Supporting Information

Dual-label STED nanoscopy of living cells using photochromism

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Material & Methods

Generation of new termini

New termini (N- and C-) for Dronpa-M159T and Padron were generated by amplifying the respective coding sequence by PCR using the primers:

ACGGATCCAATGGTGAGCAAGGGCGAGGAGAACAACATGGCCGTGATTAAACC AGAC

and

ATTAAGCTTCGAATTCTTACTTGTACAGCTCGTCCATGGCCTGCCTCGGCAG.

The PCR fragments were digested with BamHI and HindIII and inserted into the vector pQE31 (Qiagen, Hilden, Germany).

The muatation E218G was introduced using the QuikChange Site Directed Mutagenesis Kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions.

The modified Padron and Dronpa-M159T were named Padron$^{v2.0}$ and Dronpa-M159T$^{v2.0}$, respectively.

Spectral analysis

Fluorescence and absorption spectra of purified Dronpa-M159T and Padron (with modified termini) protein were recorded with a Varian Cary Eclipse fluorescence spectrophotometer (Varian, Palo Alto, CA) and a Varian Cary 4000 UV/VIS spectrophotometer, respectively.

Plasmid generation

For the generation of fusion constructs between Dronpa-M159T$^{v2.0}$ / Padron$^{v2.0}$ and the microtubule associated protein 2 (Map2), the coding sequence of the respective RSFP was amplified by PCR using

the primers:

GATCCGCTAGCGCTAATGGTGAGCAAGGGCGAGGAG

and

CACTCGAGATCTGAGTCCGGACTTGTACAGCTCGTCCATGCC.

The products were digested with NheI and BglII and inserted into the vector pEGFP-Tub (Clontech, Mountain View, CA), replacing the EGFP sequence. Subsequently, the Tubulin coding sequence was exchanged against the Map2 coding sequence (obtained from pDONR223-Map2 [1]) using the primer:

GATCTCGAGTGATGGCAGATGAACGGAAAGACGAAGC

and

GGTGGATCCTTATCACAAGCCCTGCTTAGCGAGTGCAGC.

The PCR fragment was digested with XhoI and BamHI and inserted into the vectors Dronpa-M159T$^{v2.0}$-α-tubulin and Padron$^{v2.0}$-α-tubulin replacing the α-tubulin sequence.

Fusion constructs of Dronpa-M159T$^{v2.0}$ and Padron$^{v2.0}$ with gap junction protein Connexin37 (Cx37), Keratin 19 (Krt19), Peroxisome protein 16 (Pex16) and Caviolin 1 (Cav1) were generated by gateway cloning. The RSFPs were amplified by PCR using the primers:

GATCCACCGGTCGCGGCGTGAGCAAGGGCGAGGAG

and

ACAACTTAAGAACAACAATTGTTACTTGTACAGCTCGTCCAT

The PCR product was digested with AgeI and AflII and inserted into the gateway destination vector pH-ACP-N. The final plasmids were constructed by gateway vector conversion (Invitrogen, Carlsbad, CA) using the donor vectors pDONR223-Cx37, pDONR223-Krt19, pDONR223-Pex16 and pDONR223-Cav1[1].

Cell culture and transfection

PtK2 (*Potorous tridactylus*, kidney) and Vero (*Cercopithecus aethiops*, kidney) cells were cultured in DMEM supplemented with 10% FCS, 1mM sodium pyruvate, 100 units/ml penicillin, 100µg/ml streptomycin (all GIBCO-Invitrogen), and 1 mM pyruvate (Sigma) at 37°C, 90% humidity and 5% CO2. 24 hours after seeding the cells on cover glasses, they were transfected with endotoxin-free DNA by using Nanofectin (PAA). For double transfection with Padron $^{v2.0}$ and Dronpa-M159T $^{v2.0}$ constructs equal amounts of the plasmids were mixed before transfection. Only Ker19-Padron $^{v2.0}$ and Pex16-Dronpa-M159T $^{v2.0}$ were transfected in a 6:1 plasmid ratio. Cells were incubated for at least 24 h before imaging.

STED microscopy

The home built STED microscopy setup has been detailed previously[2, 3]. The excitation laser emits pulses of 70 ps duration at a wavelength of 488 nm (Toptica Photonics, Graefelfing, Germany). The 300 ps STED pulses at 595 nm originated from an optical parametric oscillator (OPO, APE, Berlin) pumped by a Ti:Sapphire laser. A helical phase ramp was imprinted on the STED light by a polymeric phase plate (RPC Photonics, Rochester, NY) to produce a doughnut shaped intensity profile in the focal plane of the 1.3 NA objective lens (PL APO, CORR CS, 63x, glycerol, Leica, Wetzlar, Germany). Images were recorded with a resonant mirror scanning (15 kHz, SC-30, EOPC, Glendale, NY) along the x-axis; scanning along the y-axis was performed by a piezo-stage (P-733, Physik Instrumente, Karlsruhe, Germany). For switching the RSFPs, a 405nm laser line was inserted into the optical path by means of a custom made dichroic mirror. The diffraction focal spots of 405 nm, 488 nm and 595 nm were matched in space by detecting the back scattered light of each wavelength with a gold bead of 150 nm diameter. To avoid the autofluorescence background elicited by 405 nm illumination, we alternated the 405 nm illumination (which is used for switching only) with that at 488 nm. Therefore, a signal reflecting the resonant mirror deflection was used to trigger a fast shutter in the 488 nm and another one in the 405nm path. The 405 nm light was turned on in one half of the duty cycle of the resonant mirror and 488 nm in the other half. Fluorescence was detected only when 488 nm was on and 405nm off. The images were smoothed with a Gaussian low pass filter of 1.5pixels. The fluorescence lifetime data on living cells was acquired with a time-correlated single-photon counting module (SPC-140, Becker & Hickl GmbH, Berlin, Germany).

References

1. Lamesch, P.; Li, N.; Milstein, S.; Fan, C. Y.; Hao, T.; Szabo, G.; Hu, Z. J.; Venkatesan, K.; Bethel, G.; Martin, P.; Rogers, J.; Lawlor, S.; McLaren, S.; Dricot, A.; Borick, H.; Cusick, M. E.; Vandenhaute, J.; Dunham, I.; Hill, D. E.; Vidal, M., h0rfeome v3.1: a resource of human open reading frames representing over 10,000 human genes. *Genomics* 2007, *89* (3), 307-315.

2. Westphal, V.; Rizzoli, S. O.; Lauterbach, M. A.; Kamin, D.; Jahn, R.; Hell, S. W., Video-Rate Far-Field Optical Nanoscopy Dissects Synaptic Vesicle Movement. *Science* 2008, *320* (5873), 246-249.

3. Hein, B.; Willig, K. I.; Hell, S. W., Stimulated emission depletion (STED) nanoscopy of a fluorescent protein-labeled organelle inside a living cell. *Proceedings of the National Academy of Sciences of the United States of America* 2008, *105* (38), 14271-14276.