Photoactivation in Fluorescence Microscopy

David W. Piston[1]*, Gert-Jan Kremers[1], Richard K.P. Benninger[1], and Michael W. Davidson[2]
[1]Department of Molecular Physiology and Biophysics, Vanderbilt University, Nashville, Tennessee 37232
[2]National High Magnetic Field Laboratory and Department of Biological Science, Florida State University, Tallahassee, Florida 32310

* dave.piston@vanderbilt.edu

Introduction

The use of photoactive compounds in microscopy has a long history. Caged compounds have been used for almost forty years, not only to elicit chemical reactions in cells, but also to mark specific cells or regions within cells by photoactivation of fluorescence. During the last seven years, the advent of photoactivatable GFP (PA-GFP) and its successors has opened up a myriad of new applications. All of this work has, of course, been greatly facilitated in live cells through the possibility of genetic labeling that is given by the fluorescent proteins. However, even as more photo-activatable and photo-switchable proteins are discovered, they are still limited in terms of wavelength ranges and photophysical properties. Thus, there has been a resurgence of interest in small organic photoactive molecules for cell biology experiments. In this short introductory overview, we will present the basic concepts of photoactivation and discuss many of the strengths and limitations of various approaches. We will also provide a general description of the kinds of applications for which these probes can be used.

Caged Compounds

Caged compounds were originally developed in order to rapidly introduce effector molecules with known concentration and localization. Such precision is needed for many investigations into mechanisms of action of organized biological systems. In most cases, simple chemical time-resolved measurements on such systems are impossible because the diffusion of applied chemicals into the specimen is slow or the effector molecule is degraded in the sample. Further, it is often the case that pharmacological applications lead to desensitization of the biological response. One approach to these problems is to rapidly introduce biological substrates by photoactivation of precursor or "caged" compounds that are biologically inactive [1]. The term "caged" originated with caged ATP and was coined by Jack Kaplan and co-workers in 1978 [2]. Even though it is not chemically correct, the term has stuck, probably because it communicates the concept in a simple and imaginative way.

Photoactive compounds have been based on both photoisomerization and photolysis, with the latter being the most common approach. Photolysis is based on the general field of photosensitive protection during organic synthesis. Upon photolysis, the caged molecule translates the photon energy into the breaking of chemical bonds that results in the release of the cage from the active molecule. Photolysis of 2-nitrobenzyl derivatives constitutes the major class of caged compounds, although many new and promising compounds have been invented over the years. One important consideration for most of these reactions, though, is their "dark" chemistry where several internal reactions follow the initial photon absorption, and these reactions delay the final release of the active product. For example, 2-nitrobenzyl derivatives undergo a multi-step internal redox reaction, which gives rise to a 2-nitrosobenzaldehyde related by-product and the photoreleased effector molecule.

While photolysis has garnered most of the attention in caged compound development, photoisomerization has also found some uses, especially for photoswitching fluorescent probes. The emission of these probes can be repeatedly alternated between their fluorescent and nonfluorescent states. Nitrospirobenzopyran (nitroBIPS) can generate high-contrast between its dark spiro- and fluorescent merocyanine-states [3]. Photoswitching has recently found a wide range of applications for both high-contrast and high-resolution imaging, as described below.

Localized photolysis, especially as provided by two-photon excitation [4] has potential interest for investigating cellular mechanisms, particularly at inaccessible regions within biological preparations. With two-photon photolysis, both the time and spatial scales of activation can be tailored to accurately reproduce biological activity, for instance the neurotransmitters released during synaptic transmission. The dependence of the two-photon effect on the square of the light intensity allows the two-photon volume to be about 0.1 femtoliters

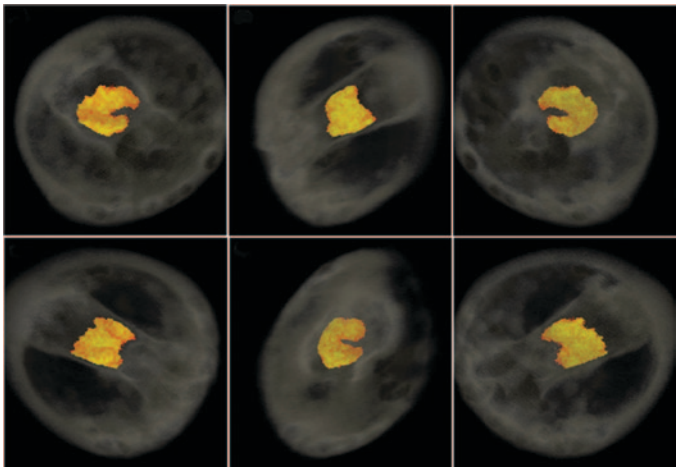

Figure 1: Six azimuthal views of a three-dimensional reconstruction of photorelease of caged-fluorescein-dextran in the sea urcin embryo gut (20 hours after fertilization). These images show a stripe of uncaged cells in the archenteron at the end of gastrulation. The uncaged region is shown in yellow, and the shadow of the rest of the embryo is represented by transparent gray. Only about three-quarters of the ring of cells in the archenteron wall has been uncaged to demonstrate the ability to mark only certain cells within a chosen region. This demonstrates that two-photon excitation photorelease can uncage dye in the middle of the embryo without any photorelease in the periphery.

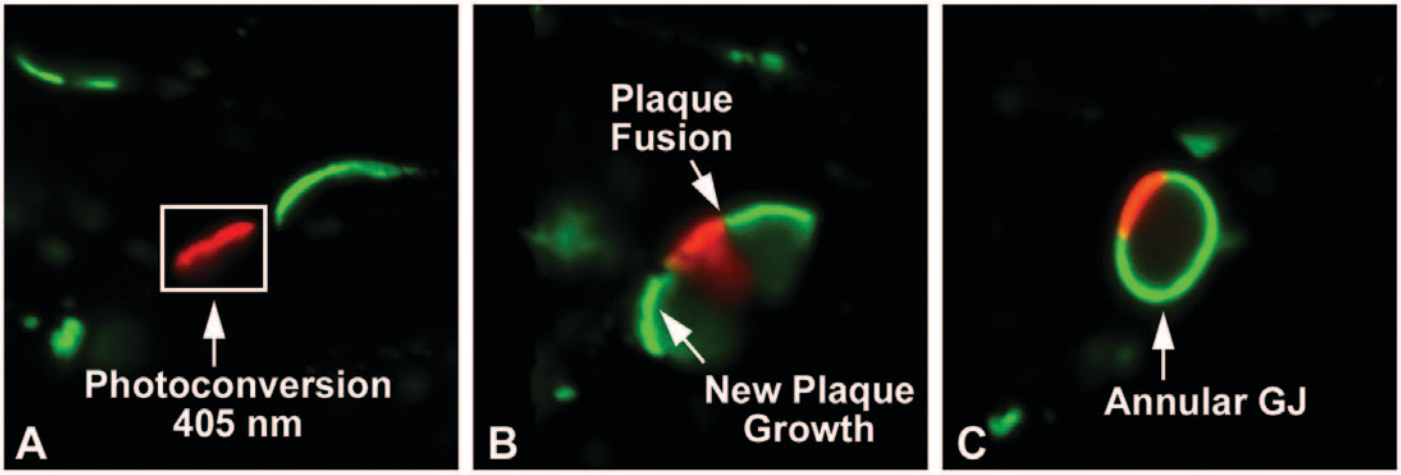

Figure 2: Optical highlighter FPs in action, imaged with laser scanning confocal microscopy. (A-C): Photoconversion of gap junctions labeled with tdEosFP-Cx43-N-7 in HeLa cells, (A) Photoconversion of a gap junction plaque (red) in a selected region (white box) with 405 nm illumination at t = 0; (B) New plaque growth and fusion of a non-converted plaque, t = 50 min; (C) Formation of annular gap junction with photoconverted region, t = 80 min.

(10^{-16} liters). Thus, localized two-photon photolysis can in principle mimic the action of a single-secretion event. To be useful for this category of applications, the caged compounds must be stable, pharmacologically inert, and release rapidly and efficiently on photolysis. For each caging moiety to be used, the specific quantitative release rates and temporal profiles must be determined. For this reason, researchers have developed caged fluorescent molecules that could be used as mimics for other biologically active molecules. Using caged fluorophores, one has a straightforward way to assay the temporal and spatial distribution of the released product by using a fluorescence microscope. These experiments can, in turn, be used to discover the level of physiological damage that is produced by such irradiation in biological preparations in relation to the extent of photolysis.

An example of this approach can be found in [5], where the snake neuromuscular junction was used as an assay of synaptic damage associated with photorelease of caged fluorescence. The junctions were exposed to the focused near-IR pulsed two-photon excitation laser beam to assess the ability of synapses to sustain normal transmission during an experimental protocol. These experiments utilized the pyranine fluorescence generated from caged NPE-HPTS. This caged fluorophore contains the same *o*-1-(2-nitrophenyl)ethyl ether photolabile protecting group as the commonly used caged compounds, NPE-caged ATP and NP-EGTA. Thus, a direct comparison could be made of photolytic efficiency and laser damage to cellular processes with the exact illumination and photochemical system. The results of this work showed that, due to the low quantum efficiency of caged compounds, very little photolysis could be generated at laser powers that do not damage the exocytotic machinery. In addition, it was found that the photorelease of NPE-caged molecules is too slow because of the dark reactions, and this causes the photoexcited compound to diffuse away from the localized excitation spot before it becomes active. Several groups have created new caged moieties with improved uncaging properties, which have proven successful in some experimental preparations [6]. Still, three-dimensionally resolved uncaging has seen limited utility thus far.

Caged fluorophores, however, can also be used to explore many biological questions. An obvious application is for lineage tracing in developmental biology, where one can simply irradiate a cell that contains the caged fluorophore and then watch the fluorescently label progeny as development continues. Combining this approach with two-photon excitation allows labeling of single cells or small groups of cells deep within a living embryo. This strategy was used in the study of sea urchin bilateral asymmetry and gastrulation (development of the gut) [7]. In this work, single-cell embryos were injected with dextran-caged-fluorescein, and two-photon excitation uncaging was employed to mark small groups of cells throughout gastrulation. Two-photon excitation allowed for noninvasive, three-dimensionally resolved uncaging inside living cells with minimal biological damage. An example of this two-photon excited uncaging is shown in Figure 1. Development of the gut involves an initial proliferation of cells, but it was assumed that further development proceeded without further involution of epithelial cells. Using two-photon excited photorelease of caged fluorophores, cellular involution into the gut was observed throughout gastrulation. These experiments could not have been done with other approaches, and now that two-photon excitation microscopes are more readily accessible, this approach should be broadly applied to development of other embryo systems.

Photoactivation of Fluorescent Proteins

The green-fluorescent protein (GFP) was discovered in the early 1960s as a protein complexed with the bioluminescent protein, aequorin [8]. GFP fluoresces green under UV excitation and was found to work with aequorin to generate the green luminescence that is characteristic of the species. Since the initial heterologous expression of GFP [9], fluorescent proteins from other species have led to further expansion of the color palette into the orange and red spectral regions [10], and all of these have been engineered to produce a vast number of

useful mutants [11]. However, one of the most exciting developments in this field has been the creation of photoactivatable fluorescent proteins, beginning with the photoactivatable GFP (PA-GFP) [12]. PA-GFP initially has an excitation peak of ~400 nm, but after photoconversion with near UV light, the excitation peak moves to ~488 nm. This allows high contrast between the unconverted and converted pools, and thus PA-GFP is useful for tracking the dynamics of subpopulations of molecules within a cell. While photoactivatable proteins serve as a foundation for the generalized overall function of optical highlighters, many new FP variants have been engineered to be capable of photoconversion (switching from one emission wavelength to another) or photoswitching (the ability to selectively turn fluorescence on and off).

Photoactivatable Fluorescent Proteins. Photoactivatable proteins in spectral regions beyond that of PA-GFP have been developed but have found limited use. Some variants exhibit reduced dynamic range, likely because their non-activated state is significantly brighter than that of PA-GFP. A recent variant of mCherry may prove useful [13]; PA-mCherry1 features an absorption maximum at 404 nm before and 564 nm after photoactivation. This protein has been demonstrated to perform well in two-color photoactivation imaging with PA-GFP. An earlier variant, PS-CFP2, photoconverts from cyan to green fluorescence after 405 nm illumination [14]. PS-CFP2 yields a significant level of cyan fluorescence before photoactivation, which enables easier determination of regions for selective illumination. While the low dynamic range and limited brightness of PS-CFP2 renders it inferior to other variants, its dual color function led to the development of many other proteins of this photoconversion class.

Photoconvertible Fluorescent Proteins. Photoconvertible fluorescent proteins switch between two colors (typically from green to red), both of which can be visualized. Most photoconvertible proteins feature a cyclized tripeptide His-Tyr-Gly chromophore that emits green fluorescence, but it also can absorb ultraviolet light to induce cleavage between the amide nitrogen and *alpha*-carbon atoms in the histidine residue. This peptide cleavage results in a shift of emission to orange-red wavelengths. These optical highlighters undergo an irreversible photoconversion, but both states can be highly photostable. Among the current photoconvertible green-to-red highlighters, one of the best choices is mEos2 [15]. Green fluorescence from this protein has a maximum of 519 nm, and upon photoconversion, the emission maximum shifts to 584 nm. mEos2 features the highest brightness in both the initial green and photoconverted red state. Photostability of the red state is significantly higher than it is in the initial green state, but both states are suitable for imaging in widefield or confocal microscopy. The use of EosFP to label the gap junction protein, Cx-43, is shown in Figure 2, where new junctions can be visualized separately from older junctions that were previously converted to the red color.

Recently, it was shown that photoconversion is fairly common among orange and red fluorescent proteins, as more than two-thirds of these variants exhibit photoconvertion.

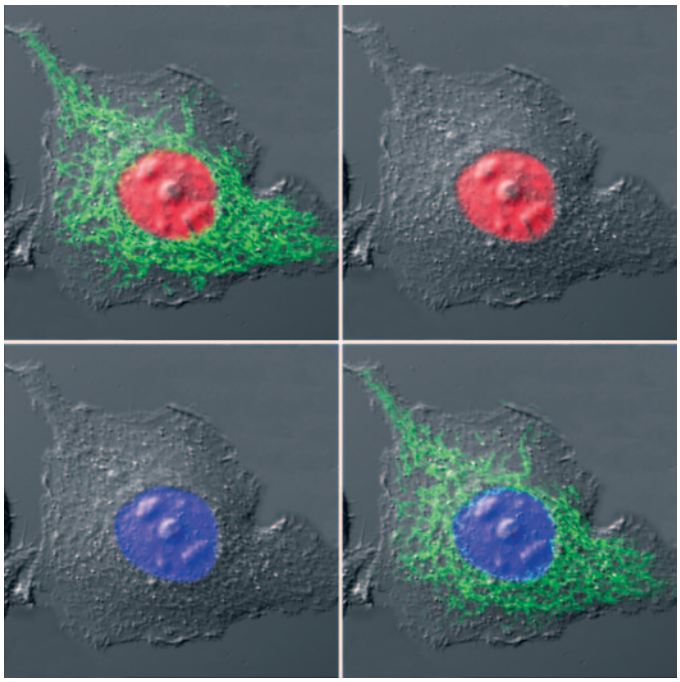

Figure 3: Dual-probe optical highlighting with Histone2B-mOrange and Dronpa-mitochondria, changing colors sequentially to give four different combinations. Upper left: Before photoconversion the cell shows green-labeled mitochondria and orange-labeled nuclei (pseudo-colored in red). Upper right: Dronpa fluorescence was switched off using low power 488 nm excitation while causing minimal photoconversion of mOrange. Lower left: High power 488 nm excitation was used to photoconvert mOrange to its far red state (pseudo-colored blue). Lower right: 405 nm illumination was used to switch the Dronpa fluorescence back on. The B/W overlays are the Nomarski DIC images of the cell.

Further, several red fluorescent proteins can be switched into a green state, and two other orange variants can be photoconverted to the far red [16]. The orange highlighters are ideal for dual-probe highlighter applications, and they exhibit the most red-shifted excitation of all autofluorescent protein described to date. Still, for many applications, it would be preferable to convert not only from one state to another, but also back to the original state. For this need, photoswitchable probes have also been developed.

Photoswitchable Fluorescent Proteins. Optical highlighters that can be toggled on or off by illumination with two different excitation wavelengths are referred to as *photoswitchable* FPs. Most of these probes have been discovered to work via a photoisomerization in much the same manner as the earliest caged compounds that were based on organic molecules. The most notable fluorescent protein of this type is Dronpa [17]. When Dronpa is irradiated at 488 nm, it emits green fluorescence with a maximum at 518 nm and a brightness level almost 2.5 times that of EGFP. However, when Dronpa is irradiated at 488 nm, its signal rapidly decreases, not from photobleaching but from photoswitching to a dark state. Dronpa can be photoswitched back on by illumination at 405 nm. This cycle can be repeated several hundred times without significant photobleaching. An example of the use of Dronpa and the mOrange photoswitchable protein for dual-color photoactivation is shown in Figure 3, where a single cell is marked in four different ways based on

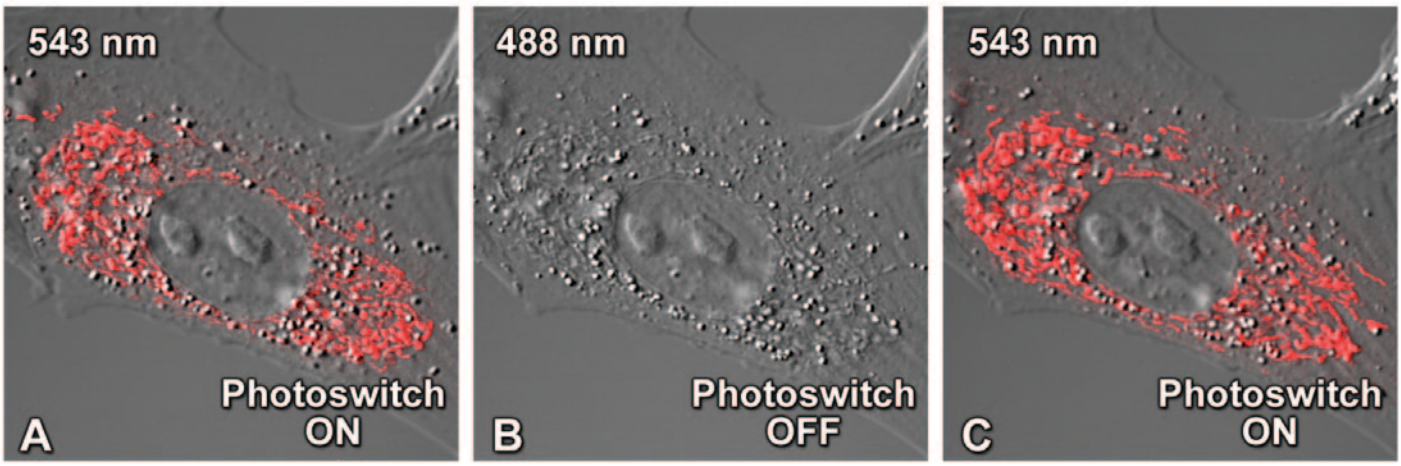

Figure 4: Photoswitching in optical highlighter fluorescent proteins (A-C): Photoswitching of the mitochondria with KFP1-mito-N-6 in fox lung cells, (A) Labeled mitochondria imaged with 543 nm laser in both fluorescence and differential interference contrast, t = 0; (B) After completely photoswitching the labeled chimera "off" with 488 nm illumination, the mitochondria now appear devoid of fluorescence, t = 3 min; (C) KFP1 label in mitochondria, reactivated with illumination at 543 nm, does not significantly photobleach after 5 rounds of photoswitching.

the on/off properties of these two probes.

Several other photoswitching fluorescent proteins have been developed from sea anemones and corals. One useful variant is the Kindling protein [18], which is available commercially as KFP1. KFP1 is a tetrameric highlighter that emits 600 nm red fluorescence after illumination with green or yellow light (525-580 nm). Irradiation with intense 488 nm light quenches KFP1 fluorescence immediately, enabling control over the photoswitching. An example of the use of KFP1 to track the localization of mitochondria in living cells is shown in Figure 4. Two other useful photoswitchable variants were recently derived from the popularly used mCherry protein [19]. Termed rsCherry and rsCherryRev, these derivatives display antagonistic switching modes such that irradiation of rsCherry with yellow light induces the bright state and blue light drives the FP to the dark state, whereas the reverse is observed with rsCherryRev. Both FPs appear to be less bright as mCherry when expressed in cells, but they should still find considerable use because of their photoswitching properties and red emission spectra.

Applications

The need for novel approaches to visualize molecules in cells continues to be driven by our lack of insight into the precise temporal and spatial dynamics that dictate the growth, function, and survival of living cells. Although many of the fundamental molecular processes that occur within cells are understood on the biochemical level, a cell is not a bag of chemicals. Hypotheses regarding precise protein interactions at the right time and the right place are becoming increasingly common. The use of fluorescent probes for single-molecule experiments *in vitro* is well-established. However, in living cells, such detection is degraded by autofluorescence and light scattering, and instrumentation advances are approaching their physical limits. A major challenge in live-cell imaging, therefore, is to develop probes and imaging techniques that are capable of resolving fluorescence signals from small numbers of molecules within large background signals under conditions of temporal and spatial variations. Two recently developed approaches utilizing photoactivatable fluorophores yield such capabilities and, thus, hold promise to revolutionize biological light microscopy. The first is super-resolution techniques where repeated rounds of photoactivation are used to beat the Abbe diffraction limit, and the second is high-contrast methods where photoactivation is used as a source of modulation in conjunction with lock-in detection to beat the shot noise limit.

Super resolution. This major advance in microscopy exploits photochemical transitions between a nonfluorescent and fluorescent state of probe-labeled structures, originally described as photoactivation light microscopy (PALM) by Eric Betzig and co-workers in 2006 [20]. This new technique has received significant publicity, for instance as *Nature Methods*' "Technique of the Year 2008," so it will only be briefly described here. In the PALM approach, many molecules are labeled, but weak photoactivation illumination is used so that only a few molecules are "turned on." This sparse population of molecules can then be imaged, and provided that each fluorophore is sufficiently separated from the rest, each fluorescent spot will represent a single molecule. After imaging this molecule, the center of the point spread function can be fit using the known microscope characteristics, and thus the molecule can be localized to ~20 nm, well under the diffraction limited value of ~200 nm. Repeatedly photoactivating small numbers of molecules allows one to build up a picture of the entire sample, but with a resolution far surpassing what is possible with conventional imaging. An example of PALM imaging is given in Figure 5 where EosFP labeling of the mitochondria is imaged with a final resolution of ~20 nm. Many of the photactivatable and photoswitchable proteins have found utility for PALM-type experiments. Among these, the PA-mCherry1, which features an absorption maximum at 404 nm before and 564 nm after photoactivation, has been demonstrated to perform well with PA-GFP for two-color PALM.

Lock-in Detection. The second recent advance in light

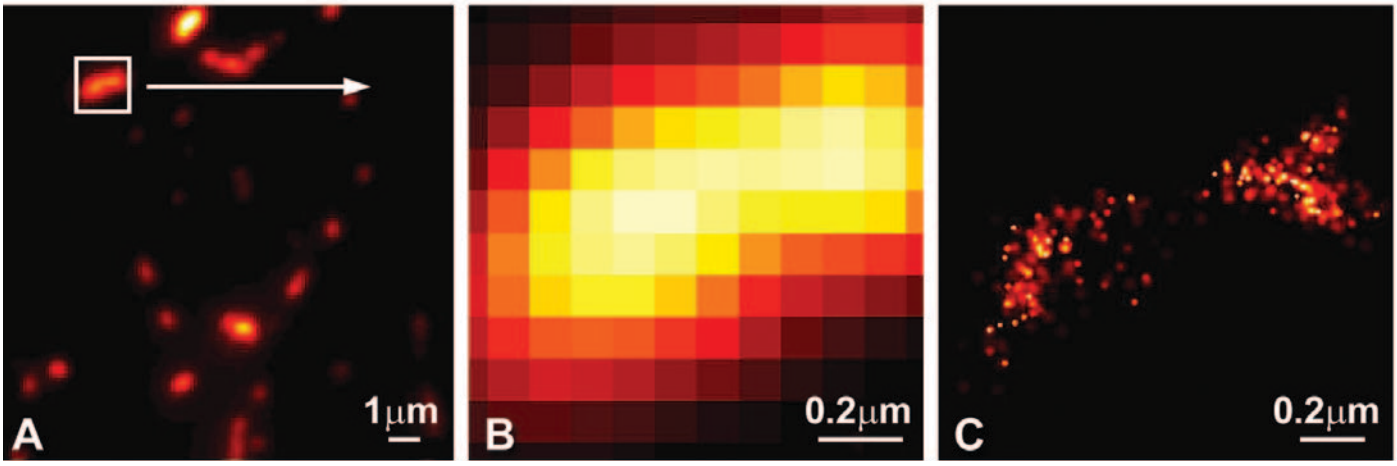

Figure 5: Superresolution microscopy using optical highlighters (A-C): PALM imaging of tdEos-mitochondria-N-7 in fox lung cells; (A) Widefield TIRF image of a mitochondria field near the nucleus; (B) summed PALM image of boxed region in A; (L) PALM image of the mitochondrial fusion.

microscopy that utilizes photoswitching probes is lock-in detection methods where photoswitching modulation is used with lock-in detection. One of the limitations of live cell imaging is the presence of significant autofluoresence background. Using lock-in detection, it is possible to isolate specific signals from background, and this can be accomplished in fluorescence imaging using photoswitchable probes. In this approach, the fluorescence emission of the probe is modulated with illumination to control its fluorescent and nonfluorescent states. Because the photoswitching is being actively controlled, one can subsequently apply lock-in detection and, in turn, detect only the modulated signal. Using lock-in detection with sufficiently bright fluorescent probes, it should be possible to image down to the single-molecule level even within living cells. Lock-in detection has been demonstrated using both Dronpa fluorescent protein and small organic NitroBIPS probes [21]. Using this approach, high-contrast images can be generated of specific structures and proteins in labeled cells. Cross-correlation analysis was also utilized to isolate the modulated signal of the probe and generate a pixel-by-pixel enhanced contrast image of the probe over background.

The usefulness of Dronpa as an optical switch was shown to be somewhat limited by its tendency to transition into a nonswitchable state, but the power of genetically encoded fusion labels yields superb specificity in living cells. Thus, Dronpa is likely to have broad impact for cellular lock-in imaging, particularly given the recently described improved versions, Dronpa-2 and Dronpa-3 [22]. To utilize small organic molecules for live cell imaging experiments, it has proven convenient to exploit newly developed tagging systems such as the SNAP-tag (Covalys), Halo-tag (Promega), and Ligand-Link (Active Motif) systems. Using these systems, specific *in vivo* labeling of proteins can be achieved with small organic caged compounds or optical switches. In the SNAP-tag system, for example, a benzylguanosine derivative of NitroBIPS (BG-PEG-NitroBIPS) is used as a substrate for a variant of alkylguanosine transferase (AGT). This labeling system is used to link NitroBIPS to AGT, which can be genetically fused to a protein of interest in living cells. An example of using NitroBIPS targeted to the AGT SNAP tag in live cells is shown in Figure 6, which also shows the time course of switching the NitroBIPS fluorescence on and off. Using either photoswitching fluorescent proteins or optically

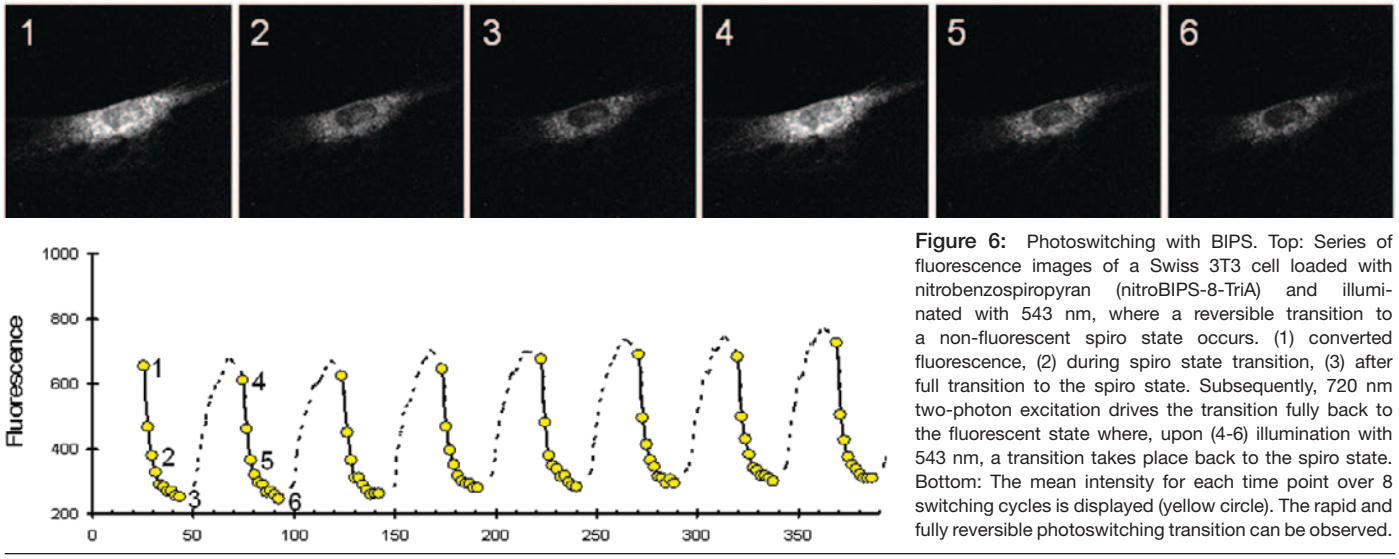

Figure 6: Photoswitching with BIPS. Top: Series of fluorescence images of a Swiss 3T3 cell loaded with nitrobenzospiropyran (nitroBIPS-8-TriA) and illuminated with 543 nm, where a reversible transition to a non-fluorescent spiro state occurs. (1) converted fluorescence, (2) during spiro state transition, (3) after full transition to the spiro state. Subsequently, 720 nm two-photon excitation drives the transition fully back to the fluorescent state where, upon (4-6) illumination with 543 nm, a transition takes place back to the spiro state. Bottom: The mean intensity for each time point over 8 switching cycles is displayed (yellow circle). The rapid and fully reversible photoswitching transition can be observed.

switched organic dyes in the ways described above can be readily employed in most laboratories on existing microscopes.

The lock-in detection method has also been used to develop a sensitive assay for Förster Resonance Energy Transfer (FRET), which is widely used to study bio-molecular dynamics and protein interactions in live cells. FRET is ideally suited for these protein-protein interaction studies, as it combines the high sensitivity and spatial resolution of fluorescence detection with the ease of introducing genetically encoded donor and acceptor probes to specific proteins. However, FRET measurements generally require a known stoichiometry between the donor and acceptor probes, which may be difficult to ascertain in live cell experiments, especially if unlabeled endogenous proteins are present. By incorporating NitroBIPS as an acceptor in a FRET pair with a donor such as GFP, NitroBIPS photoswitching can be used to modulate the FRET efficiency [3]. Recording the donor fluorescence in the presence (NitroBIPS "on") and absence (NitroBIPS "off") of the acceptor permits a quantitative assay of FRET even in the presence of excess donors, photobleaching of the donor, or time-varying background signals. In this sense, lock-in FRET is similar in concept to the photobleaching of acceptor method, which is often used to detect FRET between interacting proteins in cells. However, lock-in FRET has the added advantage of being able to be applied repeatedly, unlike acceptor photobleaching that is irreversible and can only be used for a single determination of FRET. Most importantly, lock-in FRET allows detection of small sub-populations of protein complexes undergoing FRET over a high background of non-complexed proteins, with sensitivity many times of that currently achievable with conventional techniques.

Conclusion

Photoactivation is providing powerful new tools for high-resolution and high-contrast fluorescence imaging. In particular, the use of these probes allows specific resolution of the fluorescence signals within live-cell systems, which contain substantial background signals. The active dynamic approaches, such as PALM or lock-in detection, rely on the properties of the optical switches whether they are genetically encoded proteins or small organic molecules. Use of photoactivation produces a stimulated change that is distinct from the native sources of cellular fluorescence. Simple analysis methods, such as point-spread fitting or cross-correlation of images, can be used to obtain significant spatial resolution and contrast enhancements. Best of all, most photoactivation can be easily performed on existing automated widefield or confocal microscopes without extra modifications. Thus, use of these approaches should continue to grow as ever-increasing biological applications for them are found.

Acknowledgements

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