability, compared to other genetically encoded PAFPs. PAmCherry1 also has a favorable maturation time and pH-dependence, which are needed for both fluorescent proteins and PAFPs. One major advantage of PAmCherry1 is that it can work together with PA-GFP and other green PAFPs, and potentially many other PAFPs with distinct spectral properties. In particular, PAmCherry1 is not activated by blue (~480 nanometer) light, which could potentially be

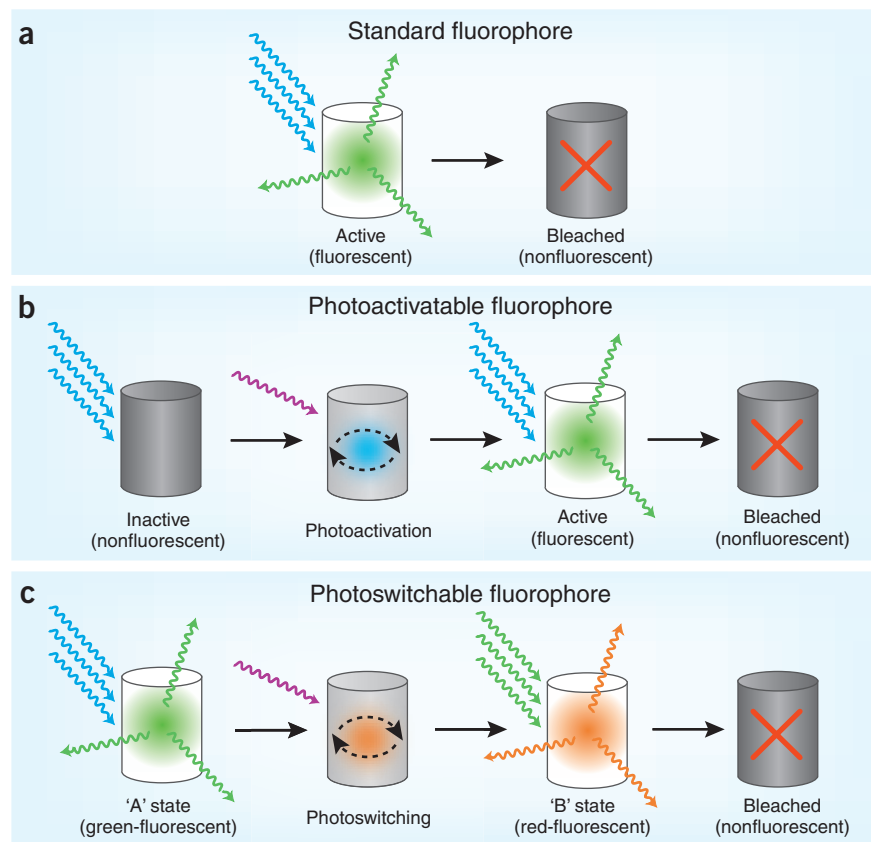


Figure 1 | Probes for photoactivation microscopy. (a–c) Localization microscopy depends on photoactivatable and photoswitchable fluorophores, which are different from standard fluorophores. The purple arrow in **b** and **c** indicates light of the activating (b) or switching (c) wavelength. Some steps shown may be reversible.

exploited in combination with Dendra2. It is also advantageous that both irreversibly and reversibly activated forms are available with high brightness, high contrast and independent control of activation and readout.

Also in this issue, McKinney *et al.*² from the Looger laboratory describe an improved version of monomeric EosFP (mEosFP), called mEos2. This new protein has all of the advantages of EosFP, with similar or better contrast, maturation and photoconversion efficiency. The tendency for self-aggregation of mEos2 is also quite low, which helps minimize probe-induced aggregation of the tagged protein of interest. Indeed, Looger and colleagues show that fusions that have proven difficult with the tandem dimer, tdEos—such as fusions to histones, tubulin, intermediate filaments or connexin—localize properly with mEos2 as the fusion tag. Also, they characterize and compare characteristics of mEos2 with those of other PAFPs reported in the literature both *in vitro* and in cellular PALM experiments, providing invaluable practical data for localization microscopy users.

These two reports help satisfy a need for better probes for localization microscopy. An almost endless set of possible pairs of proteins can now be imaged with nanometer resolution. Perhaps the most important next question is: what is the most interesting biological question to attempt to answer?

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