Supplementary Information for:

Dual-Activatable Cell Tracker for Controlled and Prolonged Single-Cell Labeling

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METHODS

General procedure for the synthesis of DACTs via acyl substitution of compound 5. 2'-diazo-12-hydroxy-2,3,6,7-tetrahydro-1H,5H-spiro[chromeno[2,3-f]pyrido[3,2,1-ij]quinoline-9,1'-inden]-3'(2'H)-one **5** (25 mg, 0.06 mmol)[27] was dissolved in dry CH_2Cl_2 (230 µL) in a flame-dried flask. Then the corresponding acyl chloride and DIPEA (34 µL, 0.20 mmol) were sequentially added. After 10 min the volatiles were evaporated under reduced pressure. The residue was taken up in CH_2Cl_2. The organic phase was extracted three times with water and once with brine. The organic layer was dried over $MgSO_4$, filtered and concentrated under reduced pressure. The residue was purified by FC (SiO_2; CH_2Cl_2 to CH_2Cl_2/CH_3OH 98:2) taking care of keeping the compound always shielded from light exposure.

2'-diazo-3'-oxo-2,2',3,3',6,7-hexahydro-1H,5H-spiro[chromeno[2,3-f]pyrido[3,2,1-ij]quinoline-9,1'-inden]-12-yl acetate (DACT-1): Following the general procedure, addition of acetyl chloride (20 µL, 0.20 mmol) gave DACT-**1** as a yellow solid (9.5 mg, 0.02 mmol, 35%) after pentane wash. ^{1}H NMR (300 MHz, $CDCl_3$) δ = 8.01 (d, J = 7.0 Hz, 1H, H12), 7.66 (td, J = 7.4, 1.5 Hz, 1H, H13), 7.60 (td, 1H, H14), 7.20 (d, J = 7.5 Hz, 1H, H15), 7.09–7.05 (m, 1H, H8), 6.74 (d, J = 0.7 Hz, 1H, H11), 6.72 (d, J = 2.0 Hz, 1H, H10), 6.14 (s, 1H, H1), 3.16 (dt, J = 11.3, 5.6 Hz, 4H, H4 and H5), 2.92 (t, J = 6.6 Hz, 2H, H7), 2.61–2.45 (m, 2H, H2), 2.30 (s, 3H, H9), 2.07–1.97 (m, 2H, H6), 1.92–1.82 (m, 2H, H3) ppm. HRMS (ESI) calcd. for $[C_{29}H_{24}N_3O_4]^+$: 478.1761, found: 478.1759.

2'-diazo-3'-oxo-2,2',3,3',6,7-hexahydro-1H,5H-spiro[chromeno[2,3-f]pyrido[3,2,1-ij]quinoline-9,1'-inden]-12-yl propionate (DACT-2): Following the general procedure, addition of propionyl chloride (20 µL, 0.23 mmol) gave DACT-**2** as a beige solid (8 mg, 0.016 mmol, 28%) after pentane wash. ^{1}H NMR (400 MHz, $CDCl_3$) δ = 7.83 (d, J = 7.5, 1.4, 0.7 Hz, 1H, H13), 7.48 (td, J = 7.5, 1.4 Hz, 1H, H14) , 7.42 (td, J = 7.4, 1.1 Hz, 1H, H15), 7.08 (dt, J = 7.7, 0.9 Hz, 1H, H16), 6.99 (d, J = 2.3 Hz, 1H, H8), 6.88 (d, J = 8.6 Hz, 1H, H12), 6.69 (dd, J = 8.6, 2.3 Hz, 1H, H11), 6.28 (s, 1H, H1), 3.20–3.04 (m, 4H, H4 and H5), 2.88 (t, J = 6.6 Hz, 2H, H7), 2.63–2.44 (m, 2H, H2), 2.58 (q, J = 7.5 Hz, 2H, H9), 2.02 (p, J = 6.4 Hz, 2H, H6), 1.95–1.82 (m, 2H, H3), 1.26 (t, J = 7.5 Hz, 3H, H10) ppm. ^{13}C NMR (101 MHz, $CDCl_3$) δ = 187.21, 172.63, 155.83, 151.93, 150.69, 146.98, 143.39, 134.69, 134.35, 128.79, 128.46, 125.59, 124.65, 122.29, 119.10, 118.14, 116.80, 110.44, 108.00, 107.01, 49.90, 49.42, 27.77, 27.21, 21.88, 21.33, 21.13, 9.05.ppm. HRMS (ESI) calcd. for $[C_{30}H_{26}N_3O_4]^+$: 492.1918, found: 492.1915

2'-diazo-3'-oxo-2,2',3,3',6,7-hexahydro-1H,5H-spiro[chromeno[2,3-f]pyrido[3,2,1-ij]quinoline-9,1'-inden]-12-yl cyclobutanecarboxylate (DACT-3): Following the general procedure, addition of cyclobutanecarbonyl chloride (26 µL, 0.23 mmol) gave DACT-**3** as a beige solid (17 mg, 0.033 mmol, 57%) after pentane wash. ^{1}H NMR (400 MHz, $CDCl_3$) δ = 7.82 (d, 1H, H15), 7.45 (dtd, *J* = 26.5, 7.4, 1.2 Hz, 2H, H16 and H17), 7.08 (dt, *J* = 7.7, 0.9 Hz, 1H, H18), 6.99 (d, *J* = 2.3 Hz, 1H, H8), 6.88 (d, *J* = 8.6 Hz, 1H, H14), 6.68 (dd, *J* = 8.6, 2.3 Hz, 1H, H13), 6.28 (d, 1H, H1), 3.37 (p, *J* = 8.5, 1.1 Hz, 1H, H9), 3.18–3.05 (m, 4H, H4 and H5), 2.88 (t, *J* = 6.6 Hz, 2H, H7), 2.63–2.47 (m, 2H, H2), 2.47–2.26 (m, 4H, H10 and H12), 2.11–1.93 (m, 4H, H3 and H11), 1.92–1.83 (m, 2H, H6) ppm. ^{13}C NMR (101 MHz, $CDCl_3$) δ = 187.38, 173.74, 155.99, 152.08, 150.92, 147.12, 143.58, 134.83, 134.48, 128.91, 128.59, 125.72, 124.78, 122.43, 119.17, 118.23, 116.92, 110.54, 108.11, 107.10, 50.02, 49.53, 38.26, 27.37, 25.45, 22.04, 21.49, 21.28 ppm. HRMS (ESI) calcd. for $[C_{32}H_{28}N_3O_4]^+$: 518.2074, found: 518.2073.

2'-diazo-3'-oxo-2,2',3,3',6,7-hexahydro-1H,5H-spiro[chromeno[2,3-f]pyrido[3,2,1-ij]quinoline-9,1'-inden]-12-yl pivalate (DACT-4): Following the general procedure, addition of pivaloyl chloride (28 µL, 0.23 mmol) gave DACT-**4** as a beige solid (13 mg, 0.025 mmol, 44%) after pentane wash. ^{1}H NMR (400 MHz, CDCl3) δ = 7.83 (d, *J* = 7.5, 1.4, 0.7 Hz, 1H, H14), 7.48 (td, *J* = 7.4, 1.4 Hz, 1H, H15), 7.41 (td, *J* = 7.4, 1.1 Hz, 1H, H16), 7.07 (dt, *J* = 7.7, 1.0 Hz, 1H, H17), 6.97 (d, *J* = 2.4 Hz, 1H, H8), 6.88 (d, *J* = 8.6 Hz, 1H, H13), 6.66 (dd, *J* = 8.6, 2.4 Hz, 1H, H12), 6.28 (s, 1H, H1), 3.17–3.08 (m, 4H, H4 and H5), 2.89 (t, *J* = 6.6 Hz, 2H, H7), 2.63–2.44 (m, 2H, H2), 2.07–1.97 (m, 2H, H6), 1.94–1.81 (m, 2H, H3), 1.34 (s, 9H, H9, H10 and H11) ppm. ^{13}C NMR (101 MHz, $CDCl_3$) δ = 187.23, 176.77, 155.88, 151.94, 151.06, 146.98, 143.42, 134.69, 134.33, 128.75, 128.45, 125.56, 124.66, 122.29, 118.96, 118.10, 116.76, 110.39, 107.98, 106.98, 49.89, 49.43, 49.39, 27.23, 27.10, 21.90, 21.34, 21.15 ppm. HRMS (ESI) calcd. for $[C_{32}H_{30}N_3O_4]^+$: 520.2231, found: 520.2231.

HeLa cells plating. HeLa cells were grown to 90% confluence and seeded onto 8-well Nunc Lab-Tek II chambered cover glass plates (50,000 cells per well) for confocal microscopy or onto Ibidi µ-slide 8-well plates (50,000 cells per well) a day prior to imaging experiments.

Imaging experiments with confocal microscopy. Fluorescent images of cells stained with DACTs and eFluor670 were taken using a Nikon Eclipse Ti light microscope equipped with a Yokogawa spinning-disk confocal scanner unit CSU-W1-T2, two sCMOS cameras (Orca Flash

4.0 V2), and a LUDLPrecision2 stage with a piezo focus. Diode-pumped solid-state lasers (DPSS) were used as light sources: 405 nm (120 mW), 561 nm (200 mW) and 647 nm (150 mW). Confocal images of cells stained with CFDA-SE and CTV were obtained with a Leica SP8 microscope operated with the Leica LAS-X software. As light sources a supercontinuum white light pulsed laser (470 – 670 nm) at 488 nm with emission at 415 – 470 nm and a diode-pumped solid-state laser 405 nm with emission at 500 – 550 nm were used. The images were acquired using an HC PL Apo, oil-immersion, 63x magnification, NA = 1.40 objective.

Staining procedure for HeLa cells. A staining solution containing: DACT (10 μM), eFluor670 (5 μM), CFDA-SE (5 μM), or CTV (5 μM) was prepared in FluoroBrite DMEM. Immediately after, 300 μL of the corresponding solution was added to the wells containing the plated HeLa cells. The cells were incubated for 30 min at 37 °C. The staining solution was subsequently removed, the cells were washed with PBS (2x) and replaced with fresh FluoroBrite DMEM for imaging. Cells stained with DACT were irradiated for 1.5 min with the staining solution, washed and incubated for the corresponding time. For longer incubation times (>1 h), FluoroBrite DMEM was replaced with growth medium. The cells were incubated for the desired time and imaged with a confocal microscope.

iFRAP experiments. A ROI within a cell or around a cell was selected and irradiated using a 405 nm (1 s, 30 mW) laser. Images of the same field of view were taken in the 561 nm channel (0.5 s, 120 mW) at experiment-specific timepoints after photoactivation.

Isolation of splenocytes. A murine spleen was harvested and placed in a 70 μm cell strainer over a 50 mL Falcon tube. It was subsequently smashed and rinsed with leukocyte medium (RPMI-1640 (ThermoFisher), 10% FBS, 1% penicillin-streptomycin, 1 mM HEPES, 1 mM sodium pyruvate, 2 mM L-glutamine, 50 μM β-mercaptoethanol). The cells were centrifuged and resuspend in ACK buffer (1 mL, 15 mM NH_4Cl, 1 mM $KHCO_3$, 0.1 mM, 150 mM Na_2-EDTA at pH = 7.2–7.4) and left for 5 min for lysis of red blood cells. Then the solution was diluted to 10 mL with leukocyte medium. Cells were spun, the supernatant was removed, and the pellet was re-suspended in leukocyte medium. The cells were kept on ice until the start of the experiment.

Isolation of cells from lymph nodes. The auricular, axillary and inguinal LNs were harvested and placed in 70 μm cell strainers over 50 mL Falcon tubes. Each pair was then smashed and

rinsed with FACS buffer, spun down and the pellet was resuspended in FACS buffer and kept on ice ready for FACS analysis.

FACS analysis of HeLa cells stained with DACTs 1-4. HeLa cells were cultured in 96-well plates before incubating 4-wells on two 96-well plates per DACT **1**-**4** for 5 min under shaking at 37 ºC and 30 min under standard incubation. One plate of cells was irradiated for 10 min using a conventional UV lamp (364 nm) and further incubated for 1 h, the second plate was kept in the dark as a background reference. HeLa cells were detached from the glass by addition of trypsin (0.25%, 20 μL) for 5 min. The cells were diluted in growth medium (200 μL) and 4 wells per sample were combined. The sample was centrifuged (10 min at 1800 x g), the supernatant was removed, and the pellet was resuspended in FACS buffer (300 μL, PBS, 2% FBS, 1 mM EDTA, 0.05% sodium azide). The obtained cell solution was filtered into FACS vials (Corning™) and acquired by FACS.

Fixation and permeabilization experiments. To assess DACT-**1** stability after cell fixation, a 1:1 ratio of DACT-**1**-labeled splenocytes and unlabeled splenocytes were subjected to eBioscience Foxp3 fixation and permeabilization (ThermoFisher) kit following manufacturer's instructions. Cells were subsequently acquired by FACS and fluorescent signal retention was determined by analyzing the fluorescent signal in fresh and fixed and permeabilized cells.

Splenocytes toxicity tests. Freshly harvested splenocytes were plated in two 96 well plates. Each well contained approximately 300,000 cells in leukocyte medium (200 μL) at different concentrations 10, 20 and 40 μM of DACT-**1** and 0.5% DMSO plus a negative control containing only DMSO. Both plates were incubated in the dark for 30 min at 37 ºC. One of the two plates was irradiated for 1.5 min at 410 nm, while the other one was left in the dark. Cells were analyzed by FACS after 5, 10 and 24 h after irradiation. For the analysis ~50,000 cells of each condition were transferred into a new FACS plate, washed with FACS buffer and resuspended in staining solution (30 μL: Annexin V Binding Buffer (BioLegend), 1:500 Zombie Aqua and 1 μL APC-Annexin V per well). After 10 min in the dark, Annexin V Binding Buffer (70 μL) was added for FACS analysis.

Homing studies. Freshly isolated splenocytes were labeled with eFluor670 following the manufacturer's instructions and incubated with 10 μM of DACT-**1** during 30 min. Half of the cells were photoconverted (1.5 min UV exposure) and pooled with the rest in a 1:1 ratio. 1.5 million

cells of the resulting cell suspension were diluted to 150 μL with PBS and injected into the mouse tail vein. After 19 h mice were sacrificed and the auricular, axillary and inguinal LNs were harvested and processed for FACS analyses.

FACS acquisition. All FACS experiments were acquired on a BD FACSCanto (BD Biosciences) using FACSDiva software or on a CytoFLEX S (Beckman Coulter, Inc.) using CytExpert software. Data were analyzed using FlowJo software (Treestar).

FACS analysis of splenocytes. Splenocytes were stained in 200 μL of staining solution (FACS buffer with fluorescent reagents) during 30 min on ice. To study DACT signal in CD4 and CD8 T cells, the staining solution contained; 1:500 ZombieAqua (BioLegend), 1:200 rat anti-mouse CD45 APC/Cy7 (BioLegend), 1:200 armenian hamster anti-mouse CD3e APC (BioLegend), 1:200 rat anti-mouse CD4 A700 (BioLegend), 1:200 rat anti-mouse CD8 BV650 (BioLegend). To study DACT signal in dendritic cells, the staining solution contained; 1:500 ZombieAqua (BioLegend), 1:200 rat anti-mouse CD45 APC/Cy7, 1:200 armenian hamster anti-mouse CD11c APC (BioLegend) and 1:200 rat anti-mouse MHCII BV421 (BioLegend). After incubation, cells were spun, washed with FACS buffer and prepared for FACS analysis.

Preparation of the imLECs monolayer. Cells were seeded in culture dishes coated with 10 μg mL^{-1} of collagen (PureCol, Advanced Biomatrix) and 10 μg mL^{-1} fibronectin (Millipore) and grown at 33 °C in the presence of IFNγ (10 units mL^{-1}, Peprotech). For functional assays the cells were cultured for 48 h at 37 °C without IFNγ prior the experiments [44] and the monolayer formation was confirmed under the microscope.

FACS analysis of imLECs for leakage experiments. Cells (imLECs) were treated with Accutase solution (Sigma) and the suspension was stained for 30 min on ice with a solution containing; 1:500 ZombieAqua (BioLegend), 1:200 rat anti-mouse CD31 BV421 (BioLegend) and 1:200 rat anti-mouse CD45 APC/Cy7 (BioLegend). After incubation, cells were spun, washed with FACS buffer and prepared for FACS analysis.

***In vitro* crawling experiments**. Splenocytes were isolated from the spleen of a mouse and kept on ice in leukocyte medium. Cell solutions were counted (Triptan Blue in a Neubauer chamber, Marienfeld) and diluted to 12 million mL^{-1} in leukocyte medium. Splenocytes were stained separately with: i) eFluor670 5 μM) ii) DACT **1** (10 μM), or with a iii) 1:1 mixture of eFluor670 and

DACT-**1**. After 30 min incubation, the wells were irradiated (1.5 min, 410 nm) and transferred to the confluent imLEC monolayer to a final concentration of 125 000 cells per dye treatment. For single cell staining experiments using iFRAP, unstained splenocytes (125 000 cells) in wells containing a confluent imLECs monolayer for 19 h to allow interaction and adhesion. Before imaging, the leukocyte medium containing DACT-**1** (10 μM) was added and incubated for 10 min. For confocal imaging, the splenocytes stained with eFluor670 or DACT-**1** were detected in the 561 and 647 nm channel respectively. For FACS analysis, the cells were detached after 19 h of incubation with Accutase solution (Sigma), labeled with the corresponding antibodies (CD31 BV421 eBioscience, CD45 APC-Cy7 BioLegend, Zombie Aqua, BioLegend) and analyzed.

Generation of BM-DCs. DCs were generated from BM isolated from C57BL/6 mice as described.[1] In brief, 5 Mio BM cells were seeded into bacterial dishes (Greiner Bio-One) in DC-medium (leukocyte medium with 80 ng mL^{-1} GM-CSF purified in the lab from the supernatant of myeloma cells (X63 Ag8.653) transfected with murine GM-CSF cDNA).[2] On day 9-10 non-adhering cells were harvested and used in proliferation experiments.

Isolation of CD4 T cells and proliferation experiments. CD4 T cells were isolated from spleens of OT-II mice using anti-CD4 MACS beads (Milteny Biotec) following the manufacturer's protocol. Cells were labeled with one of the following dyes; CFDA-SE (5 μM), DACT-**1** (10 μM), CTV (5 μM) and eFluor670 (5 μM) during 20-30 min in leukocyte medium at 37 °C. 50,000 CD4 T cells were subsequently co-cultured with 10,000 BM-DCs in medium containing the OVA-derived peptide pOVA323-339 (100 μM, EMC Microcollections) and LPS (10 ng mL^{-1}). After 72 h cells were harvested and stained with anti-CD4 APC (BioLegend) for 20 min at 4 °C, washed with FACS buffer and analyzed by FACS analysis.

Quantification and statistical analysis. Image quantification was performed with Fiji software.[3] All subsequent analyses were performed using GraphPad Prism 7 (GraphPad software). FACS data was analyzed using FlowJo software (Treestar) all relevant statistical details are included in the figure captions, and text or the Methods section.

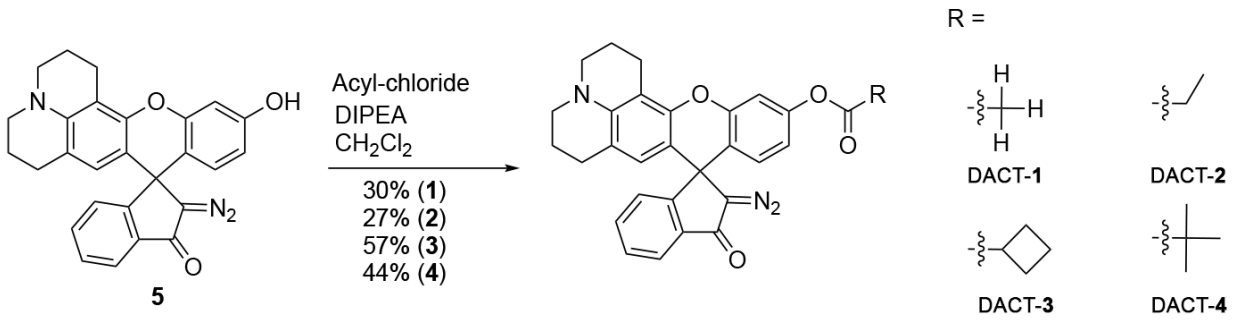

Figure S1. Chemical synthesis. Synthesis of the DACTs (**1**–**4**) from precursor **5**.

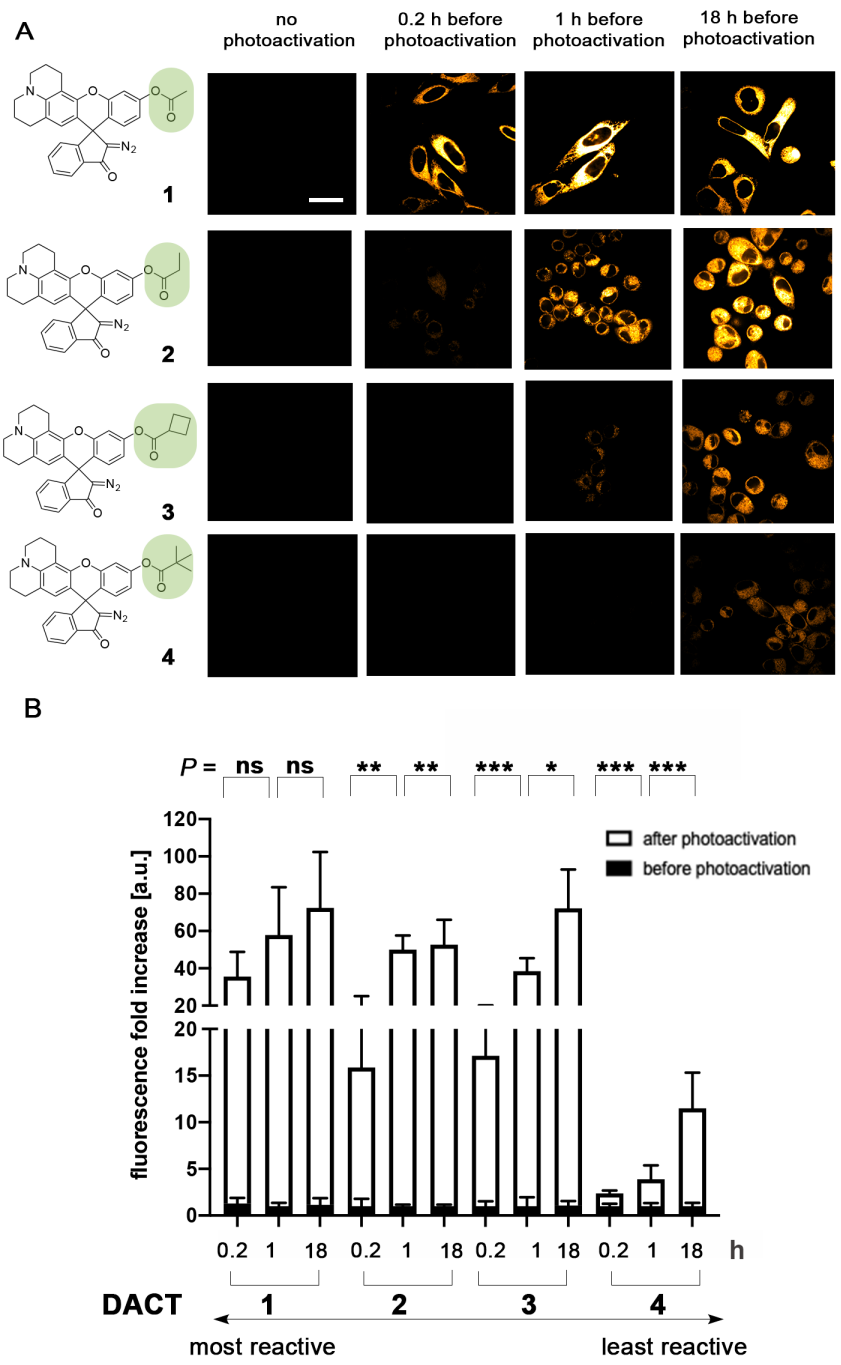

Figure S2. Photoactivation experiments in HeLa cells with varying incubation times before photoirradiation. (A) Confocal images of HeLa cells treated with DACTs **1**-**4**, before irradiation, and after irradiation (405 nm, 1 s, 60 mW) at *t* = 0.2, 1 and 18 h of incubation before photoactivation. Images were obtained using 561 nm laser (0.5 s, 120 mW). Representative images from one out of three biological replicates are displayed. (B) Fluorescence fold increase obtained from the experiments displayed in panel A. n > 8 measurements from three biological replicates. $P < 0.0001$ (***), $P < 0.001$ (**), $P < 0.01$ (*), $P < 0.1$ (ns). Scale bar = 10 μm.

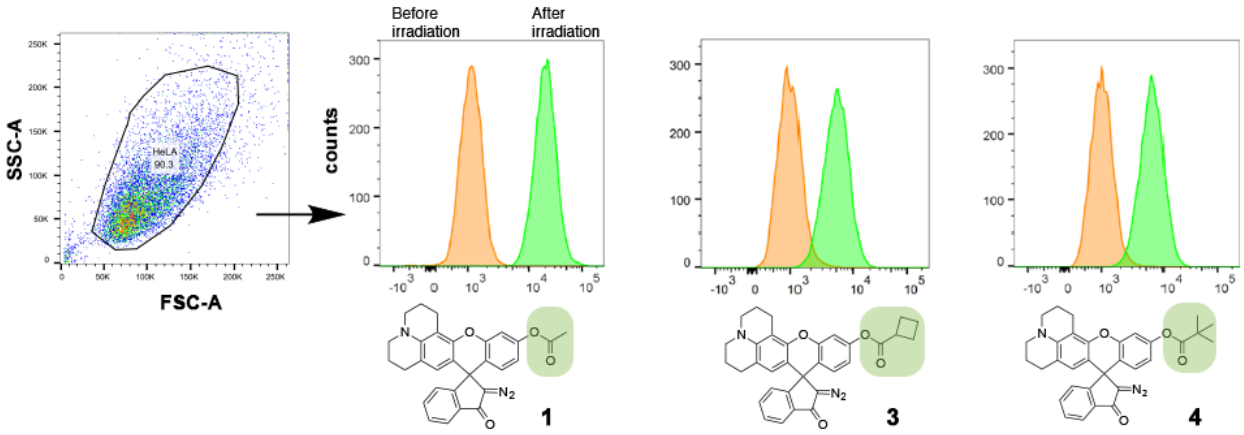

Figure S3. FACS analysis of HeLa cells treated with selected DACTs (1, 2 and 4). HeLa cells were incubated for 30 min with the different compounds (10 μM), irradiated and mixed with non-irradiated cells. Representative graphs of three independent experiments are displayed.

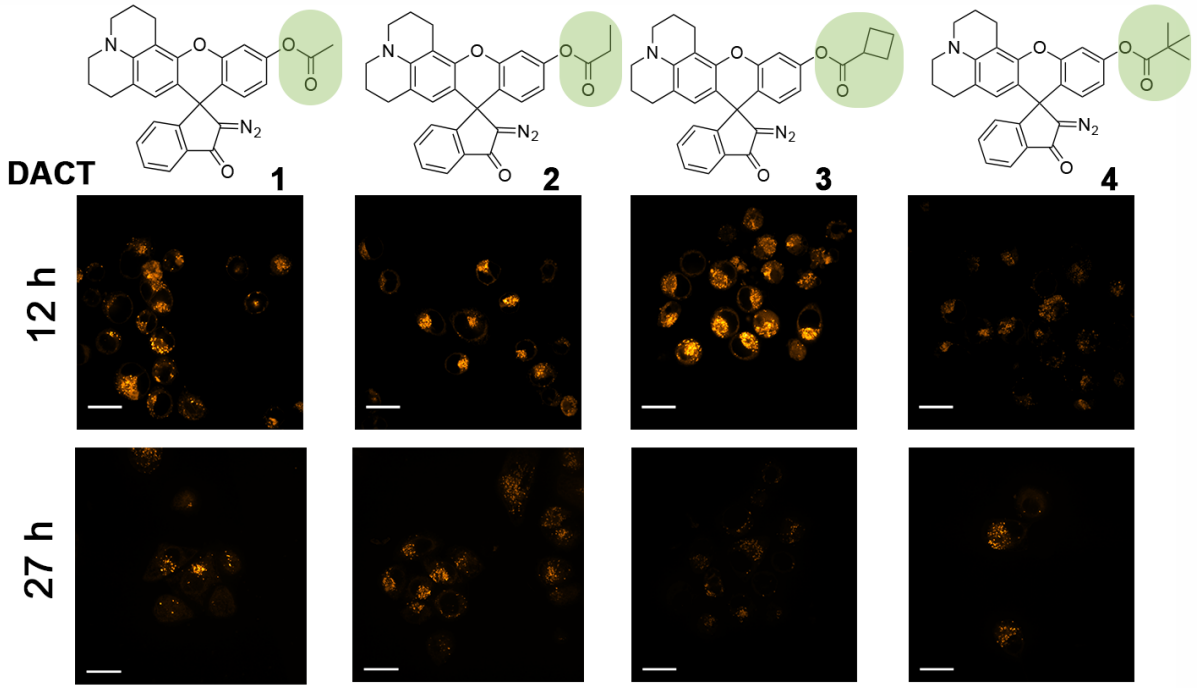

Figure S4. Fluorescent signal durability with varying incubation times after irradiation. Comparison of the fluorescence images taken after 12 and 27 h after irradiation in HeLa cells incubated with 10 μM DACT (**1**-**4**) for 1 h. Photoirradiation was achieved using a home built transilluminator (410 nm, 10 min). After photoactivation, the cells were washed with PBS and incubated with fresh growth medium without phenol red and images recorded with a 561 nm laser (0.5 s, 120 mW). Representative images from one out of three independent experiments are displayed. Scale bars = 10 μm.

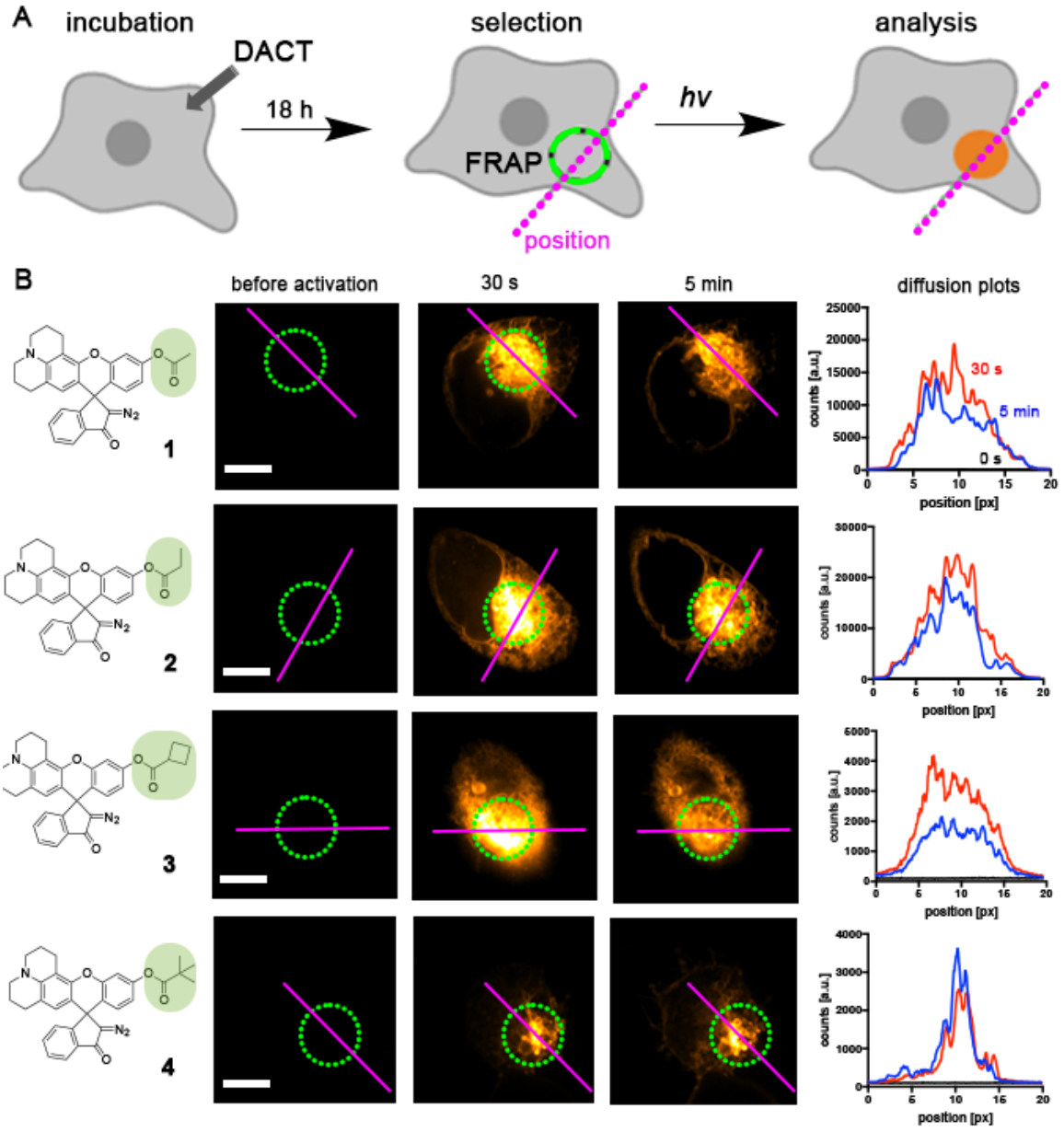

Figure S5. Fluorescent signal durability by iFRAP. (A) Schematic representation of iFRAP method to determine diffusion of fluorescent signal. (B) HeLa cells treated with DACTs (**1-4)** at 10 μM and incubated for 18 h to ensure complete hydrolysis of the ester groups by hCEs. The iFRAP area was selected (green circle) and was irradiated with 405 nm laser (1 s, 30 mW). Read-out emission was performed with a 561 nm laser (0.5 s, 120 mW) 30 seconds and 5 minutes after initial iFRAP. The position (magenta line) was selected and the corresponding position plots were created using Prism. Representative images from one out of three independent experiments are displayed. Scale bars = 5 μm.

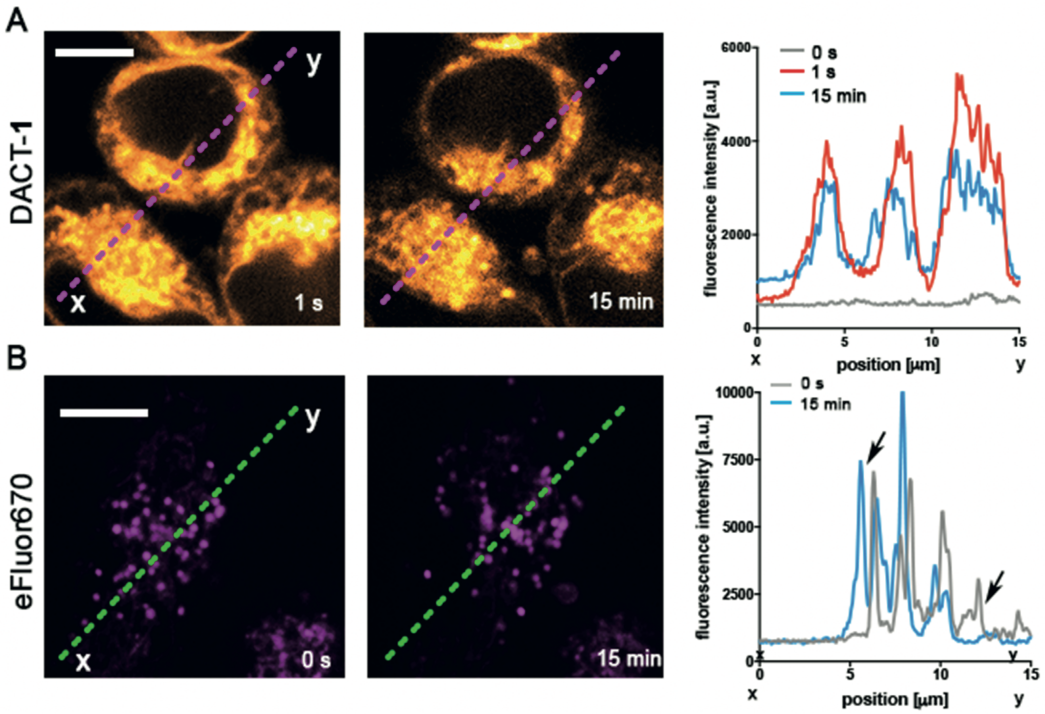

Figure S6. Intracellular staining, diffusion and comparison of DACT-1 with eFluor670. Confocal images of HeLa cells treated with (A) DACT-**1** (10 μM) and (B) eFluor670 (5 μM) 1 s and 15 min after irradiation. Diffusion plots (fluorescence intensity along the dotted lines displayed in the images) obtained from cells in panel C and D respectively. Black arrows indicate appearing and disappearing fluorescent signals within the plotted line. Scale bars 5 μm. Representative images form three independent experiments are displayed.

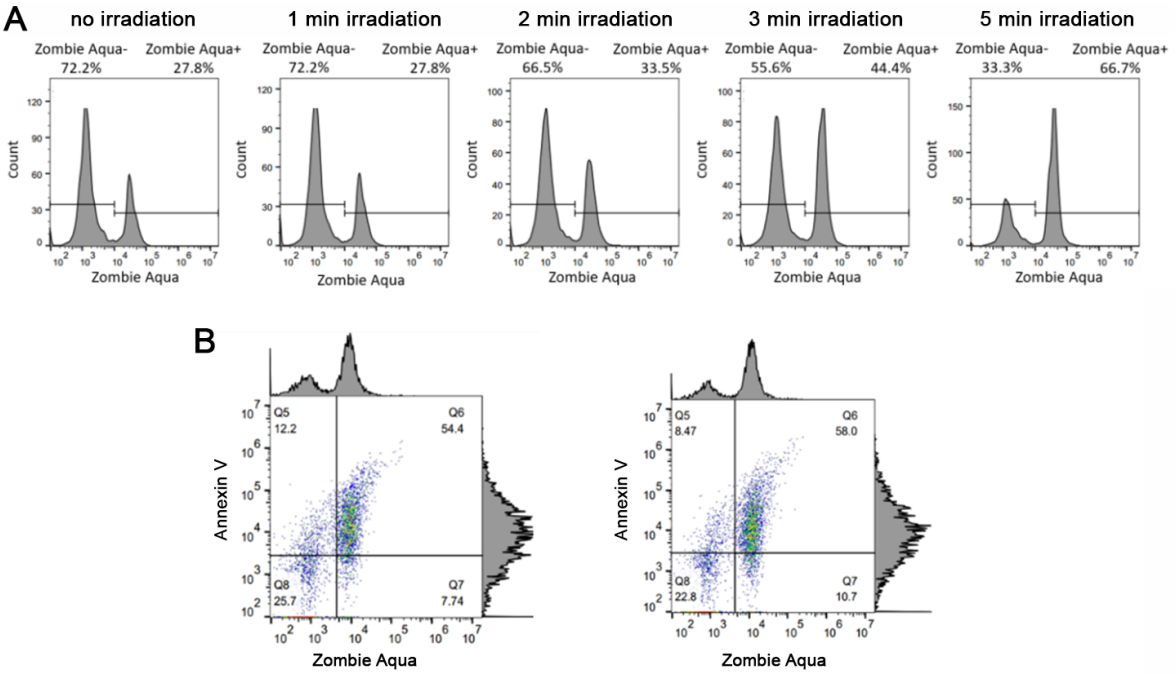

Figure S7. UV phototoxicity assessed on primary cells. (A, B) Splenocytes were UV irradiated for varying time periods, stained with Zombie Aqua and Annexin V and analyzed by FACS. Representative graphs of three independent experiments are displayed.

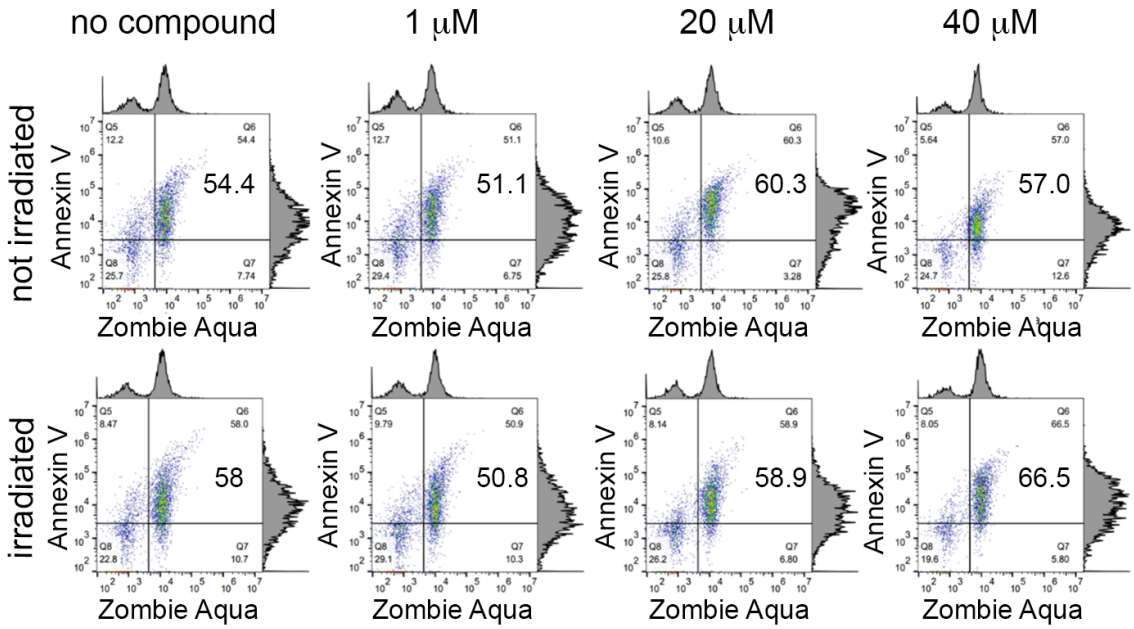

Figure S8. DACT cytotoxicity assessed on primary cells. Splenocytes were incubated with different concentrations of DACT-**1**, irradiated during 1.5 min, stained with Zombie Aqua and Annexin V and analyzed by FACS. Representative graphs of three independent experiments are displayed.

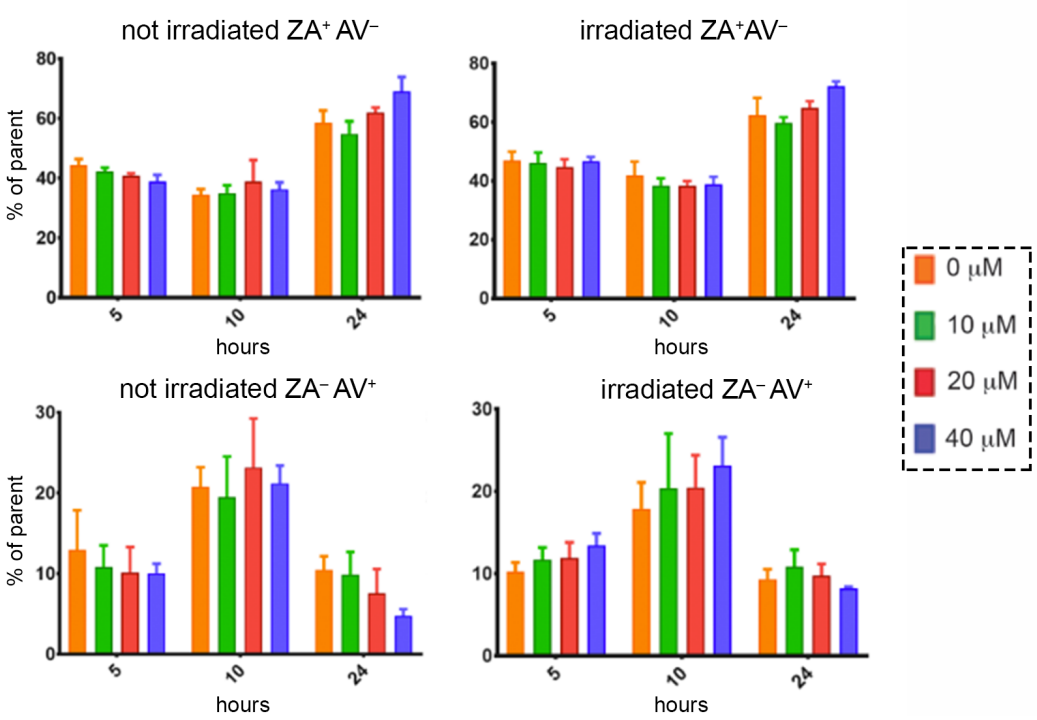

Figure S9. Cell viability assessed over time using optimized conditions. Splenocytes were incubated with different concentrations of DACT-**1**, irradiated during 1.5 min and kept at 37 °C and 5% CO_2 in leukocyte medium. Zombie Aqua (ZA) and Annexin V (AV) stained cells were analyzed by FACS and results are displayed as mean ± SEM (n = 3 independent experiments).

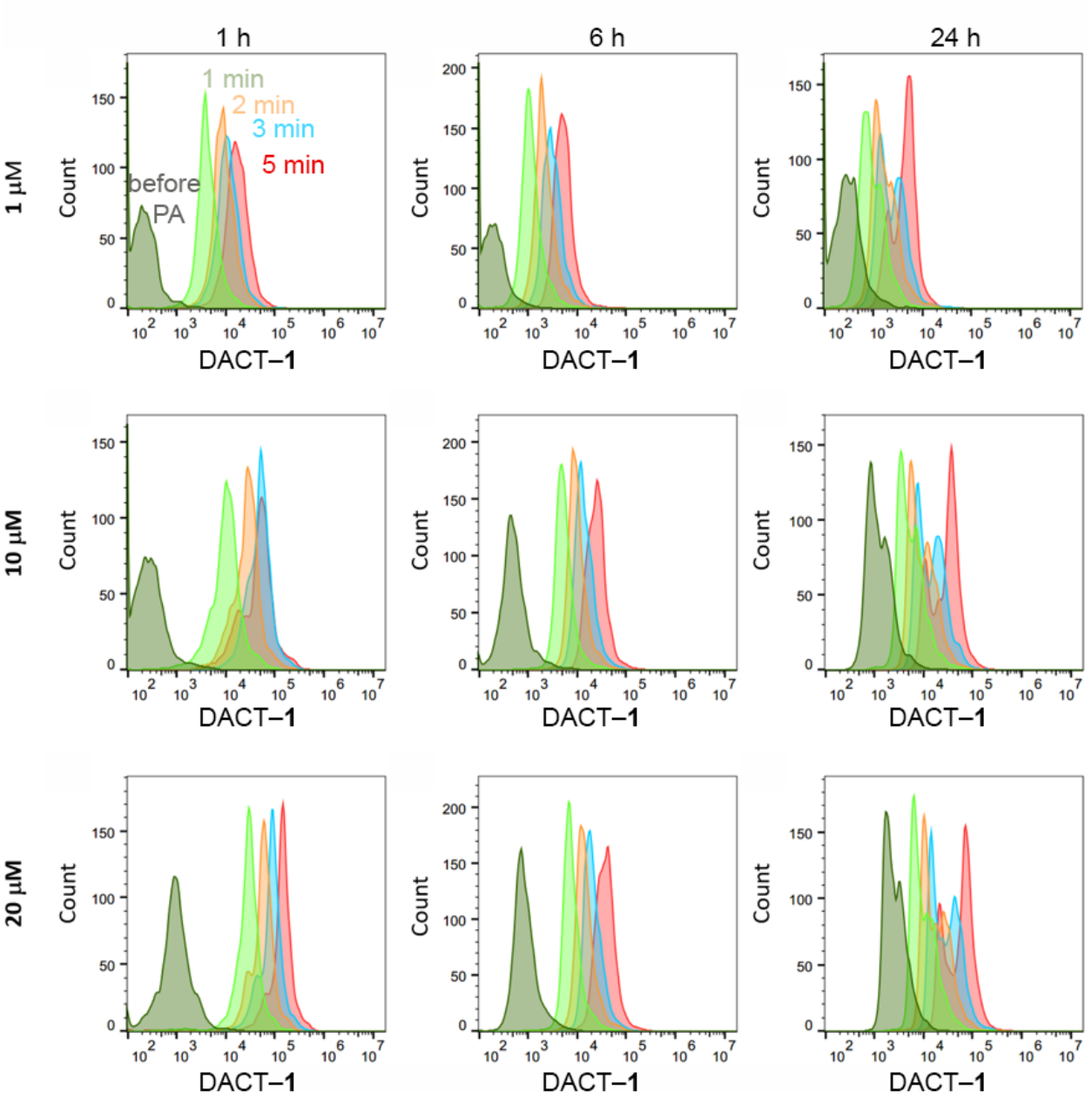

Figure S10. Fluorescent signal optimization of DACT-1. Splenocytes were incubated with 1, 10 or 20 µM of DACT-**1** and irradiated during 1, 2, 3 or 5 min or not (before photoactivation (PA)). DACT-**1** signal was assessed 1, 6 or 24 h later by FACS. Representative graphs of three independent experiments are displayed.

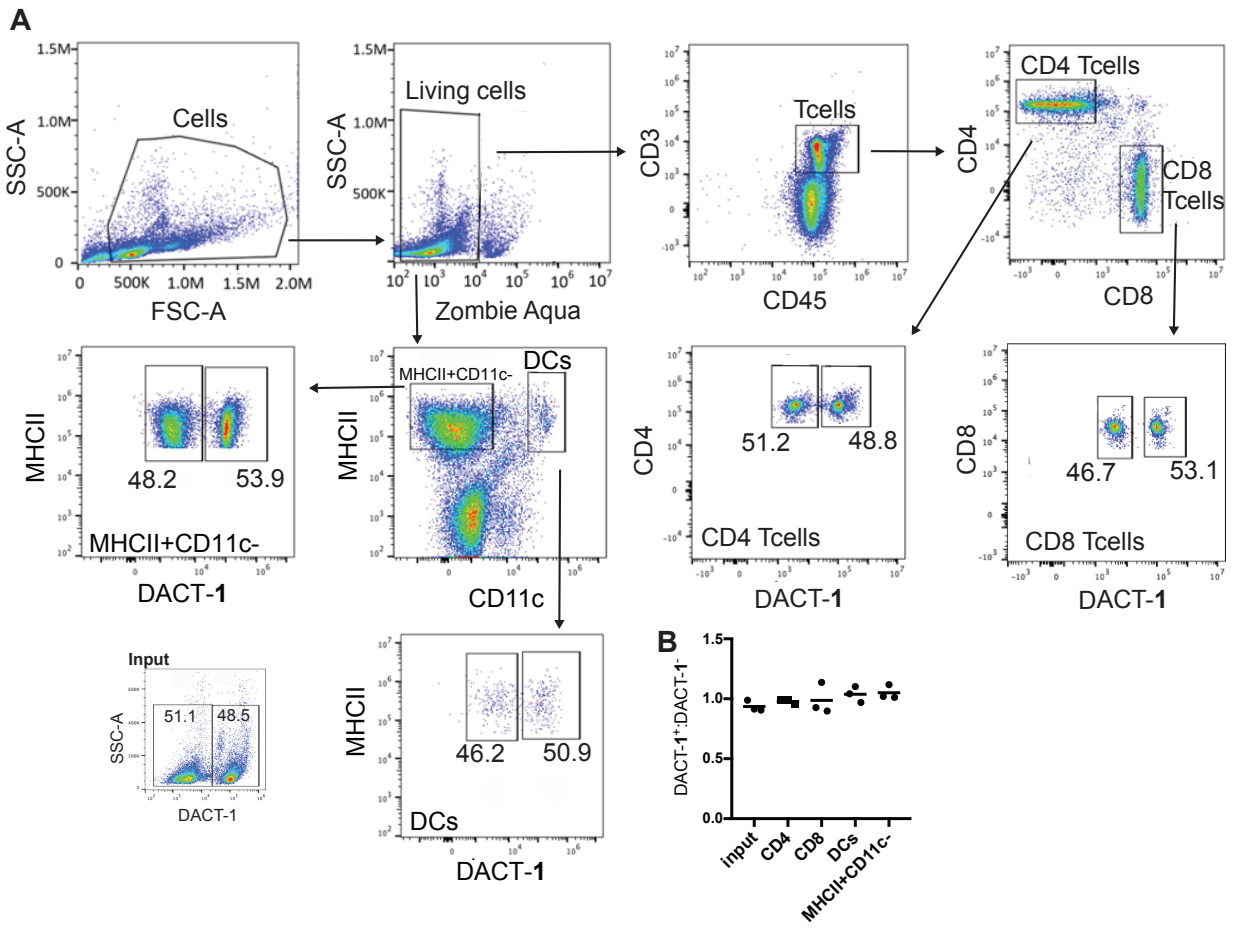

Figure S11. DACT-1 efficiently labels different cell populations. DACT-**1**$^{+}$ and DACT-**1**$^{-}$ splenocytes (ratio 1:1) were stained with ZombieAqua, anti-CD45, anti-CD3, anti-CD4, anti-CD8, anti-MHCII and anti-CD11c antibodies and acquired by FACS. DACT-**1**$^{+}$ and DACT-**1**$^{-}$ cells are displayed gating on CD4 T cells, CD8 T cells, DCs and MHCII^{+}CD11c^{-} cells. (A) Representative graphs of three independent experiments are shown. (B) Quantification of three independent experiments. The ratios were calculated from the percentage of cells obtained employing the gating strategy displayed in (A).

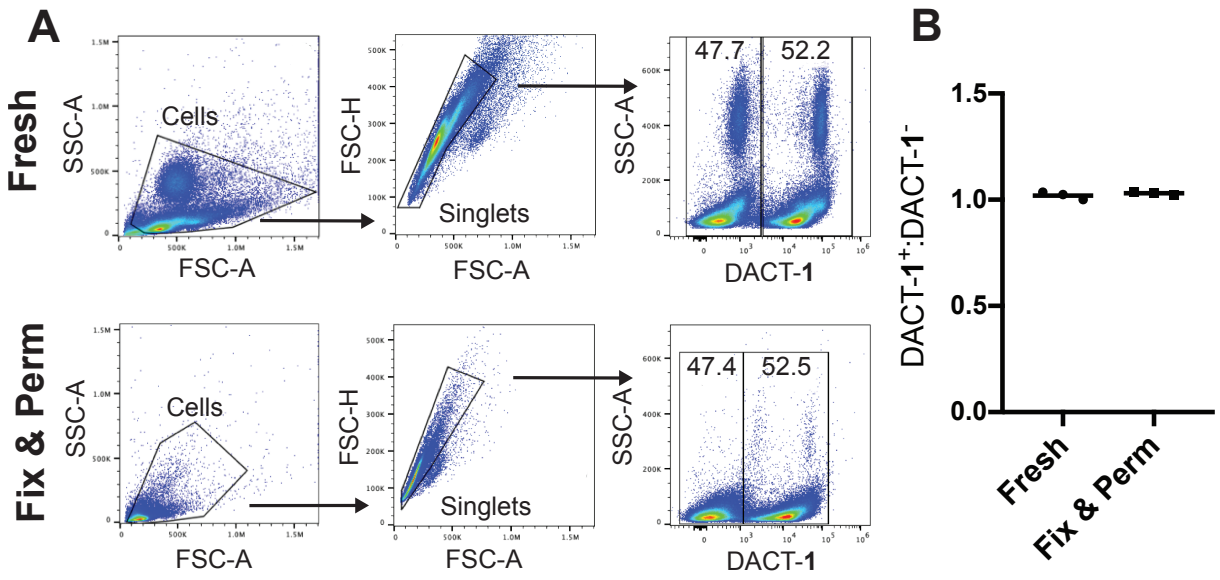

Figure S12. DACT-1 signal is retained in fixed and permeabilized cells. Splenocytes were incubated for 30 min with DACT-**1** (10 μM), irradiated and mixed with non-irradiated cells. One half of the mixture was kept at 4 °C (fresh) and the other half was fixed (Fix) and permeabilized (Perm). Both halves were subsequently acquired by FACS and quantified. (A) The results of a representative experiment out of three independent measurements are displayed. (B) Quantification of three independent experiments, line indicates the average value. The ratios were calculated from the percentage of cells obtained employing the gating strategy displayed in (A).

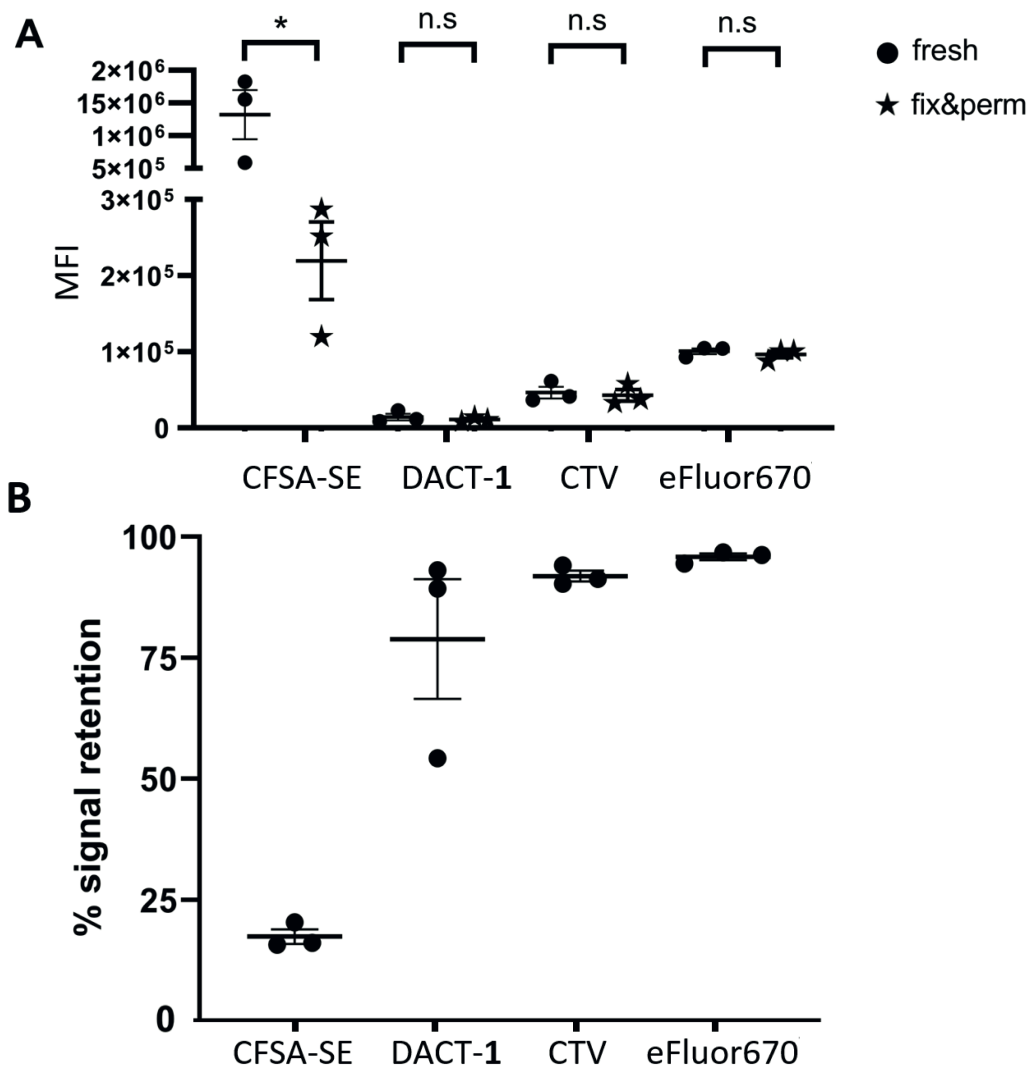

Figure S13. Signal durability after fixation and permeabilization. Analysis of median fluorescent intensities (MFI) of the cells in the gated population (A) and % of signal (MFI) retention compared to the signal of the freshly stained splenocytes (B). Data are displayed as mean ± SEM (n = 3 independent experiments pooled). Unpaired t test *P* = *< 0.05).

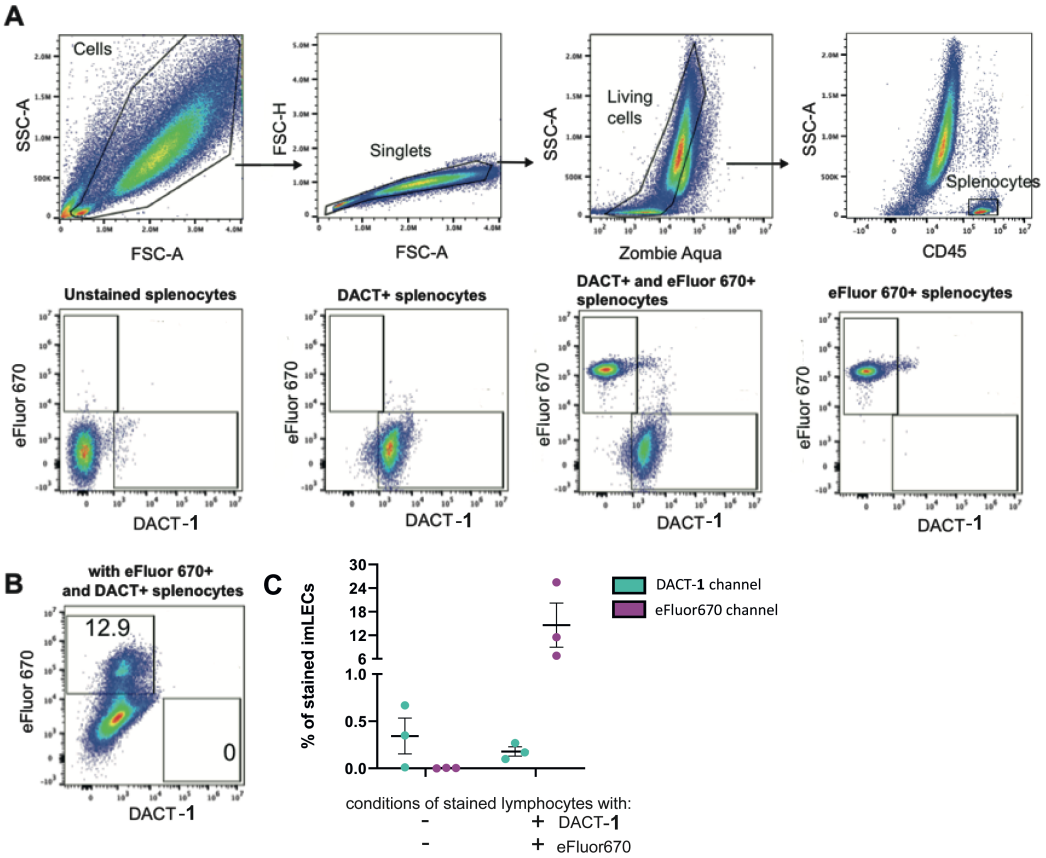

Figure S14. Analysis of DACT-1 and eFluor670 dye transfer in co-culture studies. (A) Gating strategy applied to the cells of an imLEC monolayer that was incubated with either unstained splenocytes or splenocytes stained with eFluor670 (5 μM), DACT-**1** (10 μM) or with a 1:1 mixture of eFluor670 and DACT-**1** stained cells during 19 h. The gate was set using unstained splenocytes. Cell aggregates were excluded by selecting individual cells, or singlets, in the FSC-A/ FSC-H panels. (B) Representative FACS analysis of co-culture studies using DACT-**1** and eFluor670 labeled splenocytes mixed in a 1:1 ratio. (C) Analysis of % of imLECs stained with DACT-**1** and eFluor670 in these studies. For the calculation of these values, we used the numbers obtained through the gating strategy displayed in (A). Data are displayed as mean ± SEM (n = 3 different experiments).

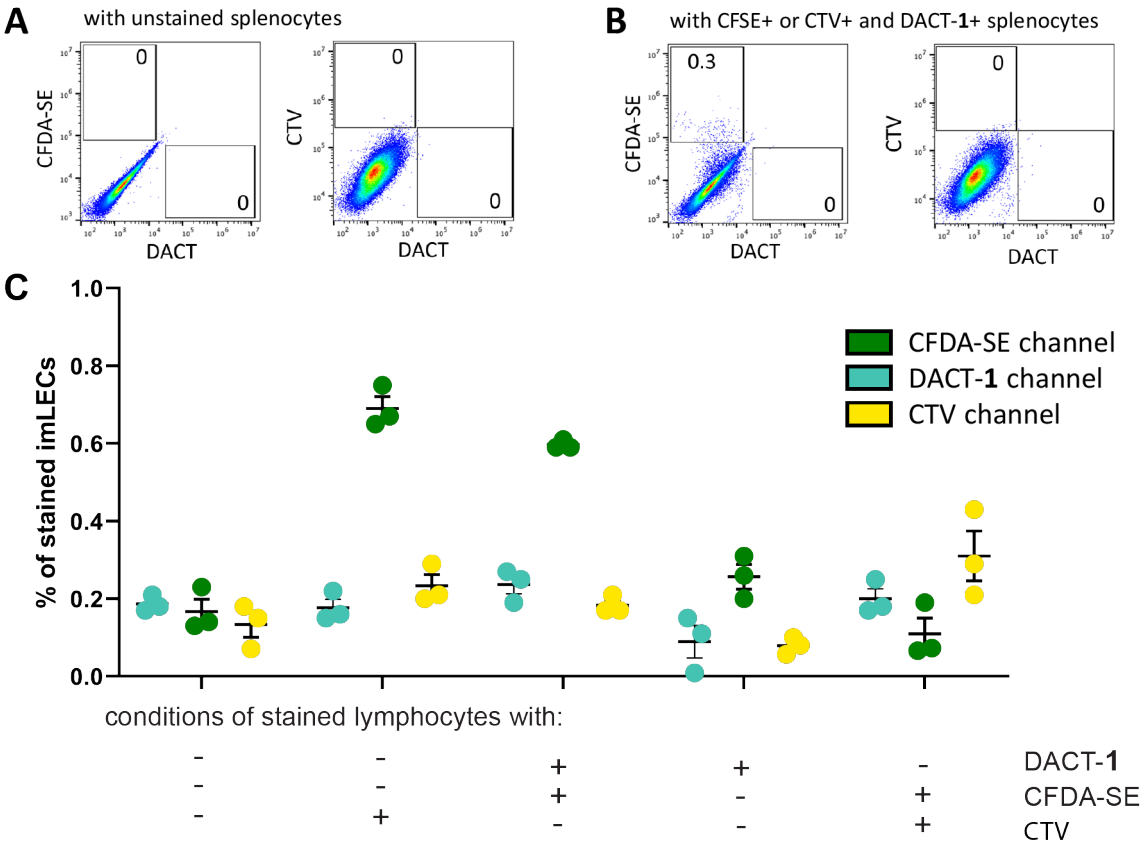

Figure S15. Analysis of DACT-1, CFDA-SE and CTV dye transfer in co-culture studies. The same experiment that was carried out for eFluor670 (Figure S14) was conducted with CFDA-SE and CTV. (A) The imLECs co-cultured with unstained cells were analyzed by FACS to define the gating strategy. (B) Representative FACS acquisitions of imLECs co-cultured with doubly stained splenocytes. (C) The % of imLECs stained with DACT-**1**, CFDA-SE and CTV. Data are displayed as mean ± SEM (n = 3 independent experiments pooled).

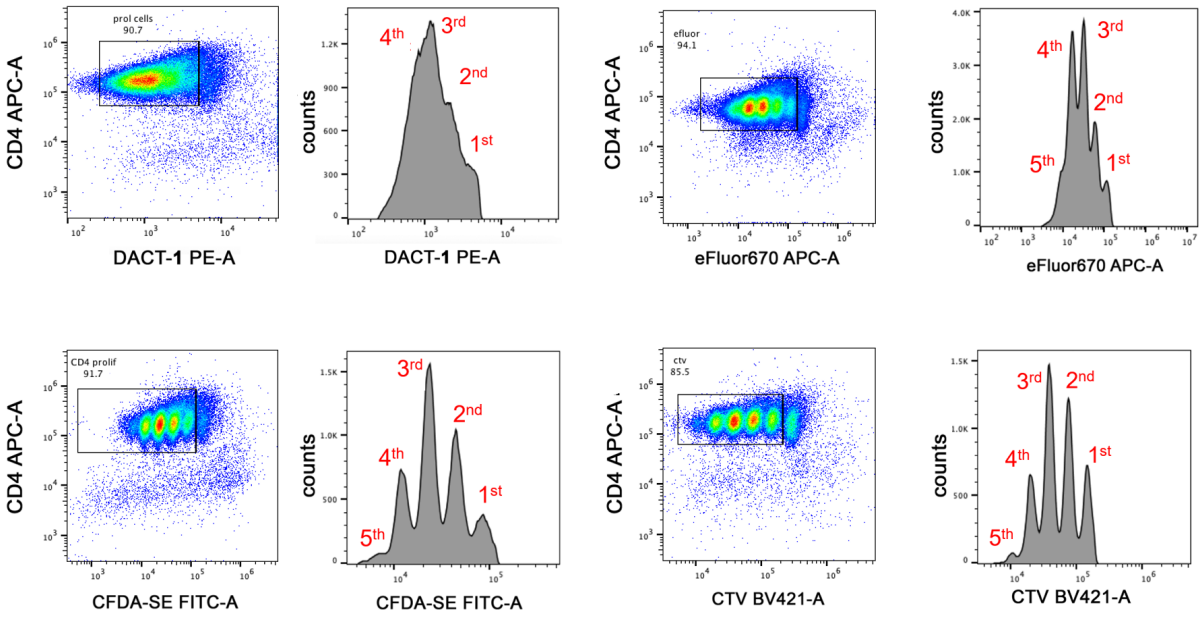

Figure S16. Cell proliferation plots of different staining treatments. Representative FACS plots (n = 3 independent experiments) of proliferated (from the first to the youngest generation) CD4 T cells initially stained with CFDA-SE, DACT-**1**, CTV and eFluor670. Numbered peaks corresponding to different CD4 T cell divisions are presented. Control experiments without induction of proliferation but stained with different dyes did not show any proliferation (data not shown).

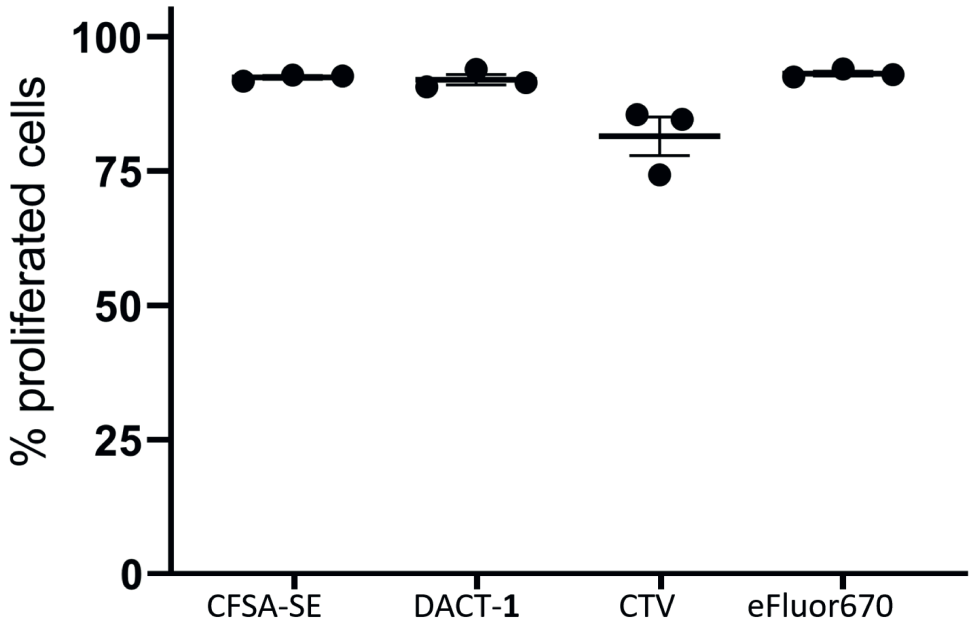

Figure S17. Analysis of the % of proliferated cells with different staining treatments. % of CD4 T cells proliferated cells stained with CFDA-SE, DACT-**1**, CTV and eFluor670. These values were obtained using the gating strategy displayed in Figure S16, including all cells from the first to the youngest generation. Data are displayed as mean ± SEM (n = 3 independent experiments pooled).

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