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Supplementary Material

In Vivo Labeling Method using a Genetic Construct for Nanoscale Resolution Microscopy

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Supplementary Material for "In Vivo Labeling Method using a Genetic Construct for Nanoscale Resolution Microscopy"

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MATERIALS AND METHODS

The HaloTag® 655 ligand was created by conjugating Atto655 dye (ATTO-TEC GmbH, Siegen, Germany) to the Halo Tag® amine (O4) ligand building block (Promega Corporation) and purified using HPLC (2).

HeLa cells (ATCC CCL-2) or U2-OS cells (ATCC HTB-96™) stably expressing β1-Integrin-HaloTag® fusion protein were maintained respectively in DMEM/F12 or McCoy 5A media (Gibco®) supplemented with 10% fetal bovine serum (FBS, ATCC) at 37°C and 5% CO_2.

HeLa cells were transfected with β1-Integrin-HaloTag® construct using the TransIT®-LTI Transfection Reagent (Mirus). Twenty four hours post-transfection cells were labeled with the Atto655 ligand (5μM, 15 min, 37°C; cell impermeant), fixed with 2% paraformaldehyde to prevent cell movement during imaging, and image cells using a Leica TCS STED microscope.

U2-OS cells stably expressing β1-Integrin-HaloTag® fusion protein were labeled with the HaloTag® AlexaFluor®488 ligand (1μM, 15 min, 37°C; cell impermeant) then HaloTag® TMR ligand (5 μM, 15 min, 37°C; cell permeant) and were imaged live on a confocal microscope FluoView500 (Olympus) using appropriate filter sets.

CONTROL EXPERIMENTS

To demonstrate that the HaloTag® 655 ligand can be used to study localization of the β1-Integrin-HaloTag fusion protein in cells, we first had to: a) test ability of the ligand to form highly specific covalent bond with the HaloTag® reporter protein in living cells; b) assess the nature of the ligand-cell interaction (e.g. cell-permeability of the ligand); and c) assess effect of the ligand on cell viability and cell morphology. To address these issues we have used U2OS cells stably expressing the β1-integrin-HaloTag® fusion (1,2). When these cells are labeled in pulse-chase mode with the HaloTag®AlexaFluor®488 ligand (cell impermeant) then with the HaloTag®TMR ligand (cell permeant) two separate protein pools are detected, i.e. surface exposed and intracellular pools (Fig. 1).

We incubated live U2OS cells expressing β1-integrin-HaloTag® with the HaloTag® 655 ligand, quickly washed out unbound ligand, relabeled cells with cell impermeant HaloTag® AlexaFluo®488 ligand, and imaged cells. The images show that the HaloTag® 655 ligand efficiently binds all available β1-integrin-HaloTag®, as shown by the lack of Alexa-488 label (Fig. 2A). Further, the HaloTag® 655 ligand labeling pattern is similar to the labeling pattern observed with cell impermeant ligands (Fig.1).

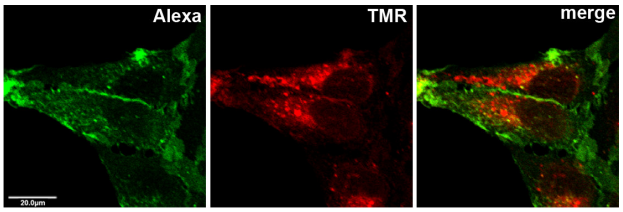

FIGURE 1: Two distinct pools of β1-integrin are labeled in single cells using Halo Tag®. U2OS cells expressing β1-integrin-Halo Tag® were consecutively labeled with the Alexa Fluor® 488 ligand and then the TMR ligand and imaged live by confocal. The cell impermeant Alexa ligand (green) labels the plasma membrane (surface pool), while the cell permeant TMR ligand (red) labels the internal protein pool.

The reverse pulse-chase labeling experiment in which cells were first incubated with the HaloTag®AlexaFluor®488 ligand and then with the HaloTag®655 ligand, shows efficient HaloTag®AlexaFluor®488 ligand labeling and no labeling with the HaloTag® 655 ligand (Fig. 2B). This confirms the impermeability of the HaloTag® 655 ligand.

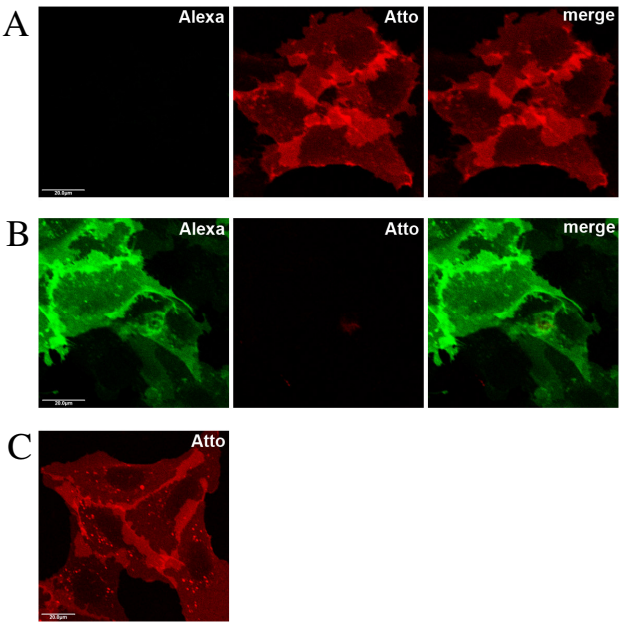

FIGURE 2: Atto655 ligand exhibits specificity of labeling, cell impermeability and is non-disruptive to cells. U2OS cells expressing β1-integrin-Halo Tag® were labeled in various ways and imaged live by confocal. Cells labeled with Atto655 ligand followed by Alexa Fluor® 488 show no Alexa labeling (green); indicative of complete and specific labeling of Halo Tag® on the plasma membrane by the Atto655 ligand (red) (A). Conversely, labeling with Alexa Fluor® 488 ligand followed by Atto655 ligand shows no Atto655 ligand (red) labeling, confirming the cell impermeability of this ligand (B). Labeling with the Atto655 ligand and incubating the cells for 24 hours prior to imaging shows the expected internalization of some β1-integrin protein (red punctae within cells) (C).

Taken together these data indicate that the HaloTag® 655 and HaloTag®AlexaFluor®488 ligands compete for the same binding cites on the cell surface and that the HaloTag®655 labeling is therefore highly specific. Most importantly for this study, the HaloTag® 655 ligand is cell impermeant (at least under tested conditions). The bond between the Atto655 ligand and the β1-Integrin-HaloTag fusion protein is stable under the denaturing conditions used for SDS-PAGE (0.1% SDS; 95 °C; 5 min; data not shown) or immuno-cytochemistry (treatment with paraformaldehyde; Fig. 2A, 2B and 2C). In addition, the ligand has no detectable effect on cell viability (data not shown), cell morphology or internalization of β1-Integrin-HaloTag fusion protein (Fig. 2C).

STED IMAGING

STED images were acquired with a Leica TCS STED confocal microscope (Fig. 3) using a Leica HCX PL APO CS 100 x 1.40 STED-lens and the pinhole was adjusted at one airy unit.

The fixed cells were excited by a single wavelength 635 nm diode laser and for depletion Spectra-Physics MaiTai Broadband multiphoton laser was used at 780 nm (laser power ~70mW in focus). Scan frequency was set to 100 Hz in combination with 6 fold line average in a 2048 x 512 format (pixel size 30 nm). For image acquisition the avalanche photo diode was used.

The STED images are deconvolved by a single step linear Tikhonov filter.

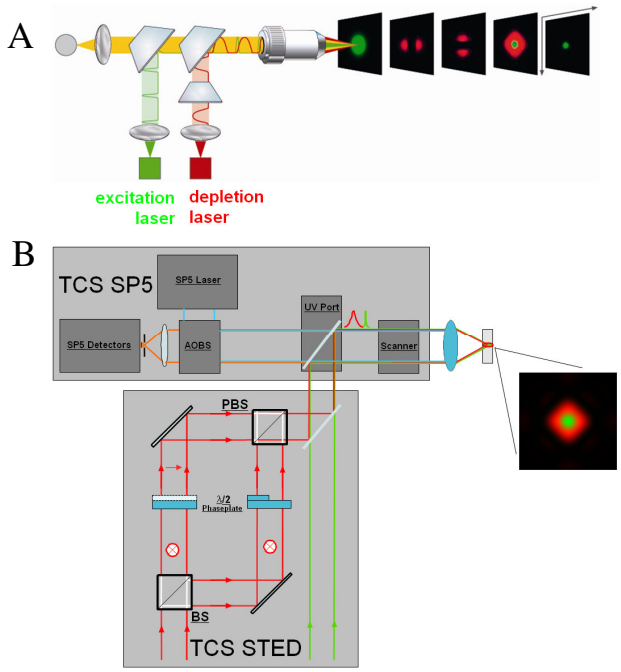

FIGURE 3: Beampath of the Leica TCS STED microscope. The excitation laser is shown in green, depletion laser in red (A). The depletion laser is divided in to parts (B) by a beamsplitter (BS) and the two parts are polarized perpendicularly by a λ/2 Phaseplate. The polarizing beamsplitter (PBS) merges the two parts and a bagel like shaped depletion pattern is generated.

REFERENCES AND FOOTNOTES

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