

Supporting Information

Functional blood-brain barrier crossing by biomimetic M13 phage vectors for targeted neuronal delivery

Silvia Vercellino^{a,b§}, Lucia Pappagallo^{a,f§}, Francesca De Chirico^{c§}, Luca Boselli^{d,e}, Hazem Ahmed^{a,d}, Luca Spagnoletti^a, Giovanni Pittaluga^a, Paolo Emidio Costantini^{f,g}, Matteo Calvaresi^{g,h}, Pier Paolo Pompa^{d,i}, Alberto Danielli^{f,g,j*}, Fabio Benfenati^{a,b*}, Valentina Castagnola^{c*}

- a. Center for Synaptic Neuroscience and Technology, Istituto Italiano di Tecnologia, Largo Rosanna Benzi 10, Genova, 16132 Italy.
- b. IRCCS Ospedale Policlinico San Martino, Largo Rosanna Benzi 10, Genova, 16132 Italy.
- c. Fondazione Pisana per la Scienza ONLUS, Via Ferruccio Giovannini, 13, San Giuliano Terme (Pisa), 56017 Italy.
- d. Nanobiointeractions & Nanodiagnostics, Istituto Italiano di Tecnologia (IIT), Via Morego 30, Genova, 16163 Italy.
- e. Dipartimento di Promozione delle Scienze Umane e della Qualità della Vita, Università Telematica San Raffaele, Via di Val Cannuta, 247, 00166 Rome, Italy
- f. Dipartimento di Farmacia e Biotecnologie, Alma Mater Studiorum – Università di Bologna, via Francesco Selmi 3, 40126 Bologna, Italy.
- g. IRCCS AOUBO Sant’Orsola – Laboratory of Preclinical and Translational Research in Oncology (PRO), Bologna 40138, Italy.
- h. Dipartimento di Chimica “Giacomo Ciamician”, Alma Mater Studiorum – Università di Bologna, Via Francesco Selmi 2, 40126 Bologna, Italy.
- i. College of Optical Science and Engineering, Zhejiang University, Hangzhou, 310058, P. R. China
- j. Consorzio Interuniversitario Biotecnologie, 34148 Trieste, Italy.

§ equal contribution

* corresponding authors: alberto.danielli@unibo.it, fabio.benfenati@iit.it, valentina.castagnola@iit.it

Supplementary Figures

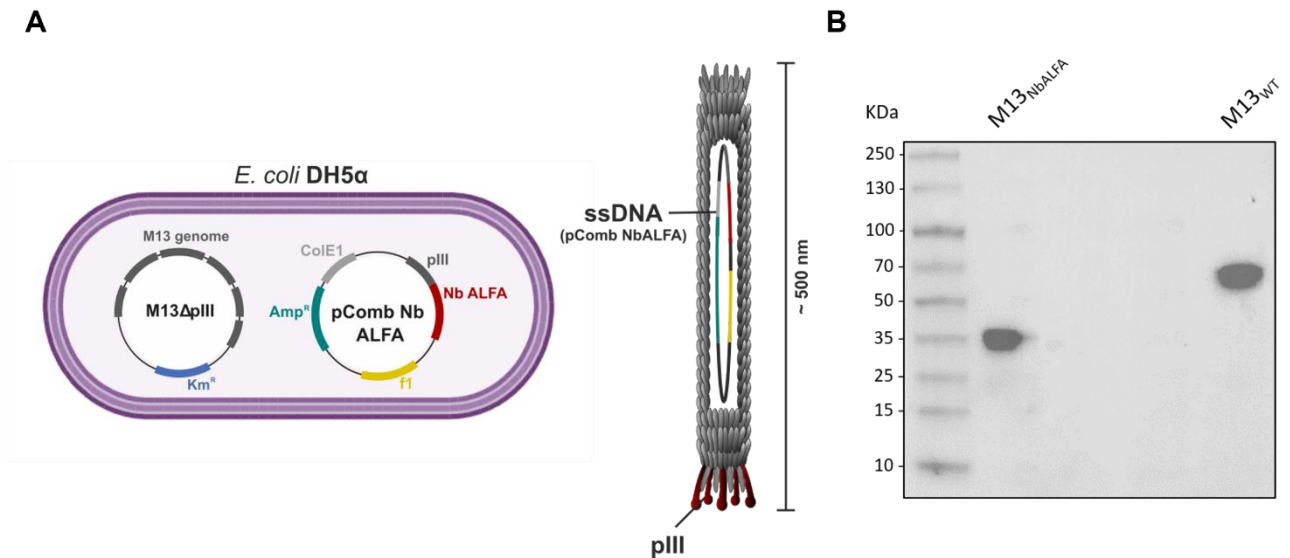


Figure S1. M13_{NbALFA} preparation strategy. (A) M13_{NbALFA} was obtained through the co-transformation method, using a phagemid encoding for an anti-ALFA-Tag nanobody on the pIII protein and the helper phage vector HyperΔori in *E. Coli* DH5α cells. (B) Immunoblot analysis of phage pIII coat protein in M13_{WT} and M13_{NbALFA}, confirming that the pIII protein in M13_{NbALFA} was successfully engineered.

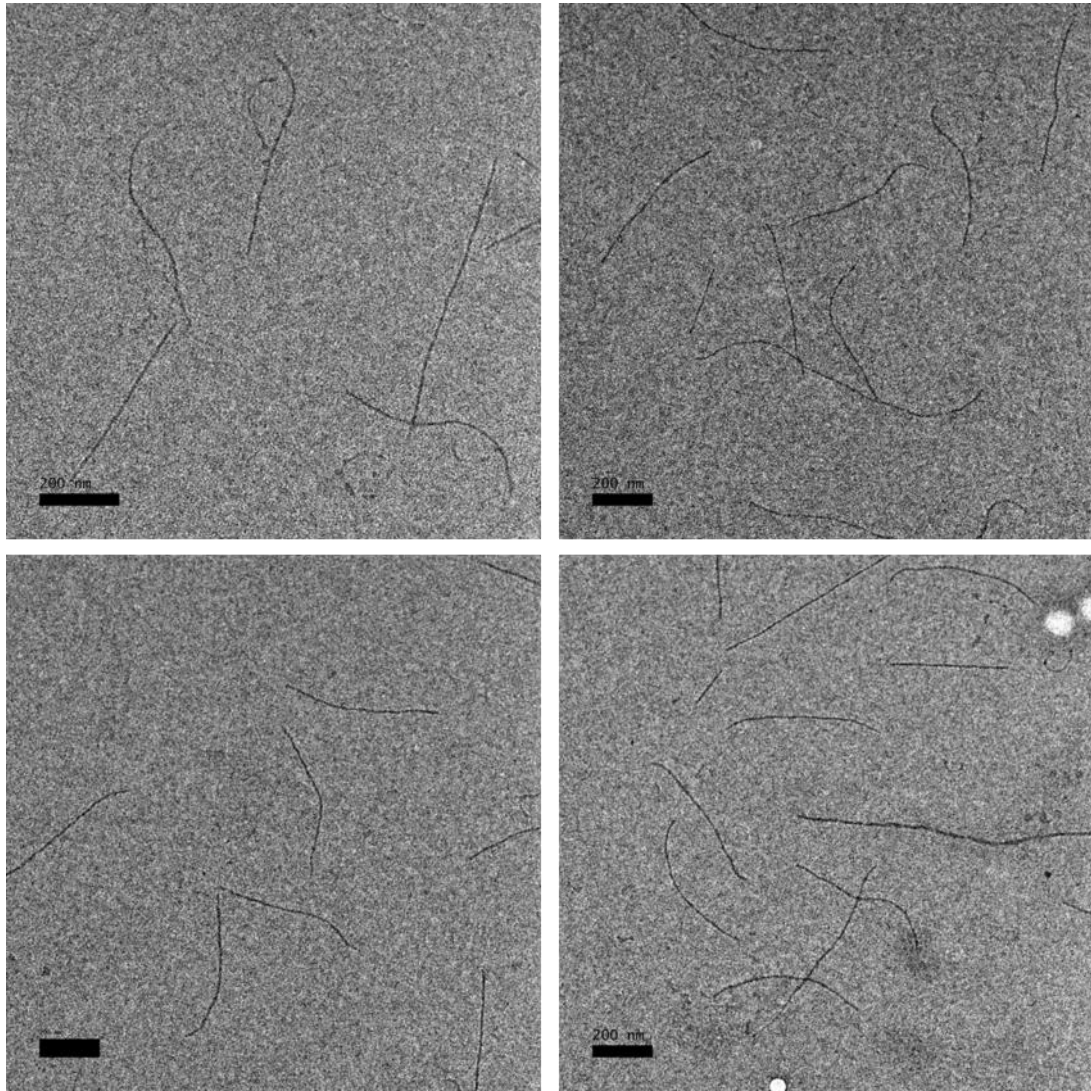


Figure S2. M13_{NbALFA} representative TEM micrographs. Negative-stained M13_{NbALFA} TEM images showing phages with heterogeneous lengths (mainly two populations). Scale bars: 200 nm.

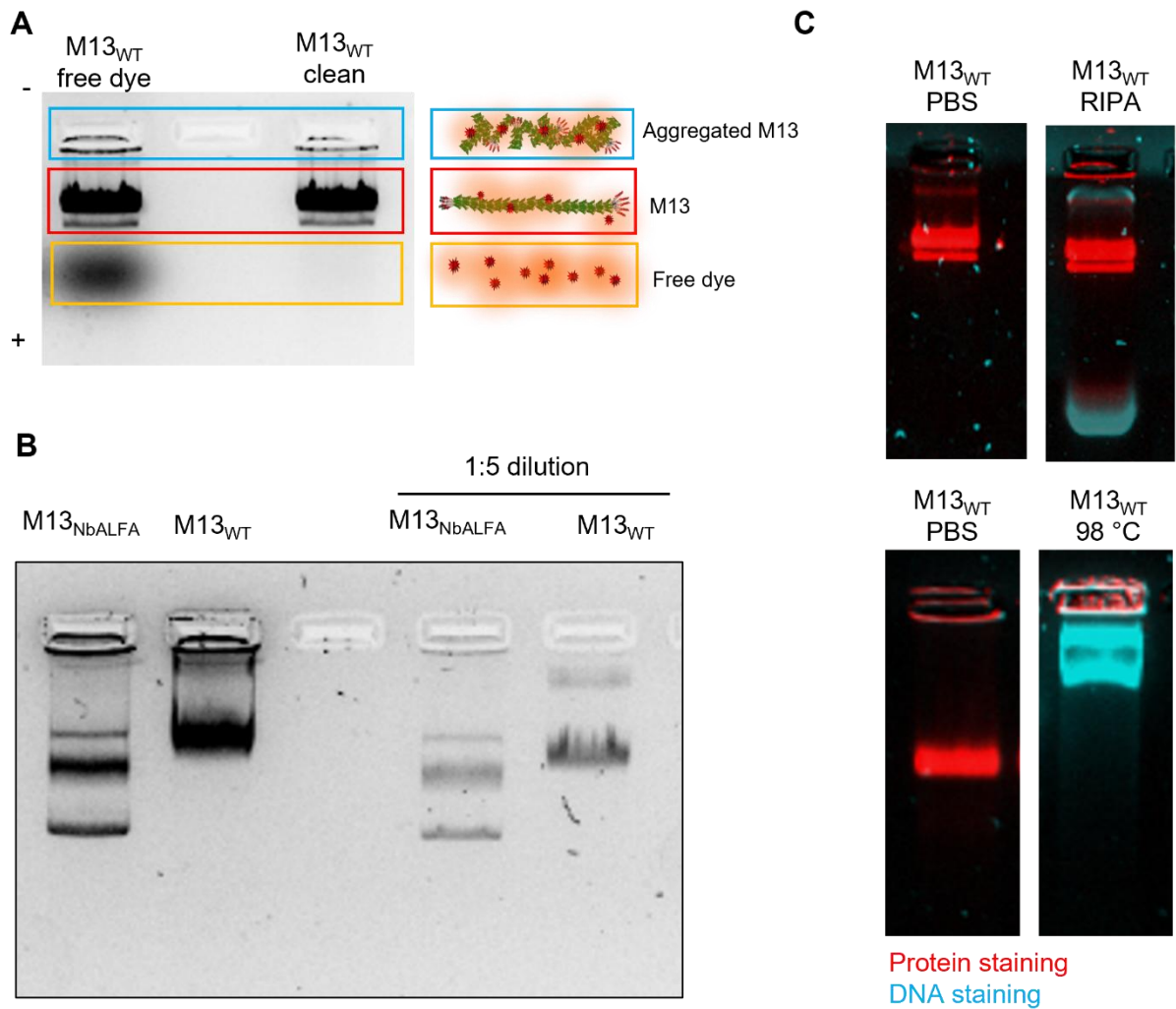


Figure S3. M13_{WT} labeling and evaluation of the aggregation/degradation state. (A) 2% agarose gel to characterize M13_{WT} labeling with CF594 dye. Three populations are visible in the gel: (i) aggregated phage trapped in the gel well (light blue square); (ii) intact phage labeled with a fluorescent dye (red square); and (iii) free dye contamination (yellow square). The signal is generated by the CF594 label. (B) 2% agarose gel to characterize M13 aggregation state. Two concentrations of both M13_{WT} and M13_{NbALFA} were loaded in the gel. The signal of the in-gel phage bands visualized by SybrGold DNA staining decreases proportionally (lanes on the left), while the signal in the wells completely disappears after 1:5 dilution (lanes on the right), confirming that the reversibly aggregated phage can be re-suspended by dilution, thus excluding irreversible aggregation or degradation. (C) 2% agarose gel to characterize M13 degradation by comparing intact M13_{WT} (left, PBS) and denatured M13_{WT}. Denaturation was performed by two strategies: incubation in RIPA buffer (above) or boiling at 98 °C for 15 min (below). When the protein coat is degraded, a free DNA smear is visible (red = CF594 protein staining; cyan = SybrGold staining).

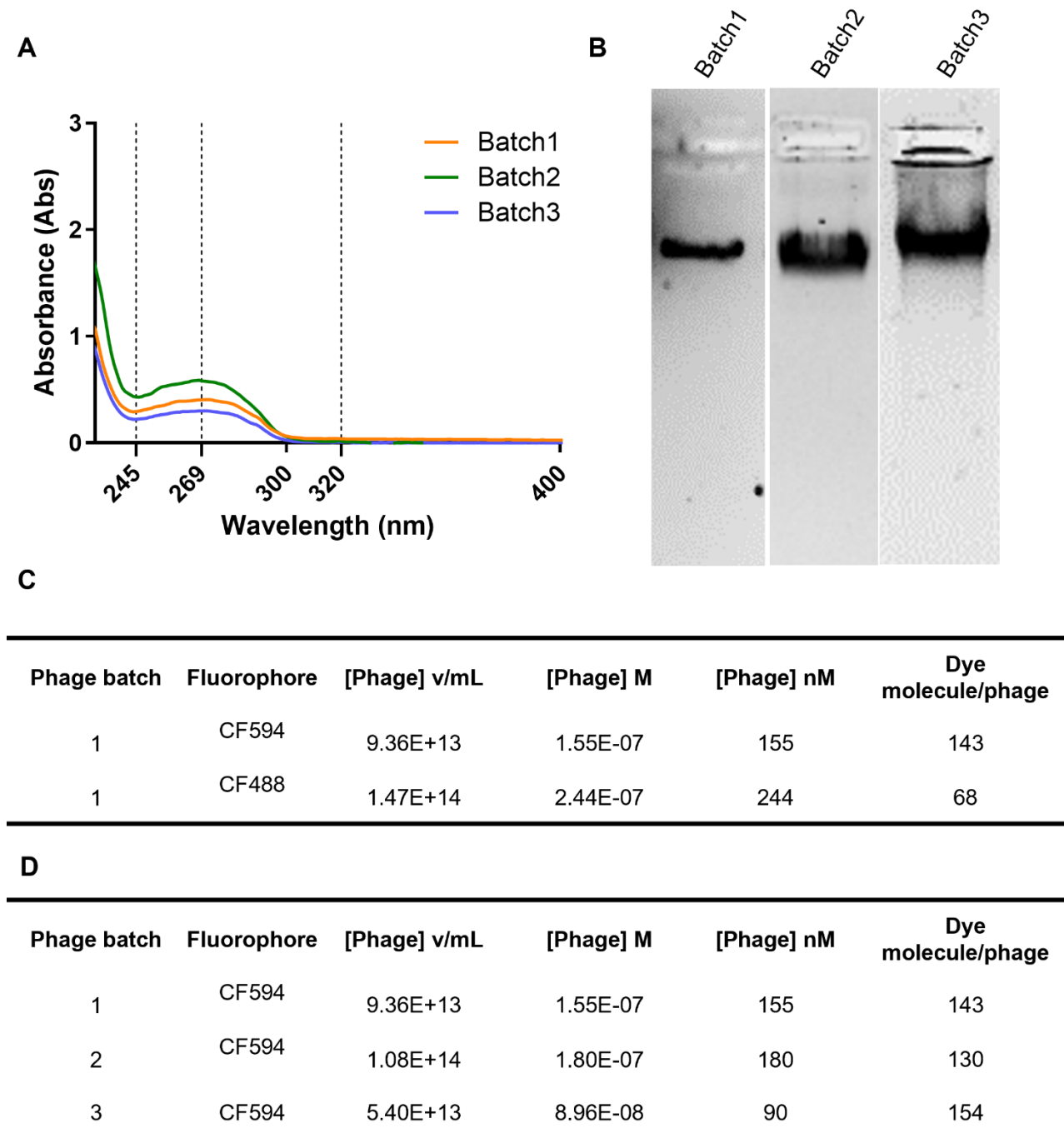


Figure S4. Reproducibility of the M13_{WT} production. **(A)** UV-VIS spectra of three batches after production and purification. **(B)** 2% agarose gel showing three M13_{WT} batches visualized by SybrGold staining that intercalates the phage DNA. **(C)** M13_{WT} phages can be conjugated to different dyes through the NHS reaction, resulting in variable phage-to-dye ratios. **(D)** Reproducibility of the phage-dye conjugation reaction yielding similar dye/phage ratios.

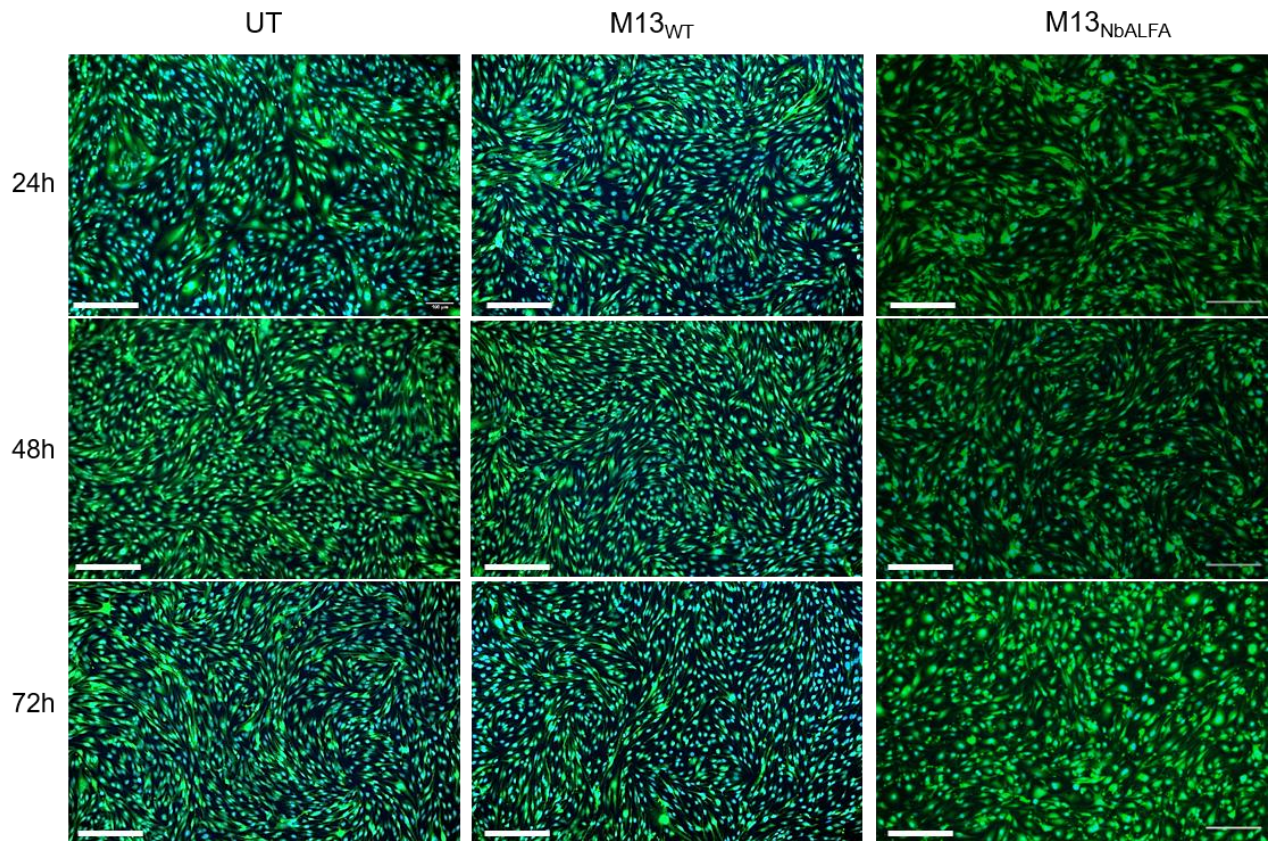


Figure S5. bEnd.3 cell viability in the presence of M13_{WT} and M13_{NbALFA}. The bEnd.3 cells were treated for 24, 48, 72 h with 5×10^{11} v/mL and stained with PI and Calcein-AM. As it can be seen from the representative images, the cell viability was not affected by the M13 phage treatment (blue: nuclei; green: Calcein-AM; red: PI; scale bar: 200 μ m) and the quantification for n=3 independent replicates resulted nearly 100% viability for both conditions.

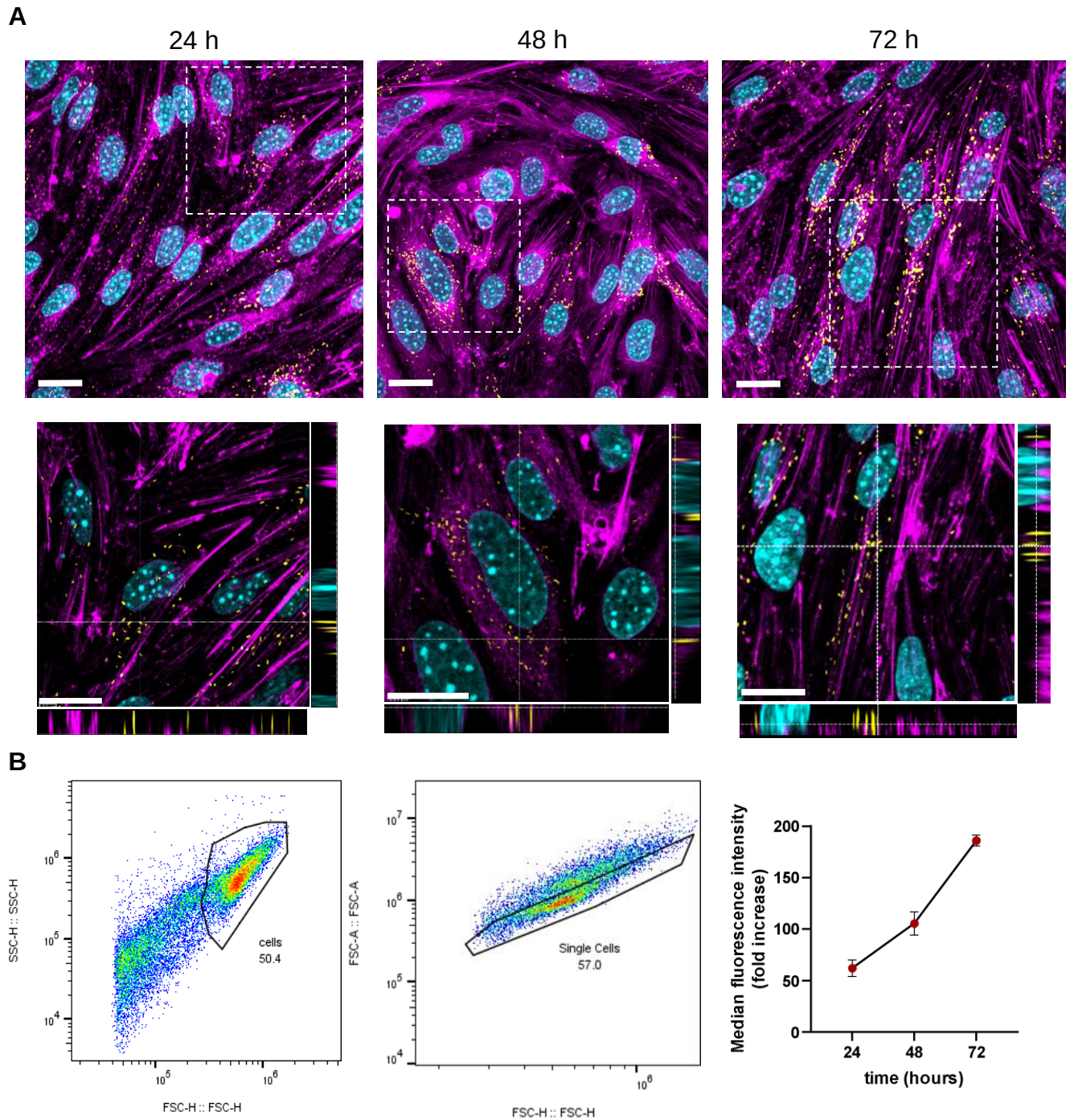


Figure S6. M13_{WT} uptake in bEnd.3 cells. (A) Representative max projection confocal images and single stack orthogonal **view insets** at higher magnification (below) showing M13_{WT}-594 internalization in bEnd.3 cells at different time points (cyan: nucleus; magenta: actin; yellow: M13_{WT}-594; scale bar: 20 μ m). (B) Flow cytometry gating strategy (left and center panels) and internalization kinetics expressed as per-cell median fluorescent intensity (right panel, n=3).

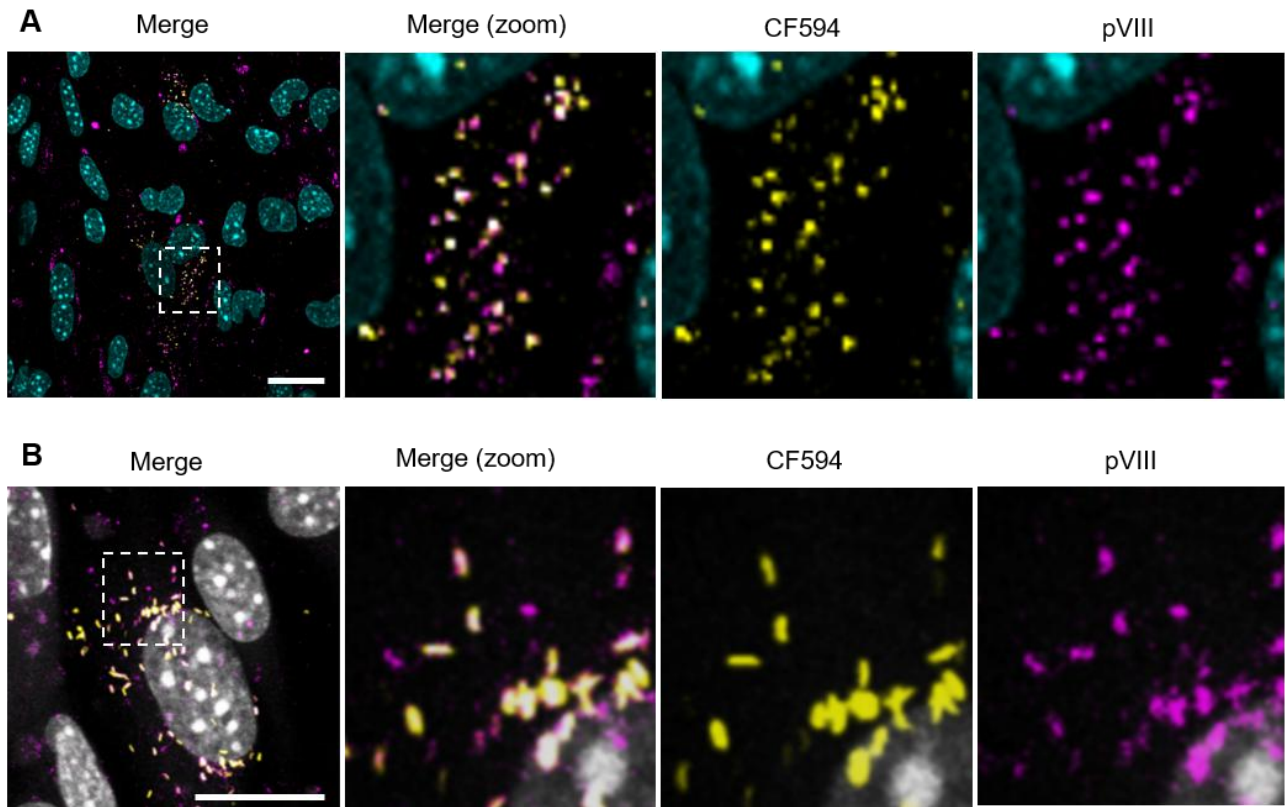


Figure S7. Colocalization analysis of the M13_{WT}-594 signal and anti-pVIII antibody. Representative confocal images and higher magnification insets. Cells were treated with 5×10^{11} v/mL of M13_{WT}-594 for 24 h (**A**) or 72 h (**B**), fixed, and immunostained for the M13 pVIII major coat protein. The CF594 signal (yellow) and the anti-pVIII (magenta) completely colocalized, as indicated in the zoomed areas of the image (cyan/grey: nuclei; scale bar: 20 μ m). Colocalization analysis at different time points is reported in Figure S11.

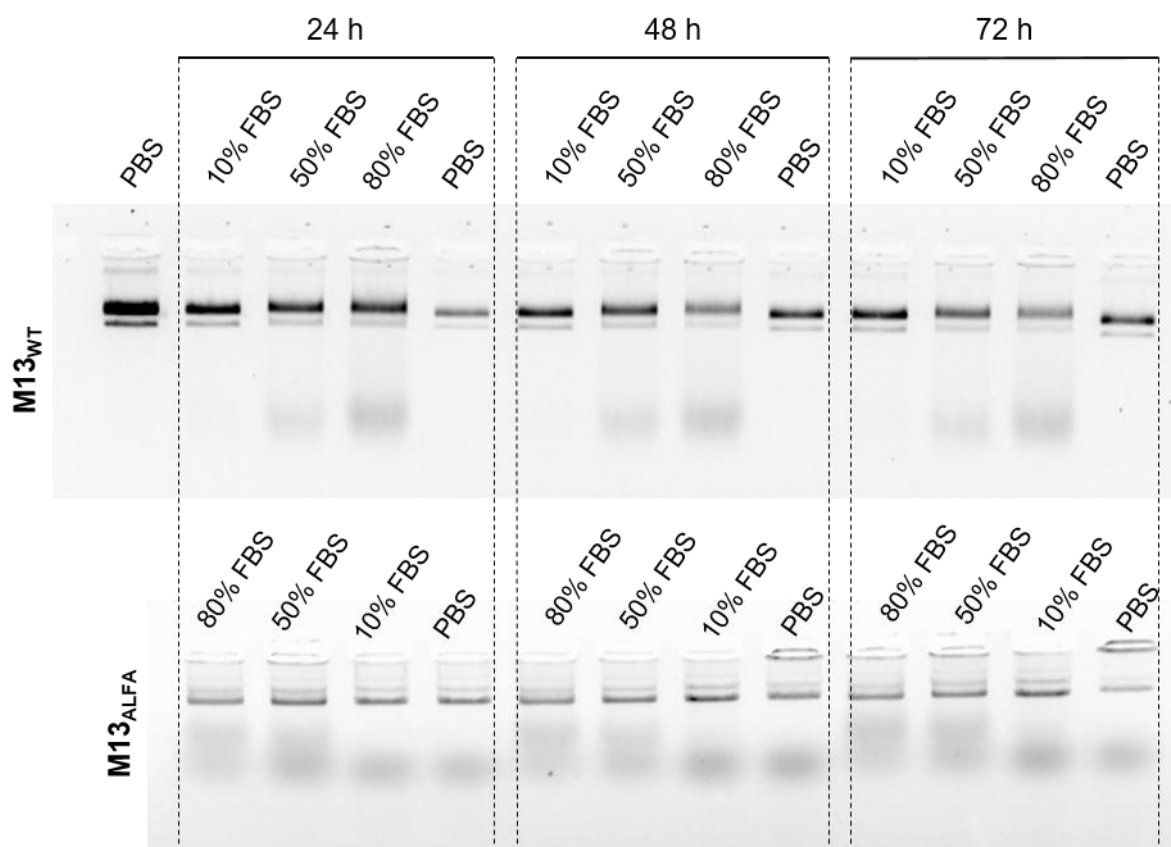


Figure S8. M13_{WT}-594 and M13_{NΔALFA}-594 stability in complex media. To mimic *in vivo* conditions, M13_{WT} and M13_{NΔALFA} were incubated for 24, 48, and 72 h in PBS or 10, 50, or 80% FBS at 37 °C. The phages were then loaded and run in a 2% agarose gel and visualized with an iBright gel imaging system in fluorescence mode. Both phages showed excellent stability in all the tested conditions.

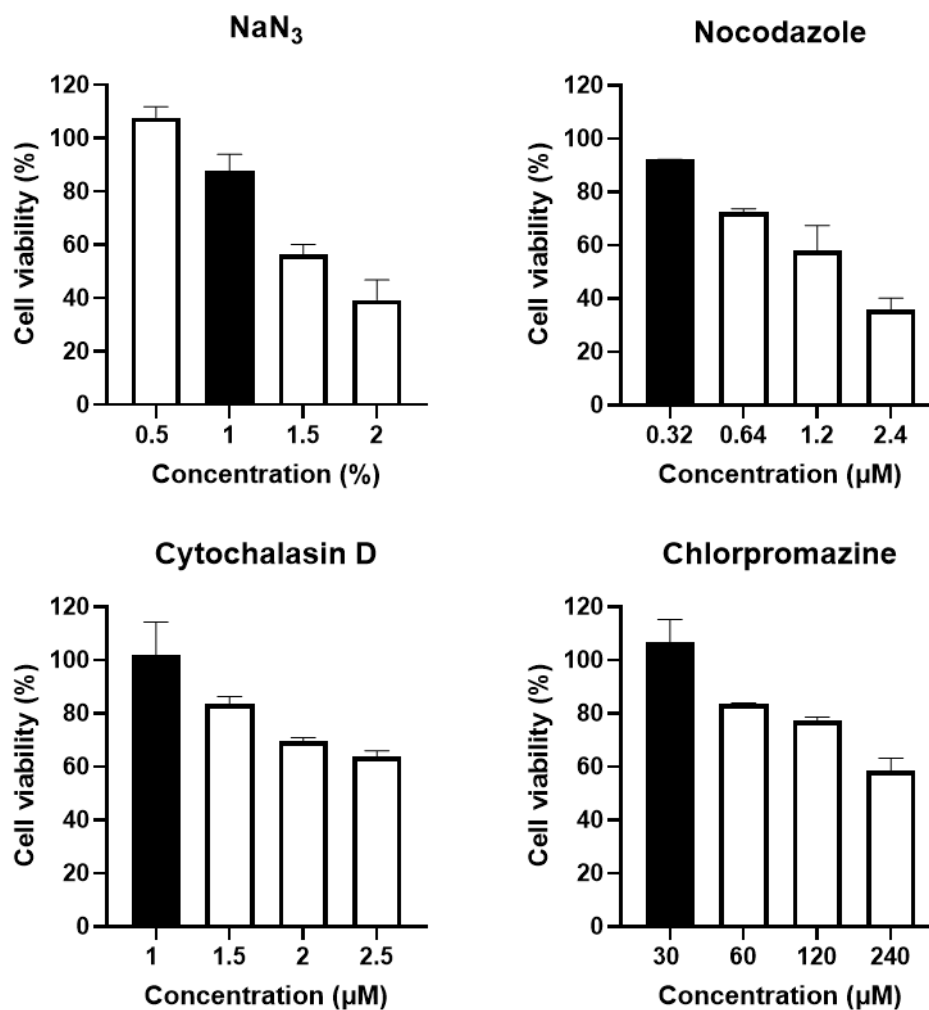


Figure S9. Cytotoxicity assessment for uptake inhibitors. The MTT cytotoxicity assay confirmed that the chemical inhibitor maintained a cell viability over 80 % at the concentration used in the cell entry experiments (black bar, means $\pm$ SEM, n=2).

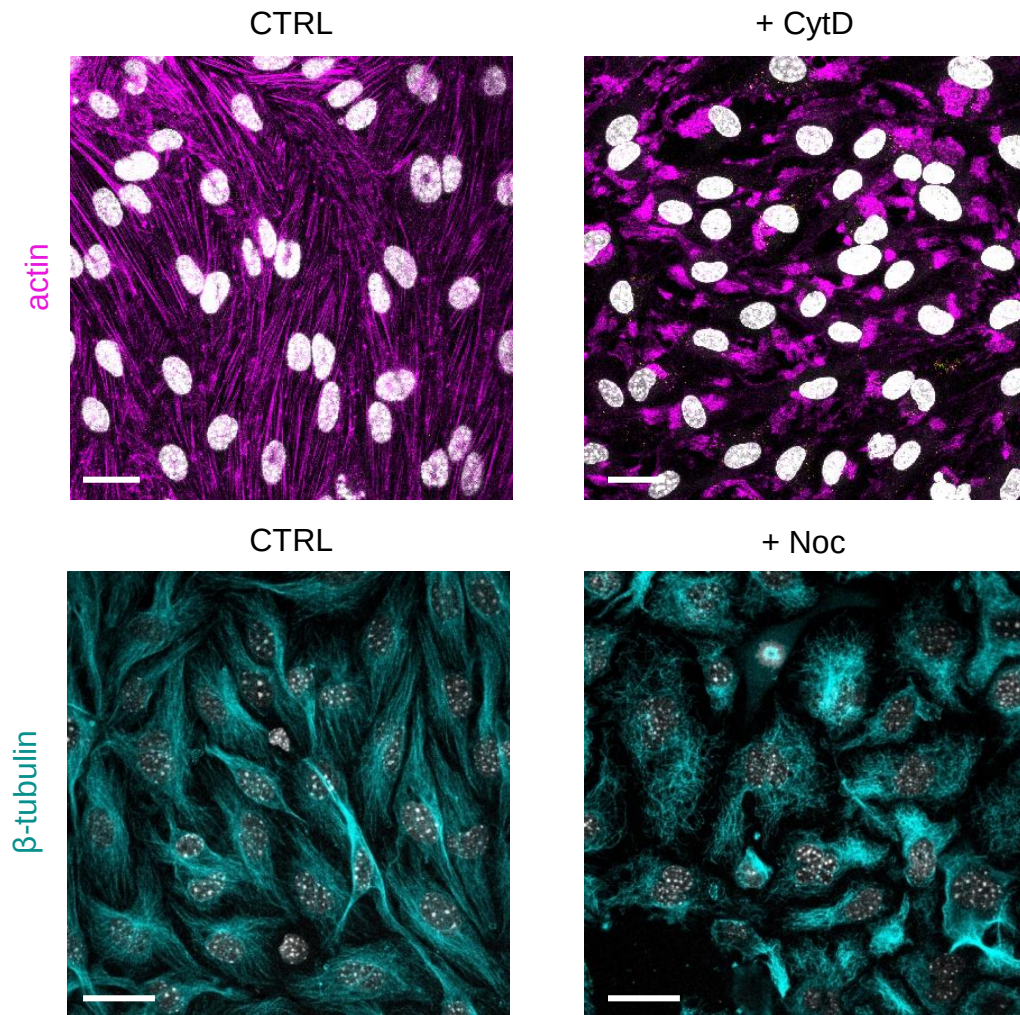


Figure S10. Uptake inhibition platform. bEnd.3 cells treated with either Cytochalasin D (CytD, 1 μ M) or Nocodazole (Noc, 320 nM) for 30 min. The staining shows disruption of actin filaments (pink) and depolymerization of β -tubulin (cyan). **Scale bar: 20 μ m.**

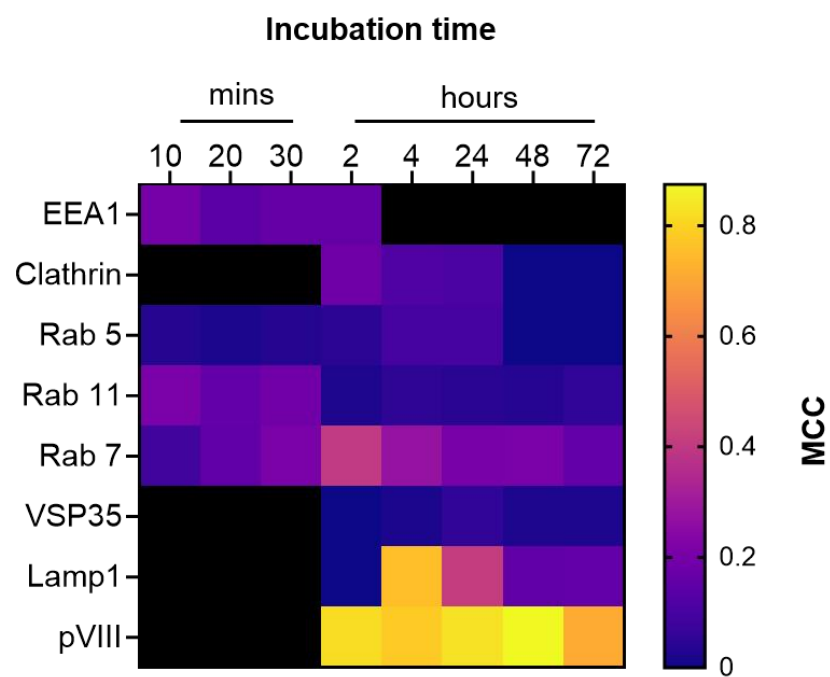
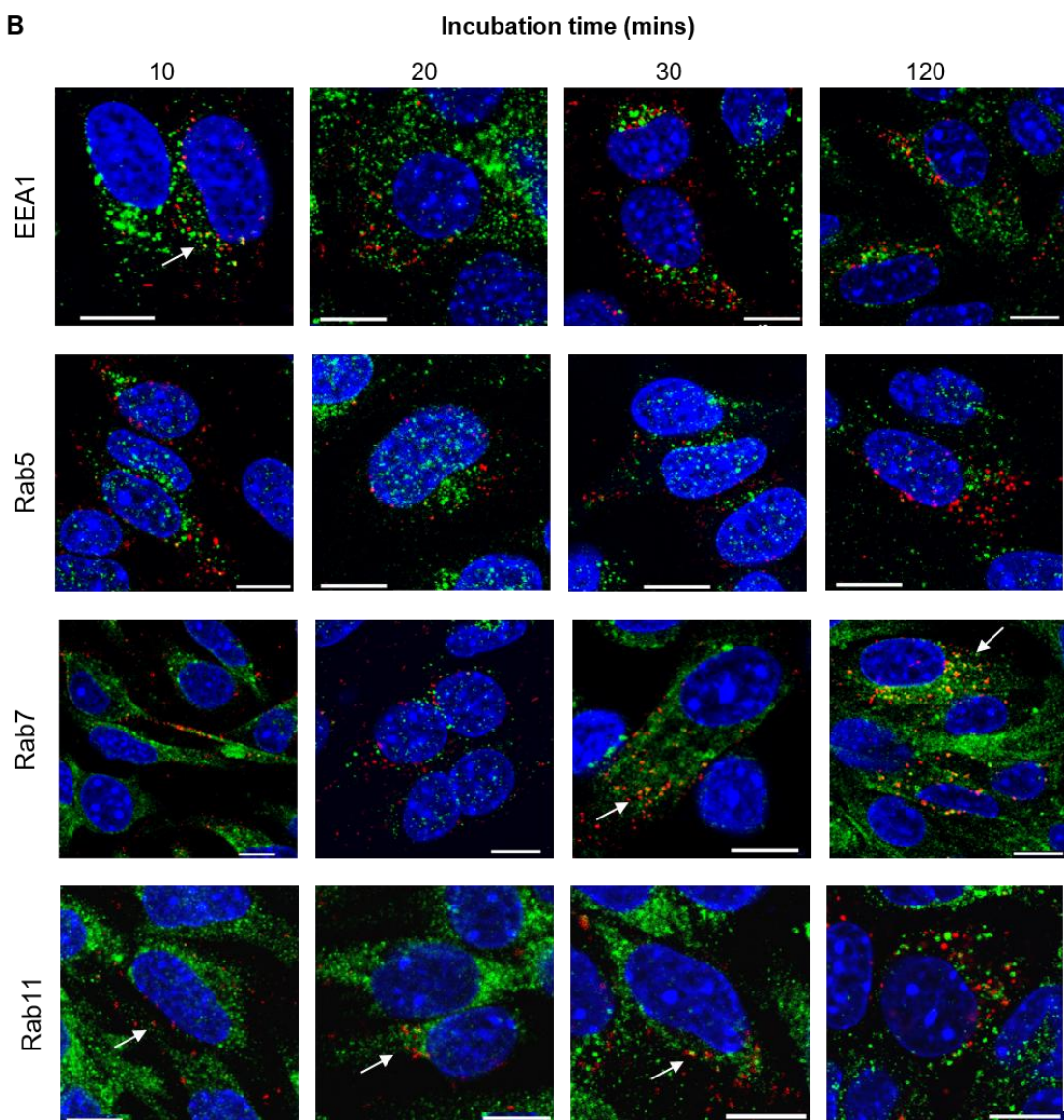
A**B**

Figure S11. M13_{WT} co-localization with intracellular markers. (A) Heat-map showing the Manders' colocalization coefficient (MCC) for M13_{WT}-594 for distinct intracellular markers at different time points (10, 20, 30 and 120 min and 2, 4, 24, 48 and 72 hours). Analyzed markers are: EEA1 and Rab 5 (early endosomes), Rab 7 (late endosomes), and Rab 11 (recycling endosomes), clathrin (endocytic uptake), VSP35 (retromer complex), LAMP1 (lysosomes), as measured from confocal images on n = 2 biological replicates (minimum 10 fields per condition, minimum 40 cells per field on each replicate). MCC for M13_{WT}-594 over Ab anti-pVIII shows control values > 0.7 for all time points. **Scale bar: 10µm.**

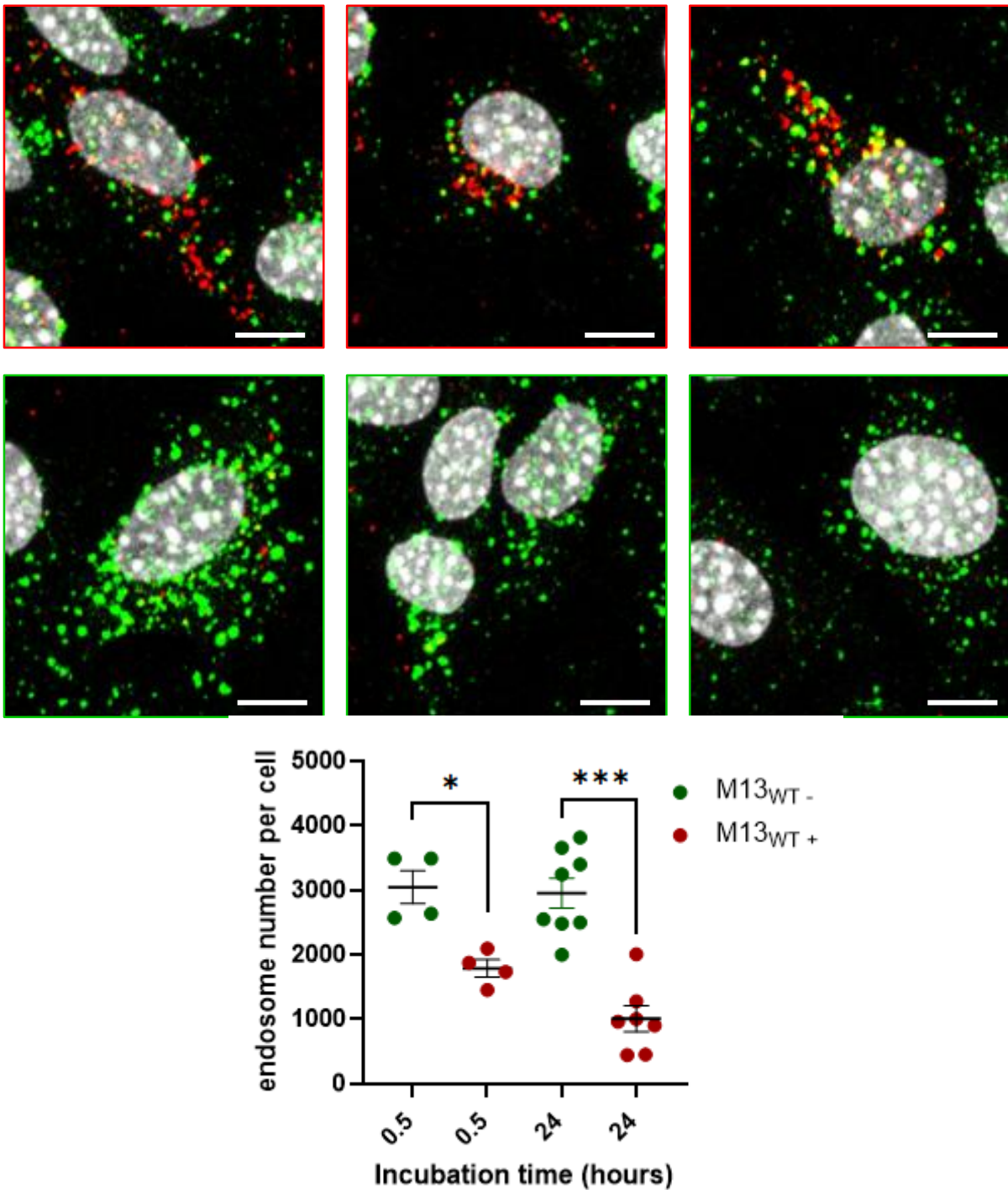


Figure S12. Early endosomes analysis. Top: Representative single-stack confocal images of bEND.3 cells incubated with 5×10^{11} v/mL of M13_{WT} (red) for 30 min and immunostained for EEA1 (green). The first line show cells with high amount of internalized phages, while the second line show cells with scarce phage uptake. Bottom: Quantitative analysis of total early endosomes per cell in M13_{WT} treated samples. The cells were classified as phage poor (M13_{WT} -, green dots) or phage rich (M13_{WT} +, red dots) based on the intracellular phage quantity (measured as phage fluorescence intensity *per cell*). The endosomes were then counted in the two cell populations and compared. Data are means $\pm$ SEM of 4-8 fields per condition (150 total cells on average per condition). Statistical analysis: * $p < 0.05$, *** $p < 0.001$ two-tailed Mann-Whitney *U*-test. Scale bar: 10 μ m.

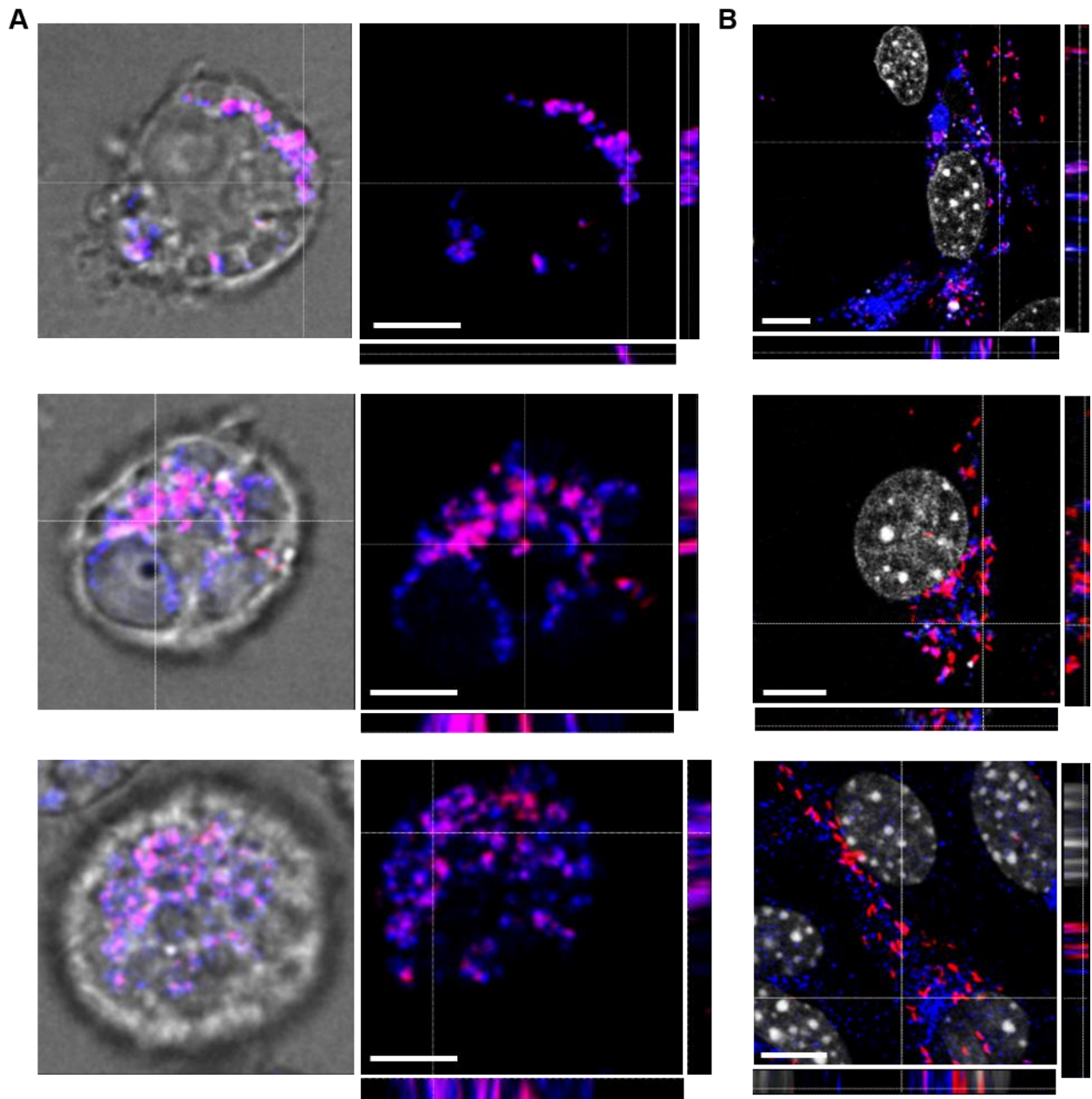


Figure S13. Representative imaging of M13_{WT} intracellular lysosomal distribution in various cell lines. (A) RAW267.4 cell and (B) bEnd.3 cells showed different intracellular distribution, with high colocalization with lysosomes in RAW264.7 cells, and low colocalization in bEnd.3 cells. The confocal images are here shown as orthogonal view to confirm the different distribution on the z-axis (grey: bright field (RAW 267.4) or nuclei (bEnd.3); red: M13_{WT}; blue: lysosomes. Scale bars: 5 μm).

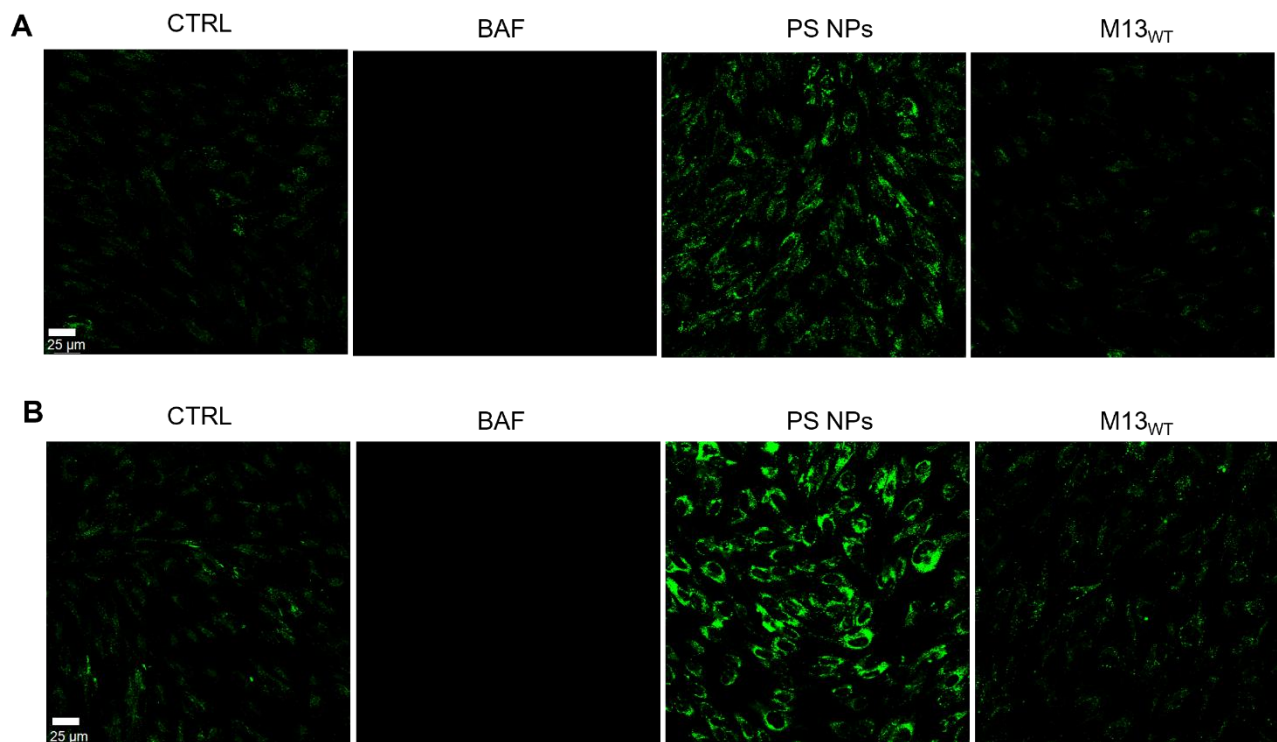


Figure S14. Representative imaging of bEnd.3 cells. Cells were treated with bafilomycin A1 (BAF; 100 nM), 100 nm PS NPs (100 μg/mL), and M13_{WT} (5x10¹¹ virions/mL) for 24 (**A**) and 72 (**B**) hours and stained with Lysotracker green (scale bar: 25 μm).

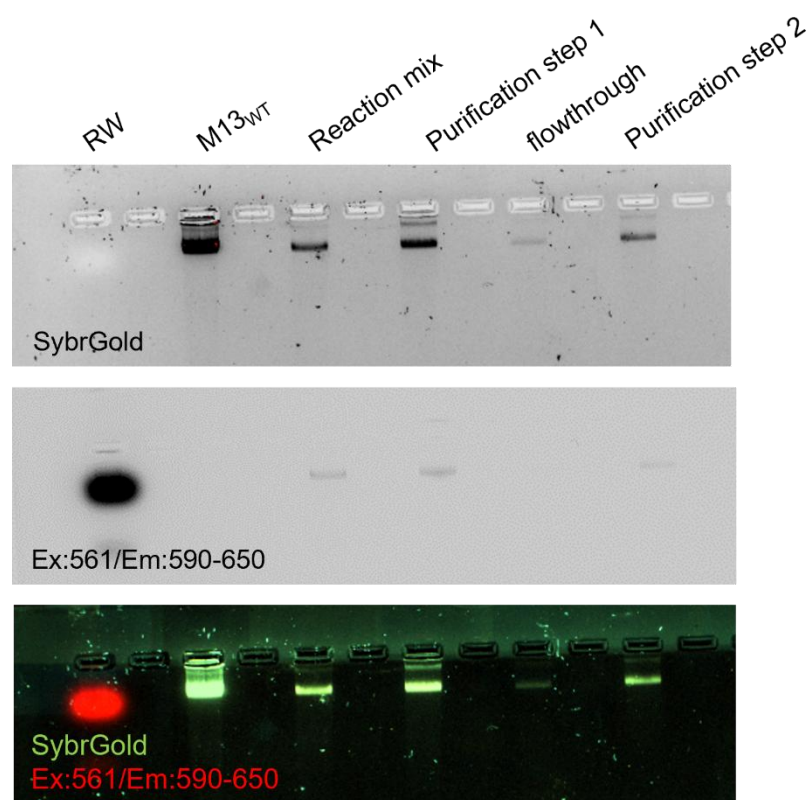


Figure S15. M13_{WT} conjugation with RatioWork dye. 2% agarose gel showing the electrophoretic run of RatioWork dye (RW), M13_{WT}, reaction mix, M13_{WT}-RW after the first passage through Zeba column (purification step 1), column flowthrough, and M13_{WT}-RW after the second passage through Zeba column (purification step 2). The agarose gel is reported as stained by SybrGold (DNA, upper panel), RW fluorescence (middle panel), and the merge of the two (lower panel) to appreciate the overlapped DNA signal (M13_{WT}) and RW signal, and the absence of free dye in the flowthrough, indicating efficient conjugation under the used conditions.

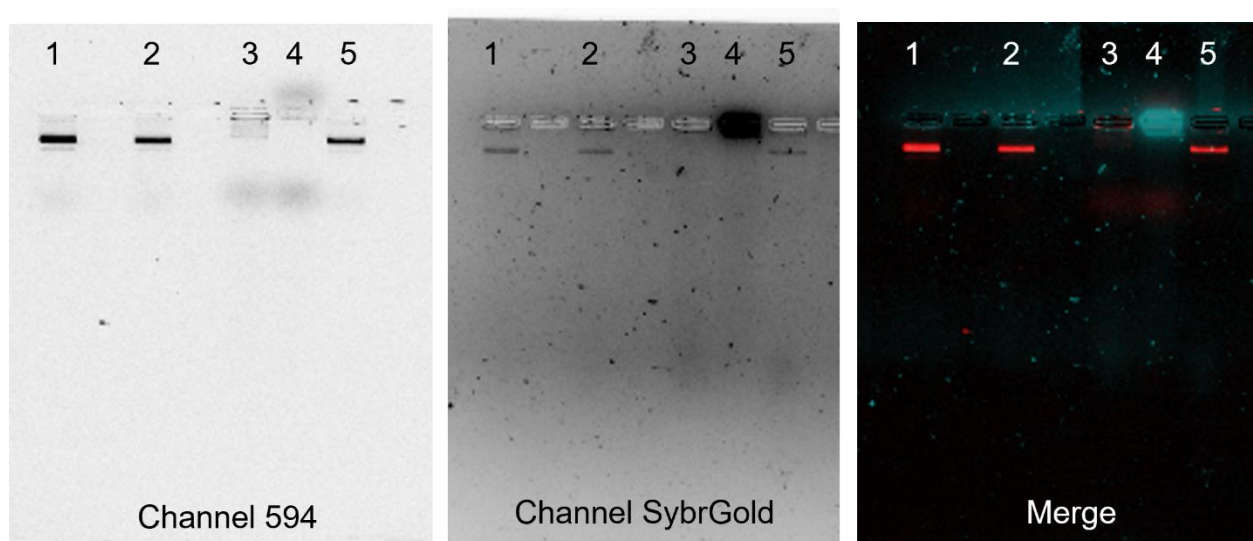


Figure S16. M13_{WT} stability in artificial lysosomal fluid (ALF). 2% agarose gel electrophoretic run for: 1) M13_{WT} stock; 2) M13_{WT} in PBS at 37 °C; 3) M13_{WT} in PBS solution of Pronase (0.2 units/mL) at 37 °C; 4) M13_{WT} in ALF solution (pH 4.5) of Pronase (0.2 units/mL) at 37 °C; 5) M13_{WT} in ALF solution (pH 4.5) at 37 °C; M13_{WT} concentration is 1×10^{12} v/mL for all conditions. M13_{WT} label channel, DNA label channel, and the merge of the two are presented.

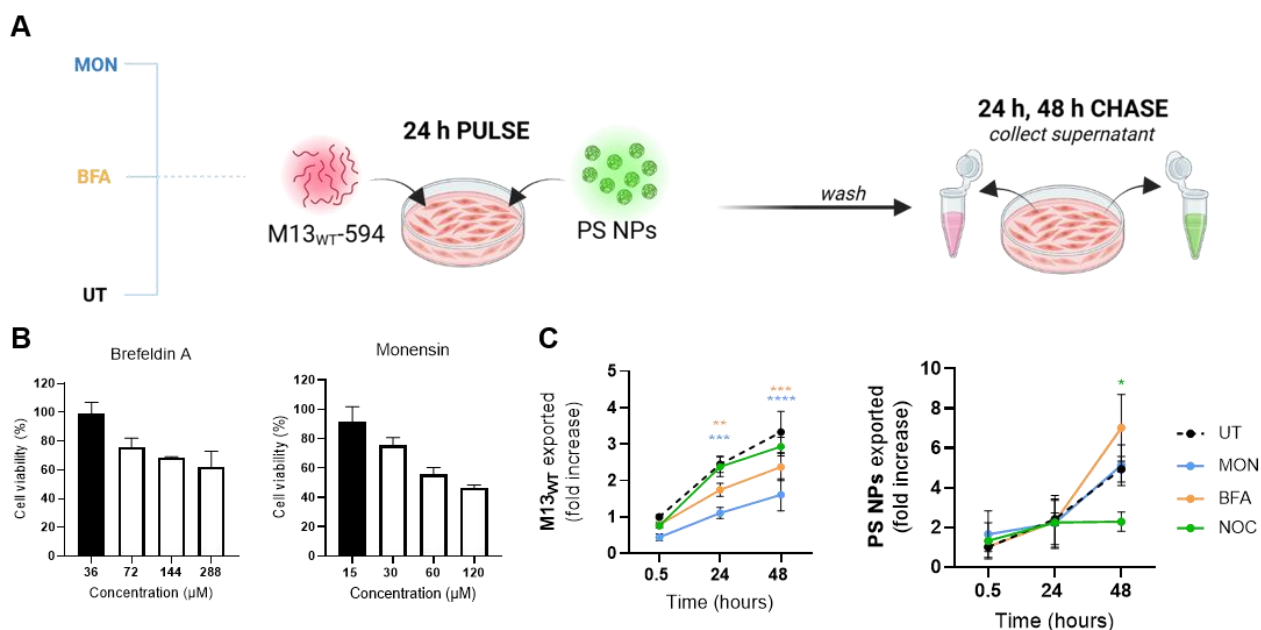


Figure S17. Pulse-chase experiment to study export mechanisms. (A) Schematic of the experimental setup. (B) MTS cytotoxicity assay (means $\pm$ SEM, $n=3$) confirming that the inhibitors employed in the experiments do not affect the cell viability. The black bars represent the concentrations selected for the experiments. (C) The export is quantified by measuring the fluorescence values of the apical culture media in a bEnd.3 confluent culture. Following 24 h incubation with either M13_{WT}-594 (left) or PS NPs (right), cells were washed and the media replaced. Then, the fluorescence signal was measured at 30 min, 24 h, and 48 h and normalized to control conditions. The experiment was performed in the absence or presence of brefeldin A (BFA – endoplasmic reticulum-Golgi transport inhibitor), monensin (MON- protein transport inhibitor), and nocodazole (NOC – microtubule depolymerization), respectively. While export for PS NPs is not affected by the presence of either inhibitor, M13_{WT} export was significantly decreased. Data are means $\pm$ SEM, $n=3$. Statistical analysis: *** $p<0.001$, **** $p<0.0001$, two-way ANOVA/Tukey's tests.

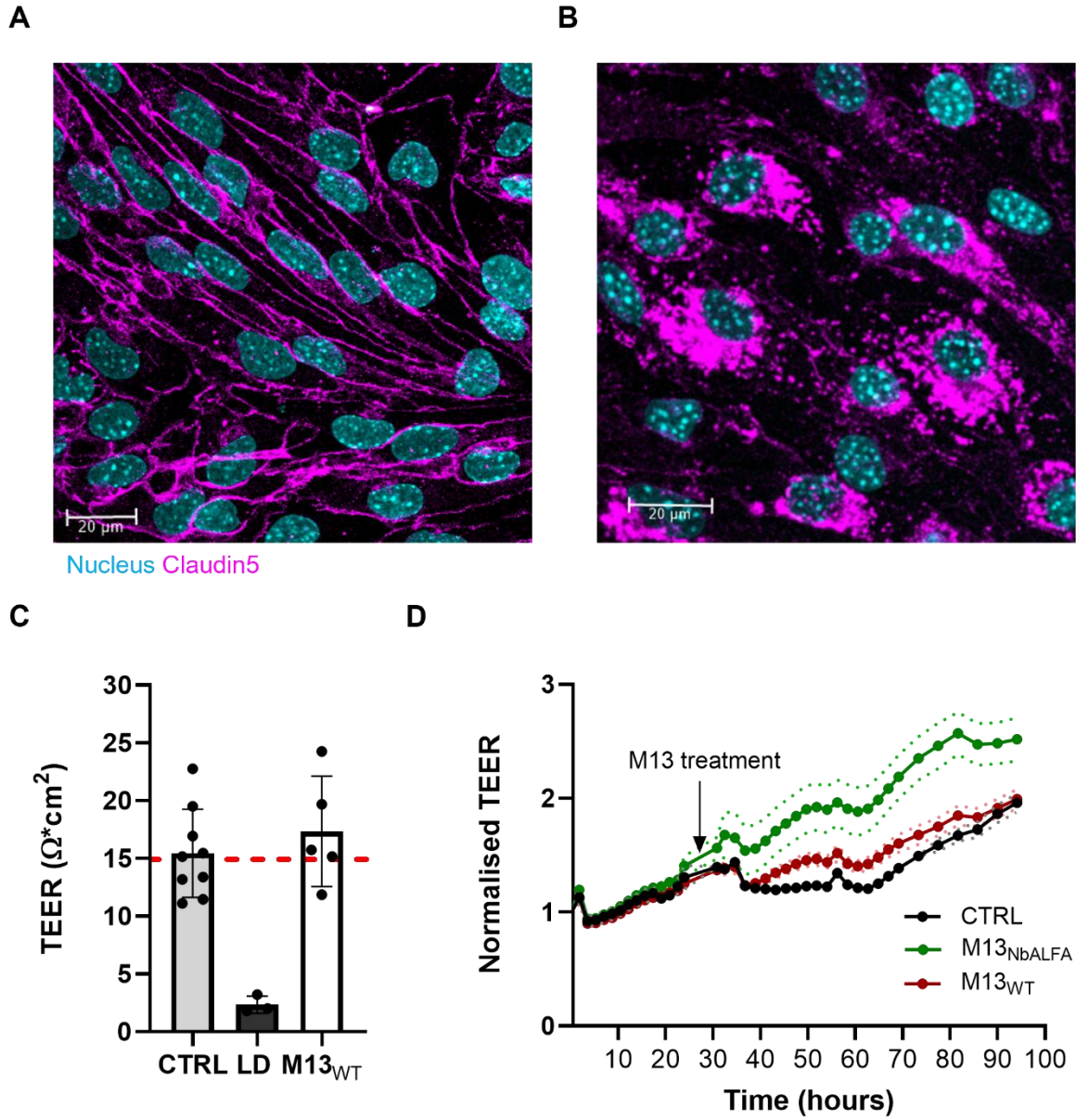


Figure S18. Validation of the 2D-Transwell BBB model. (A,B) Confocal imaging of bEnd.3 cells seeded onto transwell inserts at high (A; 60000 cells/well) and low (B; 10000 cells/well, LD) density and kept in culture for 72 h, showing a different tight junction spatial organization, visualized through immunostaining with anti-claudin5 antibody (magenta). LD transwells do not possess a formed barrier (cyan: nuclei; scale bar: 20 μm). (C) Average TEER values reached before the phage treatment in BBB crossing experiments. (D) Representative time profile of TEER values (normalized over the value at T=0) monitored throughout the experiments, showing that the barrier was stable and was not altered after M13 treatment. Values are presented as means $\pm$ SEM.

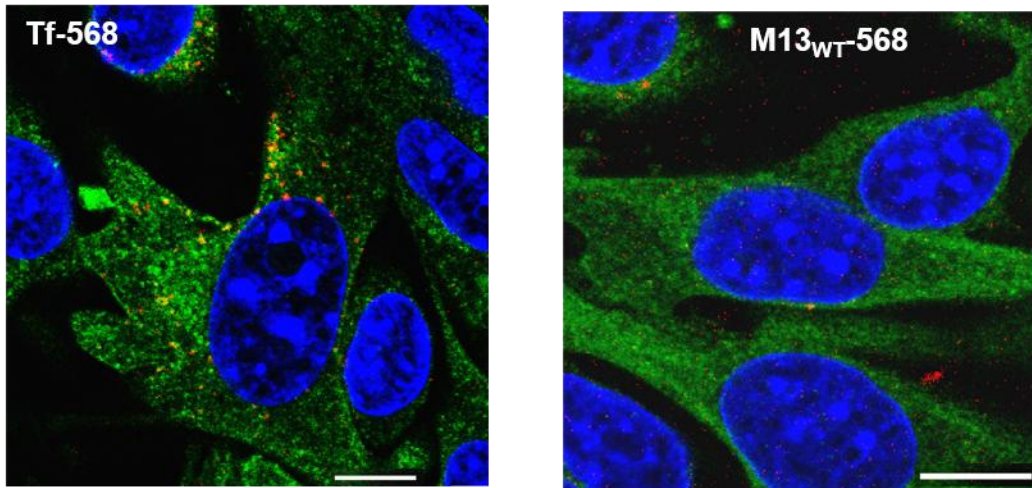
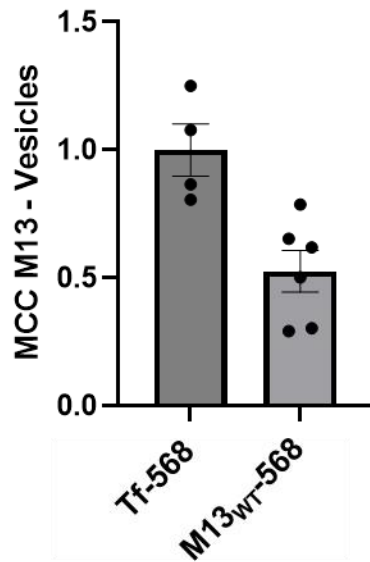
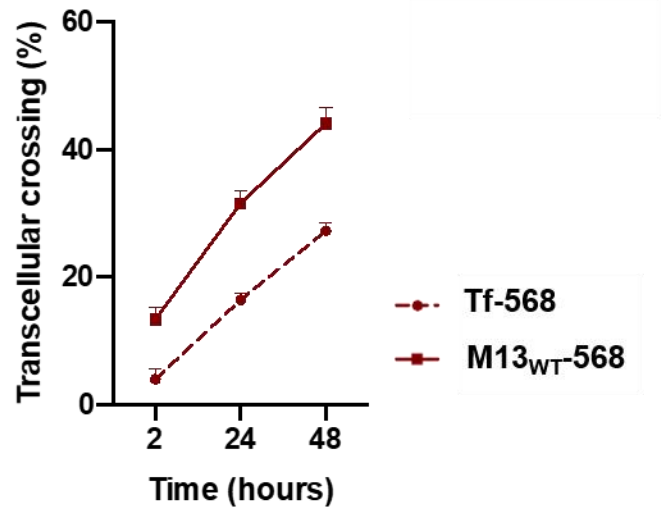
A**B****C**

Figure S19. Comparison with Transferrin transport at the BBB. (A) Representative confocal imaging and (B) Normalized Mander's colocalization coefficient (MCC) for fluorescently labeled transferrin (Tf-568) and M13_{WT}-568, respectively, with clathrin (4-6 fields, 15 cells per condition). (C) Comparison of the kinetics of permeability for Tf-568 and M13_{WT}-568 through a 2D-Transwell BBB model evaluated by fluorescent quantification in the basolateral fraction and expressed as a percentage of crossing through a fully formed barrier (transcellular crossing), normalized against a non-confluent cell layer (paracellular crossing). bEnd.3 cells were treated with 5×10^{11} v/mL of M13_{WT}-568 and 5 μ g/mL of Tf-568 (n=3, mean $\pm$ SEM).

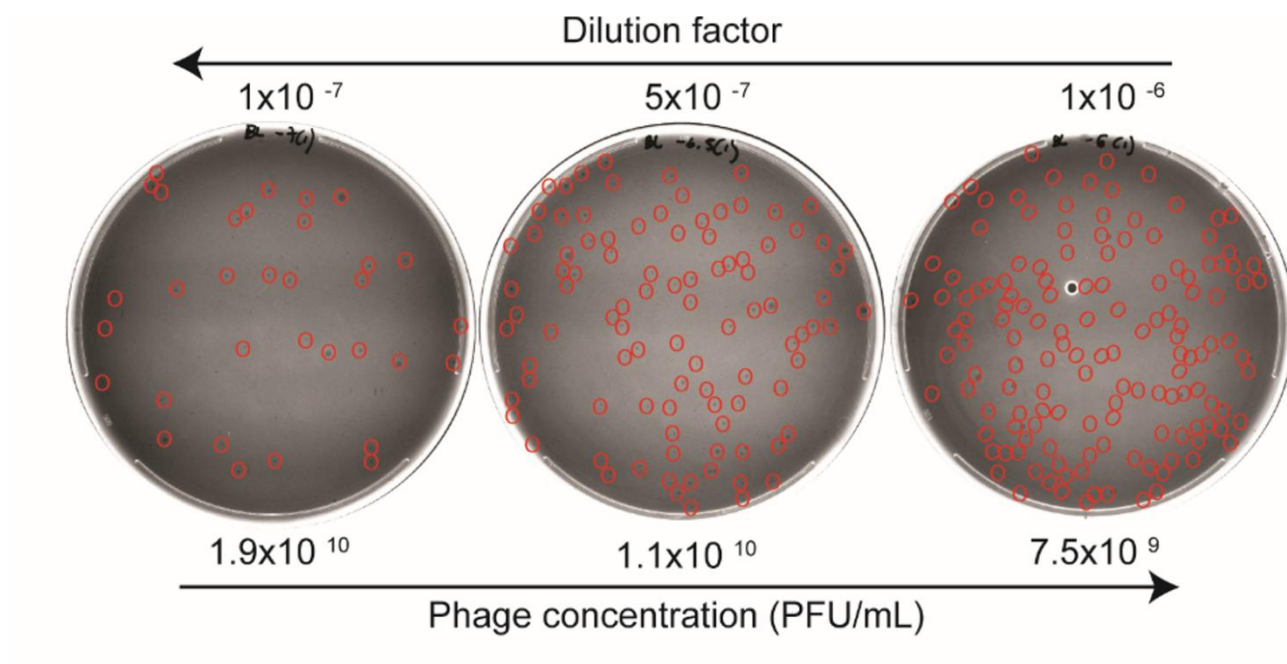


Figure S20. Representative plaque assay dishes. The dishes show three serial dilutions of M13_{WT} collected in the basolateral compartment after 72 h of incubation with bEnd.3 cells from the apical compartment.

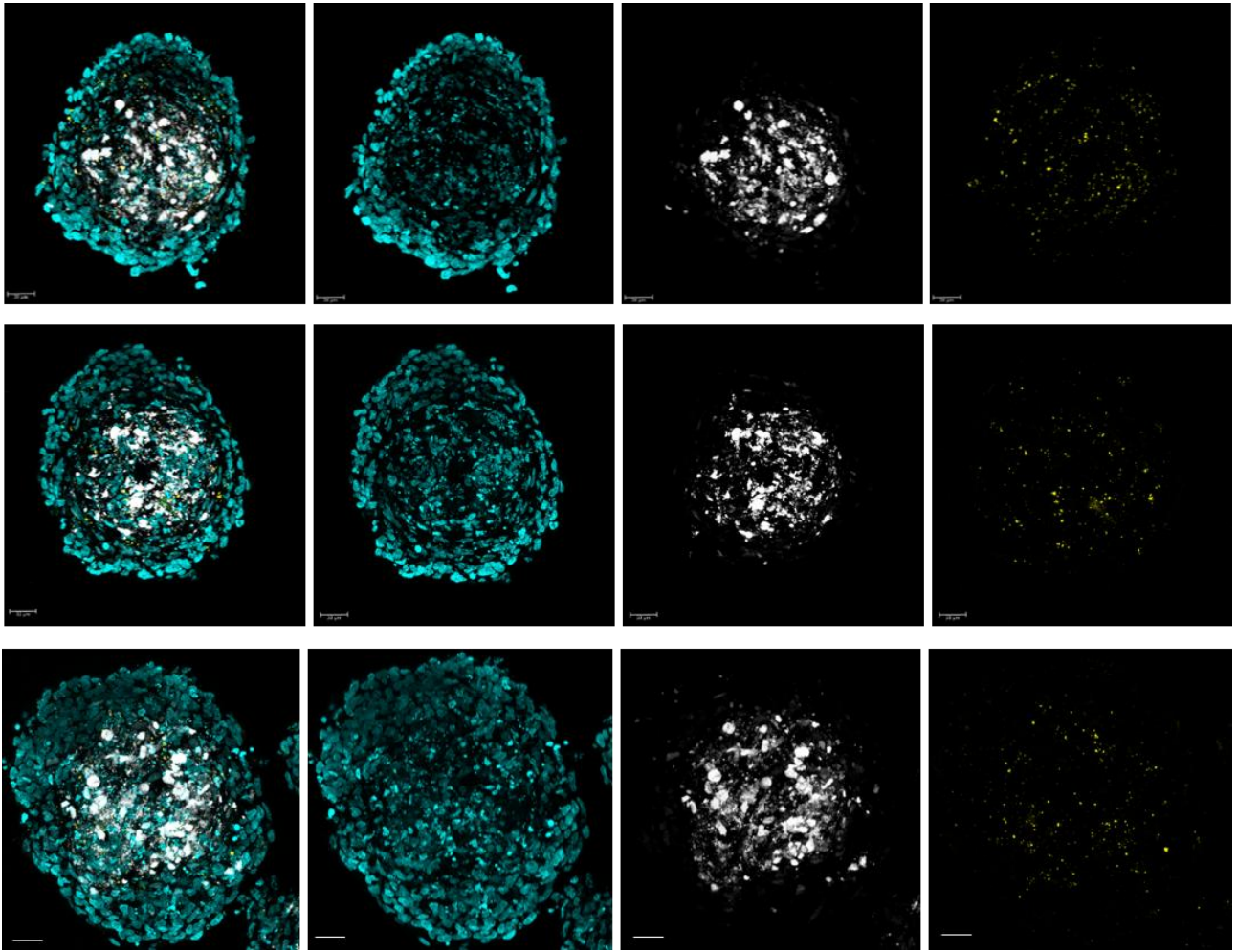


Figure S21. M13_{WT} permeability into BBB assembloids. Additional representative confocal images from independent biological replicates showing the phage permeability into human assembloids after 72 hours incubation (cyan: nuclei; white: pericytes and astrocytes; yellow: M13_{WT}. Scale bar: 50 μ m).

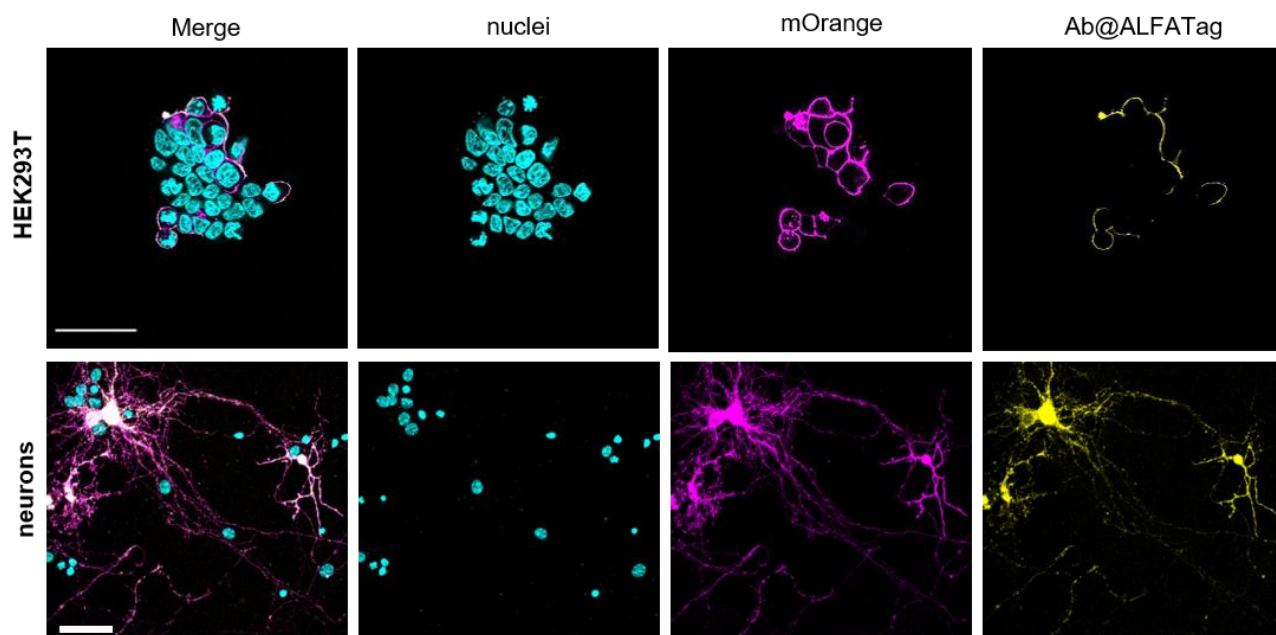


Figure S22. Transfection of HEK293T cells and mouse cortical primary neurons with the hCD4 ALFA-Tag plasmid. HEK293T cells and neurons were transfected, as detailed in the Method section, fixed, and imaged to verify the colocalization of the intracellular mOrange reporter signal (magenta) and the extracellular ALFA-Tag signal (yellow) visualized through immunostaining with an anti-ALFA-Tag antibody (cyan: nuclei; scale bar: 50 μ m).

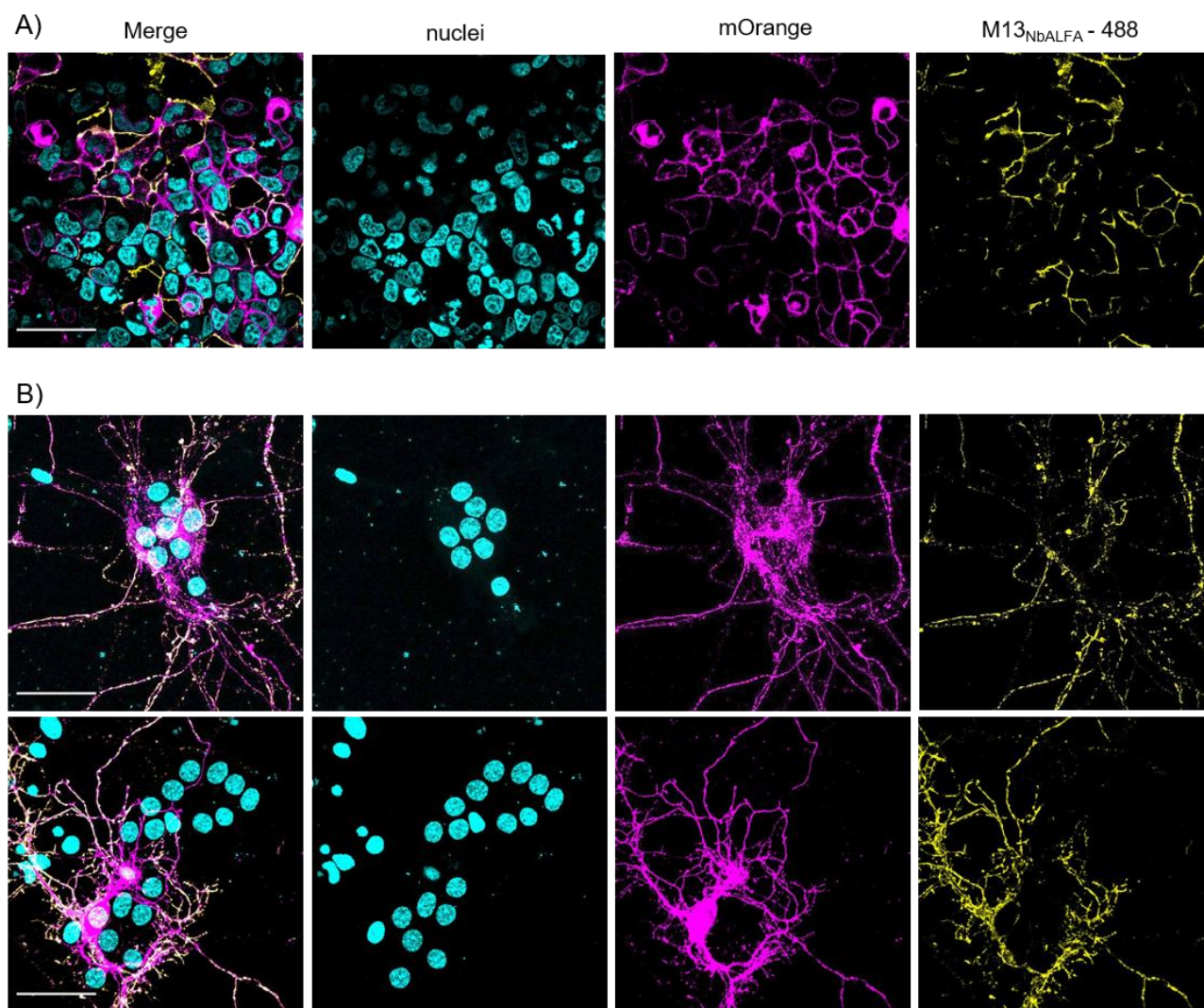


Figure S23. M13_{NbALFA} – 488 targeting of hCD4 ALFA-Tag. (A) ALFA-Tag-transfected HEK293T and (B) primary neurons were incubated for 4 h with M13_{NbALFA} - 488, washed, and fixed. M13_{NbALFA}–488 signal (yellow) colocalization with the intracellular mOrange reporter (magenta) was observed (cyan: nuclei; scale bar: 50 μ m).

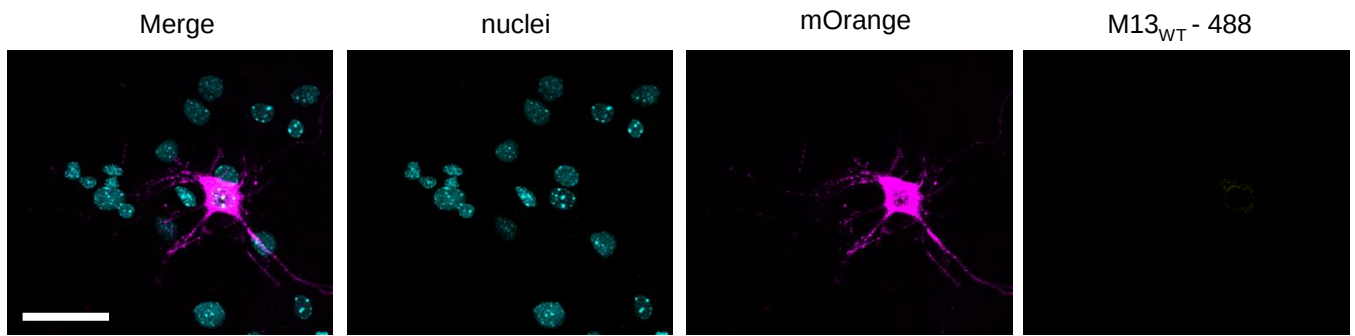


Figure S24. Control conditions for targeting of primary neurons expressing hCD4 ALFA-Tag. ALFA-Tag-transfected neurons were incubated for 4 h with M13_{WT}-488, washed, and fixed. No M13_{WT}-488 signal (yellow) or colocalization with the intracellular mOrange reporter (magenta) was observed (cyan: nuclei; scale bar: 50 μ m).

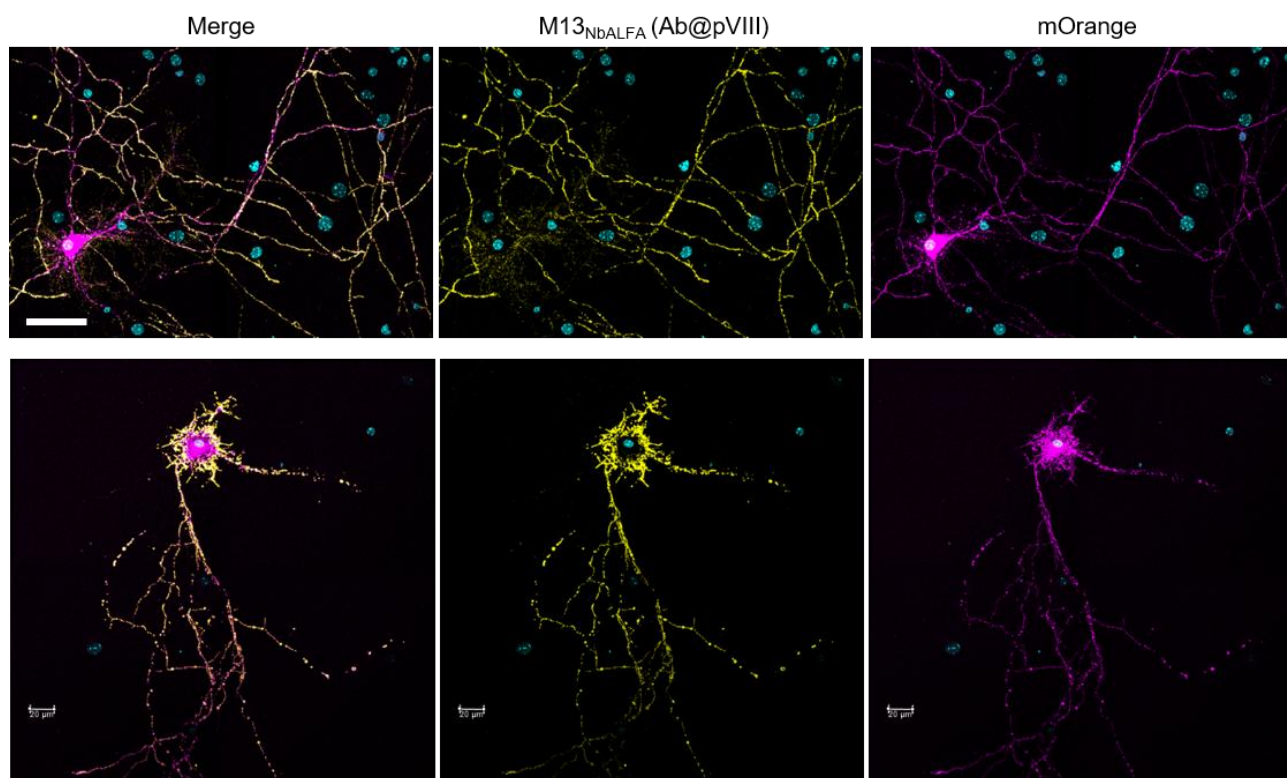


Figure S25. Targeting in a complex biological environment. M13_{NbALFA} was preincubated for 1 h at 37 °C in a solution of FBS 50% v/v in PBS to explore possible coronation phenomena, then diluted in Neurobasal media to a final concentration of 5×10^{11} v/mL (FBS 2.5% v/v, cyan: nuclei, yellow: M13_{NbALFA}, magenta: mOrange reporter; scale bar: 20 μ m).

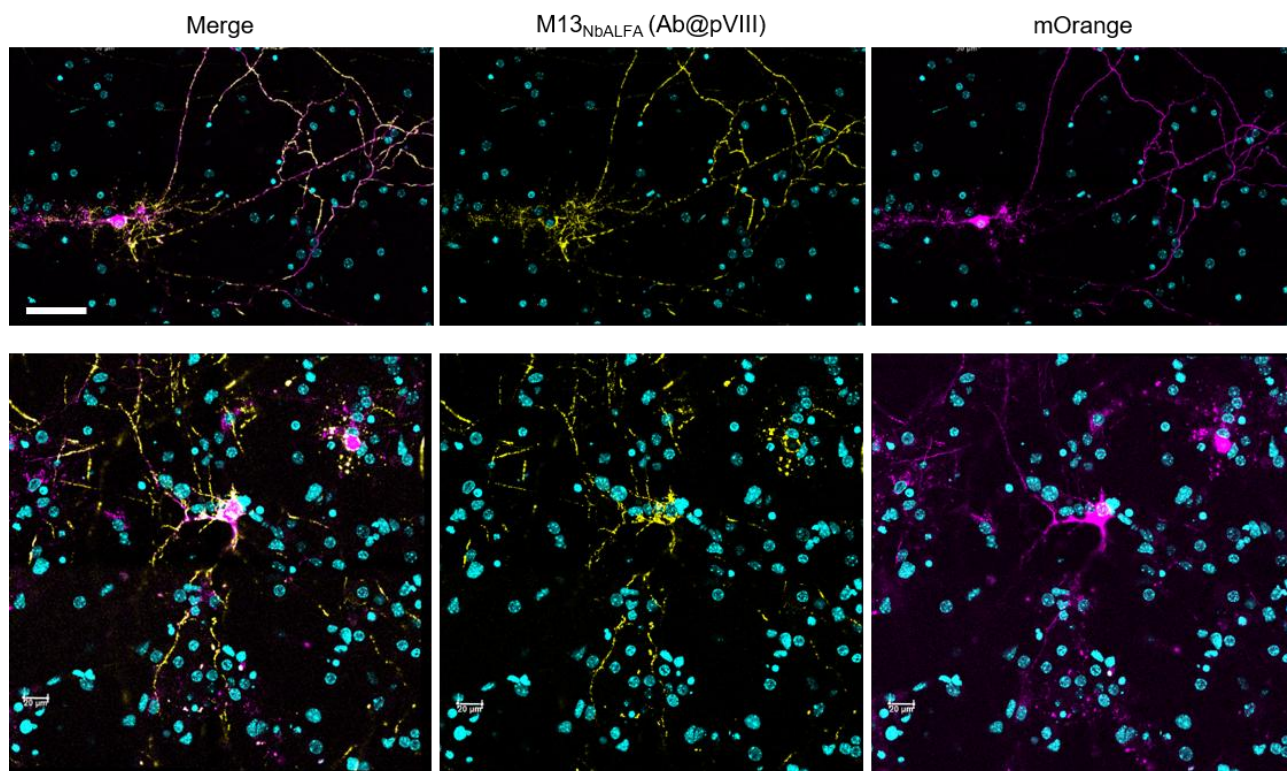


Figure S26. Targeting in a complex biological environment. Confocal representative images showing M13_{NbALFA} (5×10^{11} v/mL) targeting efficiency after preincubation in 50% FBS-supplemented Neurobasal medium. The ALFA-Tag-transfected neurons were treated for 1 h. M13_{NbALFA} (yellow) is visualized through immunostaining for pVIII main coat protein (cyan: nuclei, yellow: M13_{NbALFA}, magenta: mOrange reporter; scale bar: 20 μ m).