

Supplementary Information

Nanobodies equipped with HaloTag variants enable rapid and straightforward one-step immunofluorescence lifetime multiplexing.

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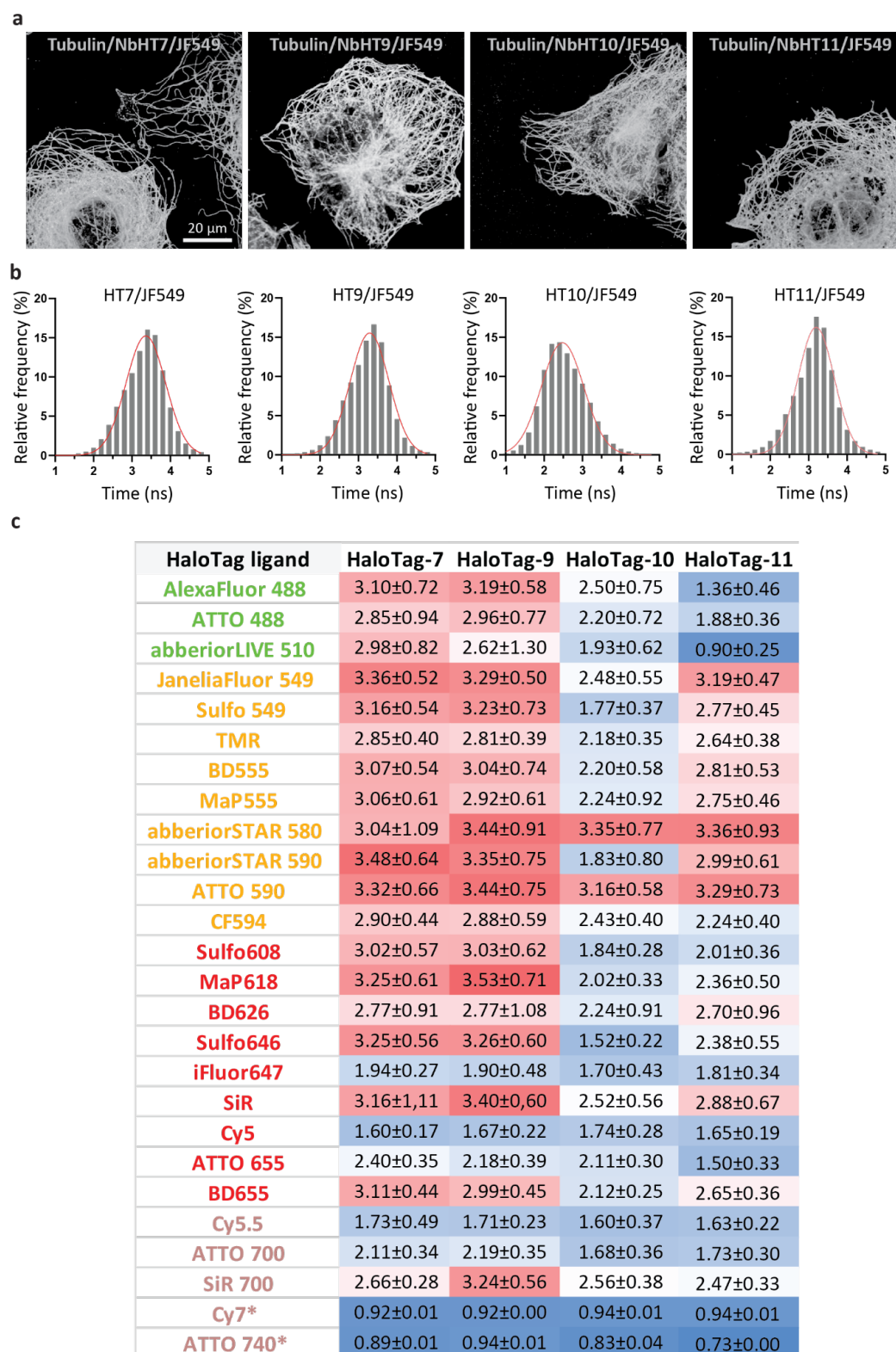
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Supplementary Table 1. List of all antibodies.

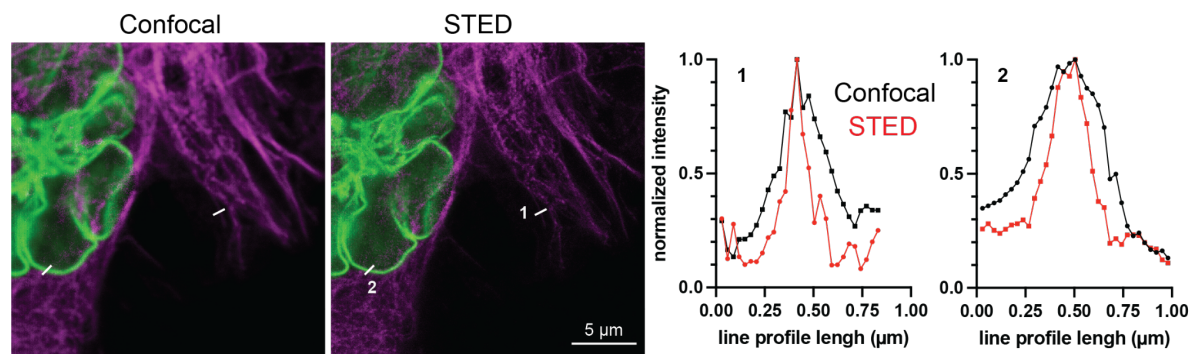
Target	Host	Isotype	Clonality	Company	Cat. No.	RRID
Tubulin	mouse	IgG1	mono	Synaptic Systems	302211	AB_887862
Vimentin	mouse	IgG1	mono	Santa Cruz	sc-6260	AB_628437
Tomm20	mouse	IgG1	mono	Sigma Aldrich	WH0009804M1	AB_1843992
PMP70	rabbit	IgG	mono	Abcam	ab85550	AB_10672335
Clathrin	rabbit	IgG	poly	Abcam	ab21679	AB_2083165
GALNT2	rabbit	IgG	poly	Sigma Aldrich	HPA011222	AB_1849446
NUP50	rabbit	IgG	mono	Abcam	ab137092	AB_2921286
Parvalbumin	mouse	IgG1	mono	Synaptic Systems	195011	AB_2619883
NeuN	rabbit	IgG	mono	Synaptic Systems	266108	AB_3662033
CTIP2	rabbit	IgG	mono	Abcam	ab240636	AB_10730034
vGlut1	rabbit	IgG	poly	Synaptic Systems	135303	AB_887875
Tyrosine hydrox.	mouse	IgG2	mono	Synaptic Systems	213111	AB_2636902
SOX2	rabbit	IgG	poly	Synaptic Systems	347003	AB_2620099

Supplementary Table 2. List of all nanobodies.

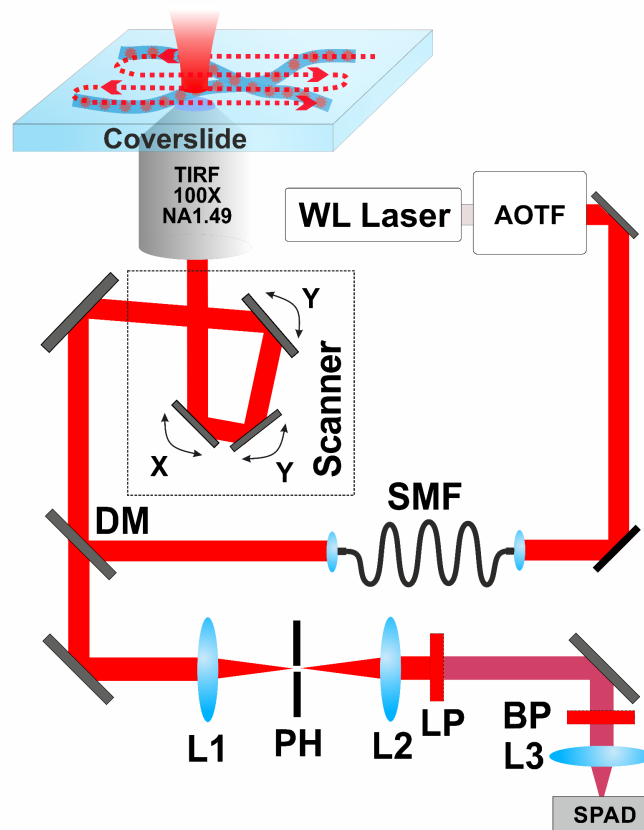
Target	Host	Clonality	Fusion/Conj.	Company	Cat. No.	RRID
Mouse IgG1	alpaca	mono	Atto643	NanoTag Biotechnologies	N2002-At643-S	AB_3076022
Mouse IgG2	alpaca	mono	azDye658	NanoTag Biotechnologies	N2702-AF568-S	AB_3076052
Rabbit IgG	alpaca	mono	AlexaFluor647	NanoTag Biotechnologies	N2402-AF647-S	AB_3076036
Rabbit IgG	alpaca	mono	AbberiorSTAR Green	NanoTag Biotechnologies	N2402-AbGREEN-S	AB_3697322
RFP/mCherry	alpaca	mono	HT7	NanoTag Biotechnologies	custom made	-
Mouse IgG1	alpaca	mono	HT7	NanoTag Biotechnologies	N2441	AB_3668679
Mouse IgG1	alpaca	mono	HT9	NanoTag Biotechnologies	custom made	-
Mouse IgG1	alpaca	mono	HT10	NanoTag Biotechnologies	custom made	-
Mouse IgG1	alpaca	mono	HT11	NanoTag Biotechnologies	custom made	-
Mouse IgG2	alpaca	mono	HT7	NanoTag Biotechnologies	N2741	AB_3668678
Rabbit IgG	alpaca	mono	HT7	NanoTag Biotechnologies	N2041	AB_3668677
Rabbit IgG	alpaca	mono	HT9	NanoTag Biotechnologies	custom made	-
Rabbit IgG	alpaca	mono	HT10	NanoTag Biotechnologies	custom made	-
Rabbit IgG	alpaca	mono	HT11	NanoTag Biotechnologies	custom made	-



Supplementary Figure 1. Benchmarking 26 fluorophores conjugated to HTL. (a) OneStep-IF using anti- α -Tubulin 1.Ab combined with 2.NbHT7, HT9, HT10, or HT11 and in the example added HTL-JF549 on COS-7 cells. (b) FL distribution of full images and fit (red line) of the JF549 when combined with the different HaloTag variants, as shown in a. (c) Mean FL from the fits in ns and error are standard deviations for all tested fluorophores. All FLs were obtained using the setup described in the Methods section, except for Cy7* and Atto 740*, which were extracted from Leica STELLARIS software directly.



Supplementary Figure 2. Proof of concept that 2 targets on the same chromatic spectrum can be separated under Tau-STED. COS-7 cells transfected with LaminA-Y71L-mCherry (non-fluorescent mCherry, here in green) were stained with 1.Nb anti-RFP-HT7 and SiR-HTL (here displayed in green). Simultaneously, anti-alpha-Tubulin 1.Ab was combined with 2.Nb-Atto643 (here in Magenta). Line profile used to demonstrate the STED effect, separation of 2 targets imaged under the chromatic line.



Supplementary Figure 3. Schematic of the custom-built time-resolved confocal laser-scanning microscope. Excitation is provided by a white-light (WL) laser and directed through a scanning confocal microscope to image microtubules immobilized on a glass coverslip. Fluorescence emission is collected confocally and detected by a single-photon avalanche diode for time-correlated single-photon counting.