

Supporting information

Combined Use of Unnatural Amino Acids Enables Dual Color Super-Resolution Imaging of Proteins *via* Click Chemistry

Kim-A. Saal, ^[a] Frank Richter, ^[b] Peter Rehling, ^[b] Silvio O. Rizzoli ^[a]

[a] Institute for Neuro- and Sensory Physiology, Center for Biostructural Imaging of Neurodegeneration, University Medical Center Göttingen, Cluster of Excellence Nanoscale Microscopy and Molecular Physiology of the Brain, Göttingen, Germany.

[b] Institute for Cellular Biochemistry, University Medical Center Göttingen, Göttingen, Germany

*correspondence to: Kim-A. Saal (kim-ann.saal@med.uni-goettingen.de), Silvio O. Rizzoli (srizzol@gwdg.de).

Göttingen

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Materials and Methods

Reagents, UAA and dyes

Chemicals and cell culture reagents were obtained either from Sigma Aldrich (now Merck, Darmstadt, Germany), AppliChem GmbH (Darmstadt, Germany) or Lonza (Cologne, Germany), unless stated otherwise.

The unnatural amino acids propargyl-L-lysine (PRK) and *trans*-Cyclooctene-L-lysine (TCO*A, following termed TCO) were purchased from Sirius Fine Chemicals SiChem GmbH (Bremen, Germany). Clickable dyes were Abberior Star635P-azide and Abberior Star580-tetrazine, provided by Abberior Instruments (Göttingen, Germany).

Epifluorescence microscopy

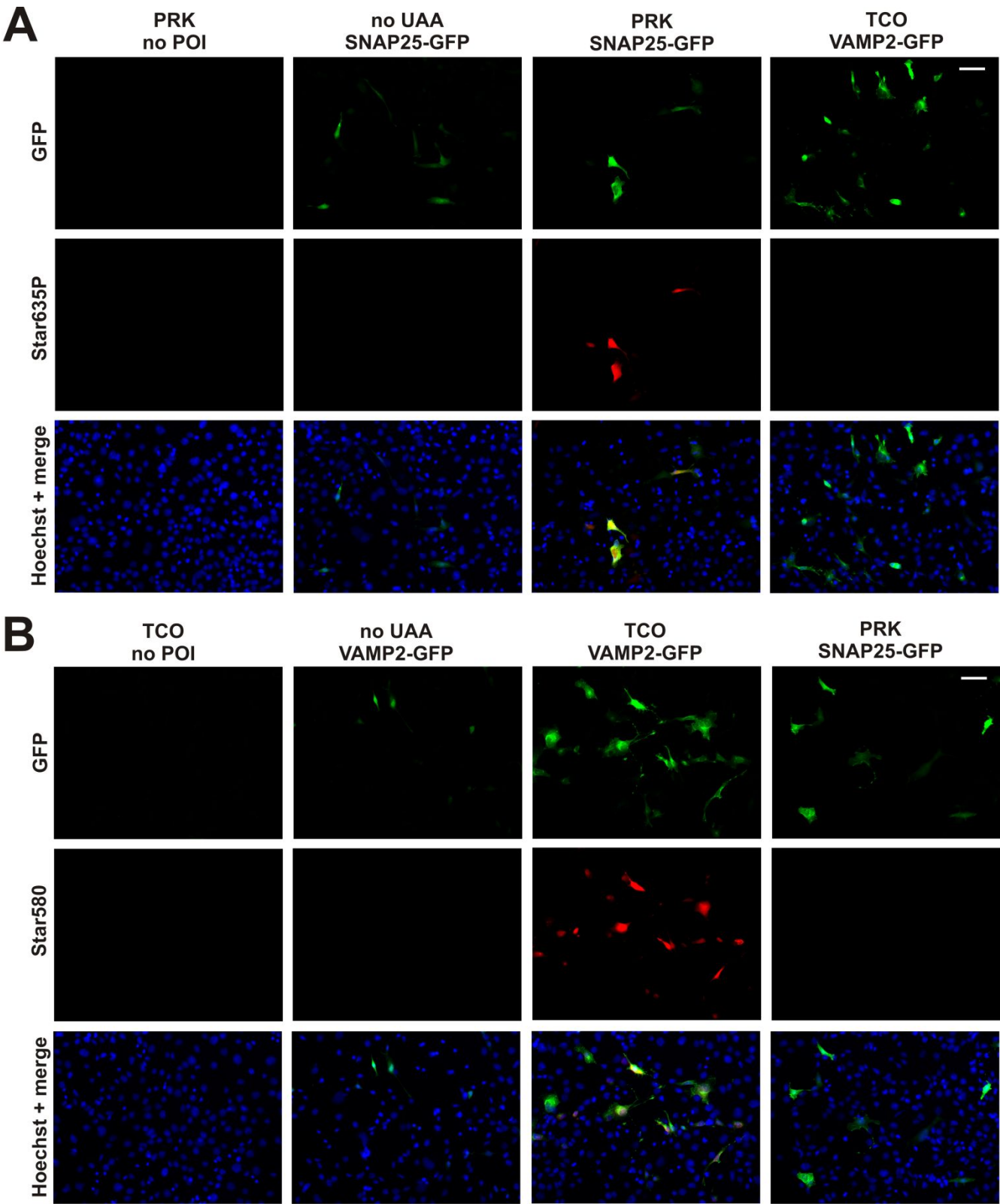
To generate overview images of cells clicked with the particular fluorophores Star635P or Star580, an Olympus IX 71 inverted fluorescence microscope (Olympus, Hamburg, Germany) equipped with a 20 × objective (Olympus) was used. Fluorescent images were acquired by a CCD camera (FView II, Olympus) and processed with the CellF software (Olympus). A 377/50 excitation filter, a 409 long pass beam splitter, and a 447/60 emission filter were used to detect Hoechst, and to visualize GFP a 480/40 HQ excitation filter, a 505 LP Q beam splitter, and 527/30 HQ emission filter were employed. To identify Star635P fluorescence, a 620/60 HQ excitation filter, a 660 LP Q beam splitter, and a 700/75 HQ emission filter were used. For the acquisition of Star580 a 545/30 HQ excitation filter, a 570 LP Q beam splitter and a 610/75 HQ emission filter were used. All filters were purchased from AHF (Tübingen, Germany).

Data analysis

For the determination of UAA cross-incorporation we performed line scans (61 pixels in length) over manually selected organelles in the imaged cells. Then the Pearson correlation coefficients were determined between the line scans in the green, red and blue channels, and were presented as box plots and bar graphs in Supplementary

Fig. S5. The line scans were performed using self-written routines in Matlab (The Mathworks, Inc, Natick, MA, USA), and the graphs were performed using SigmaPlot (Systat Software Inc, Erkrath, Germany).

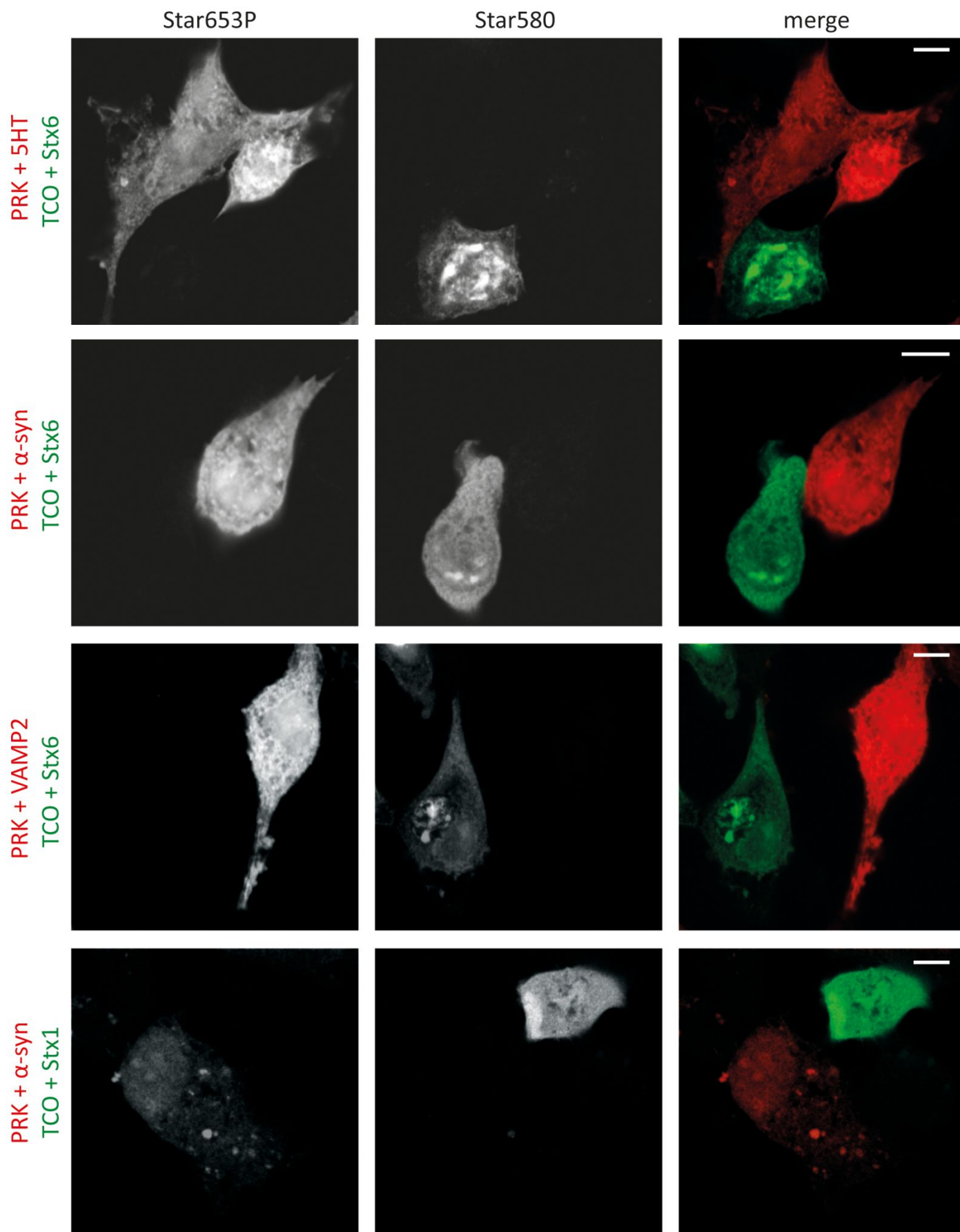
Supplementary Figures



Supplementary Figure S1: Specificity of click reactions for PRK with Star645P-azide, and for TCO with Star580-tetrazine.

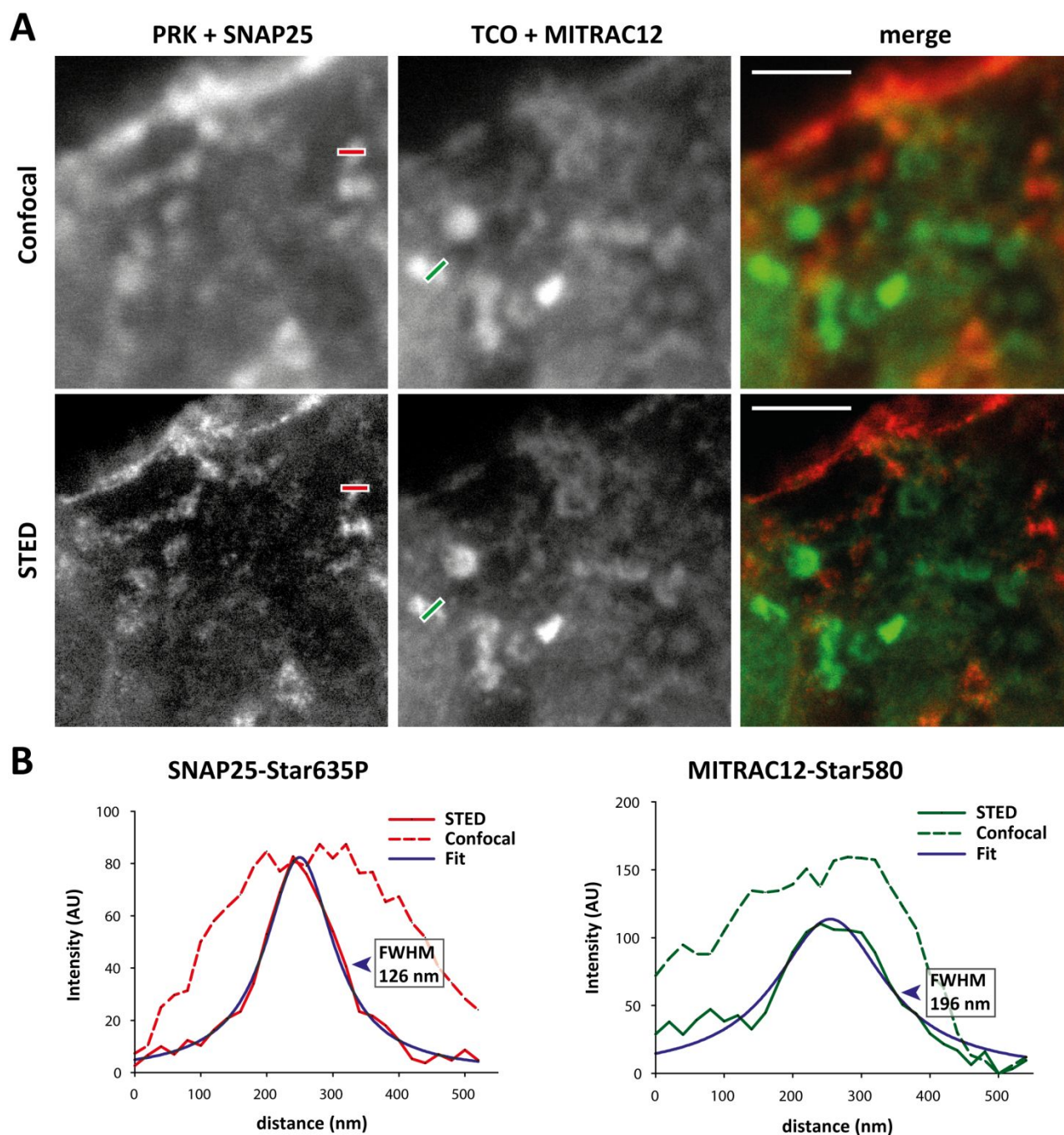
(A) Epifluorescence micrographs show different control situations for the CUAAC reaction between PRK and Star635P. In the first panel (left) PRK was added, but was not incorporated into the cells, in the absence of the DNA for the POI, so consequently there is no click with Star635P-azide. In the second panel, cells were transfected with DNA for SNAP25, including a GFP reporter sequence, but there was no UAA added. Some read-through of the Amber stop codon, without incorporating the UAA, results in scattered SNAP25-GFP expressing cells. However, no click reaction appears, due to the lack of the incorporated UAA. The specific CUAAC reaction is depicted in the third panel, where SNAP25-GFP expressing cells did incorporate the PRK at the Amber stop codon, resulting in distinct Star635P labeling. The fourth, right-most panel tests the incorrect click reaction of Star635P-azide with the other UAA, TCO. This was added, and was incorporated into the protein of interest (VAMP2-GFP, in this case), but no click reaction was seen (no Star635P labeling).

(B) Similar control experiments for the specific labeling of Star580 *via* SPIEDAC were performed, again relying on SNAP25 and VAMP2 as the POIs. Scale bar = 50 μm .



Supplementary Figure S2: Confocal micrographs of separately-transfected cells, seeded together without inducing fusion.

Four different protein combinations, containing either PRK or TCO, were clicked with Star635P-azide (first column at the left; red cells in the right column) or with Star580-tetrazine (middle column; green cells in the right column), respectively. Cells expressing the different POIs are in each other's vicinity. No fusion of membranes or intermixing of cell organelles can be seen. Both cell types show specific labeling from the appropriate click reaction. Scale bar = 5 μm .

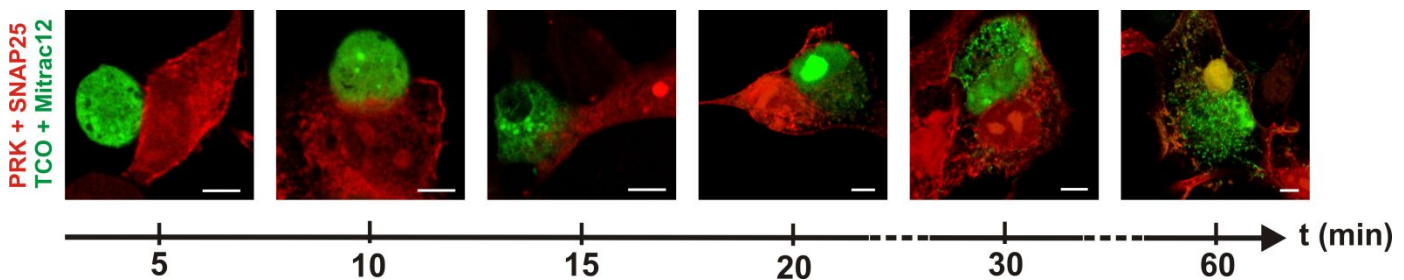


Supplementary Figure S3: Application of Fuse2Click for STED versus confocal microscopy.

(A) Representative micrographs of fused cells, either expressing SNAP25 or MITRAC12, which were clicked with Star635P or Star580, respectively, and were imaged in confocal and in STED mode (single channels = grey scale; merge = SNAP25 in red and MITRAC12 in green). Typical protein structures were chosen as

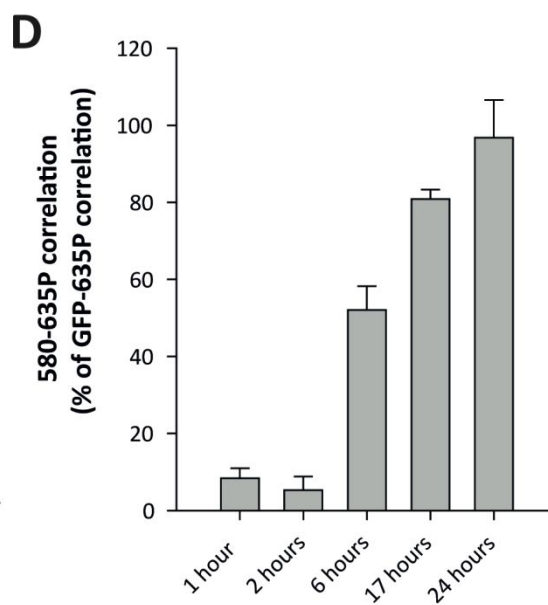
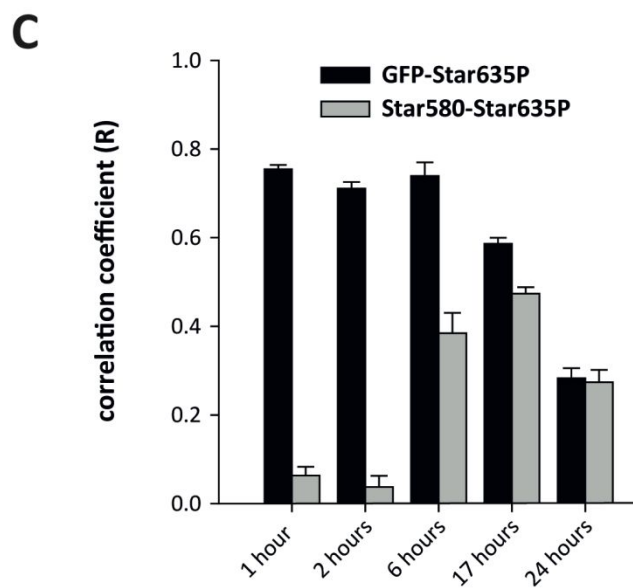
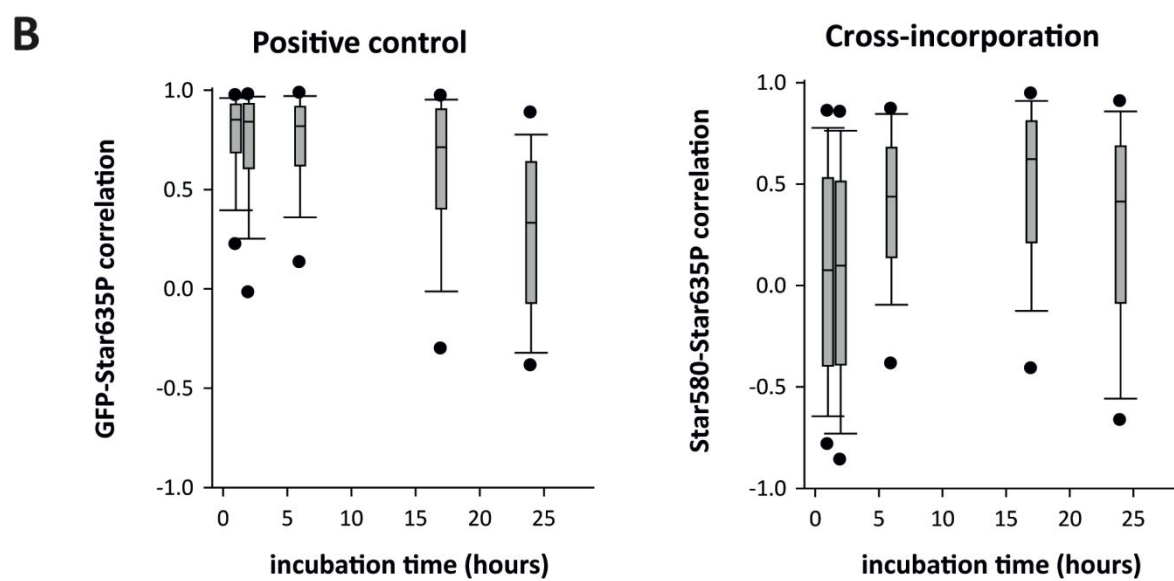
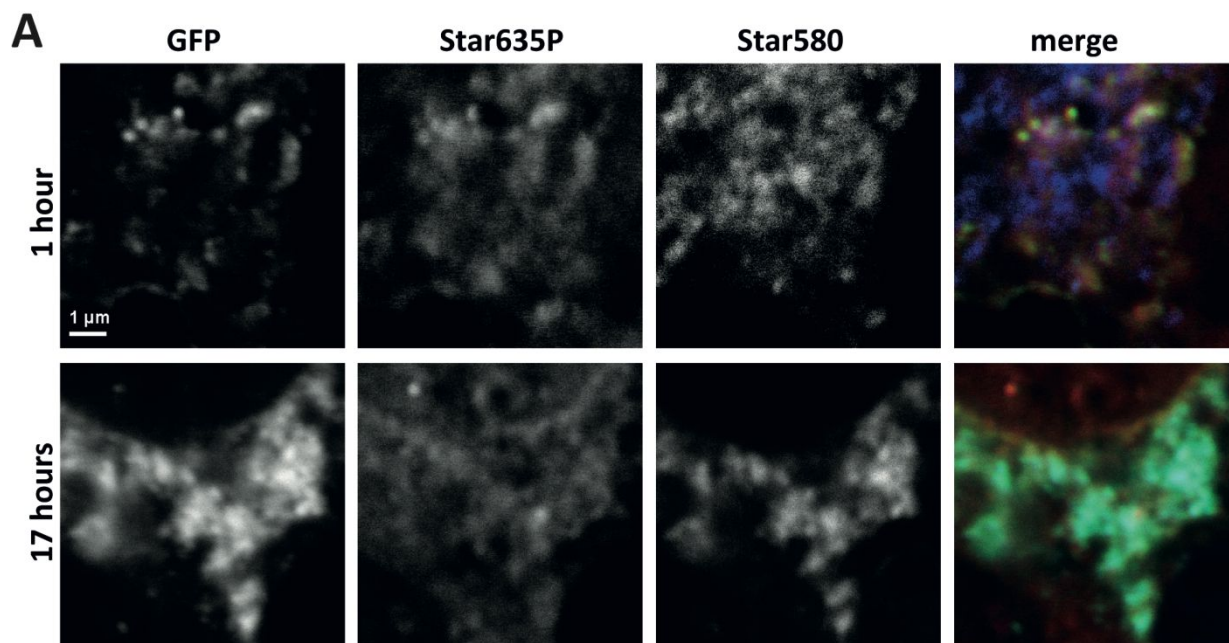
regions to perform line scans, indicated as colored lines (white framed red line for line scan through SNAP25 and white framed green line for line scan through MITRAC12), to evaluate the STED effect.

(B) The line scans are illustrated. The dotted line represents the line scans generated from confocal images, while the continuous line displays the line scans from STED images. By calculating a fit curve (indicated in blue) onto the STED line scans, the full width at half maximum (FWHM, blue arrowheads) is determined, providing the perceived size of the objects (in nm) from STED imaging. Scale bar = 2 μm .



Supplementary Figure S4: Cells expressing two different POIs are fixed at different time points after the initiation of fusion.

Cells expressing MITRAC12 were detached from a 6-well plate, were added to resident cells on coverslips expressing SNAP25, and fusion was induced using HVJ-E. Subsequently, cells were fixed 5, 10, 15, 20, 30 or 60 minutes after initiating fusion. The confocal images show that the added cells directly attach to residents, and start to connect membranes relatively rapidly. However, they seem to be stressed from the plate-detaching procedure, as indicated by their rounded shape. After 10 minutes cells seem to recover, extend their processes, and proceed to membrane fusion, while they do not yet intermix cytosolic compounds. 15 minutes after pooling the cells, fused cells start to exchange some cytosolic compounds, while a common plasma membrane encases the whole formation (see micrograph at 20 min), building a giant cell with multiple nuclei. Dispersion and intermixing of cell organelles needs up to 60 minutes or longer. However, it is not advisable to fix cells much later than 1 hour after fusion, because the synthesis of new proteins may lead to the uncontrolled incorporation of remaining free PRK or TCO into both POIs, unless protein synthesis is blocked pharmacologically. Scale bar = 5 μm .



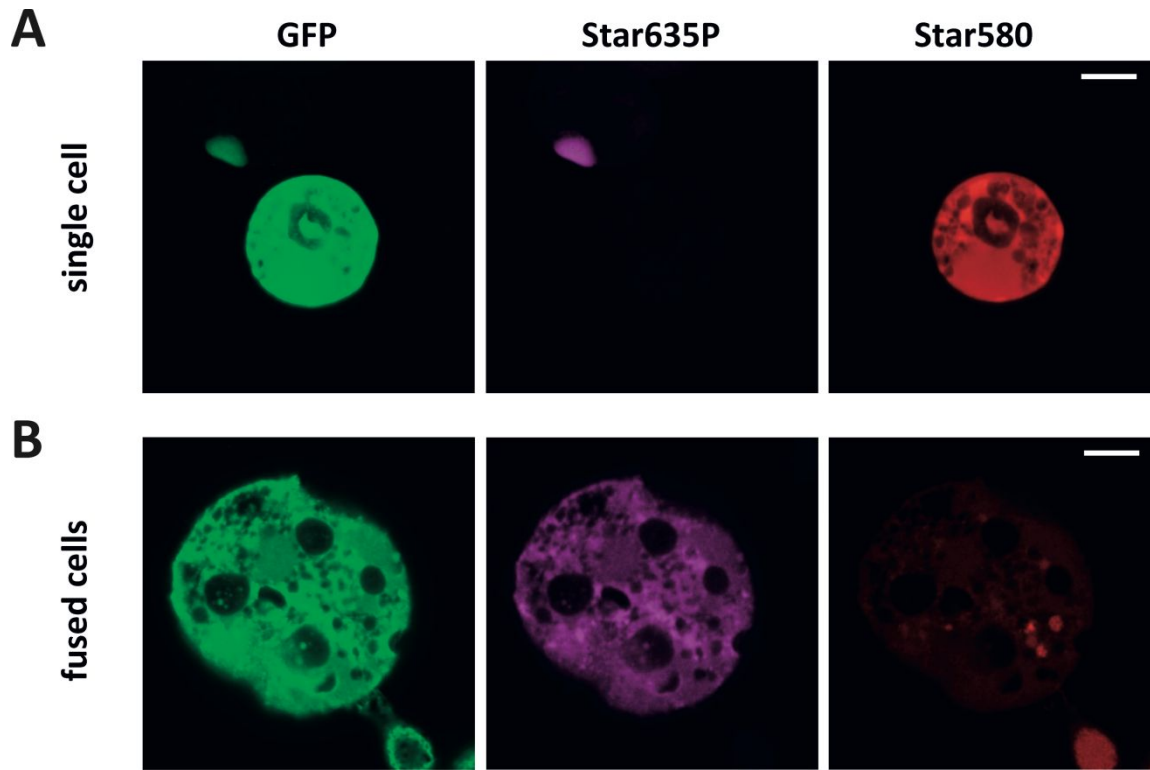
Supplementary Figure S5: Cross-incorporation of UAAs into POIs at long time periods after cell-cell fusion.

(A) Cells expressing SNAP25-GFP were incubated with PRK, and cells expressing MITRAC12 were separately incubated with TCO, before the induction of cell-cell fusion. The fused cells were then grown in the absence of UAAs for either 1 hour (top row) or 17 hours (bottom row). The cells were then fixed, and click reactions were induced to covalently link Star635P (red) to PRK, and hence to SNAP25-GFP (green), and Star580 (blue) to MITRAC12. The overlay panels indicate that SNAP25-GFP correlates well to Star635P at 1 hour after cell-cell fusion, as expected. However, at 17 hours after cell-cell fusion the Star580 signal also correlates well to the GFP signal, indicating that TCO has been wrongly incorporated in SNAP25-GFP over this time interval. Scale bar = 1 μ m. The images are representative of 5 independent experiments.

(B) A box-plot analysis of the correlation between GFP and Star635P (taken as positive control, since Star635P is linked to the UAA that is designed to incorporate in the GFP chimera), and of the correlation between Star635P and Star580 (which represents the cross-incorporation of TCO in the GFP chimera). The midlines of the box plots show the median; the box boundaries show the 25th percentiles; the error bars show the 75th percentiles; the dots show the 90th percentiles. We would like to point out that the median value for the cross-incorporation is around 0 at one or two hours after fusion, but rises afterwards. The box plots show data from 63 to 886 analyzed organelles in fused cells in the respective conditions.

(C) For convenience, we plotted the data from (B) as conventional bar graphs. The cross-correlation at 1-2 hours is not significantly different from 0 (t-tests, corrected for multiple testing by a Bonferroni procedure).

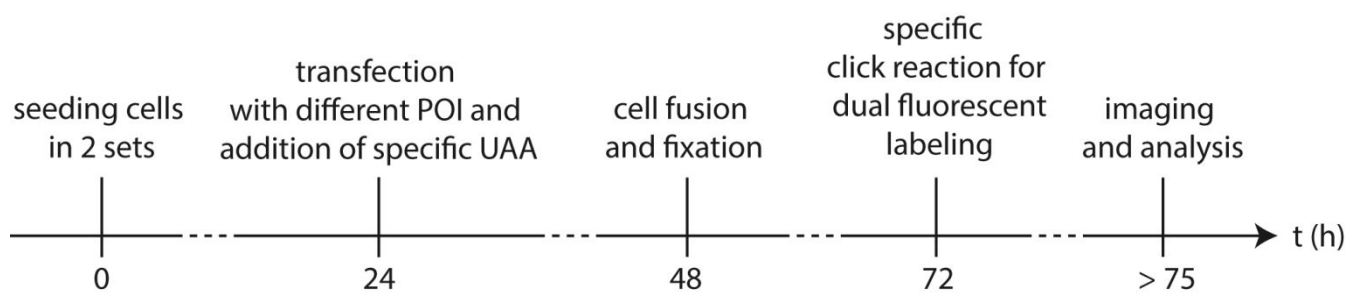
(D) To enable a simpler comparison between the positive control and the cross incorporation, we expressed the value obtained in the latter as % of the positive control value. It is evident that the cross incorporation raises over time, while the positive control also decreases, due to the continued expression of GFP-containing proteins that do not incorporate PRK, but TCO.



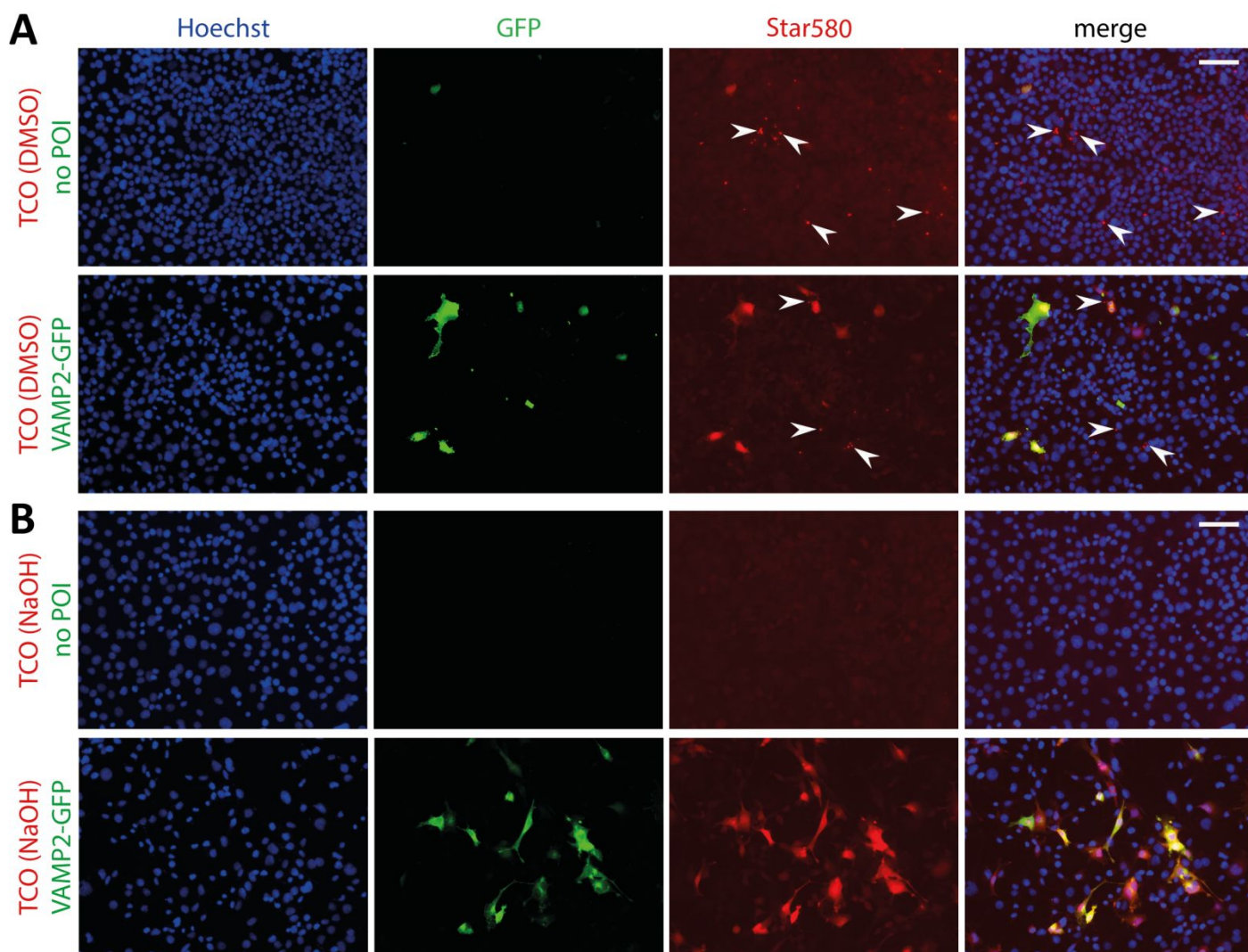
Supplementary Figure S6: Visualization of unhealthy cells in Fuse2Click. Protein over-expression can lead to cellular stress responses and cell death. The sick cells are typically identified by 1) their very high fluorescence levels; 2) a rounded cell shape and membrane bulges and bursts; 3) numerous large vacuoles.

(A) Cells expressing SNAP25-GFP were incubated with TCO, and cells expressing MITRAC12 were separately incubated with PRK, before the induction of cell-cell fusion. One hour later, cells were fixed and clicked to Star635P (for MITRAC12) and Star580 (for SNAP25-GFP). The image shows a typical sick cell, which has not been able to fuse to any other cell. Scale bar = 5 μ m.

(B) Cells expressing SNAP25-GFP were incubated with PRK, and cells expressing MITRAC12 were separately incubated with TCO, before the induction of cell-cell fusion and click reactions to Star580 (for MITRAC12) and Star635P (for SNAP25-GFP). Here we found one fused cell group, containing sick cells (note the frequent vacuoles and the membrane burst to the lower right of the image). This is an extremely rare event (probably less than 1% of all fused cell groups). Scale bar = 5 μ m.



Supplementary Figure S7: Experimental time line.



Supplementary Figure S8: Comparison of added TCO dissolved in either a mixture of formic acid and DMSO or in NaOH.

A) When TCO is dissolved in 12% formic acid in DMSO (DMSO) and added to the cells, as often suggested in the literature, it accumulates in the cells without being incorporated into the POI (top panel row, white arrowheads). It is hardly washed from the cells, and is therefore clicked by Star580-tetrazine, giving a distinct background fluorescence signal (top panels). When added in presence of the POI, the click reaction is not particularly efficient, and background is still visible (second panel row). B) The lowest two panel rows show that the addition of TCO dissolved in 0.2 M NaOH results in no background. Here, the Star580-tetrazine fluorescence signal is restricted to POI-expressing cells. The reaction appears to be overall more efficient. Scale bar = 50 μ m.

Fuse2Click user Protocol:

Cell seeding * **Timing 1 d**

1| Seed cells in appropriate number in two different sets: 20.000 cells per 18 mm coverslip (CS) and ~60.000 cells per 9.6 cm² multi well plate. **! Critical step** Do not seed cells too dense → best transfection efficiency with 40-50% confluency.

2| Perform medium change to BHK culture medium without antibiotics min. 2 h before transfection.

Transfection and incorporation of UAA * **Timing 1 d**

3| 24 h after seeding prepare the mixes of vectors: 50 µL Opti-MEM with 0.5 µg of tRNA/RS^{WT} together with 0.5 µg of the Amber stop mutagenized vector for POI1 and 50 µL Opti-MEM with 0.5 µg of tRNA/RS^{AF} together with 0.5 µg of the Amber stop mutagenized vector for POI2. Let DNA mixes equilibrate for 5 min.

4| Use 2 µL Lipofectamine2000 reagent per 1 µg DNA and dilute in 50 µL Opti-MEM. Add Lipofectamine/Opti-MEM mix to DNA/Opti-MEM mix and incubate for 20 min at RT.

5| In the meanwhile add the respective UAA to the cell sets: Dilute the UAAs 1:4 with 1 M HEPES buffer directly before use and add to the set of cells in a final concentration of 250 µM for PRK and 400 µM for TCO.

▼ **Caution** TCO should be dissolved in 0.2 M NaOH and not in 12% formic acid in DMSO for stock solution → TCO forms precipitates in 12% formic acid in DMSO (see Supplementary Fig. S8).

6| Add the DNA/Lipofectamine mixes to the respective set of cells, mix gently and incubate for 18-24 h.

7| Minimum 4 h before fusion exchange medium to normal BHK medium to remove excess of DNA/Lipofectamine and not incorporated UAAs.

Cell-cell fusion on plate * **Timing 1 d**

8| Collect TCO and POI2-expressing cells in falcon tube by harshly washing them from dish with pipette.

9| Centrifuge at 1500 rpm and 4 °C for 5 min, remove supernatant and count cells as needed.

10| Resuspend cells in appropriate amount of freshly prepared ice-cold fusion buffer (FB, Cell Fusion Kit GenomONE-CF Ex, follow manufacturer's instructions: 25 µL FB per 200.000 cells)

11| Add 2-3 µL ice-cold HVJ-E suspension per ~2 M cells and incubate on ice for 5 min.

† **Troubleshooting** Sparse amounts of HVJ-E results in just a few fused cells, while too much HVJ-E results in extremely giant cells (>10 cells fused) and can have toxic effects. **! Critical step** If many cells are fused, one has to consider the dilution effect of the POIs, because non-transfected cells will also fuse.

12| Centrifuge at 2000 rpm, and 4°C for 5 min.

13| In the meanwhile prepare PRK incorporated cell set on CSs: remove medium and wash cells with pre-warmed PBS, remove PBS and add 400 µl ice-cold FB to each CS. Leave on ice for 5 min.

14| Remove supernatant from centrifuged TCO cells and resuspend in the respective amount of ice-cold FB (for 250,000 – 500,000 TCO cells that will be combined with one CS containing PRK cells, we suggest adding 500 µL FB).

15| Remove FB from PRK cells and add TCO cells in 500 μ L FB on top.

16| Centrifuge plate at 1000 rpm, and 4 °C for 5 min

17| Afterwards, incubate plate in 37 °C incubator for 15 min, remove FB and add pre-warmed medium to each well and incubate for further 45 min. **! Critical step** Incubation time after fusion initiation depends on desired biological effect of the POIs → incubation > 60 min might lead to synthesis of POI1 and 2 with uncontrollable incorporation of PRK and TCO (see Supplementary Fig. S.5)

◆ **Additional Information** Use inhibitors for protein synthesis, as needed.

18| Remove medium, wash with ice-cold PBS and fix fused cells with ice-cold 4% PFA in PBS for 10 min at 4 °C and for further 30 min at RT.

■ **Break** Fixed cells can be kept at 4 °C for 1-2 days until being further processed.

Click reaction * Timing 3-6 h

19| After fixation quench cells with 100 mM NH_4Cl in PBS for 20 min at RT. Afterwards, rinse CS shortly with PBS, and permeabilize cells with 0.1% Triton-X 100 in PBS 3 × for 5-10 min.

20| Block 3 × 5-10 min with 5% BSA + 5% Tryptone/Peptone in 0.1% Triton-X 100 in PBS.

21| Rinse CSs with 3 % BSA in PBS before click. ▼ **Caution** Prepare click solutions directly before use.

22| Perform click reaction first with Star580-tetrazine by using a 20 nM final concentration of Star580 in 3% BSA in PBS and incubate for 10 min in a humidified chamber protected from light. **! Critical step** Do not perform first the CuAAC and the tetrazine reaction afterwards → It will not work!

23| Wash cells 5 min in 3% BSA in PBS and perform click reaction with Star635P-azide with the help of the Click-iT® Cell Reaction Buffer Kit for 30 min in a humidified chamber protected from light. Prepare click solution like suggested in the instruction manual and use 20 μ M STAR635P-azide as final concentration.

▼ **Caution** In case of GFP expression, GFP fluorescence signal could be reduced by CuSO₄ (component B). CuAAC works also with only $\frac{2}{3}$ amount of CuSO₄, and most of the GFP signal will be preserved.

24| After click reactions wash cells with 3% BSA in PBS 3x for 5 min. ♦ **Additional Information** As needed, immunocytochemistry can be applied in this protocol from here as usual.

25| Finally wash cells 3 × 10 min with PBS and embed in Mowiol. Keep samples at ≤ 4 °C and perform microscopy.