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STED nanoscopy in living cells using Fluorogen Activating Proteins

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Abstract

We demonstrate the effectiveness of a genetically encoded Malachite Green (MG) binding fluorogen activating protein (FAP) for live cell stimulated emission depletion nanoscopy (STED). Both extracellular and intracellular FAPs were tested in living cells using fluorogens with either membrane expressed FAP or as an intracellular FAP-actin fusion. Structures with FWHM of 110-122nm were observed. Depletion data however suggests a resolution of 70nm with the given instrument.

Fluorescence microscopy has experienced an explosive growth of use over the past three decades, transforming from a disparate set of optical techniques to a cohesive toolset of imaging methodologies for biological research. This transformation has been greatly facilitated by the development of bright photo-stable fluorescent probes ranging from organic dyes(1,2) to genetically expressible fluorescent proteins(3) as targeted fusions at the molecular level. This, in conjunction with the development of new lasers and low-noise photodetectors has enabled the observation of many aspects of cell structure that were previously unseen. However, despite these incredible advances, conventional optical microscopy is inherently mismatched with biological length scales due to the far-field diffraction limit of light ($\lambda/2NA$); i.e. one cannot resolve objects that are separated by less than half the wavelength of light. This is particularly restrictive when the spatial location of multiple interacting objects is desired, especially when the size of most protein complexes are on the order of 10-50 nm, and even subcellular organelles have dimensions on the order of 100 nm. To overcome this fundamental obstacle, the last decade has seen the birth of the completely new field of far field superresolution optical microscopy, which has revealed many aspects of cellular ultra-structure previously only discernible in electron microscopy. Currently there are two distinct methodologies, one of which is based on single-molecule localization approaches, PALM (Photoactivation Localization Microscopy)(4,5) and STORM (STochastic Optical Reconstruction Microscopy)

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Supporting Information

Supporting information contains the following 1). materials and methods for the growth, transfection and imaging of yeast and mammalian cells, 2). the depletion efficiency of various different malachite green binding FAP modules, 3). the survival of the intracellular FAP module through fixation and permeabilization, 4). methods used to fit Gaussian and Lorentzian functions to extracted line profiles and 5). estimating the maximum achievable resolution using the Leica TCS-STED system. This information is available free of charge via the Internet at <http://pubs.acs.org/>.

(6) and the other on ensemble imaging employing either point-spread function engineering or low-frequency structured illumination(7,8).

Ensemble methods rely on the manipulation of the point spread function (PSF) of the microscope while imaging a complete ensemble of molecules. One very successful approach is Stimulated Emission Depletion (STED) nanoscopy(9). In this method an excitation laser beam is overlaid with a depletion laser that has an optically engineered point spread function, resulting in a much smaller effective fluorescing spot. Currently, the best attained STED resolution is 6 nm, obtained for photonic crystal centers(10).

Compared to localization, such ensemble methods do not routinely yield the same spatial resolution enhancements (especially in biological samples), but do provide much higher temporal resolution. In the case of STED, video rate imaging of 28 frames/second has been achieved allowing the observation of synaptic vesicle movement in living neurons(11). This is almost three orders of magnitude faster than the fastest PALM-based dynamic imaging reported to date at 0.04 frames/second(12). Further live cell STED with fluorescent proteins has been successfully demonstrated in imaging both dendritic spines(13) and the endoplasmic reticulum(14), both making use of Yellow Fluorescent Proteins (YFP). To minimize potential phototoxic effects in long-term live cell imaging experiments, especially with the high laser powers required for effective depletion, it is highly desirable to operate at wavelengths far to the red.

The far-red organic dye Atto647N has been applied in the case of video-rate STED of synaptic vesicle movement in living neurons(11) and to explore the nanoscale dynamics of lipid membranes using FCS-STED methodology(15). In addition, Atto655 has been used in conjunction with the Halo-Tag labeling technology to visualize β 1-integrin in non-living HeLa cell filopodia(16). Both of these dyes are compatible with the commercial Leica TCS STED system. Unfortunately these live cell approaches are inherently restrictive as they require either the uptake of a dye labeled lipid or antibody or internalization of an impermeant dye pre-bound to a genetic tag, which is not always biologically convenient. At present, no fluorescent protein has been reported to work with the commercial STED system in the same spectral range. As such, STED imaging of living cells in the far red has been limited to specific applications compatible with these labeling approaches. In order for the STED method to become generally useful for live cell imaging in the far red, it is highly desirable to have genetically targetable far-red fluorescent probes with quantum yields and spectral characteristics similar to those of the Atto Dyes. In this brief communication we describe the first use of genetically expressed fluorogen activating proteins (FAPs) that bind and activate the far-red fluorogenic dye Malachite Green for live-cell STED nanoscopy. These results using extra and intracellular compatible FAPs have widespread implications in the development of live cell STED nanoscopy for high-speed dynamic superresolution imaging.

Malachite Green is a far-red non-fluorescent organic dye that has been used to generate fluorescent signal when bound specifically to selected proteins (such as FAPs(17)) and nucleic acid aptamers(18). Because the dye exists in a non-fluorescent form it can be added to cells in media and/or buffer with no appreciable background signal. When the dye interacts specifically with its target, the fluorescence is activated up to ~20,000 fold resulting in genetically targetable, bright far-red emission. The photo-physical properties of Malachite Green are well-suited to excitation and depletion in the red and near infra-red spectral range as is available on the Leica TCS-STED system.

The STED potential for different Malachite Green binding extracellular FAP modules was evaluated using yeast cells displaying the FAP on the cell surface. Yeast cells were grown and induced as described in Szent-Gyorgy *et. al.* (17) and imaged as described in the Supplementary

Data. Figure 1A illustrates the implementation of the STED microscope in a commercial apparatus. Briefly a visible red excitation spot is overlapped with a “donut” shaped engineered PSF of the infrared laser to enable shrinkage of the effective fluorescing spot size by stimulated emission. A variety of MG binding FAPs with 100nM of the MG-2p fluorogen were tested throughout a range of wavelengths (730-750nm) to elucidate which gave the most efficient depletion of fluorescence. The most promising module was L5-MG-L90S which is a single-point mutation of the native single-chain FAP (L5-MG). Figure 1B illustrates both confocal and STED images with the extracellular L5-MG-L90S expressed on the surface of yeast (average image acquisition times were 5 s at a 100 Hz scan speed, and images were averaged 4 times to improve the signal-to-noise ratio). This FAP has a quantum yield of 0.23 and excitation and emission characteristics comparable to the organic dye Atto647N which makes it amenable to commercial STED nanoscopy (see Table 1 for the characteristics of different FAP modules and Atto dyes). In order to quantify the gain in lateral resolution a 10 pixel wide region of interest was designated in the zoomed confocal and STED images (denoted by hollow white bars) and the fluorescence intensity was averaged using NIH ImageJ to generate the line profiles depicted in Figure 1C. The line profiles were fitted to one-dimensional Gaussian (confocal) or Lorentzian (STED) functions which yielded full-width half-maximum (FWHM) of 236nm and 122nm respectively. This measured FWHM is somewhat higher than the expected subdiffraction limited STED resolution from the measured depletion curves which is expected to be ~70nm for the given system and dye (see Supplementary Data) but is consistent with current estimates of the yeast cell wall thickness(19).

As stated previously, the major benefit of using MG FAPs for STED nanoscopy is the unique ability to produce genetically targeted fusions in living cells. But, despite this advantage, to make the most use of STED as a high-speed superresolution imaging technology for cellular applications it is necessary to have a fluorescent probe that functions within the cytoplasm of living cells. Given the noted folding problems of scFvs in reducing environments due to the loss of the stabilizing disulfide bridges(20), the H6-MG FAP has been modified via site-directed mutagenesis to remove the cysteine residues and subsequent affinity maturation to regain fluorogenic activation (H6-MG-AFM—Manuscript in Preparation). This FAP has been expressed transiently as an N-terminal fusion to actin in living HeLa cells. Figure 2A illustrates visible stress fibers after labeling with 300nM of the permeant MG-ester fluorogen (average image acquisition times were 4 s at a 400 Hz scan speed, and images were averaged 8-10 times to improve the signal-to-noise ratio.). The optically zoomed panels show the increase in the lateral resolution with many features obscured in the confocal image becoming distinct in the STED image. To determine structure size, a 5 pixel wide region of interest was designated (denoted by the hollow white bar) and the fluorescence intensity averaged in NIH ImageJ to generate the line profiles depicted in Figure 2B. As before, the profiles were fitted to one-dimensional Gaussian and Lorentzian functions, yielding the FWHM of each peak. In the confocal image the fitted FWHM was 327nm and in the STED image was 110nm. This resolution is consistent with previous measures of actin stress fiber diameter via transmission electron microscopy (TEM)(21). In addition, living NIH 3T3 fibroblast cells also expressing the H6-AFM-MG FAP fused to actin, which were fixed and permeabilized and then labeled with 100nM MG-2p, still showed visible stress fibers despite the fixation process (see supplementary data). Hence these probes can be used for live cell STED and combined with conventional immunofluorescence assays in fixed cells.

We have demonstrated the first use of fluorogen activating proteins for STED nanoscopy in living cells. The biological structures imaged exhibited FWHMs in STED that were reduced by up to a factor of three over confocal microscopy. In this case, the measured FWHM is likely due to the sizes of the actual biological structures measured(21) and does not reflect the true sub-diffraction limit of the probe and instrument. The ultimate achievable resolution as indicated by the depletion curves is equivalent to that of Atto647N which was determined to

be ~70nm for the system used (see supplementary data). These new genetically targetable far-red probes coupled with STED nanoscopy have the potential for making long-term dynamic measurements of living cells at near video-rate and high resolution with reduced phototoxicity relative to conventional fluorescent proteins.

Synopsis

STED nanoscopy in living cells has been demonstrated using a genetically encoded fluorogen activating protein (FAP) which binds and activates the fluorescence of the “dark” fluorogenic dye Malachite Green (MG). A greater than two-fold enhancement over the diffraction limit was observed in the structures measured, for both extracellular and intracellular targets. Imaging was accomplished in the presence of excess fluorogenic dye without any required washing.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. STED nanoscopy in living yeast cells

A. Schematic of commercial Leica TCS STED optical setup. A pulsed 635nm excitation diode laser is overlapped with the “donut shaped” pulsed depletion infrared laser beam to produce a subdiffraction limited fluorescing spot whose diameter is approximated by

$\Delta r \approx \lambda / 2NA \sqrt{1 + I_{\text{Dep}} / I_{\text{Sat}}}$ (22) where NA is the objective numerical aperture, I_{Dep} is the depletion intensity and I_{Sat} is the intensity of saturation which is fluorophore dependent. **B.** Confocal and STED images of living yeast cells surface expressing the extracellular L5-MG-L90S FAP module labeled with MG-2p fluorogen. Hollow white bar denotes a 10 pixel wide region of interest. **C.** Line profiles generated from the averaged fluorescence intensity within the region of interest plotted as a function of distance. Solid lines indicate a fit to one-dimensional Gaussian (confocal) or Lorentzian (STED) functions. FWHM are 236nm (confocal) and 122nm (STED). Solid white lines indicate 1 μ m scale bars.

Figure 2. STED nanoscopy in living HeLa cells

A. Living HeLa cells expressing the H6-MG-AFM disulfide free FAP module expressed as an N-terminal fusion to actin labeled using the MG-ester fluorogen (upper left panel confocal, upper right panel STED, with a zoomed in region denoted by the dashed line box below each image). Hollow white bar denotes a 5 pixel wide region of interest. **B.** Line profiles generated from the averaged fluorescence intensity within the region of interest plotted as a function of distance. Solid lines indicate a fit to a one-dimensional Gaussian (confocal) or Lorentzian (STED) functions. The fitted FWHM are 327nm (confocal) and 110nm (STED). Solid white lines indicate 5 μ m scale bars in the upper images, and 1 μ m scale bars in the cropped images.

Table 1

Comparison of the photo-physical characteristics of Malachite Green binding Fluorogen Activating Proteins, MG FAPs (obtained from Szent-Gyorgyi *et al* (17)) and the Atto-Tec organic dyes (obtained from <http://www.atto-tec.com/>) used in far red STED nanoscopy.

Dye/Fluorogen	Fluorogen activating protein	Excitation maximum (nm)	Emission maximum (nm)	Extinction coefficient ($M^{-1}cm^{-1}$)	Quantum yield (Φ)	Brightness $\epsilon * \Phi$
MG-2p	HL4-MG	629	649	133,000	0.16	21
	H6-MG	635	656	105,000	0.25	26
	L5-MG (wt)	640	668	103,000	0.048	5
	L5-MG-L90S	632	664	128,000	0.38*	49
Atto647N	-	644	669	150,000	0.65	97
Atto655	-	663	684	125,000	0.30	37

* QY determined relative to native L5-MG