Autofluorescence Suppression by Optically Controlling Dark States of Photoswitchable Fluorescent Proteins on Commercial Confocal Microscopes

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The specificity and biocompatibility of fluorescent proteins (FPs) have revolutionized cellular imaging by providing multiple colors for investigating biological interactions *in vivo*. However, inhomogeneous autofluorescence limits the applications of FPs, resulting in false positive signals that obscure interactions of interest. Autofluorescence background might be avoided by red-fluorescent proteins, but this limits the ability to carry out multi-color imaging.

The ability to optically control the photophysical, bright and dark states of photoswitchable fluorescent proteins (PS-FPs) provides an alternative means to avoid the background. For example, the bright state of the PS-FP rsFastLime is excited at 488nm and emits green fluorescence. After periods of emission, rsFastLime shuttles to a dark state, which can be depopulated by 405nm co-illumination. Since only rsFastLime fluorescence responds to both 405nm and 488nm lasers, single wavelength-generated autofluorescence background can be excluded.

We have developed synchronously amplified fluorescence image recovery (SAFIRe) microscopy, which involves modulating the intensity of both 488nm and 405nm lasers and extracting the signal shifted to the combinations of both laser modulation frequencies. After a proof-of-concept demonstration on a customized optical setup, we adapted SAFIRe to a commercial confocal spinning disk confocal microscope (PerkinElmer). Since the visualization of relatively dim HIV-1 virus-like particles (VLPs) entry into cells suffers from a comparable autofluorescence background, we utilize rsFastLime with SAFIRe for background removal. In brief, the HIV-1 VLPs labeled with rsFastLime are illuminated with alternating 488nm and 405nm lasers. While only rsFastLime is photoactivated by a 405nm laser, the autofluorescence background shows no photoactivation. Thus, we discriminate between rsFastLime and background fluorescence with 405nm laser illumination. After 3 cycles of emission and photoactivation in ~0.3 second, SAFIRe recovers background-free VLP-rsFastLime signals in cells. This cell-type free method enables more reliable VLP tracking and general applications to investigate nanoparticles, drug delivery, and intracellular molecular events in pharmaceutical product development.