Dual Channel RESOLFT Nanoscopy by Using Fluorescent State Kinetics

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Supporting Information

ABSTRACT: We show that RESOLFT fluorescence nanoscopy, a low light level scanning superresolution technique employing reversibly switchable fluorescent proteins (rsFPs), is capable of dual-channel live-cell imaging that is virtually free of chromatic errors and temporal offsets. This is accomplished using rsEGFP and Dronpa, two rsFPs having similar spectra but different kinetics of switching and fluorescence emission. Our approach is demonstrated by imaging protein distributions and dynamics in living neurons and neuronal tissues.

KEYWORDS: Photoswitching, nanoscopy, lifetime, low intensities

Epi-fluorescence and confocal microscopy are widely used to quantify molecular localizations in living cells, albeit with a resolution limited by diffraction. To investigate how different molecular species are colocalized in space, the species are tagged with fluorophores having different absorption or emission spectra. The advent of far-field fluorescence nanoscopy (or superresolution) techniques boosted the utility of multiple channel recording because they can discern molecules or molecular aggregates at the relevant nanoscale. Therefore, both the scanning nanoscopy techniques called STED and RESOLFT as well as their single molecule based counterparts called PALM or STORM have already been realized in multicolor formats.1−4 However, each of these realizations have their own limitations: they require relatively high light intensities, cannot record the color channels simultaneously, or are limited by the number of bright switchable fluorescent proteins available for living cells.

Of these techniques, emergent RESOLFT nanoscopy is very appealing for superresolving living cells and tissues5 because it requires light intensities and operates with reversible switchable fluorescent proteins (rsFPs), such as rsEGFP,6,7 Dreiklang,8 and various variants of Dronpa.9 The absorption and emission spectra of these rsFPs strongly overlap, prohibiting separation by color. This problem can be solved by tagging proteins with red-emitting rsFP rsCherryRev,10 but this rsFP tends to dimerize, is dim, and switches slowly. The latter is particularly disadvantageous for a point scanning RESOLFT microscope. Furthermore, the multiple (concretely five) beams required to repeatedly activate, deactivate, and excite both the green and red emitting proteins not only increase complexity but also the need to compensate for chromatic measurement errors. Here, we demonstrate the overcoming of these limitations by employing two monomeric green-emitting rsFPs whose fluorescence can be easily discerned by their different switching kinetics and fluorescent lifetime decays. Our experiments were performed with a single-point scanning RESOLFT microscope featuring a time correlated single photon counting card, a pulsed laser for excitation (100 ps pulses, 80 MHz repetition rate, and 491 nm wavelength), and continuous wave on- and

Received: August 8, 2014
Revised: November 5, 2014
Published: November 25, 2014
off-switching beams (Figure S1, Supporting Information). We named this dual-channel modality τ-RESOLFT.

Some Dronpa and rsEGFP mutants exhibit similar spectral properties but different fluorescence lifetimes, which vary slightly depending on the specific rsFP-tag construct. Both protein families can be activated by near-UV light (405 nm), whereas blue light (491 nm) causes fluorescence emission and switches them back into a long-lived dark state (Figure 1). We selected the variant DronpaM159T due to its rapid switching kinetics and short fluorescence lifetime \( \tau \approx 0.6 - 0.9 \) ns. For the second channel we opted for the mutant rsEGFPN205S because of its longer lifetime of \( \tau \approx 1.5 - 1.8 \) ns (Figure S3, Supporting Information). Furthermore, the two rsFPs have markedly different switching off kinetics (Figure 1D): for a given intensity, Dronpa converts 2–16 times faster to the dark state than rsEGFP. This can be used to further enhance the separation of the two channels (Figure S5, Supporting Information).

For imaging, we used off-switching intensities and exposure times that were sufficiently high to switch even the majority of the slow switching rsEGFP off. This enabled spatial resolutions of <40 nm in both channels (Figure S6, Supporting Information). Although 0.3–1 kW/cm\(^2\) would have been fully sufficient to convert Dronpa rapidly into the dark state, we applied 3–6 kW/cm\(^2\) in order to complete the rsEGFP off-switching as well. Note that this additional intensity does not necessarily photostress the Dronpa molecules because they turn dark rapidly. With these parameters, a single off-switching doughnut-shaped beam of blue light was capable of simultaneously confining the emission of both proteins around the same central point in the sample. The arrival time of the fluorescence photons originating from this subdiffraction region was determined and then used to separate the two rsFP.

We tested our method by imaging neurons located 30–50 μm deep inside living organotypic hippocampal brain slices (Figure 2). Imaging living neurons greatly benefits from the comparatively low light levels at which RESOLFT operates, as they reduce phototoxicity. We used 1 kW/cm\(^2\) for on-switching (0.64 μW, Gaussian shaped beam with fwhm = 220 nm, applied for 50 μs), 3.7 kW/cm\(^2\) for excitation (2.4 μW Gaussian shaped beam with fwhm = 240 nm, for 50 μs), and 2 kW/cm\(^2\) for off-switching (equaling 4 μW to create a ring-shaped beam 300 nm peak to peak, it was applied for 400 μs). We infected the neurons with viruses coding for the fusion proteins homer1c-rsEGFPN205S and LifeAct-DronpaM159T (Figure 2A). Homer is a protein present in the postsynaptic densities
(PSDs) of spine heads, whereas LifeAct is a short peptide that binds to actin. The tagging was also reverted, as indicated in Figure 2B. The imaging was performed 16–48 h after viral infection. The raw RESOLFT data was recorded in a single scan, yet the different kinetics allow both Dronpa and rsEGFP to be separated clearly (Figure 2A).

The resolution increase provided by RESOLFT becomes apparent when comparing the resulting images with their confocal counterparts (Figures 2C,D and S2, Supporting Information). Regardless if DronpaM159T or rsEGFPN205S were used as the label, both LifeAct and homer were identified in the dendritic spine heads, with LifeAct further highlighting the actin bundles in the dendritic spine necks, dendritic shafts, and in cell bodies (Figure 2). Some colocalization was also visible in the dendritic shafts, presumably because of the presence of g-actin and/or unbound LifeAct and homer molecules.

Dendrites from two different neurons can be recognized in Figure 2B, one of them expressing homer–DronpaM159T and the other one expressing LifeAct–rsEGFPN205S.

The efficiency of signal separation, here accomplished with 5–20% crosstalk, slightly varies depending on the fluorescent photon counts detected (Figure S4, Supporting Information) and the actual lifetimes of the Dronpa and rsEGFP constructs (Figure S7, Supporting Information), which depend on the local molecular environments. Nonetheless, the separation of the two channels is robust and crosstalk low (Figure S8, Supporting Information).

Next we imaged neuronal mitochondria in dissociated cultured primary hippocampal neurons. For this, the rsFPs were expressed in the mitochondrial lumen, in fusion with a mitochondrial localization sequence. The mitochondria displayed typical size and morphology (diameters 100–230 nm) and exhibited regular fusion and fission behavior. Because these organelles are particularly sensitive to light, the low light intensities (0.8–5 kW/cm²) employed enabled imaging their dynamics over 1.5 h without any sign of fragmentation or swelling (Figure 3). In dual-stained neurons we observed the expected morphological changes and dynamics of mitochondria along actin networks. Thus, the comparatively fast dwell time (300–500 μs switching-off time per pixel) and the inherently coaligned scanning scheme of τ-RESOLFT enabled these processes to be resolved with high precision. We observed constant fluctuations of the diameter of the mitochondria in the image over time. In one particular case, we observed the width of a mitochondria thinning down to 61 nm at the ends (Figure 3 green arrow), presumably following fission activities.

In summary, our τ-RESOLFT approach provides a fast and direct solution for colocalization analysis at the nanometer scale in living cells and tissues. τ-RESOLFT imaging is inherently offset-free because the colocalized molecular species are recorded with virtually no spatial and temporal offset. Another considerable advantage of using a single off-switching beam is that it can be easily corrected for aberrations, which is important for imaging complex tissues, such as living brain slices or the brain of living mice. So far, τ-RESOLFT has been performed with the fastest rsFPs currently available, but it can be expanded to use other rsFPs without difficulty. Furthermore, channel separation by kinetics can be readily expanded to commercial RESOLFT or confocal setups with relatively minor adaptations. In conclusion, RESOLFT nanoscopy can now be expanded to multiple coaligned channels that leave the yellow and red part of the spectrum free for additional channels and also for other colocalization approaches, such as FRET.

**ASSOCIATED CONTENT**

Supporting Information

Additional methods and figures. This material is available free of charge via the Internet at http://pubs.acs.org.

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**Notes**

The authors declare no competing financial interest.

**ACKNOWLEDGMENTS**

We thank J. Jethwa and T. Gilat for technical assistance, A. Schöne for the software ImSpector, and G. Vicipidini for data analysis codes. This work was supported by the Center for Nanoscale Microscopy and Molecular Physiology of the Brain (Göttingen) and the Körber Foundation (Hamburg).

**ABBREVIATIONS**

RESOLFT, reversible saturable fluorescent optical transition; GFP, green fluorescent protein; τ, fluorescent lifetime

**REFERENCES**


dx.doi.org/10.1021/nl503058k | Nano Lett. 2015, 15, 103–106