Facile autofluorescence suppression enabling tracking of single viruses in live cells

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Live cell fluorescence imaging is the method of choice for studying dynamic processes, such as nuclear transport, vesicular trafficking, and virus entry and egress. However, endogenous cellular autofluorescence masks a useful fluorescence signal, limiting the ability to reliably visualize low-abundance fluorescent proteins. Here, we employed synchronously amplified fluorescence image recovery (SAFIRe), which optically alters ground versus photophysical dark state populations within fluorescent proteins to modulate and selectively detect their background-free emission. Using a photoswitchable rsFastLime fluorescent protein combined with a simple illumination and image-processing scheme, we demonstrate the utility of this approach for suppressing undesirable, unmodulatable fluorescence background. Significantly, we adapted this technique to different commercial wide-field and spinning-disk confocal microscopes, obtaining >10-fold improvements in signal to background. SAFIRe allowed visualization of rsFastLime targeted to mitochondria by efficiently suppressing endogenous autofluorescence or overexpressed cytosolic unmodulatable EGFP. Suppression of the overlapping EGFP signal provided a means to perform multiplexed imaging of rsFastLime and spectrally overlapping fluorophores. Importantly, we used SAFIRe to reliably visualize and track single rsFastLime-labeled HIV-1 particles in living cells exhibiting high and uneven autofluorescence signals. Time-lapse SAFIRe imaging can be performed for an extended period of time to visualize HIV-1 entry into cells. SAFIRe should be broadly applicable for imaging live cell dynamics with commercial microscopes, even in strongly autofluorescent cells or cells expressing spectrally overlapping fluorescent proteins.

Fluorescent proteins (FPs)§ are widely used in biology because of their high biocompatibility and labeling specificity that enable visualization of cellular proteins and compartments (1–4). However, the modest brightness and photostability of FPs can limit their application (3–5). A fundamental limitation of live-cell imaging is the presence of intrinsic autofluorescent species that create an undesired, broad spectral background that confounds detection of FP-tagged molecules expressed at low levels. For example, cellular metabolites, such as flavins and NAD(P)H, autofluoresce between 500–550 and 450–500 nm, respectively (6). Freely diffusing metabolites increase the overall autofluorescence background, whereas cellular compartments with abundant metabolites can create bright spots that can be mistaken for specific fluorescence signals. This is especially true for imaging of small FP-tagged objects, such as single viruses, in living cells (7, 8). The development of red and far-red FPs alleviates the green/blue autofluorescence background problem, but limits the choices of FPs for multicolor imaging. Therefore, autofluorescence suppression is essential to improve the signal-to-background ratio and sensitive multicolor imaging in living cells.

Several approaches have been used to suppress autofluorescence, including spectral unmixing, which takes advantage of a broader emission spectrum of autofluorescence compared with FPs (6, 9, 10). However, if the intensity of fluorescence signal is comparable with the background, spectral unmixing tends to diminish the useful signal. Also, autofluorescence often comes from several metabolites that may be present at varying concentrations and/or ratios in different cell types or even within the same cell (11, 12). An alternative autofluorescence suppression technique employs time-gated fluorescence signals that blocks >95% autofluorescence (>20 ns after an excitation pulse), and thereby selectively registers the longer-lived fluorescence of interest (13, 14). However, this approach has limited utility for many common emitters, such as FPs with relatively short fluorescence lifetimes (1–4 ns) (6, 9). Chemical treatment or long irradiation to remove autofluorescence before imaging can also reduce background, but it is sample-dependent and not readily compatible with live-cell imaging (6, 15).

An alternative approach to discriminate between the desired signal and obscuring background is to take advantage of FP photophysics. Because of the development of photoswitchable fluorescent proteins (PS-FPs) (e.g. Dronpa, rsFastLime), the dark state population can be reversibly and efficiently modulated by primary and secondary excitations, with minimal fluo-
rescence loss (16–19). Furthermore, the chromophore environment also makes some FPs unmodulatable (e.g. EGFP) (20, 21). PS-FPs offer unique signal recovery opportunities as they exhibit long-lived, optically modulatable dark states governed by photoisomerization between cis (bright, anionic) and trans (dark, neutral) chromophore conformations that result from differentially stabilizing the two differently absorbing, optically interconvertible chromophore charge states (18, 22). Thus, fluorescence of PS-FPs can be optically recovered from the population of photophysical dark states. Such optically recoverable dark states have been widely utilized in super-resolution microscopy through stochastic recovery and proved to be photocontrollable in both fixed and live imaging conditions (23–26). Recently, autofluorescence suppression by externally controlling the dark state population of PS-FPs using a second optical excitation has been demonstrated (21, 27–33). Optical lock-in detection must use an independent, internal pure dye reference to determine the modulation waveform, which leads to difficulties in signal recovery (27, 29, 34) and reconstructed intensities that are not linear with protein concentration (27, 34). By contrast, optical modulation techniques, such as synchronously amplified fluorescence image recovery (SAFIRe) (20, 21, 28, 30, 35, 36) and out-of-phase imaging after optical modulation (OPIOM) (32, 33), suppress autofluorescence signals without the need for a reference point for cross-correlation. In the past, OPIOM and SAFIRe have been implemented using home-built microscopes to control the laser intensity or synchronize detection to recover modulated fluorescence from unmodulatable autofluorescence.

Here, we harness PS-FP photophysics to adapt SAFIRe to suppress autofluorescence using commercial fluorescence microscopes. We design a simple image acquisition and data processing protocol that markedly improves the ability to reliably visualize faint PS-FP–tagged objects in living cells. Our modulation scheme utilizes existing acquisition software without additional electronics or the need for high-frequency laser modulation. Successful implementation of SAFIRe using commercial microscopes and software will make this technique readily accessible to a wide array of applications by microscopists.

**Results**

**Rationale**

PS-FPs can be efficiently photoisomerized and their signals selectively recovered because of their high absorption cross-sections and large reverse isomerization quantum yields. These properties allow considerable alteration of emissive and dark populations by mild illumination intensities (~10 W/cm²) (21, 37). Importantly, this enables PS-FP modulation using commercial confocal and wide-field microscopes. To implement SAFIRe imaging on commercial fluorescence microscopes, we utilized rsFastLime (18, 19), a PS-FP with an extinction coefficient and fluorescence quantum yield comparable to those of EGFP. The long-lived dark state of rsFastLime can slowly thermally relax back to the bright manifold of states or can be efficiently and quickly photoactivated by blue light back to the bright manifold. We reasoned that sequential excitation of rsFastLime by 488 nm light would generate both fluorescence and dark state populations, whereas 405-nm light would reactivate fluorescence of rsFastLime, but not fluorescence of endogenous autofluorescent species present in cells. Because only the bright and dark states of rsFastLime, but not autofluorescence, are interconverted upon illumination at 405 and 488 nm, rsFastLime emission should be linearly proportional to the population of 405-nm–photoactivated rsFastLime, thereby distinguishing it from autofluorescence.

**Optically controlling dark states of photoswitchable fluorescent protein on commercial microscopes**

We designed a modulation scheme for rsFastLime that principally involves exposure to 488 nm (primary) light to generate fluorescence and transient photobleaching, followed by photoactivation/fluorescence recovery with 405 nm (secondary) excitation. Specifically, the imaging scheme was comprised of two elementary images with two intervening conditioning steps to generate each final demodulated image: the 1st elementary image (denoted Image 1, Im1) was collected upon 488 nm illumination, prior to 405 nm photoactivation. Next, PS-FPs were photoactivated by 405-nm illumination followed by acquisition of the 2nd elementary image (Im2) at 488 nm to visualize activated rsFastLime fluorescence. Finally, additional 488 nm illumination was used to photo deactivate rsFastLime back to the dark state (Fig. 1A). Fig. 1 shows an example of the photodarkened (Im1, Fig. 1B) and recovered rsFastLime emission (Im2, Fig. 1C) from NIH-3T3 cells expressing mitochondria-targeted rsFastLime (20, 21). Here, the post-405 nm–activated fluorescence image (Im2) displays an enhanced signal from the mitochondria-targeted rsFastLime, as compared with a dim signal observed before 405 nm activation (Im1). Both images 1 and 2 show similar background autofluorescence patterns. After image subtraction (Im2 − Im1), the demodulated image yielded >15-fold signal-to-background improvement (Fig. 1D) compared with the fluorescence image after activation (Im2).

Photoactivation is manifested in fluorescence recovery from secondary wavelength-absorbing dark states of PS-FPs. In contrast, EGFP, which exhibits indistinguishable emission from that of rsFastLime (19), emits fluorescence irrespective of excitation by primary or secondary laser, as it lacks secondary wavelength-absorbing dark states (20, 21). Similar to autofluorescence suppression, the ratio of fluorescence signals of mitochondria-targeted rsFastLime to abundantly expressed cytosolic EGFP was improved >7-fold by the same modulation scheme (Fig. 2). Thus, modulation of PS-FPs suppresses autofluorescence and permits multiplexed fluorescence imaging by SAFIRe.

**Imaging rsFastLime-labeled HIV-1 particles with SAFIRe**

To expand the applicability of SAFIRe to single virus imaging against the high autofluorescence signal in the perinuclear region of cells, we labeled HIV-1 pseudoviruses with the integrase fused to rsFastLime, using Vpr-IN-rsFastLime or Gag-IN-rsFastLime constructs (see “Experimental procedures” and Fig. 3A) and tracked virus entry into CV-1–derived CF3 cells, essentially as described in Refs. 38 and 39.
CF3 cells often contain distinct punctate autofluorescent objects in the absence of infection (data not shown), which complicates the identification of fluorescent viruses. For the initial validation of our system, we performed SAFIRe imaging of single HIV-1 pseudoviruses labeled with IN-rsFastLime (Fig. 3, B–D) or IN-EGFP (Fig. 3, E–G) in fixed CF3 cells. After demodulation, bright false-positive autofluorescent puncta were suppressed, enabling reliable identification of single viral particles containing IN-rsFastLime (Fig. 3D). In contrast, both autofluorescent and IN-EGFP–labeled pseudovirus signals were suppressed by SAFIRe (Fig. 3G). These results demonstrate the ability of SAFIRe to unambiguously identify single virus particles against uneven backgrounds typical for CF3 and other cells.
Facile method for autofluorescence suppression

Under current imaging conditions, we observed about 30% loss of the original intensity after 100 cycles because of irreversible photobleaching of rsFastLime population. This partial loss of rsFastLime signal within the viral particles was not associated with cellular toxicity, as evidenced by continued endocytic activity and unaltered cell morphology.

Discussion

Single virus tracking is a powerful tool for understanding viral entry into cells (7, 39–43). However, modest brightness and photostability of FPs and the limited number of labeled proteins within the virus complicate single particle tracking against the cellular autofluorescence background. Here we show that, by matching FP photophysics to illumination parameters, SAFIRe optically modulates PS-FP bright and dark states, enabling recovery of high contrast, background-suppressed images on commercial microscopes. Our imaging scheme readily achieved >10-fold signal-to-background ratio improvement in different biological samples (NIH3T3, CF3 cells), using multiple commercial imaging platforms (spinning disk confocal and wide-field microscopes). This is comparable with our prior demonstration of >10-fold improvement using optically modulatable FPs and a home-built stage-scanning confocal microscope (20). Importantly, the marked improvement of signal-to-background by SAFIRe allowed tracking single viruses in high autofluorescence regions of live cells, unambiguously discriminating signals of interest from inhomogeneous background.

In addition to autofluorescence suppression, we demonstrated the ability to suppress signals from unmodulatable FPs (e.g. EGFP) with spectra that strongly overlap with that of rsFastLime. This reveals an additional dimension for multiplexed fluorescence imaging of fluorescent proteins of overlapping colors. The overlapping fluorescence emission spectra and the relatively modest range of FP emission (~400–700 nm) limit the ability to perform multicolor imaging. Thus, our simple and fast modulation scheme adapted to commercial microscopes provides an alternative way for multiplexed imaging based on photophysical dark states.

Considerable irreversible photobleaching after 100 demodulation cycles was observed under our imaging conditions (e.g. Fig. 4G). This loss of fluorescence was not associated with detectable adverse effects on cell viability, in agreement with the lack of significant phototoxicity generated by fluorescent proteins upon either extended illumination or upon photobleaching (44). In addition, rsFL is enclosed in the viral membrane, with no access to the cytoplasm. To further optimize SAFIRe with respect to autofluorescence suppression in live cells, the photophysics/photostability of PS-FPs can be improved. PS-FPs with optimized photophysics to minimize irreversible photobleaching would improve the ability to perform long-term particle tracking in living cells. Additionally, PS-FPs with larger absorption cross-sections in both bright and dark states can increase the excitation efficiency, thus lowering the excitation intensity and minimizing unnecessary photodamage.

Also, the temporal resolution in live-cell imaging can be limited by the instrumental processing time between each image...
acquisition. For example, due to software/hardware limitations, the time required to acquire 5 sequential images (used in Fig. 4) on a DeltaVision microscope (see “Experimental procedures”) was 450 ms (mostly from data processing time of the DeltaVision imaging system). Within a 450-ms acquisition time window, single virus tracking was accomplished, as judged by virtually overlapping mCherry and demodulated trajectories (Fig. 4H). This demonstrates that particle displacement during the imaging cycle was negligible compared with their apparent size, as evidenced by the lack of asymmetries in diffraction-limited virion images. As for monitoring faster cellular dynamics, the frequency of imaging cycles for SAFIRE can be improved by using commercial microscopes with faster instrumental processing time. In conclusion, we demonstrated that SAFIRE is a straightforward and robust approach to suppression of autofluorescence or any undesirable signal in cells with sufficient temporal resolution to perform single particle tracking in living cells.

Experimental procedures

Cell lines, plasmids, and chemical reagent

Mito-rsFastLime and pEGFP-C2 plasmids were prepared, as previously described (20, 21). The Vpr-IN-rsFastLime and Gag-IN-rsFastLime plasmids were constructed by swapping the
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sgF and EGFP, respectively, with rsFastLime (18, 19, 21), using BamiHI and NotI enzymes (New England Biolabs Inc.) in the Vpr-IN-sgF (45) and Gag-IN-EGFP (46) constructs. The pCAGGS vector expressing HIV-1 HXB2 Env glycoprotein has been described previously (47, 48). The HIV-Gag-imCherry plasmid encodes for the HIV-1 Gag protein tagged with an “internal” mCherry (38, 41, 49). Upon HIV-1 infection, Gag-imCherry is cleaved by the protease, generating free mCherry that remains trapped in the intra-viral space.

NIH 3T3 cells were grown as described previously (21). HEK293T/17 cells (from ATCC, Manassas, VA) and CV-1/CD4/CXCR4 (CF3 clone (48)) were grown in high-glucose Dulbecco’s modified Eagle’s medium (Mediatech, Manassas, VA) supplemented with 10% fetal bovine serum (FBS, Sigma) and 100 units/ml of penicillin-streptomycin (Gemini Bioproducts, Sacramento, CA). The growth medium for HEK 293T/17 was supplemented with 0.5 mg/ml of G418 sulfate (Mediatech).

Virus production

Fluorescently labeled pseudoviruses were produced and characterized, as described elsewhere (50). Briefly, HEK293T/17 cells grown in a 6-well culture plate were transfected with the following plasmids: pHIV-Gag-imCherryΔEnv (0.8 μg), HXB2 160wt (0.6 μg), pcRev (0.27 μg), and Gag-IN-rsFastLime or Gag-IN-EGFP (0.7 μg). Transfection was performed using the JetPrime Transfection reagent (VWR, Radnor, PA), according to the manufacturer’s protocol. At 6 h after transfection, the medium was replaced with 2 ml of fresh Dulbecco’s modified Eagle’s medium, 10% FBS without phenol red, and the transfected cells were incubated for an additional 36 h at 37 °C, 5% CO2. Viral supernatant was collected and passed through a 0.45-μm filter. Viruses were aliquoted and stored at −80 °C until use.

Fixed and live-cell imaging

For imaging mitochondria, NIH 3T3 cells were transfected with mito-rsFastLime and fixed, as described previously (21). The CF3 clone of CV-1 cells was transfected with 0.6 μg of mito-rsFastLime and 0.6 μg of pEGFP-C2 and fixed with 4% paraformaldehyde for 30 min. Samples were then washed three times with PBS + 20 mM Tris and imaged immediately or stored at 4 °C until use.

For single virus imaging in fixed or live cells, CF3 cells (5 × 105) were grown on 35-mm glass bottom dishes (MatTek Corp., Ashland, MA). HXB2 pseudoviruses co-labeled with HIV-Gagi-mCherryΔEnv and Gag-IN-rsFastLime or Gag-IN-EGFP were used to infect the cells at low multiplicity of infection, typically leading to 10–20 single virus particles bound per cell after spinoculation at 1200 × g for 30 min at 4 °C. After spinoculation, the unbound virus was removed by washing cells with cold Live Cell Imaging Buffer (LCIB, Life Technologies) supplemented with 2% FBS. For live-cell imaging, virus-decorated cells were transferred to a microscope stage maintained at 37 °C and imaged following the addition of pre-warmed LCIB.

Live-cell images were acquired in a single axial plane with a Personal DeltaVision microscope (Applied Precision, GE Healthcare), using an UPlanFluo ×40/1.3 NA oil objective (Olympus, Tokyo, Japan) and a DAPI/FITC/AF594 filter set (Chroma, Bellows Falls, VT). Multichannel fluorescence emission was sequentially recorded by an EM-CCD camera (Photometrics, Tucson, AZ). A single time point consisted of 5 images: FITC/FITC, DAPI/FITC, FITC/FITC, FITC/FITC, AF594/AF594. The exposure times for FITC/FITC, DAPI/FITC, and AF594/AF594 were 20, 5, and 5 ms, respectively. Total time to acquire a single composite time point including the instrument control overhead was 450 ms. Composite images were taken at every 5 s (0.2 Hz). During live-cell imaging an environmental chamber was used to maintain samples at 37 °C and high humidity and the UltimateFocus module (Applied Precision, GE Healthcare) was used to compensate for axial drift.

For fixed-cell imaging, rsFastLime virus were bound to cells and transferred to an air incubator maintained at 37 °C for 20 min to allow the cells to endocytose the bound virus. Samples were removed from the incubator, cooled to room temperature and treated with 4% paraformaldehyde for 30 min. Samples were then washed three times with PBS + 20 mM Tris and imaged immediately or stored at 4 °C.

Fixed cells were imaged with a spinning disk confocal system (UltraVIEW VoX, PerkinElmer Life Sciences) with a ×60/1.49 NA oil objective and set “UltraVIEW Dichroic” to “passes 405, 488, 561, and 640 nm laser lines.” Multichannel fluorescence images were recorded in series by an EM-CCD camera (C9100–23b back-thinned EM-CCD, Hamamatsu, Tokyo, Japan) with “UltraVIEW Emission Wheel” set to “430–472, 502–544, 582–618, 663–691.” A single frame consisted of 4 images: 488 nm (laser power: 100%. Exposure time: 150 ms), 405 nm (40%, 50 ms), 488 nm (100%, 150 ms), 488 nm (100%, 150 ms). Total time to acquire one demodulation frame, including instrumental processing time, was 571 ms in PerkinElmer spinning disk confocal imaging system.

Image analysis

Data were imported in Matlab (Mathworks, Natick, MA) by OME Bio-Formats (51). Demodulation images were calculated at each time point by subtracting the first 488 nm image (lm1, obtained using a standard FITC filter) from the second 488-nm image (lm2–lm1). The resulting demodulated images for rsFastLime and EGFP samples were exported as txt files without normalization and displayed with ImageJ (National Institutes of Health) with the same brightness setting for comparison. After the demodulation, single particle tracking in live cells was performed by ICY image analysis software (icy.bioimaganalysis.org) with ICY spot tracking algorithm.


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