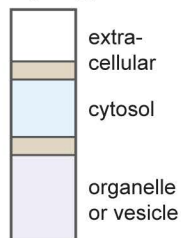


A

## topology



B

## protein length



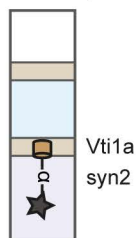
C

## construct epitopes

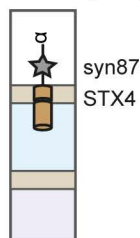


D

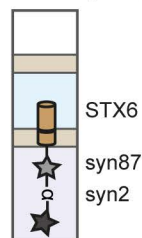
## 1. Vti1a(0001)



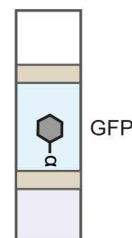
## 2. STX4(0010)



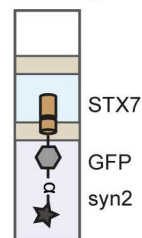
## 3. STX6(0011)



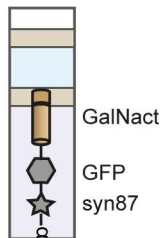
## 4. GFP(0100)



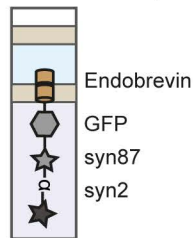
## 5. STX7(0101)



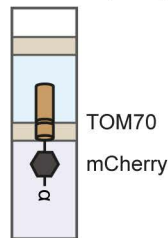
## 6. GalNact(0110)



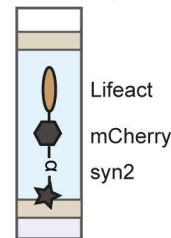
## 7. Endobrevin(0111)



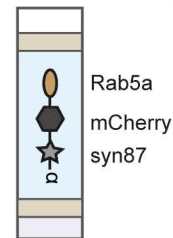
## 8. TOM70(1000)



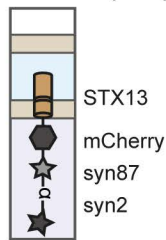
## 9. Lifeact(1001)



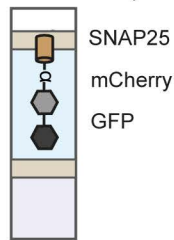
## 10. Rab5a(1010)



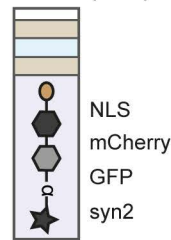
## 11. STX13(1011)



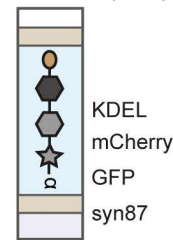
## 12. SNAP25(1100)



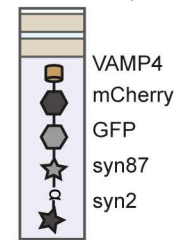
## 13. NLS(1101)

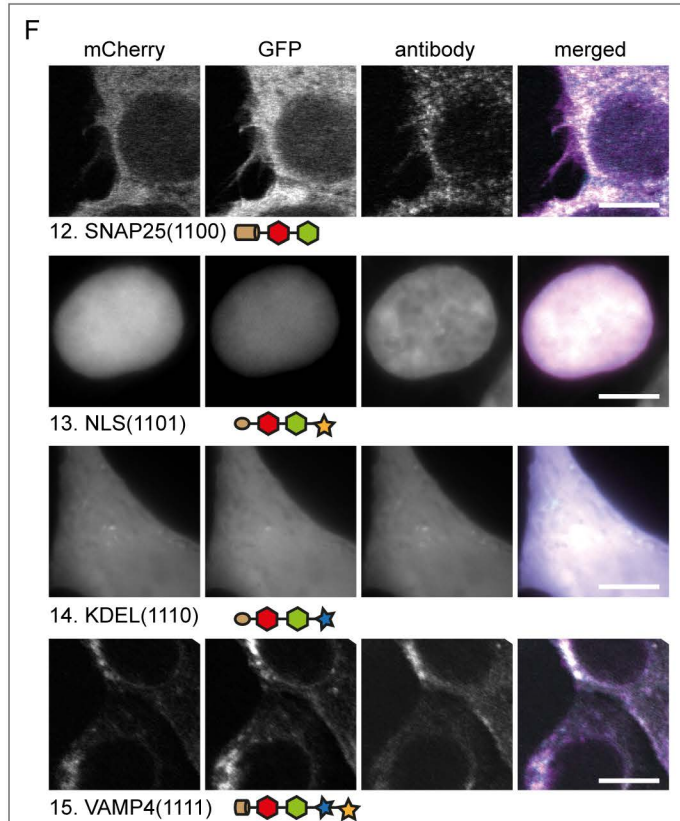
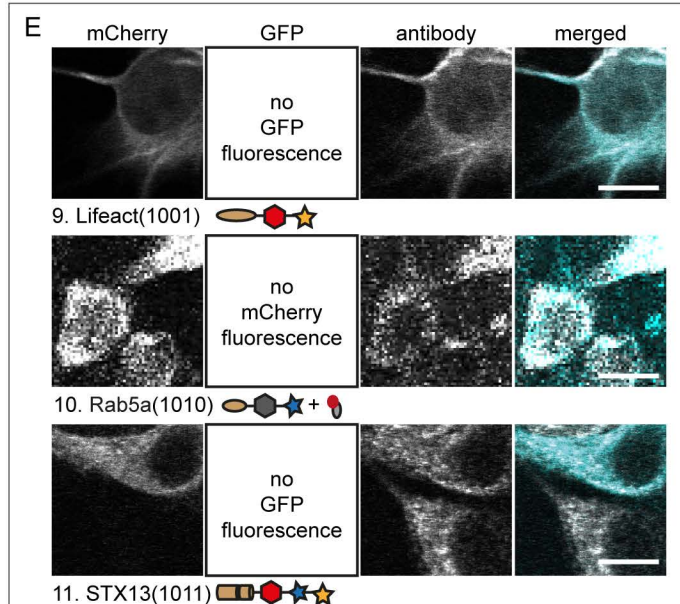
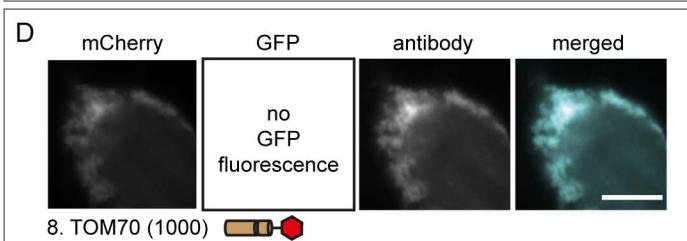
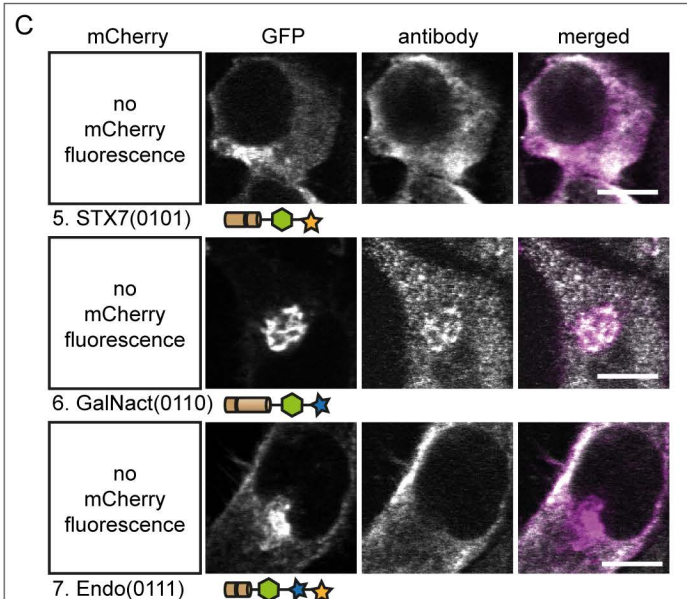
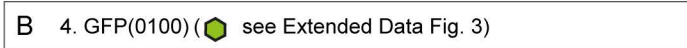
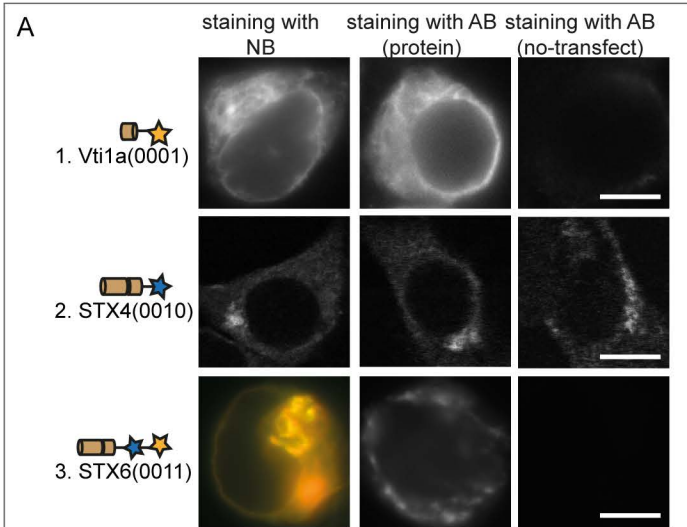


## 14. KDEL(1110)

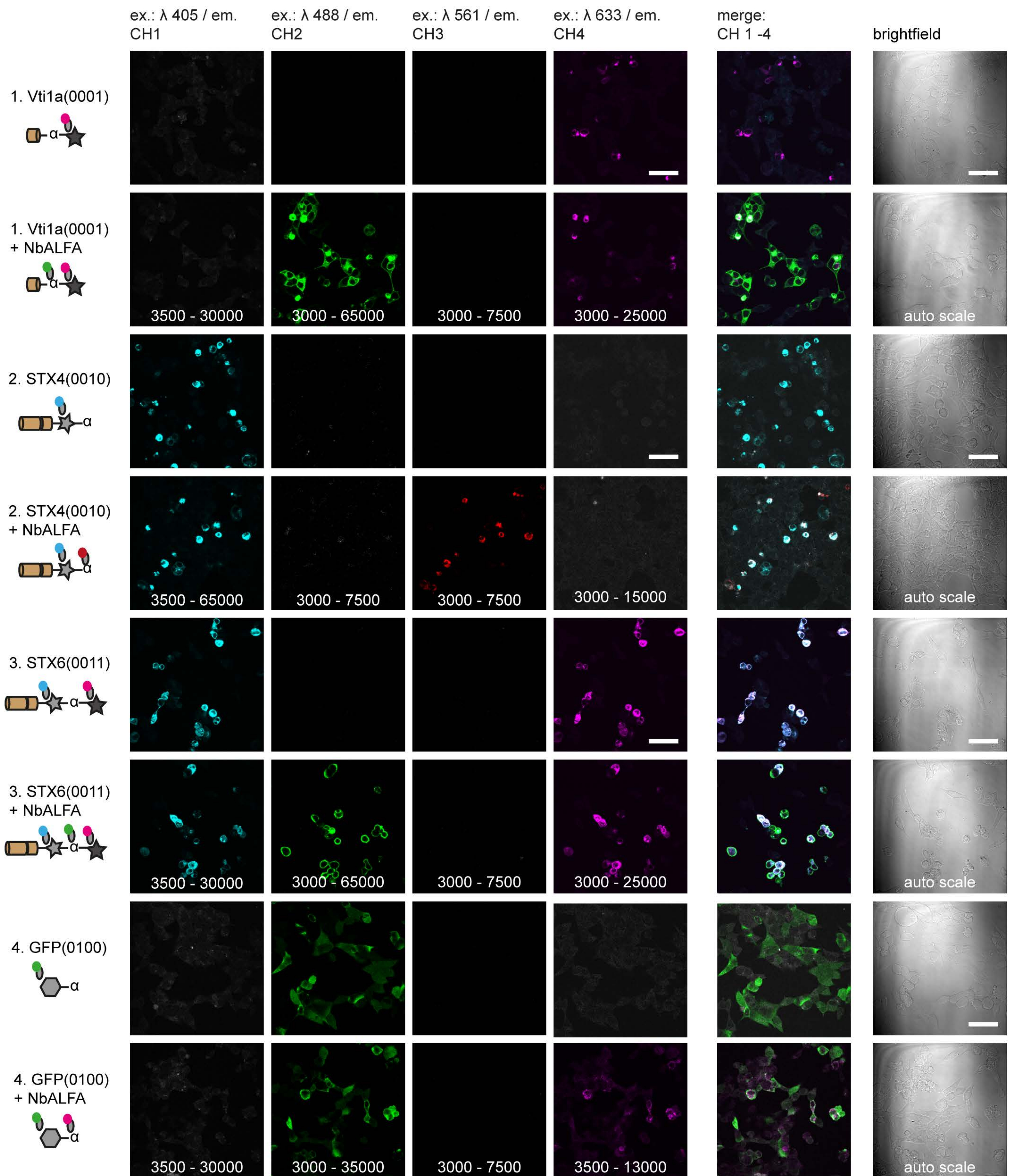


## 15. VAMP4(1111)

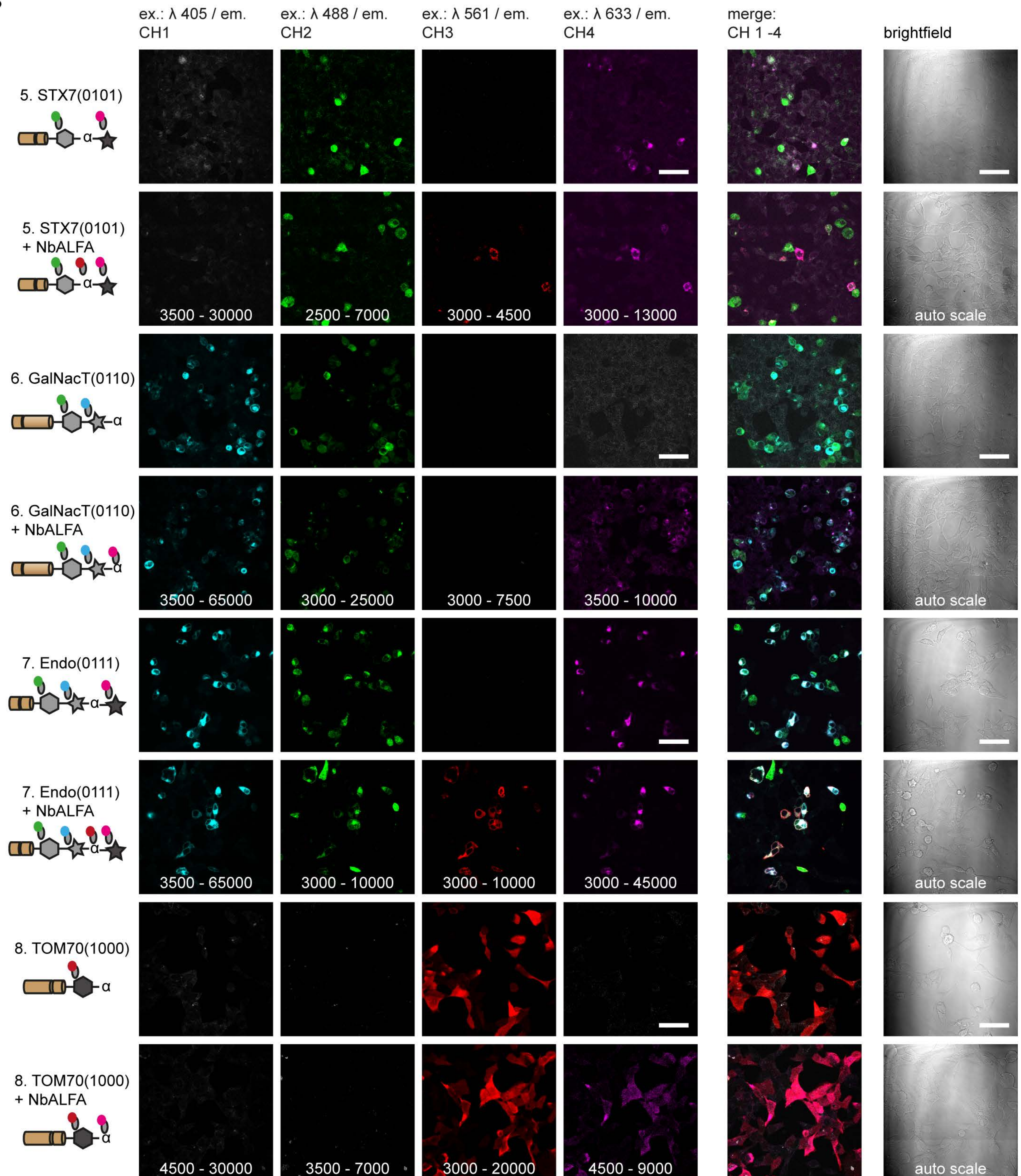




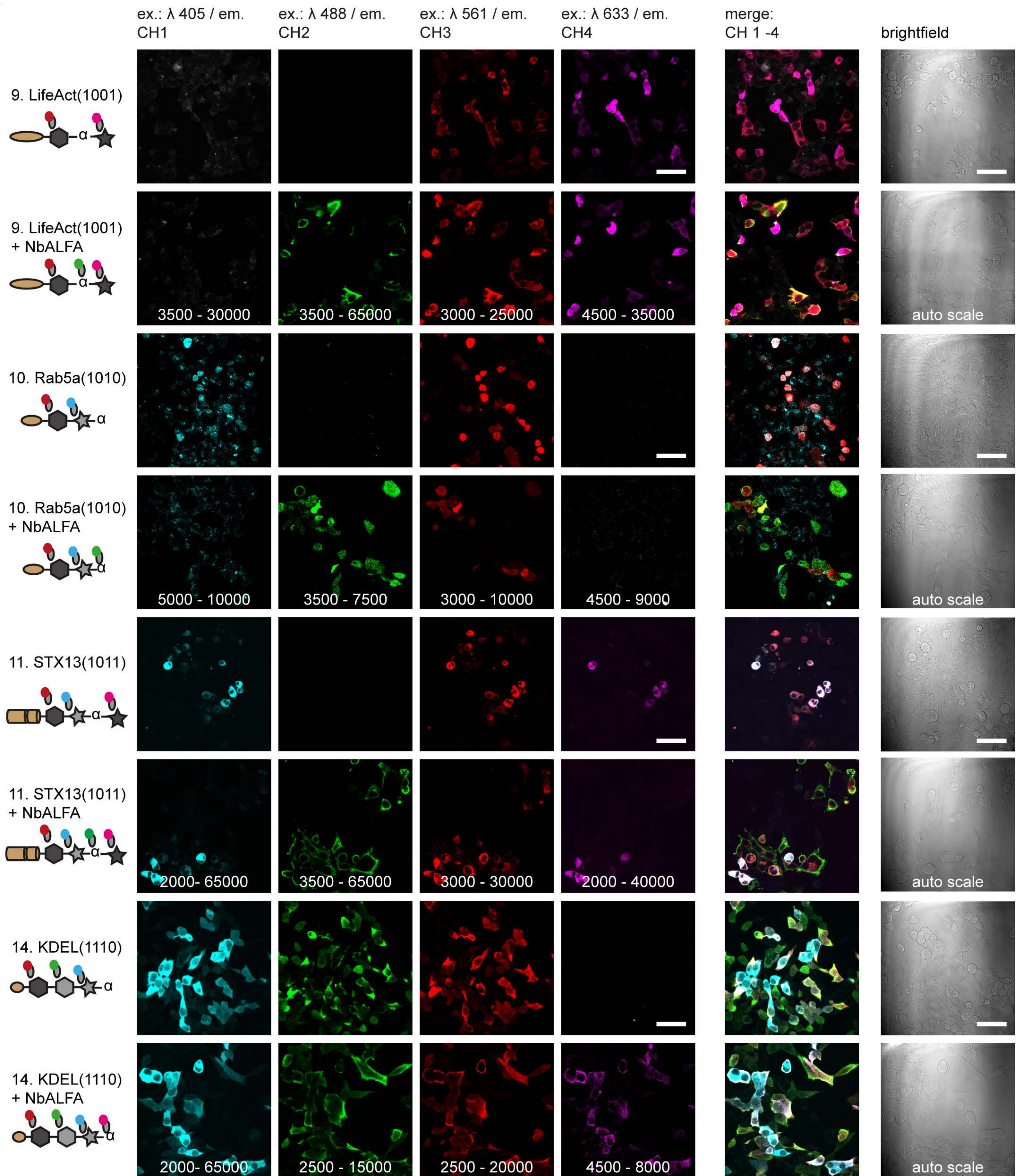
A



B

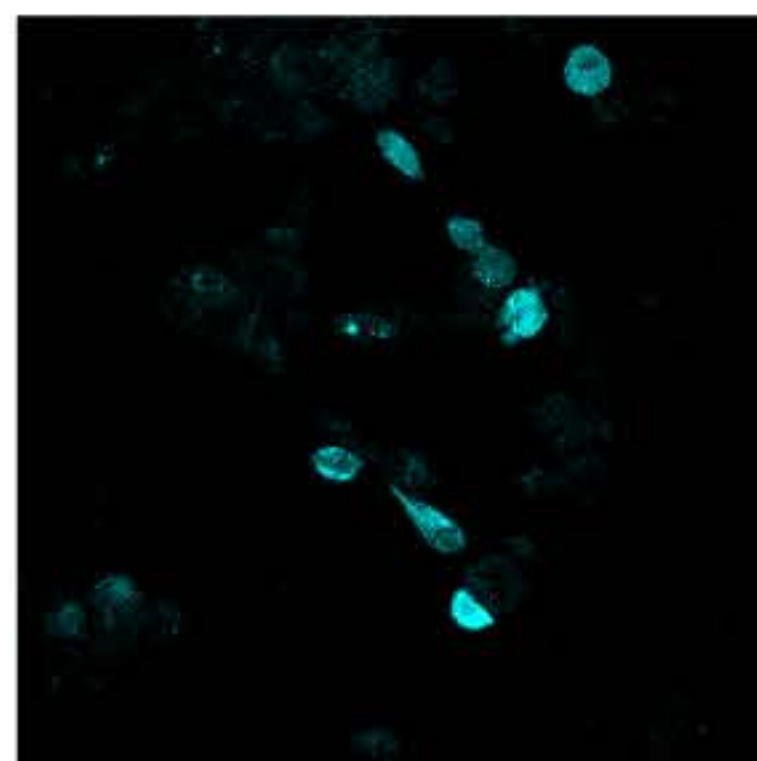
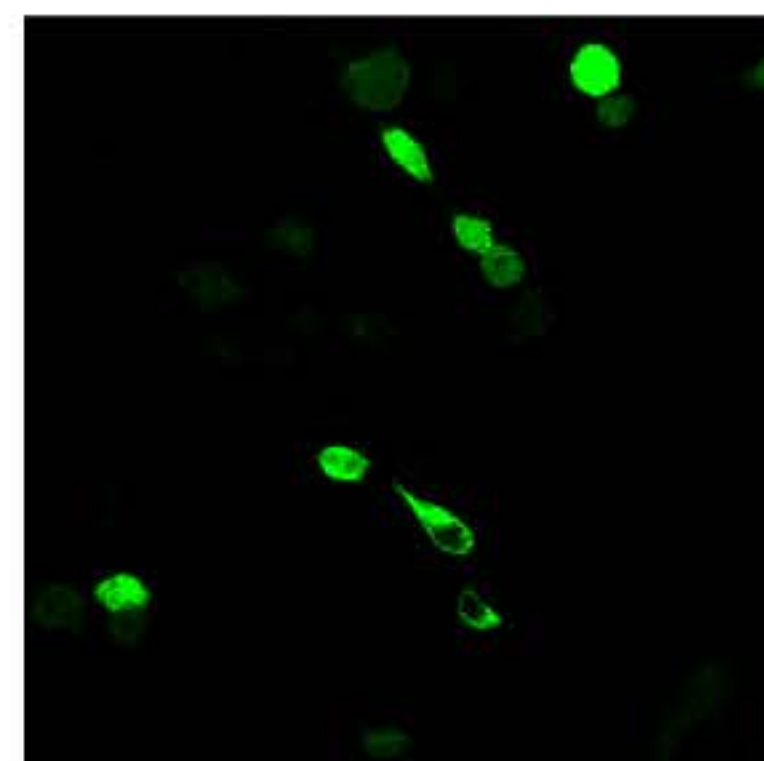
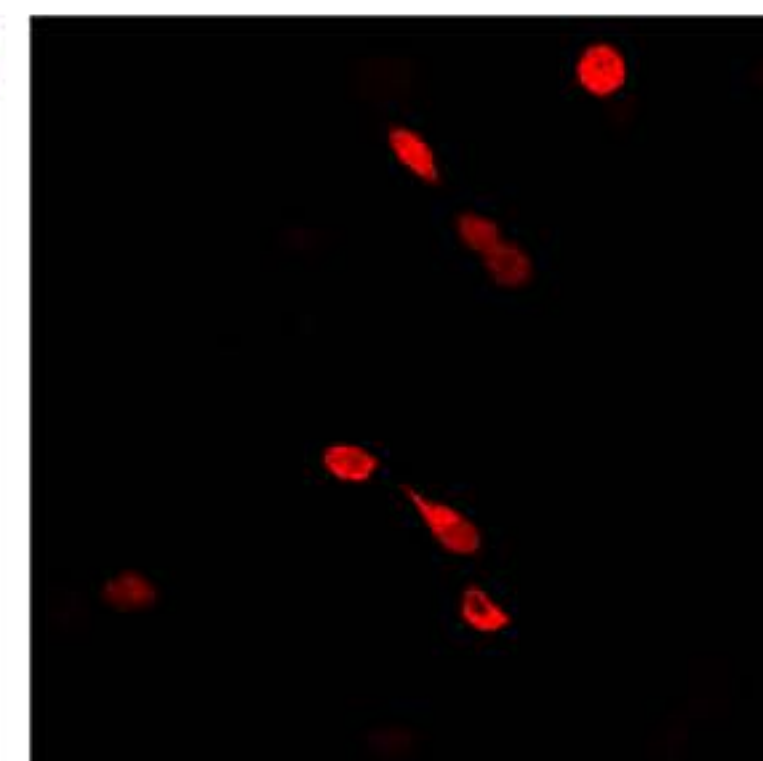
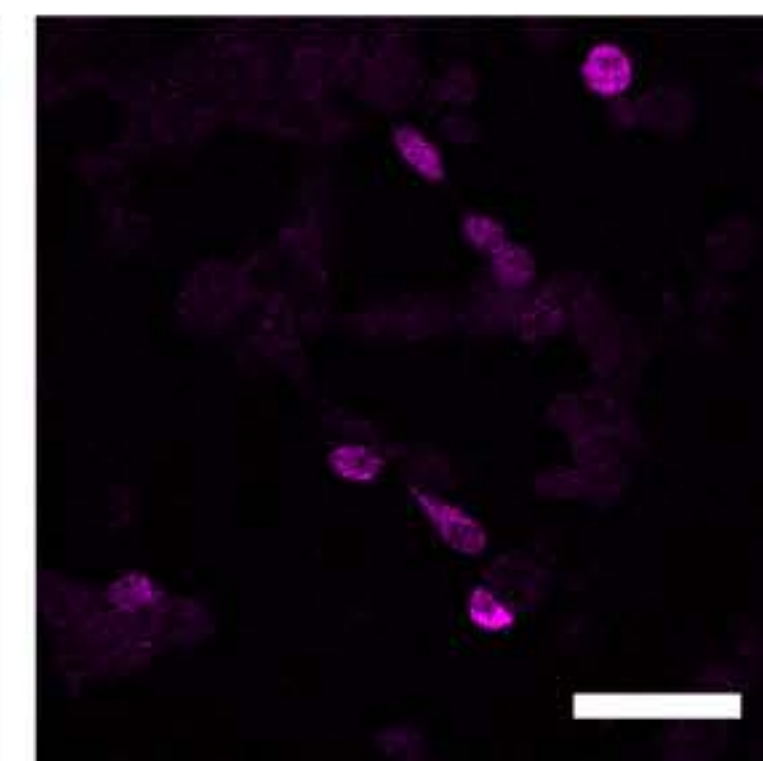
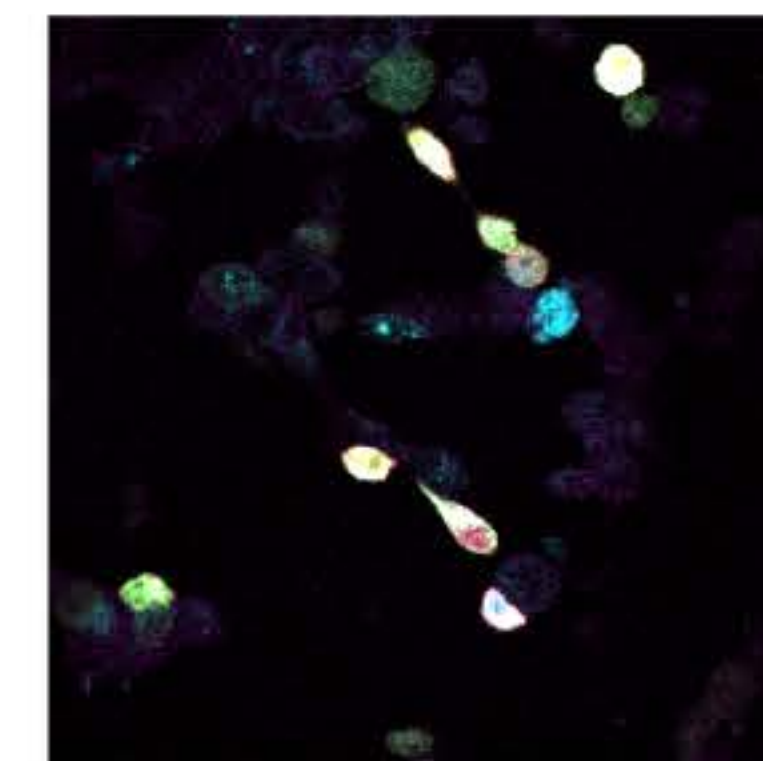


C

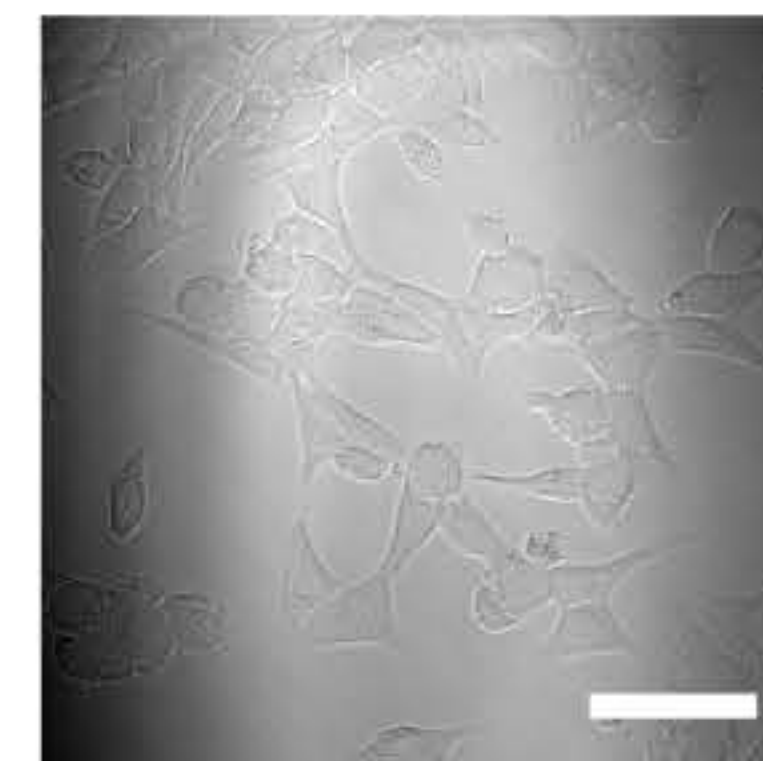


D

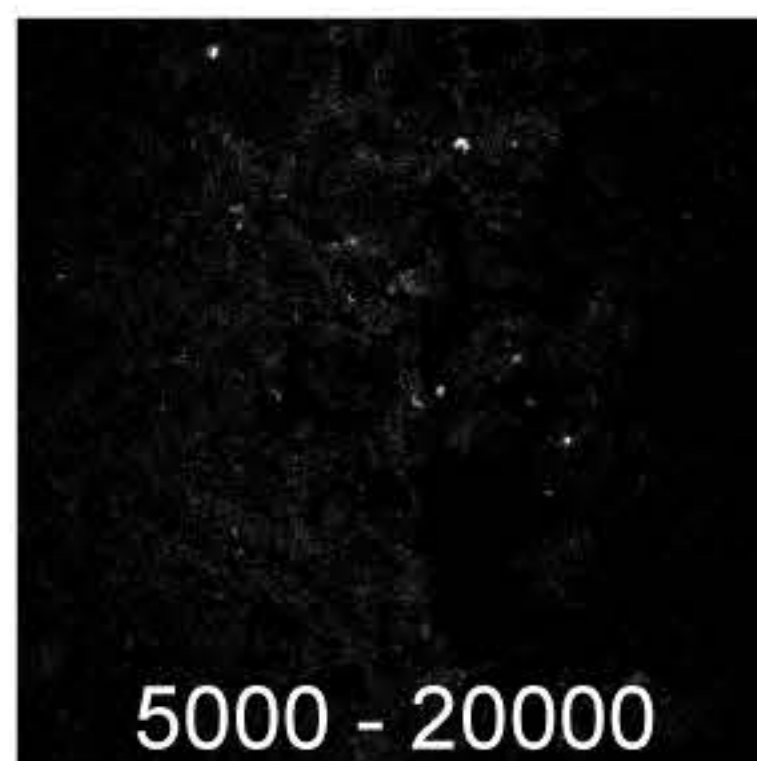
15. VAMP4(1111)

ex.:  $\lambda$  405 / em.  
CH1ex.:  $\lambda$  488 / em.  
CH2ex.:  $\lambda$  561 / em.  
CH3ex.:  $\lambda$  633 / em.  
CH4merge:  
CH 1 -4

brightfield



MOCK



5000 - 20000



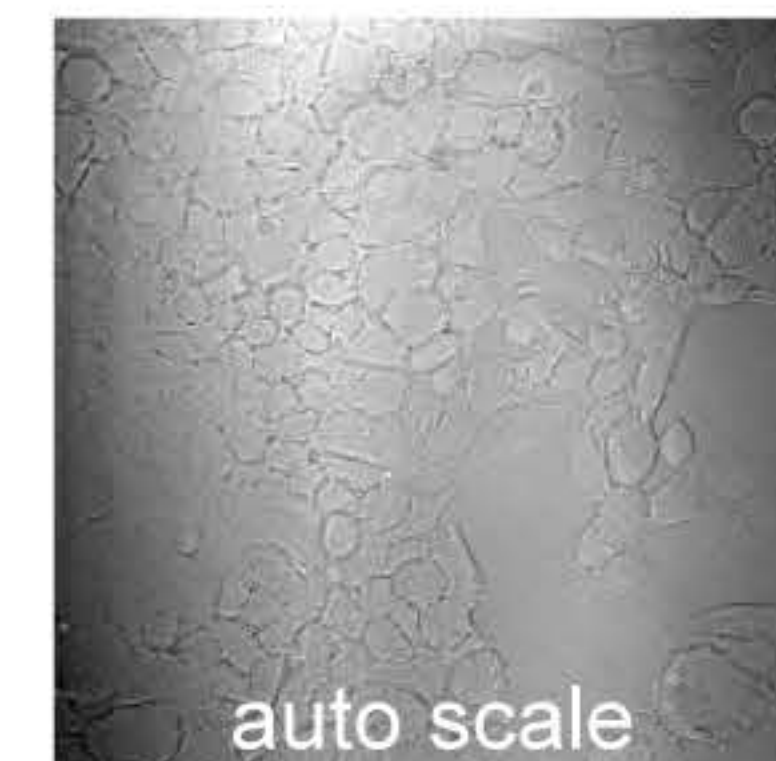
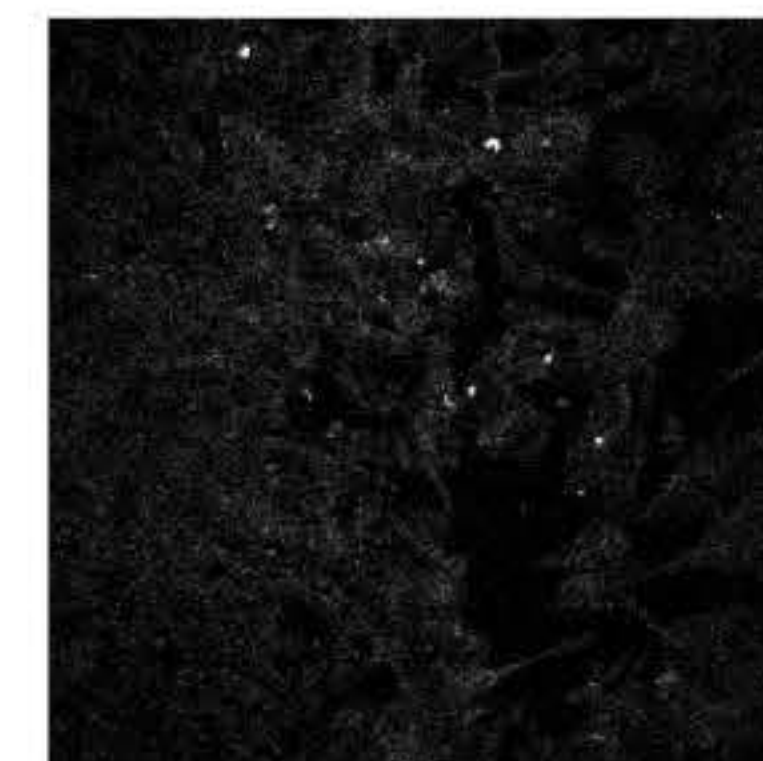
3000 - 25000



3500 - 35000

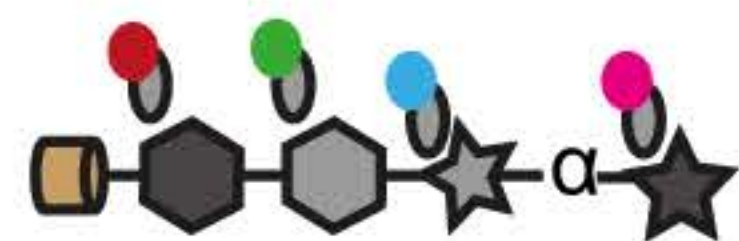
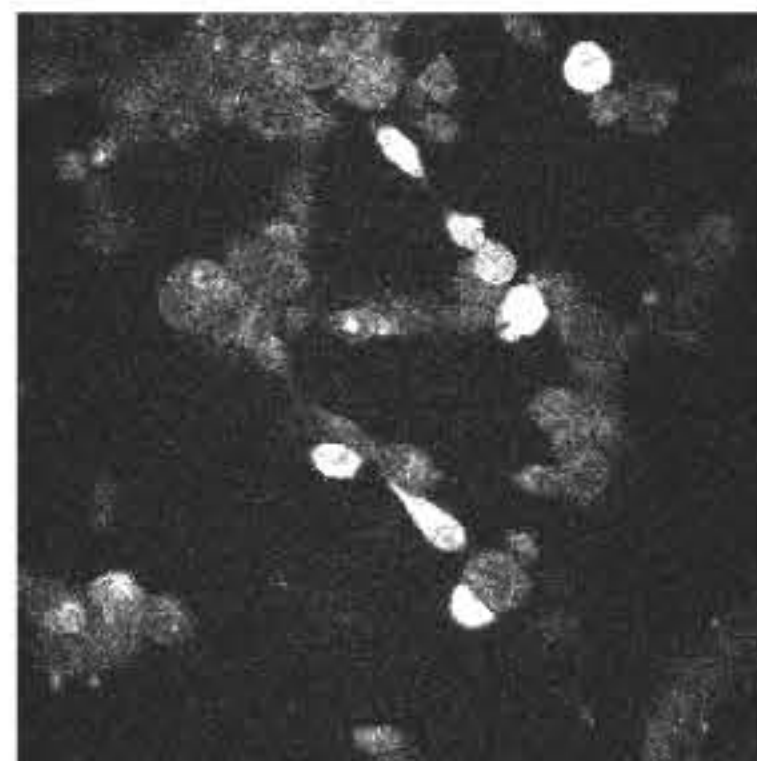
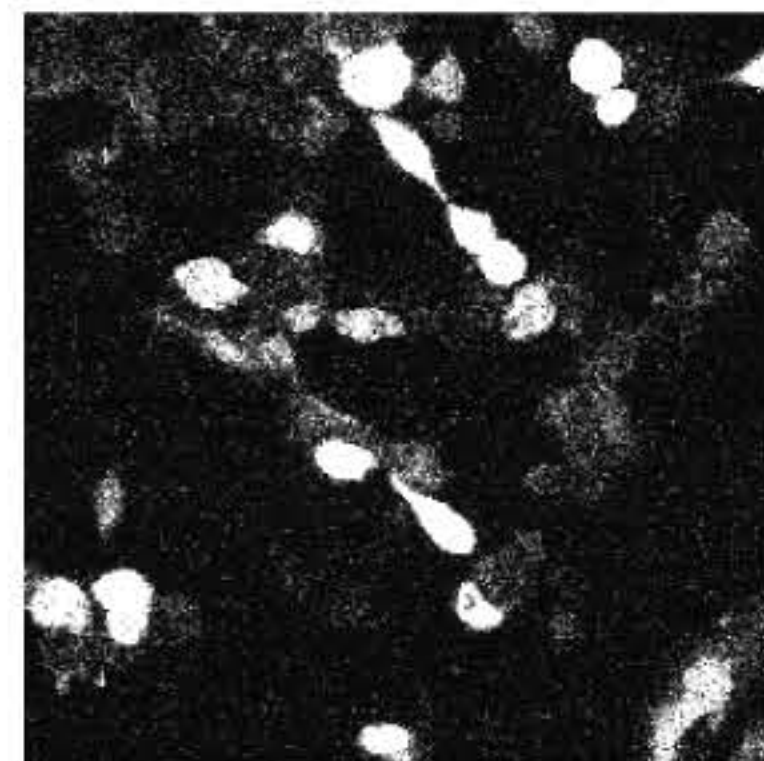
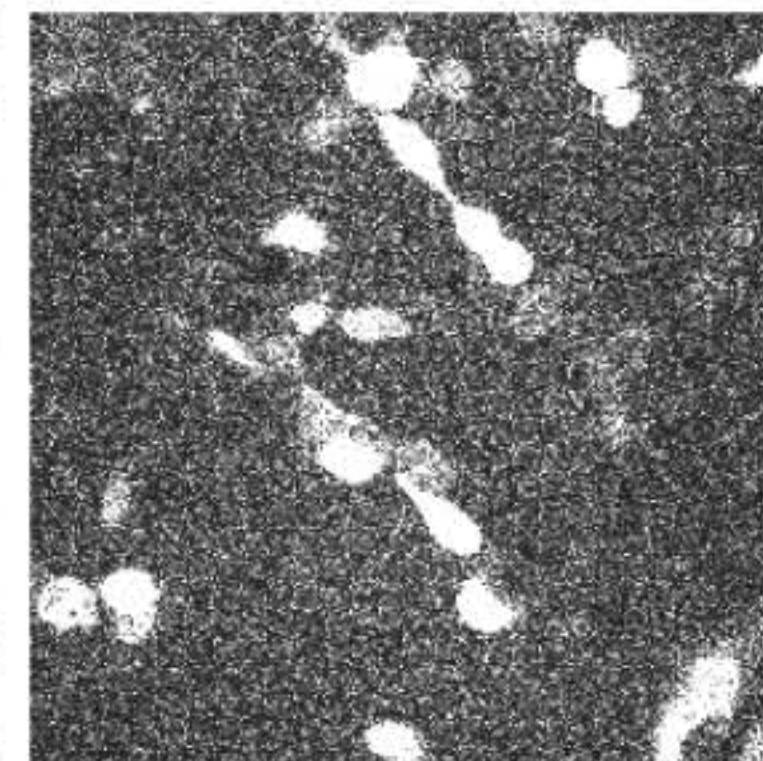
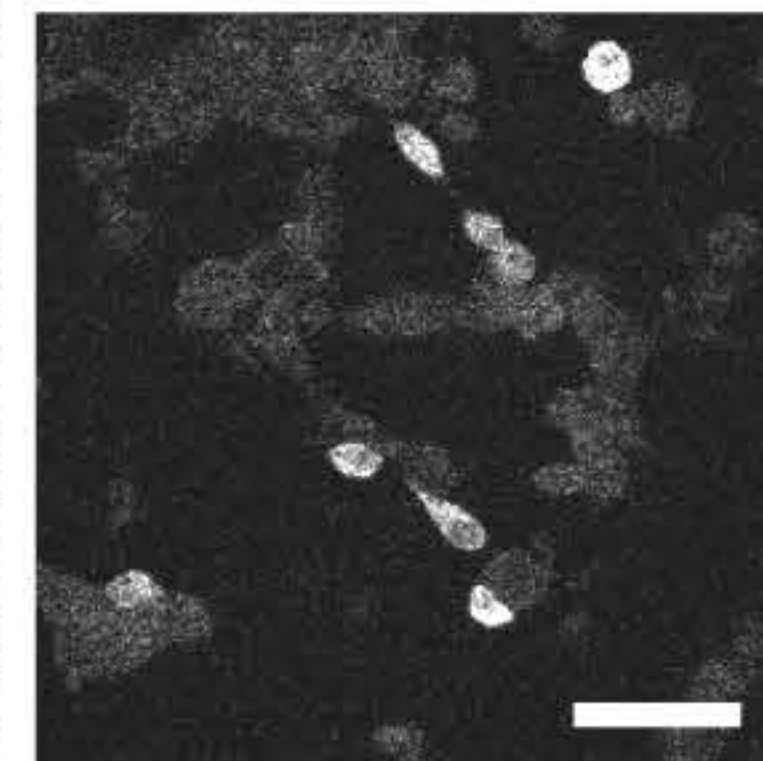


4000 - 10000

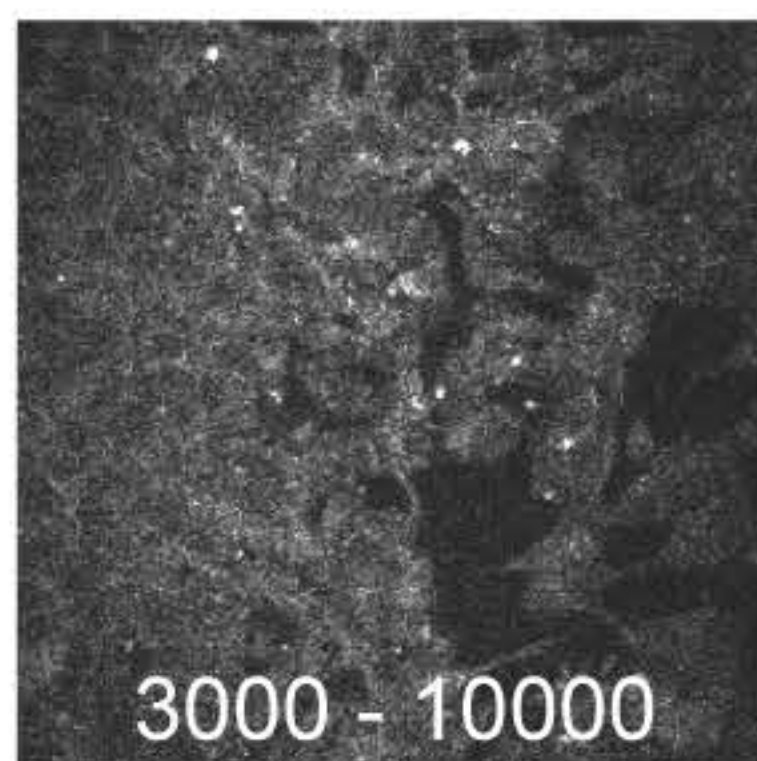


E

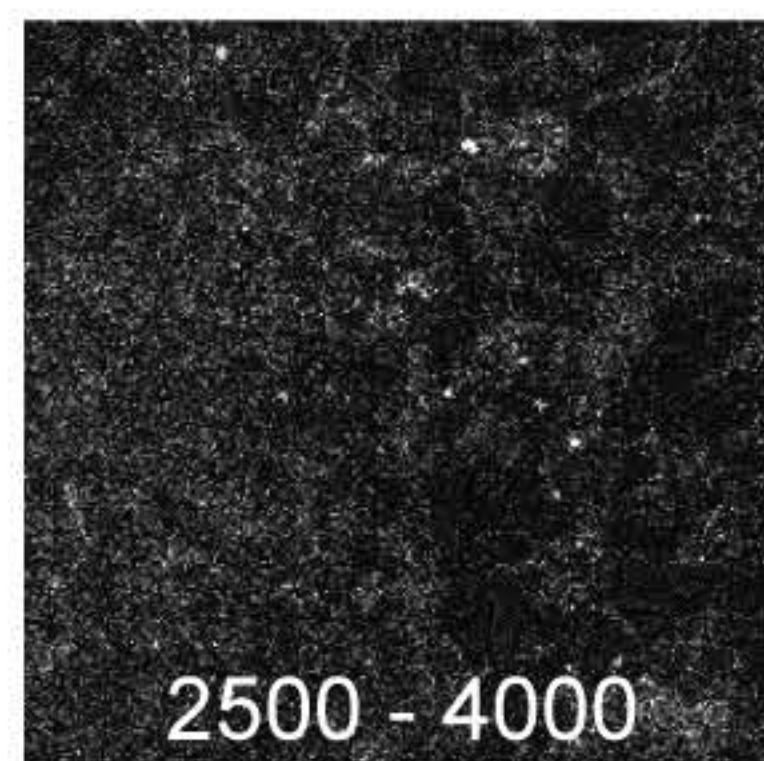
15. VAMP4(1111)

ex.:  $\lambda$  405 / em.  
CH1ex.:  $\lambda$  488 / em.  
CH2ex.:  $\lambda$  561 / em.  
CH3ex.:  $\lambda$  633 / em.  
CH4

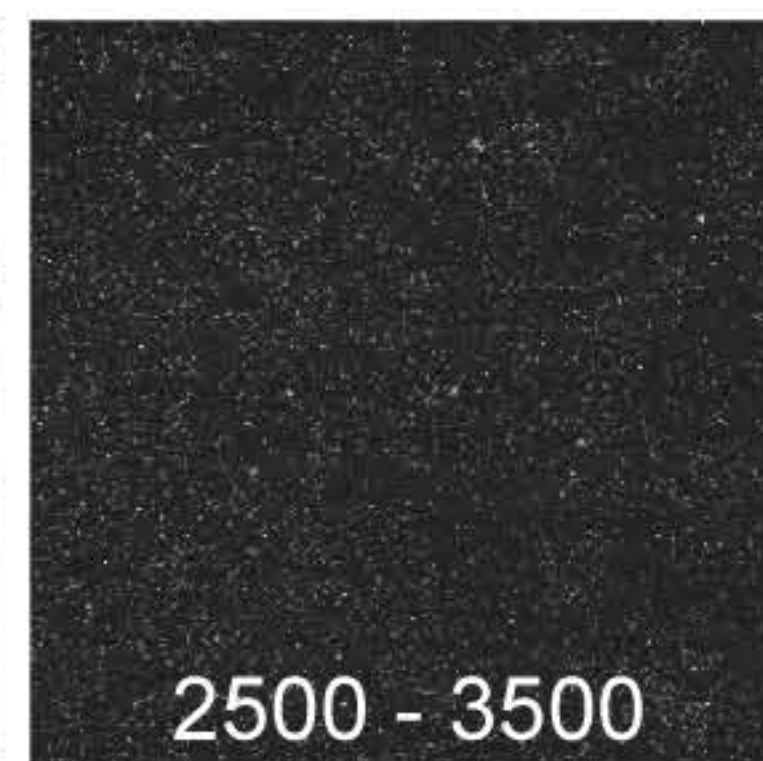
MOCK



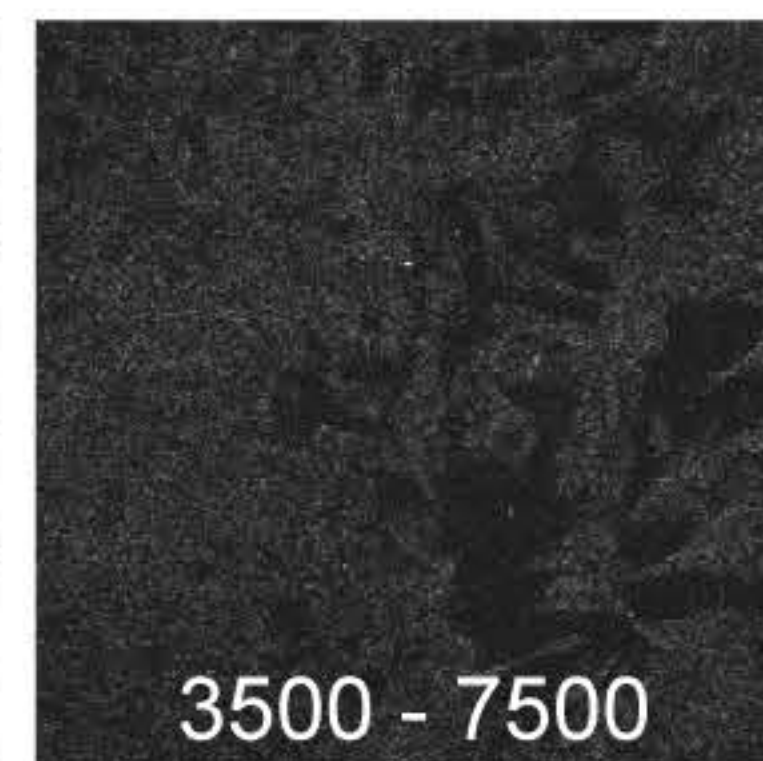
3000 - 10000



2500 - 4000

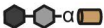
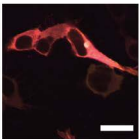
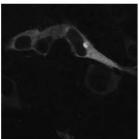
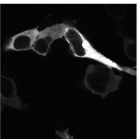
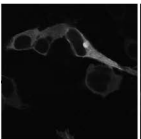
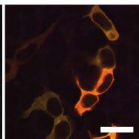
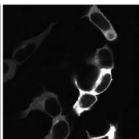
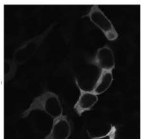
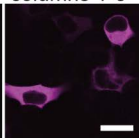
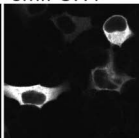
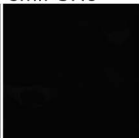
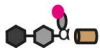


2500 - 3500

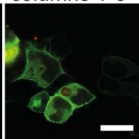
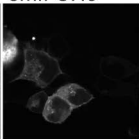
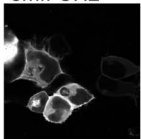


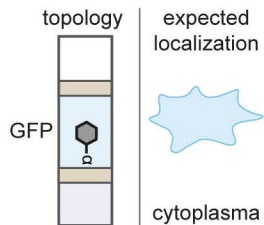
3500 - 7500

A

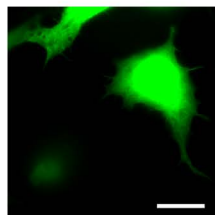
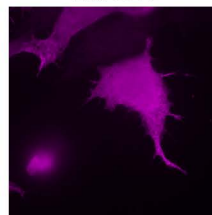
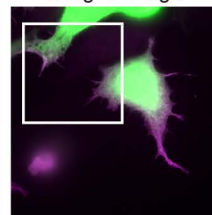
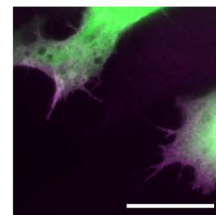
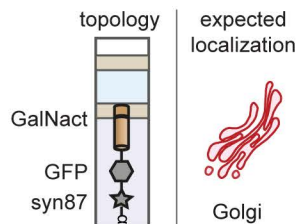
12. SNAP25(1100) ex.:  $\lambda$  488  
em.: CH2ex.:  $\lambda$  561  
em.: CH3ex.:  $\lambda$  633  
em.: CH4overlay  
columns 1-3

B

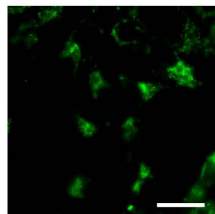
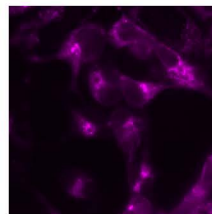
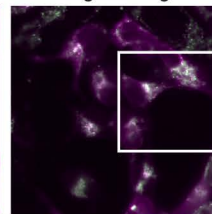
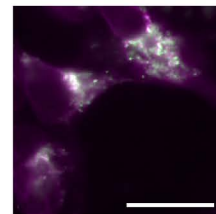
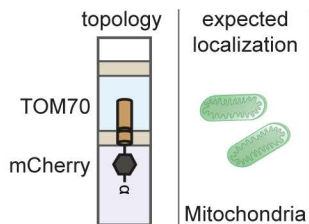
SNAP25(1100) ex.:  $\lambda$  488  
em.: CH2ex.:  $\lambda$  561  
em.: CH3ex.:  $\lambda$  633  
em.: CH4overlay  
columns 1-3

**4. GFP(0100)**

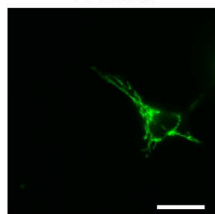
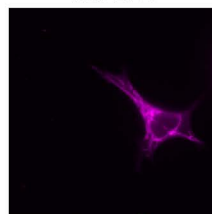
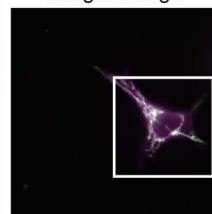
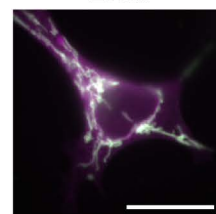
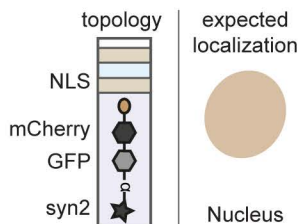
60x

**GFP(0100)  
NbALFA****Merged image****Zoom****6. GalNact(0110)**

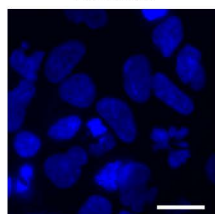
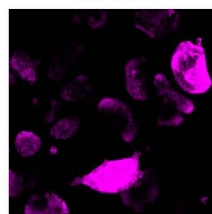
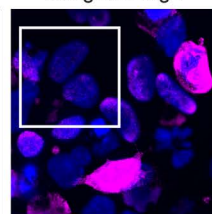
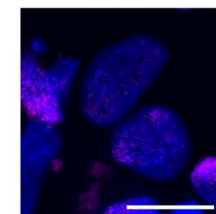
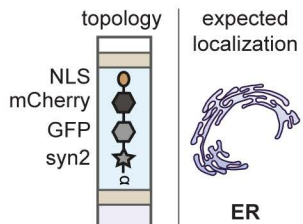
60x

**GalNact(0110)  
NbALFA****Merged image****Zoom****8. TOM70(1000)**

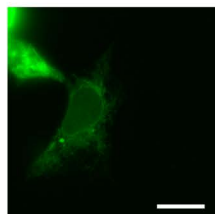
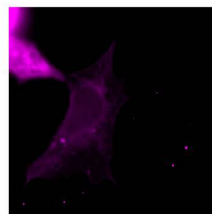
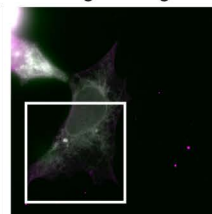
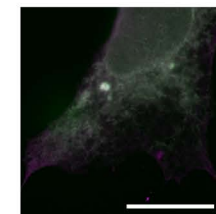
60x

**TOM70(1000)  
NbALFA****Merged image****Zoom****13. NLS(1101)**

60x

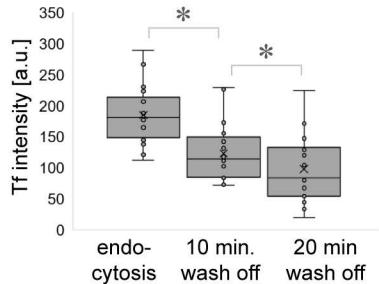
**NLS(1101)  
NbALFA****Merged image****Zoom****14. KDEL(1110)**

60x

**KDEL(1110)  
NbALFA****Merged image****Zoom**

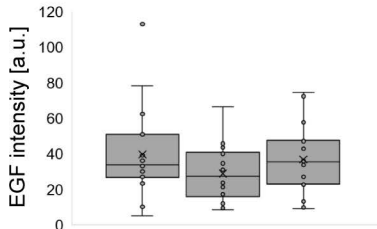
A

Tf labeling, background corrected



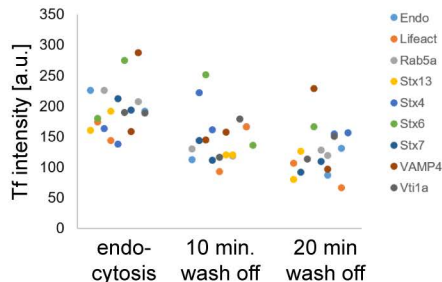
B

EGF labeling, background corrected



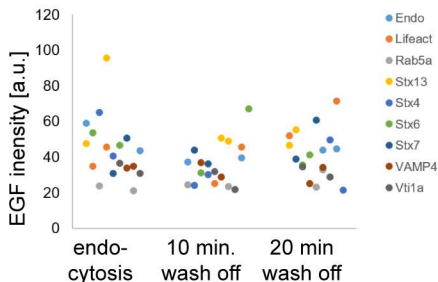
C

Tf labeling, background corrected

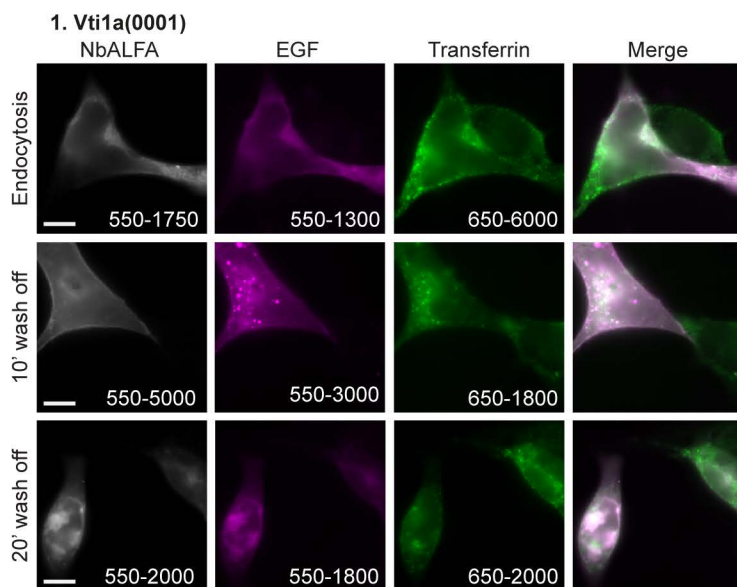


D

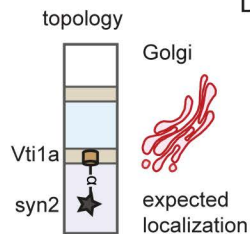
EGF labeling, background corrected



A



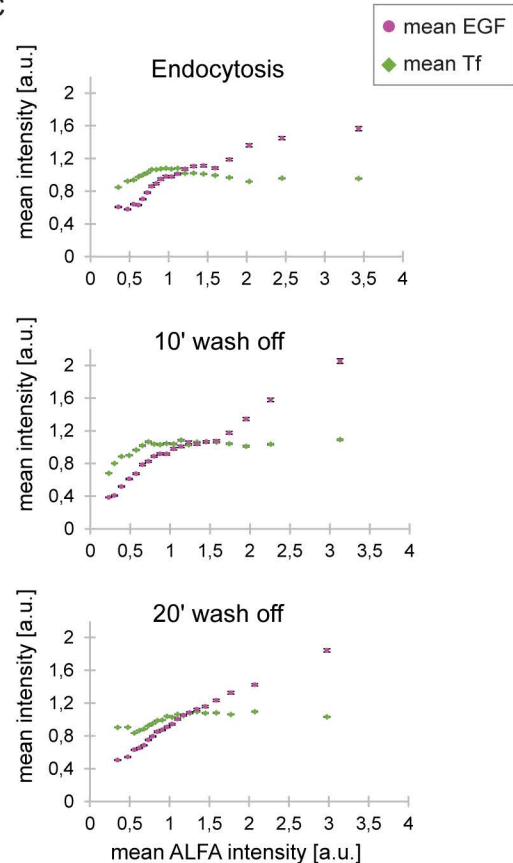
B



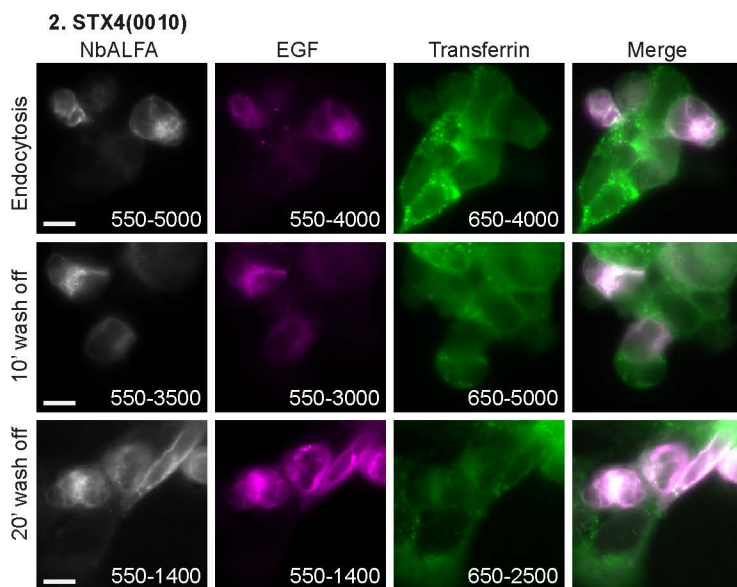
D

|                     | Transferrin |         | EGF      |         |
|---------------------|-------------|---------|----------|---------|
|                     | corr (R)    | p-value | corr (R) | p-value |
| <b>Endocytosis</b>  | 0,300       | 1,000   | 0,996    | <0,001  |
| <b>10' wash off</b> | 0,742       | 0,010   | 0,968    | <0,001  |
| <b>20' wash off</b> | 0,855       | 0,001   | 0,992    | <0,001  |

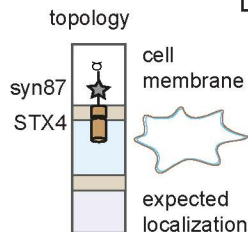
C



A



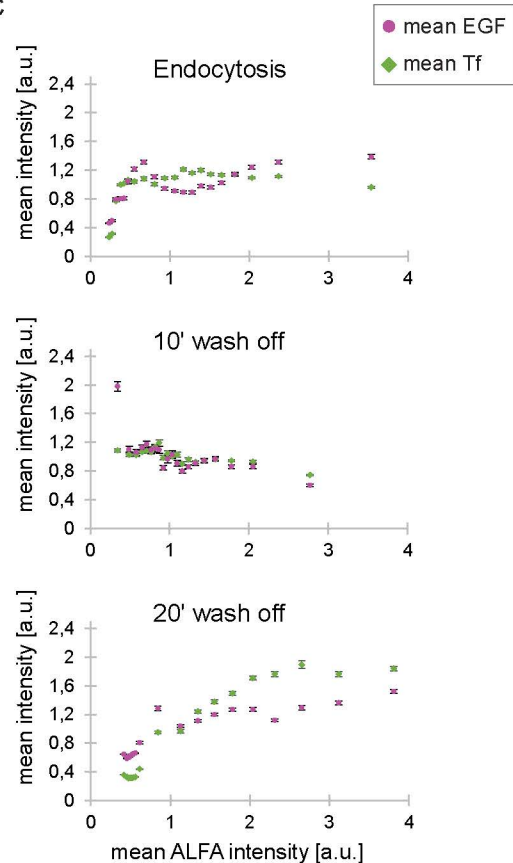
B



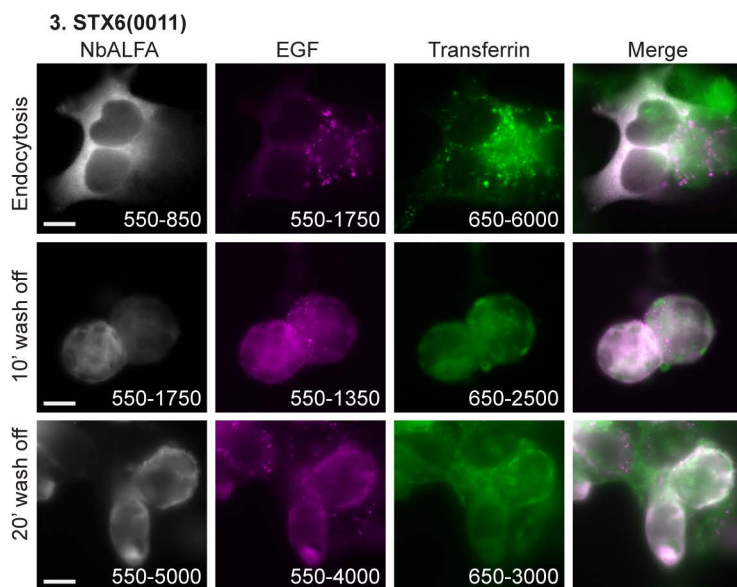
D

|                     | Transferrin |         | EGF      |         |
|---------------------|-------------|---------|----------|---------|
|                     | corr (R)    | p-value | corr (R) | p-value |
| <b>Endocytosis</b>  | 0,496       | 1,000   | 0,857    | <0,001  |
| <b>10' wash off</b> | -0,597      | 0,295   | -0,470   | 1,000   |
| <b>20' wash off</b> | 0,811       | 0,001   | 0,905    | <0,001  |

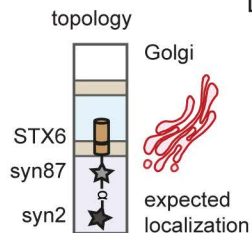
C



A



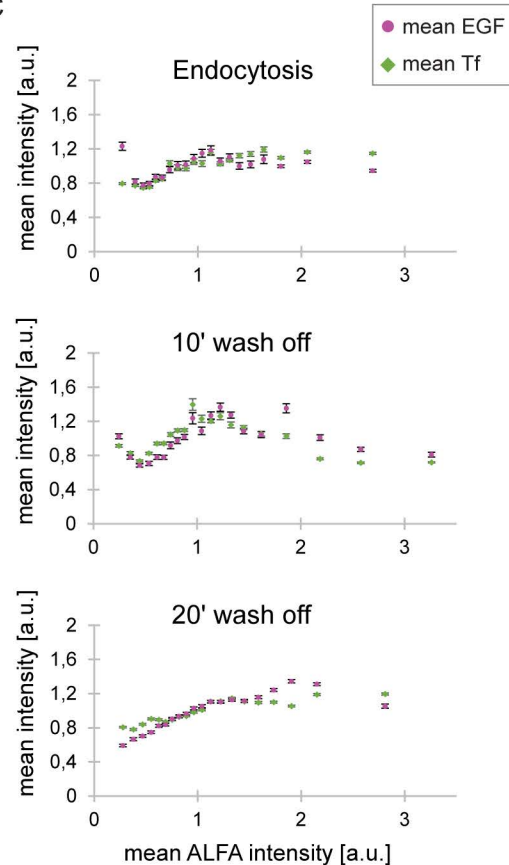
B



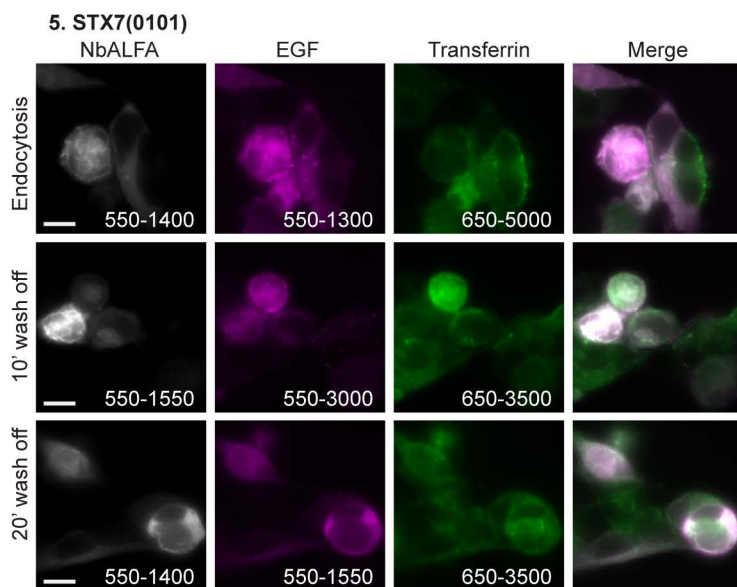
D

|                     | Transferrin |         | EGF      |         |
|---------------------|-------------|---------|----------|---------|
|                     | corr (R)    | p-value | corr (R) | p-value |
| <b>Endocytosis</b>  | 0,906       | <0,001  | 0,802    | 0,001   |
| <b>10' wash off</b> | -0,128      | 1,000   | 0,562    | 0,533   |
| <b>20' wash off</b> | 0,946       | <0,001  | 0,970    | <0,001  |

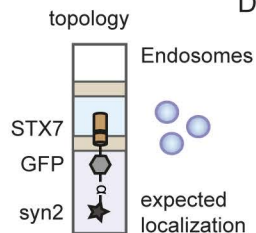
C



A



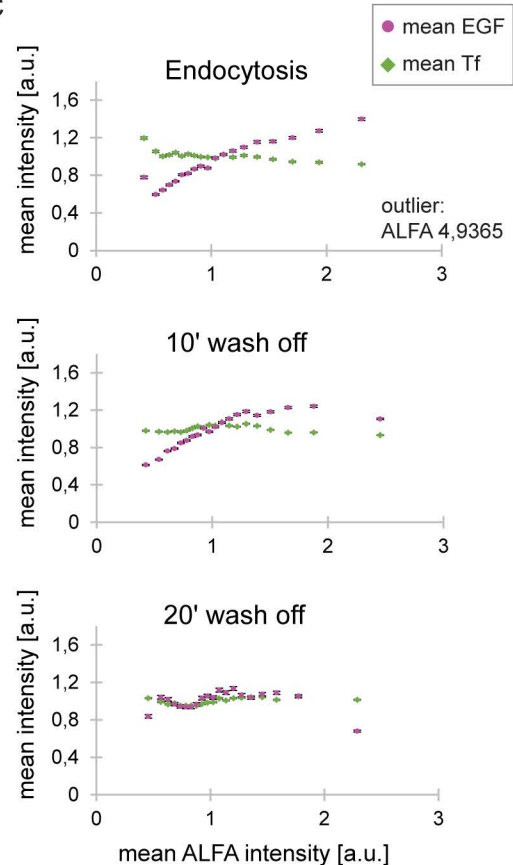
B



D

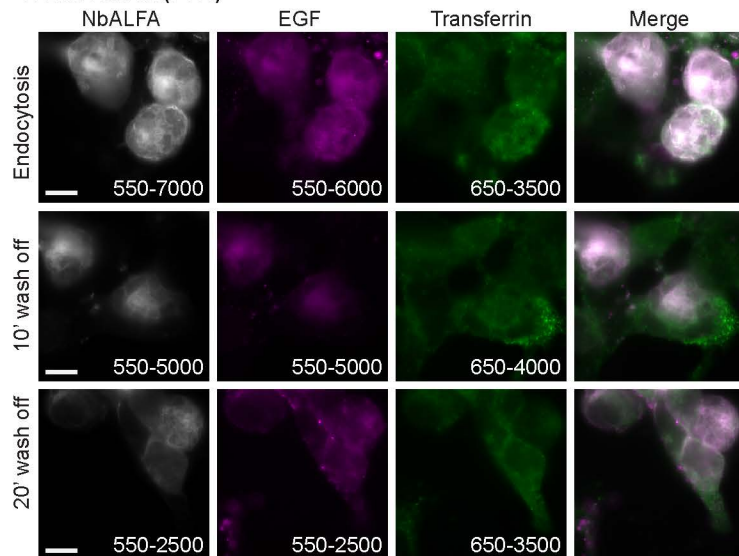
|              | Transferrin |         | EGF      |         |
|--------------|-------------|---------|----------|---------|
|              | corr (R)    | p-value | corr (R) | p-value |
| Endocytosis  | -0,345      | 1,000   | 0,963    | <0,001  |
| 10' wash off | 0,578       | 0,411   | 0,980    | <0,001  |
| 20' wash off | 0,903       | <0,001  | 0,595    | 0,305   |

C

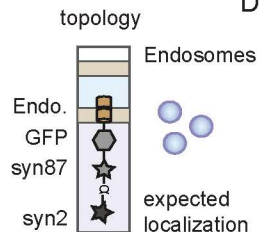


A

## 7. Endobrevin(0111)



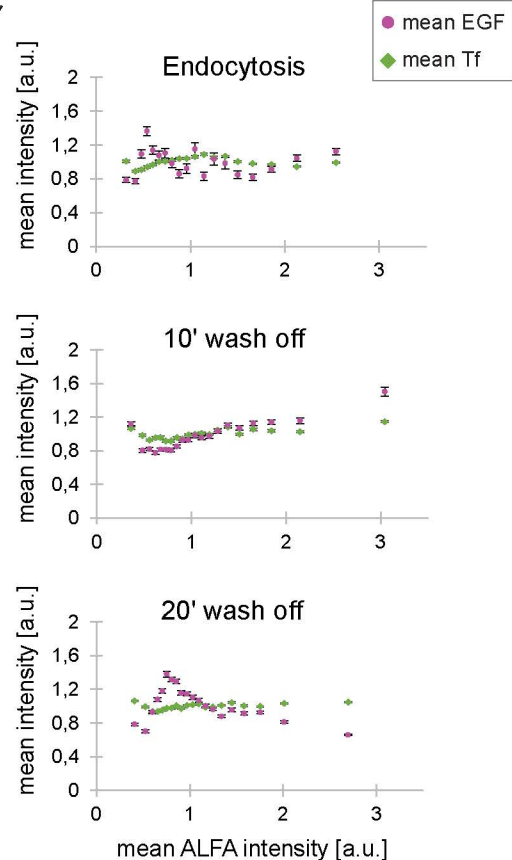
B



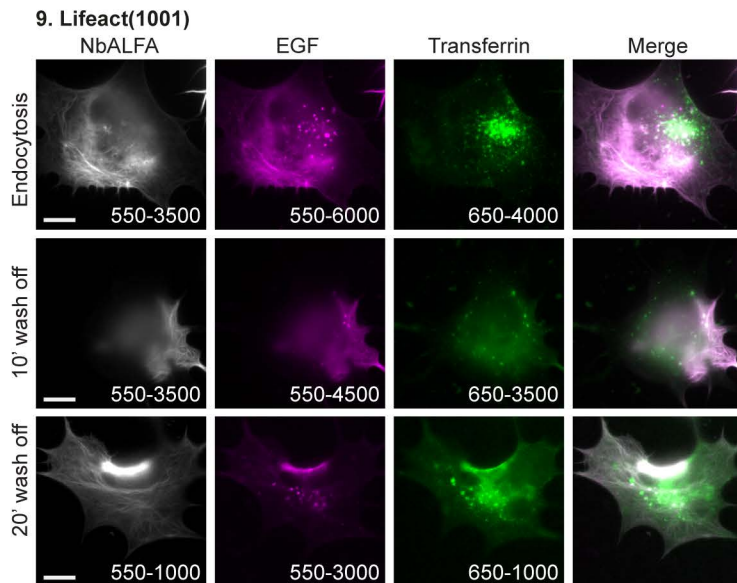
D

|                     | Transferrin |         | EGF      |         |
|---------------------|-------------|---------|----------|---------|
|                     | corr (R)    | p-value | corr (R) | p-value |
| <b>Endocytosis</b>  | 0,703       | 0,029   | 0,581    | 0,390   |
| <b>10' wash off</b> | 0,932       | 0,000   | 0,919    | <0,001  |
| <b>20' wash off</b> | 0,959       | <0,001  | -0,084   | 1,000   |

C

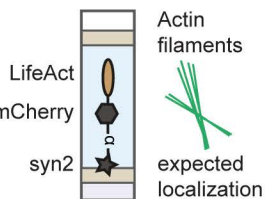


A



B

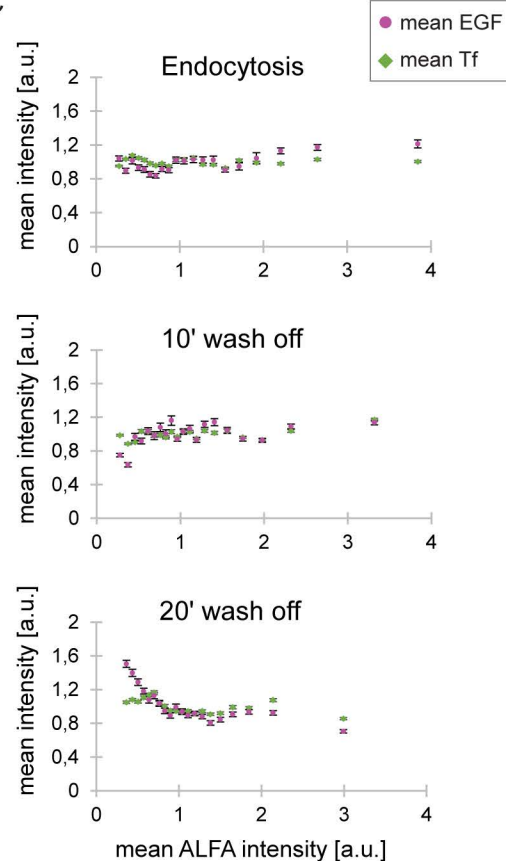
topology



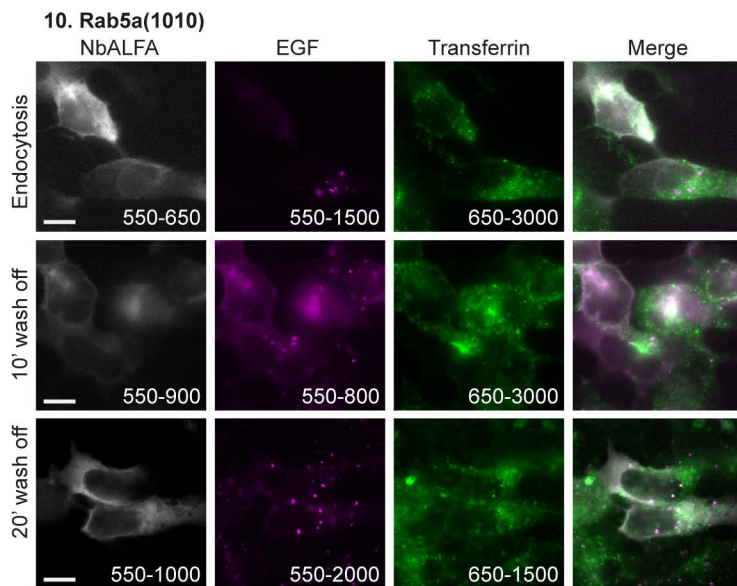
D

|                     | Transferrin |         | EGF      |         |
|---------------------|-------------|---------|----------|---------|
|                     | corr (R)    | p-value | corr (R) | p-value |
| <b>Endocytosis</b>  | 0,460       | 1,000   | 0,908    | <0,001  |
| <b>10' wash off</b> | 0,797       | 0,001   | 0,859    | <0,001  |
| <b>20' wash off</b> | -0,202      | 1,000   | -0,384   | 1,000   |

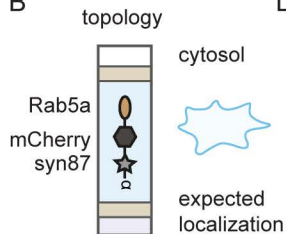
C



A



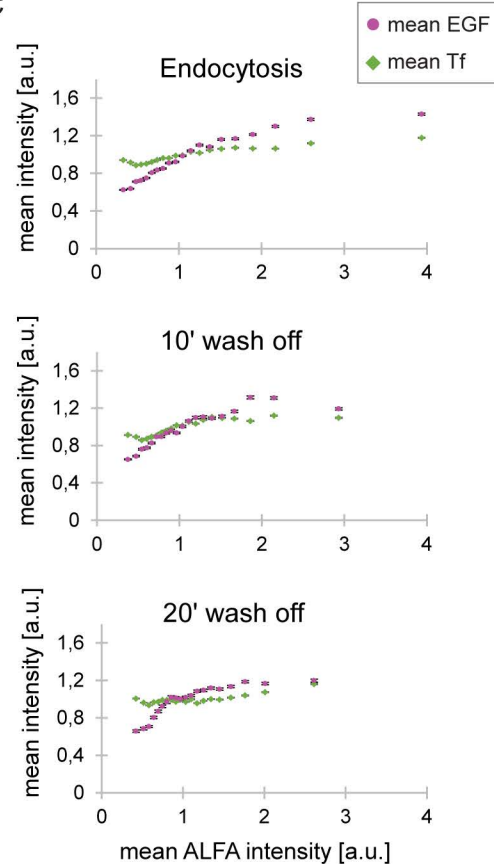
B



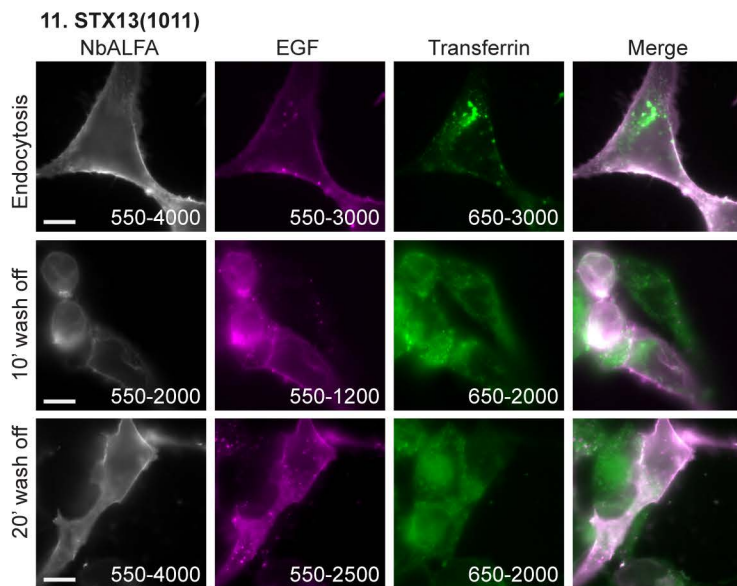
D

|              | Transferrin |         | EGF      |         |
|--------------|-------------|---------|----------|---------|
|              | corr (R)    | p-value | corr (R) | p-value |
| Endocytosis  | 0,980       | <0,001  | 0,996    | 0,000   |
| 10' wash off | 0,939       | <0,001  | 0,991    | <0,001  |
| 20' wash off | 0,916       | <0,001  | 0,986    | <0,001  |

C

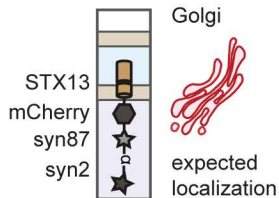


A



B

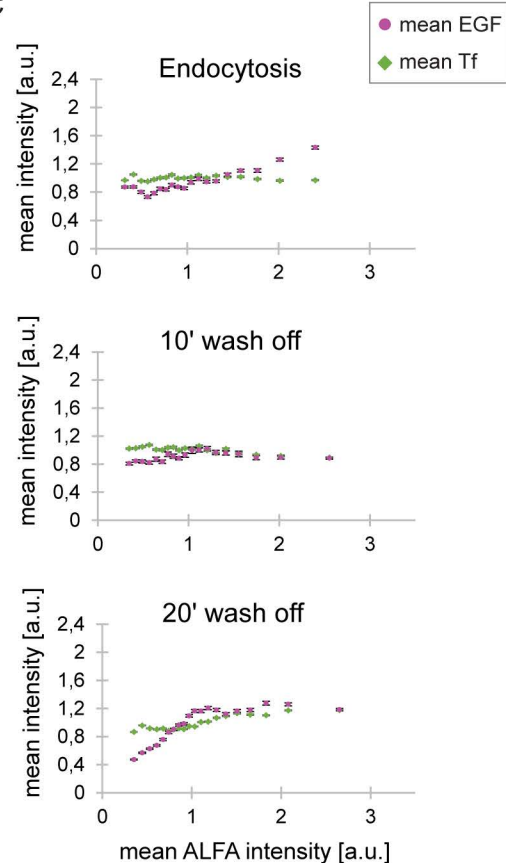
topology



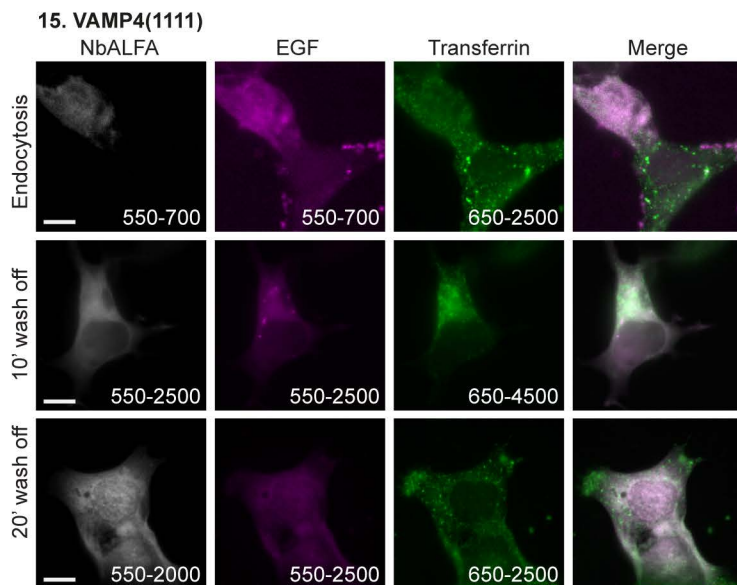
D

|                     | Transferrin |         | EGF      |         |
|---------------------|-------------|---------|----------|---------|
|                     | corr (R)    | p-value | corr (R) | p-value |
| <b>Endocytosis</b>  | 0,694       | 0,037   | 0,968    | <0,001  |
| <b>10' wash off</b> | 0,355       | 1,000   | 0,995    | <0,001  |
| <b>20' wash off</b> | 0,947       | <0,001  | 0,963    | <0,001  |

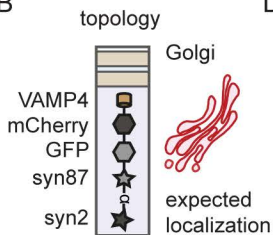
C



A



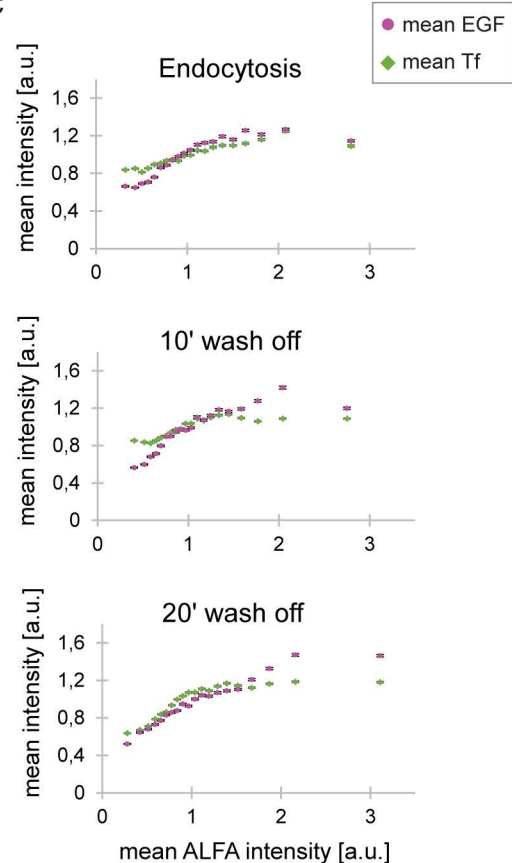
B

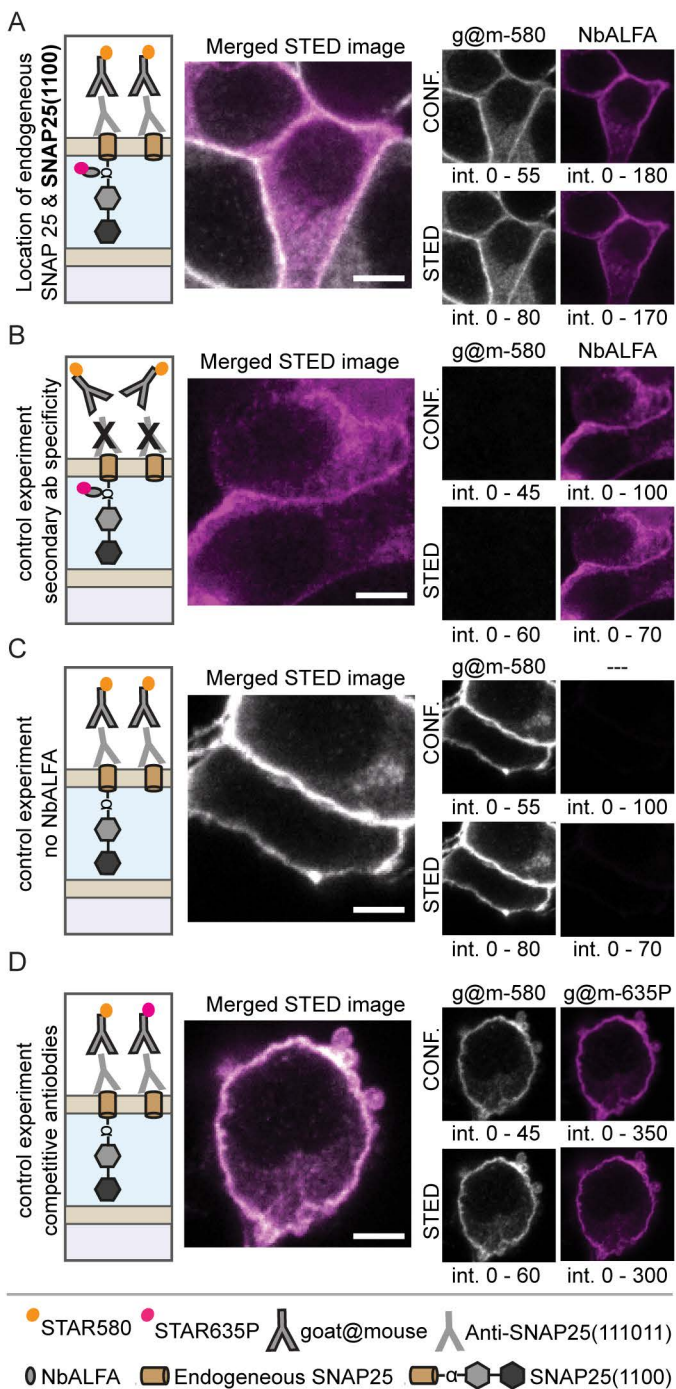


D

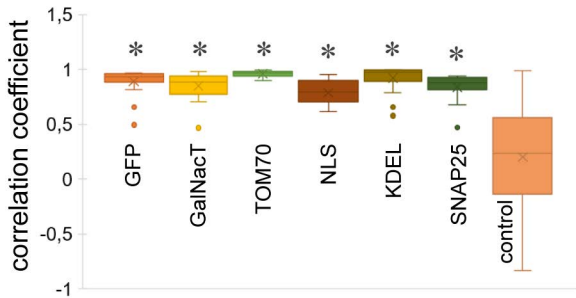
|              | Transferrin |         | EGF      |         |
|--------------|-------------|---------|----------|---------|
|              | corr (R)    | p-value | corr (R) | p-value |
| Endocytosis  | 0,918       | <0,001  | 0,981    | <0,001  |
| 10' wash off | 0,880       | <0,001  | 0,980    | <0,001  |
| 20' wash off | 0,861       | <0,001  | 0,996    | <0,001  |

C

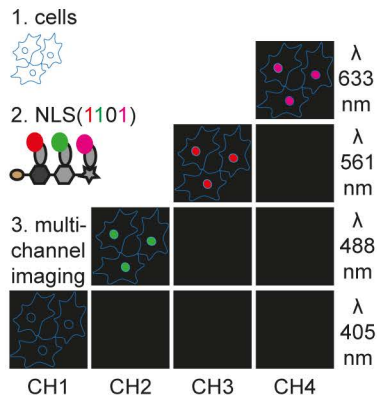




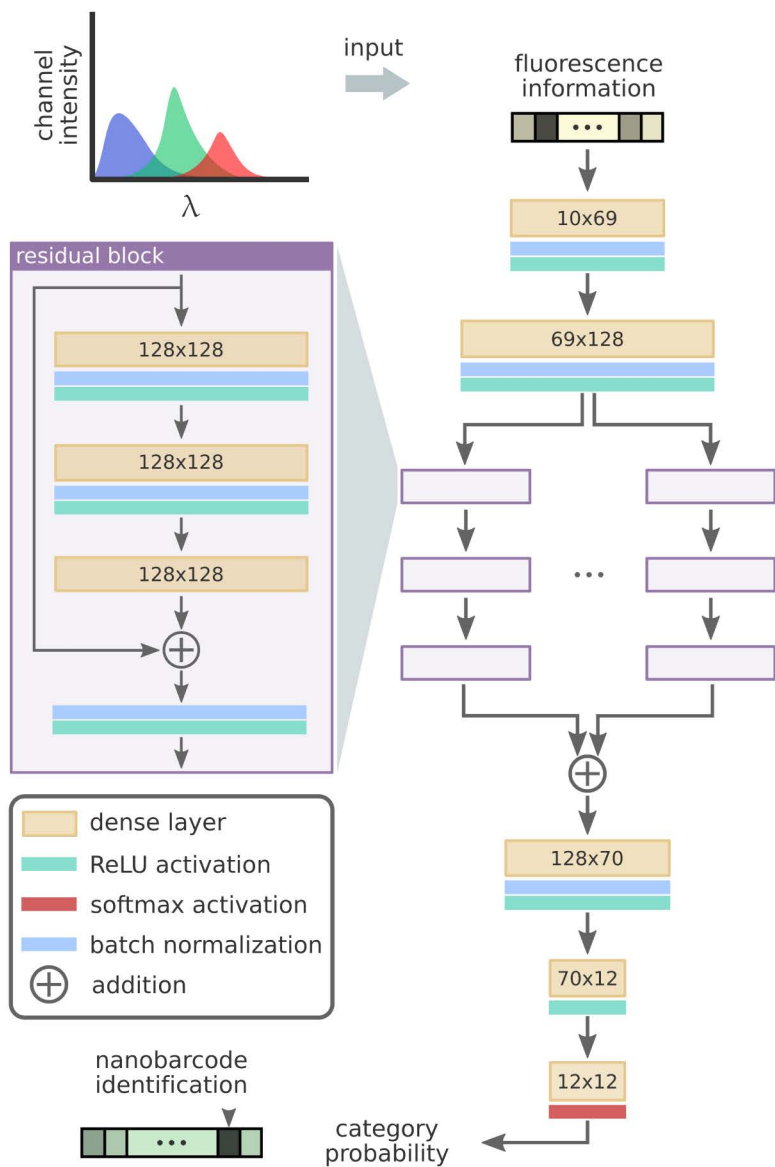
## Target protein localization



A



B



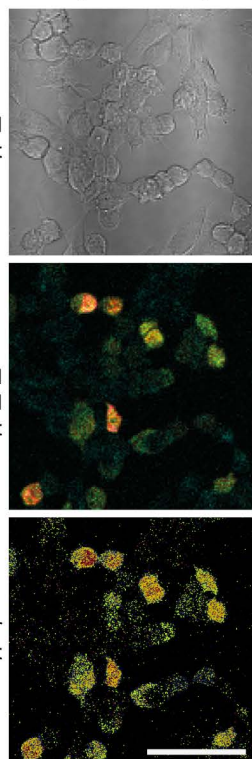
C

Example with NLS(1101)

brightfield input

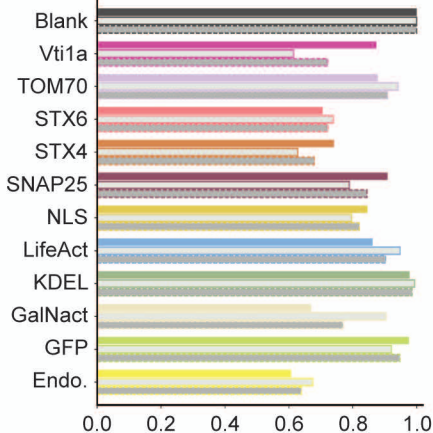
merged channel input

false color output



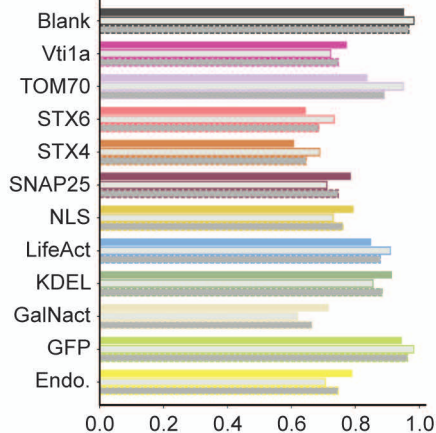
## overnight

prediction accuracy  
(over test data set) = 82,94%



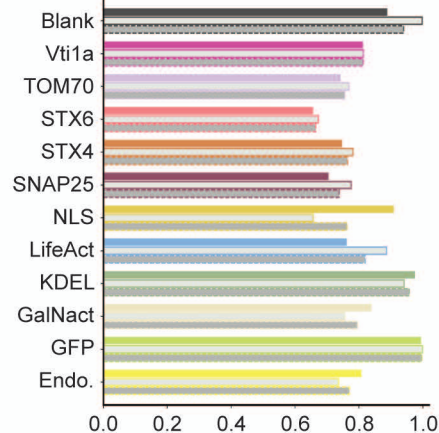
## 24 hrs

prediction accuracy  
(over test data set) = 79,98%

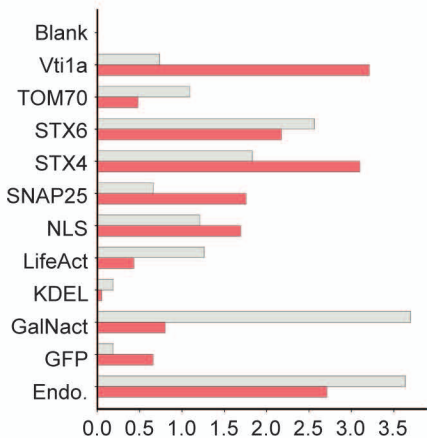


## 72 hrs

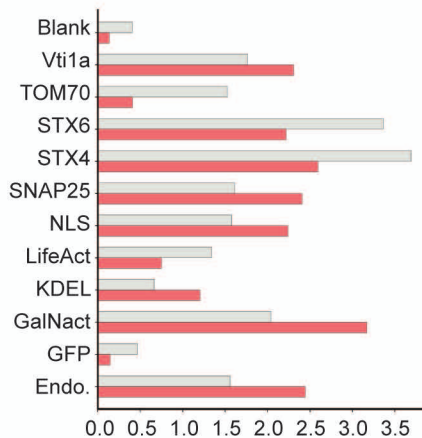
prediction accuracy  
(over test data set) = 81,54%



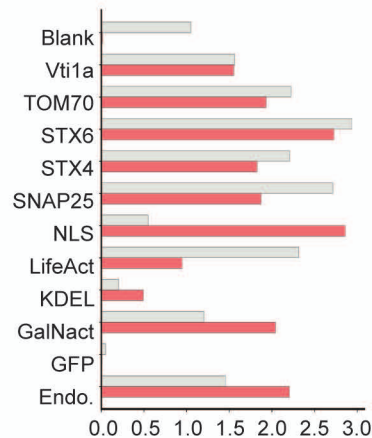
## overnight



## 24 hrs



## 72 hrs



grey false positives [%]

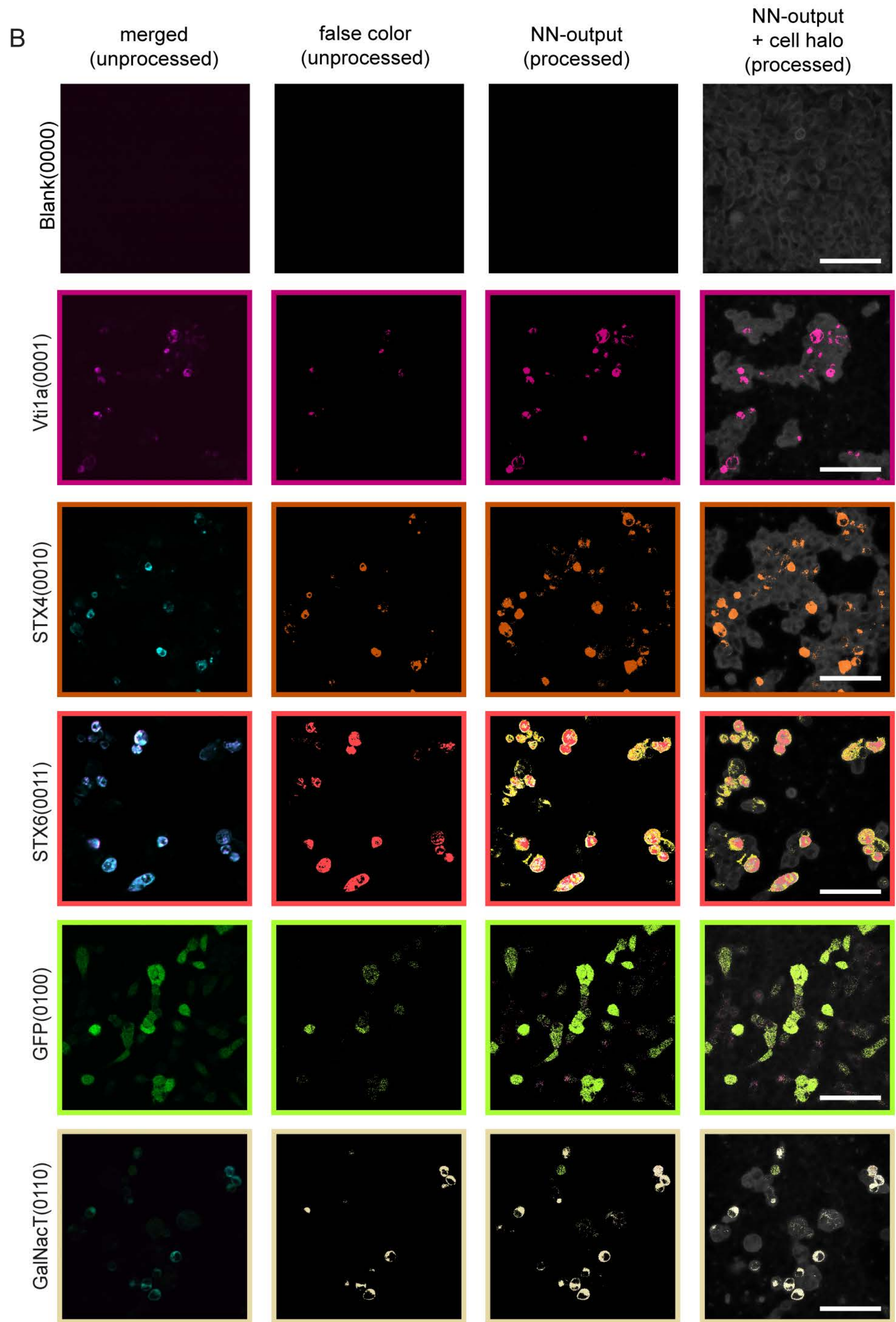
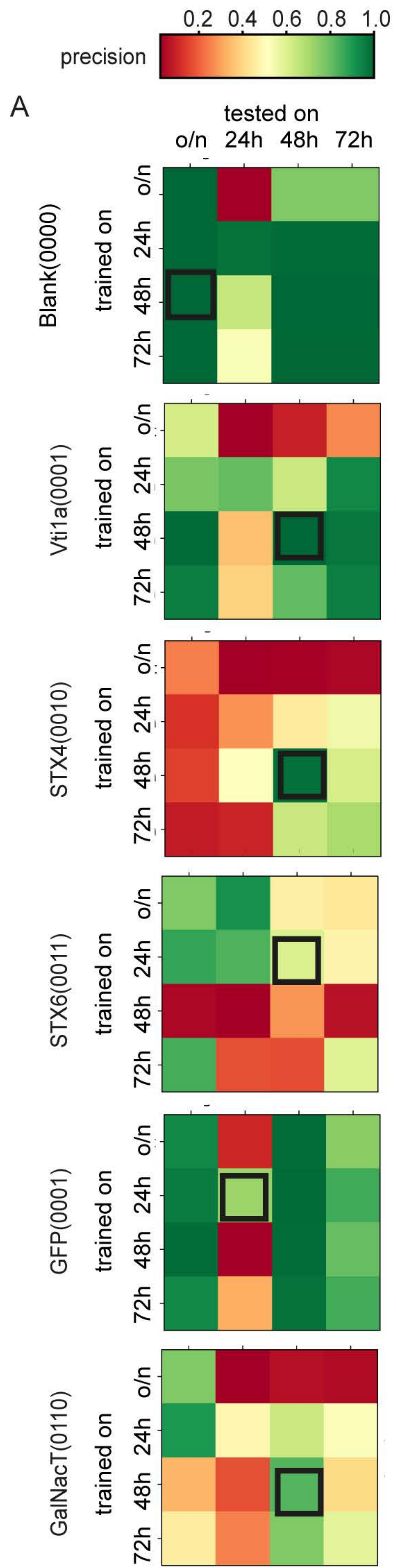
red false negatives [%]

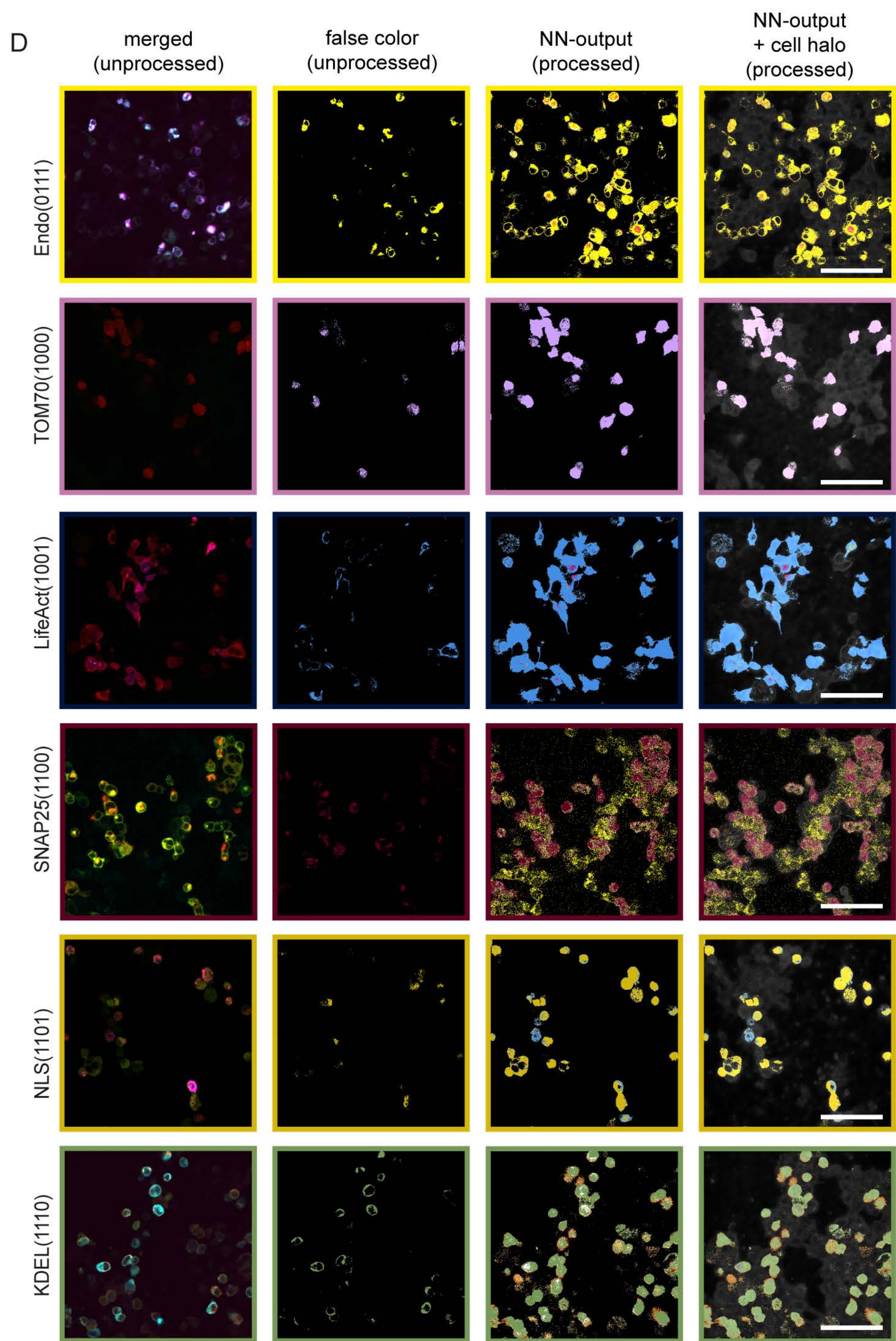
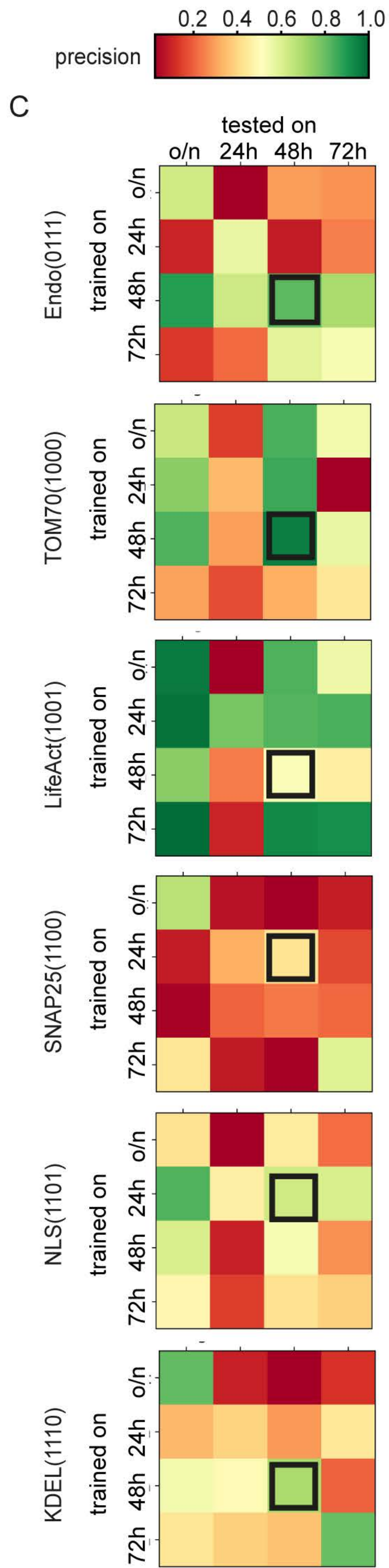
grey false positives [%]

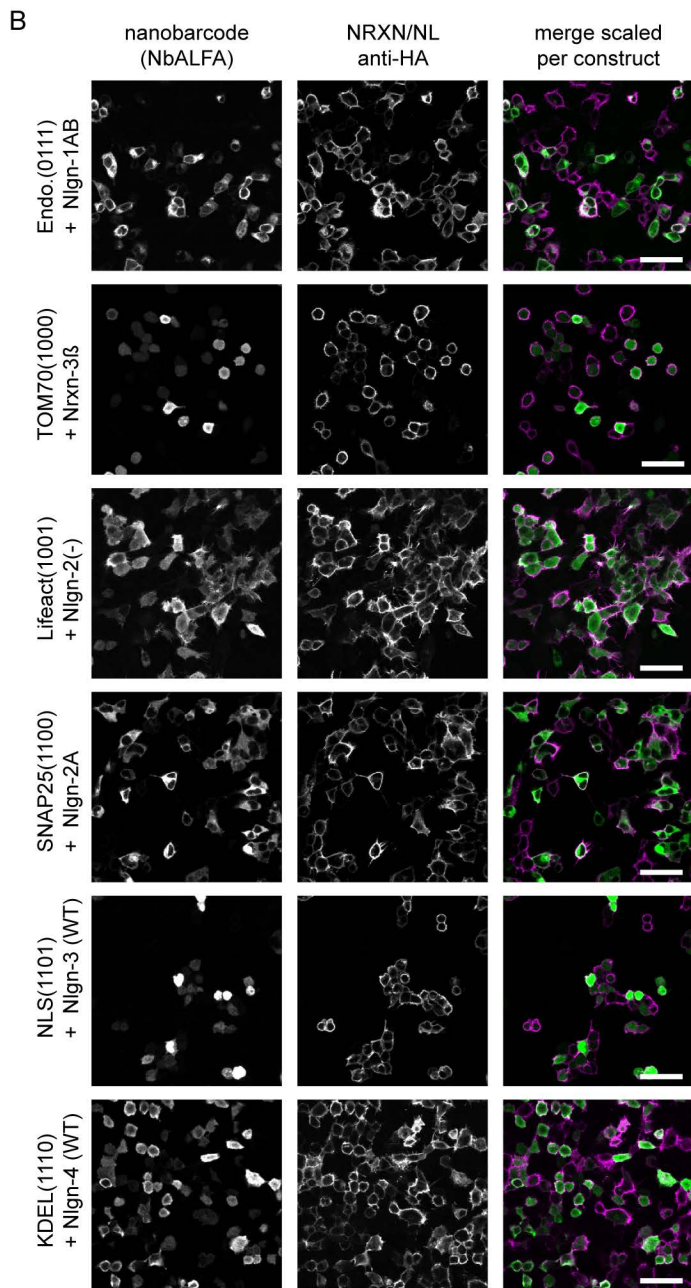
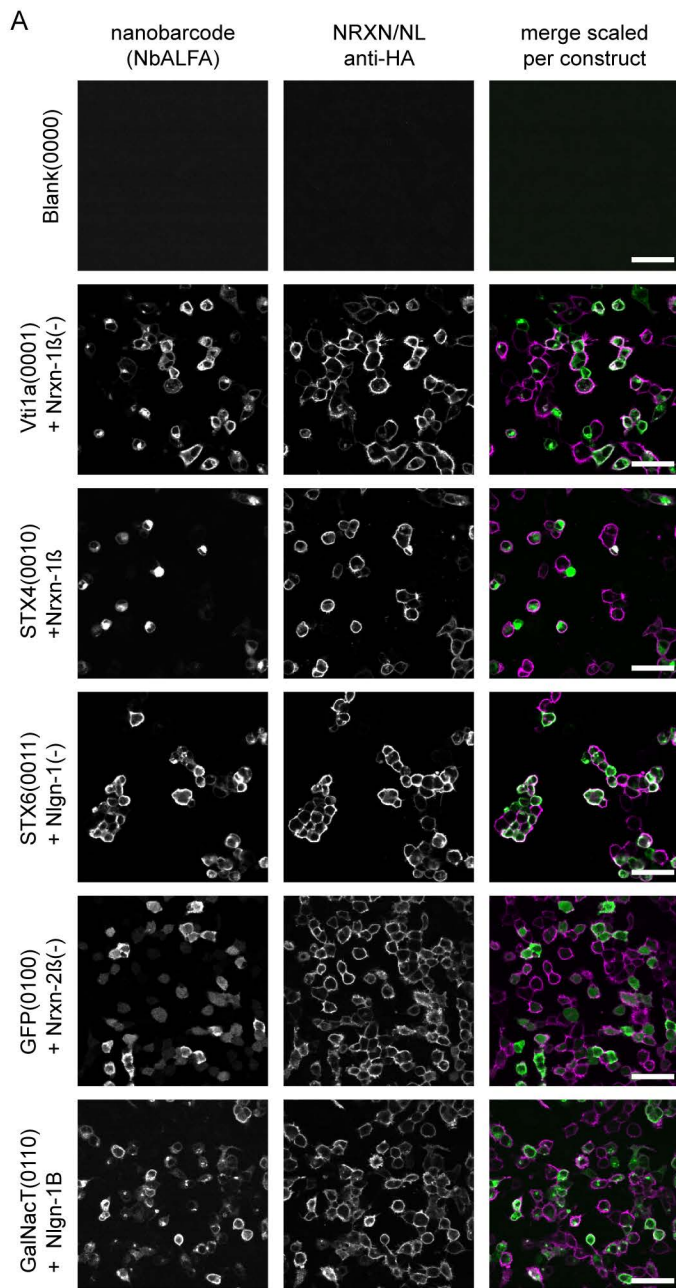
red false negatives [%]

grey false positives [%]

red false negatives [%]







| Protein             | Validation in literature (see reference list, below)                                                      | Validation in this manuscript                    |
|---------------------|-----------------------------------------------------------------------------------------------------------|--------------------------------------------------|
| 1. Vti1a(0001)      | - [1–3]                                                                                                   | EGF and Transferrin experiments (Suppl. Fig. 7)  |
| 2. Syntaxin4(0010)  | - [4,5]<br>- Synuclein Nanobody epitopes / tags are short [6–8]                                           | EGF and Transferrin experiments (Suppl. Fig. 8)  |
| 3. Syntaxin6(0011)  | - [1]<br>- Synuclein Nanobody epitopes / tags are short [6–8]                                             | EGF and Transferrin experiments (Suppl. Fig. 9)  |
| 4. GFP(0100)        | - No known function, therefore no functional validation needed; only verification of localization needed. | Colocalization assays (Suppl. Fig. 5)            |
| 5. STX7(0101)       | - [9–11] and probably others as well                                                                      | EGF and Transferrin experiments (Suppl. Fig. 10) |
| 6. GalNacT(0110)    | - Localization is related to function, so was investigated here [12].                                     | Colocalization assays (Suppl. Fig. 5)            |
| 7. Endobrevin(0111) | - [13,14]                                                                                                 | EGF and Transferrin experiments (Suppl. Fig. 11) |
| 8. TOM70(1000)      | - No known function, therefore no functional validation needed; only verification of localization needed. | Colocalization assays (Suppl. Fig. 5)            |
| 9. LifeAct(1001)    | - Actin-binding molecule, designed to be tagged [15].                                                     | EGF and Transferrin experiments (Suppl. Fig. 12) |
| 10. Rab5a(1010)     | - [1,2,16,17]                                                                                             | EGF and Transferrin experiments (Suppl. Fig. 13) |
| 11. STX13(1011)     |                                                                                                           | EGF and Transferrin experiments (Suppl. Fig. 14) |
| 12. SNAP25(1100)    | - Tested in cell lines [1,18–20]                                                                          | STED SNAP25 (PC 12 cells) (Suppl. Fig. 16)       |
| 13. NLS(1101)       | - No known function, therefore no functional validation needed; only verification of localization needed. | Colocalization assays (Suppl. Fig. 5)            |
| 14. KDEL(1110)      | - No known function, therefore no functional validation needed; only verification of localization needed. | Colocalization assays (Suppl. Fig. 5)            |
| 15. VAMP4(1111)     | - [1,21,22]                                                                                               | EGF and Transferrin experiments (Suppl. Fig. 15) |

**Supplementary Table 1. Protein tag validation in literature and/or in this manuscript.**

### Reference List for Supplementary Table 1.

1. Reshetniak S, Ußling J, Perego E, Rammner B, Schikorski T, Fornasiero EF, et al. A comparative analysis of the mobility of 45 proteins in the synaptic bouton. *EMBO J.* 2020;39.  
doi:10.15252/EMBJ.2020104596
2. Hoopmann P, Punge A, Barysch S V., Westphal V, Bückers J, Opazo F, et al. Endosomal sorting of readily releasable synaptic vesicles. *Proc Natl Acad Sci U S A.* 2010;107: 19055–19060.  
doi:10.1073/PNAS.1007037107/-/DCSUPPLEMENTAL/SM01.MOV
3. Ramirez DMO, Khvotchev M, Trauterman B, Kavalali ET. Vti1a identifies a vesicle pool that preferentially recycles at rest and maintains spontaneous neurotransmission. *Neuron.* 2012;73: 121–134. doi:10.1016/J.NEURON.2011.10.034
4. Kalwat MA, Wiseman DA, Luo W, Wang Z, Thurmond DC. Gelsolin Associates with the N Terminus of Syntaxin 4 to Regulate Insulin Granule Exocytosis. *Mol Endocrinol.* 2012;26: 128–141. doi:10.1210/ME.2011-1112
5. Takuma T, Arakawa T, Okayama M, Mizoguchi I, Tanimura A, Tajima Y. Trafficking of Green Fluorescent Protein-Tagged SNARE Proteins in HSY Cells. *J Biochem.* 2002;132: 729–735.  
doi:10.1093/OXFORDJOURNALS.JBCHEM.A003280
6. De Genst EJ, Guillems T, Wellens J, Day EM, Waudby CA, Meehan S, et al. Structure and properties of a complex of  $\alpha$ -synuclein and a single-domain camelid antibody. *J Mol Biol.* 2010;402: 326–343. doi:10.1016/J.JMB.2010.07.001
7. El-Turk F, Newby FN, De Genst E, Guillems T, Sprules T, Mittermaier A, et al. Structural Effects of Two Camelid Nanobodies Directed to Distinct C-Terminal Epitopes on  $\alpha$ -Synuclein. *Biochemistry.* 2016;55: 3116–3122. doi:10.1021/ACS.BIOCHEM.6B00149
8. Guillems T, El-Turk F, Buell AK, O'Day EM, Aprile FA, Esbjörner EK, et al. Nanobodies Raised against Monomeric  $\alpha$ -Synuclein Distinguish between Fibrils at Different Maturation Stages. *J Mol*

Biol. 2013;425: 2397–2411. doi:10.1016/J.JMB.2013.01.040

9. Prekeris R, Yang B, Oorschot V, Klumperman J, Scheller RH. Differential roles of syntaxin 7 and syntaxin 8 in endosomal trafficking. *Mol Biol Cell*. 1999;10: 3891–3908.  
doi:10.1091/MBC.10.11.3891
10. Mashima H, Suzuki J, Hirayama T, Yoshikumi Y, Ohno H, Ohnishi H, et al. Involvement of vesicle-associated membrane protein 7 in human gastric epithelial cell vacuolation induced by *Helicobacter pylori*-produced VacA. *Infect Immun*. 2008;76: 2296–2303. doi:10.1128/IAI.01573-07
11. Mori Y, Takenaka K ichiro, Fukazawa Y, Takamori S. The endosomal Q-SNARE, Syntaxin 7, defines a rapidly replenishing synaptic vesicle recycling pool in hippocampal neurons. *Commun Biol* 2021 41. 2021;4: 1–13. doi:10.1038/s42003-021-02512-4
12. Becker JL, Tran DT, Tabak LA. Members of the GalNAc-T family of enzymes utilize distinct Golgi localization mechanisms. *Glycobiology*. 2018;28: 841. doi:10.1093/GLYCOB/CWY071
13. Nagamatsu S, Nakamichi Y, Watanabe T, Matsushima S, Yamaguchi S, Ni J, et al. Localization of cellubrevin-related peptide, endobrevin, in the early endosome in pancreatic  $\beta$  cells and its physiological function in exo-endocytosis of secretory granules. *J Cell Sci*. 2001;114: 219–227.  
doi:10.1242/JCS.114.1.219
14. Okayama M, Arakawa T, Tanimura A, Mizoguchi I, Tajima Y, Takuma T. Role of VAMP8/endobrevin in Constitutive Exocytotic Pathway in HeLa Cells. *Cell Struct Funct*. 2009;34: 115–125. doi:10.1247/CSF.09013
15. Riedl J, Crevenna AH, Kessenbrock K, Yu JH, Neukirchen D, Bista M, et al. Lifeact: a versatile marker to visualize F-actin. *Nat Methods*. 2008;5: 605–607. doi:10.1038/NMETH.1220
16. Bethani I, Lang T, Geumann U, Sieber JJ, Jahn R, Rizzoli SO. The specificity of SNARE pairing

- in biological membranes is mediated by both proof-reading and spatial segregation. *EMBO J.* 2007;26: 3981–3992. doi:10.1038/SJ.EMBOJ.7601820
17. Pavlos NJ, Grønborg M, Riedel D, Chua JJE, Boyken J, Kloepper TH, et al. Quantitative analysis of synaptic vesicle Rabs uncovers distinct yet overlapping roles for Rab3a and Rab27b in Ca<sup>2+</sup>-triggered exocytosis. *J Neurosci.* 2010;30: 13441–13453. doi:10.1523/JNEUROSCI.0907-10.2010
  18. An SJ, Almers W. Tracking SNARE complex formation in live endocrine cells. *Science.* 2004;306: 1042–1046. doi:10.1126/SCIENCE.1102559
  19. Halemani ND, Bethani I, Rizzoli SO, Lang T. Structure and dynamics of a two-helix SNARE complex in live cells. *Traffic.* 2010;11: 394–404. doi:10.1111/J.1600-0854.2009.01020.X
  20. Rickman C, Medine CN, Dun AR, Moulton DJ, Mandula O, Halemani ND, et al. t-SNARE protein conformations patterned by the lipid microenvironment. *J Biol Chem.* 2010;285: 13535–13541. doi:10.1074/JBC.M109.091058
  21. Nicholson-Fish JC, Kokotos AC, Gillingwater TH, Smillie KJ, Cousin MA. VAMP4 Is an Essential Cargo Molecule for Activity-Dependent Bulk Endocytosis. *Neuron.* 2015;88: 973–984. doi:10.1016/J.NEURON.2015.10.043
  22. Tran THT, Zeng Q, Hong W. VAMP4 cycles from the cell surface to the trans-Golgi network via sorting and recycling endosomes. *J Cell Sci.* 2007;120: 1028–1041. doi:10.1242/jcs.03387

|    | Antibody                                                                                  | Target protein  | Species           | Dilution from stock | Company            | Cat#N                 |
|----|-------------------------------------------------------------------------------------------|-----------------|-------------------|---------------------|--------------------|-----------------------|
| 01 | Anti-Vti1a                                                                                | Vti1a           | Rabbit polyclonal | ICC 1:500           | Synaptic System    | 165 002               |
| 02 | Anti-Syntaxin 4                                                                           | Syntaxin 4      | Rabbit polyclonal | ICC 1:300           | Synaptic Systems   | 110 042               |
| 03 | Anti-Syntaxin 6                                                                           | Syntaxin 6      | Rabbit polyclonal | ICC 1:500           | Synaptic Systems   | 110 062               |
| 04 | GFP, does not have a target protein, see Supplementary Figure 3 for validation of epitope |                 |                   |                     |                    |                       |
| 05 | Anti-Syntaxin 7                                                                           | Syntaxin 7      | Rabbit polyclonal | ICC 1:300           | Synaptic Systems   | 110 073               |
| 06 | Anti-GM130                                                                                | GM130           | Rabbit polyclonal | ICC 1:300           | Sigma-Aldrich      | G7295                 |
| 07 | Anti-Endobrevin                                                                           | Endobrevin      | Rabbit polyclonal | ICC 1:300           | Synaptic Systems   | 104 303               |
| 08 | Anti-TOMM20                                                                               | Sigma           | Mouse monoclonal  | ICC 1:200           | Sigma-Aldrich      | WH0009804M1           |
| 09 | Anti-Beta-Actin                                                                           | Beta-Actin      | Mouse polyclonal  | ICC 1:75            | Sigma-Aldrich      | A1978                 |
| 10 | Anti-Rab5a                                                                                | Rab5a           | Rabbit monoclonal | ICC 1:250           | Abcam              | ab199530              |
| 11 | Anti-Syntaxin 12/13                                                                       | Syntaxin 12/13  | Rabbit polyclonal | ICC 1:300           | Synaptic System    | 110 133               |
| 12 | Anti-SNAP 25                                                                              | SNAP 25         | Rabbit polyclonal | ICC 1:500           | Synaptic Systems   | 111 002               |
| 13 | Anti-NFkappaB p65                                                                         | NLS             | Rabbit monoclonal | ICC 1:200           | Biomol / Rockland  | 600-401-271           |
| 14 | Anti-KDEL                                                                                 | KDEL            | Mouse monoclonal  | ICC 1:500           | Enzo Life Sciences | ADI-SPA-827-F         |
| 15 | Anti-VAMP 4                                                                               | VAMP 4          | Rabbit polyclonal | ICC 1:300           | Synaptic Systems   | N136 002              |
| 16 | Anti-HA                                                                                   | HA-Tag          | Goat              | ICC 1:250           | Novus Biologicals  | NB600-362             |
| 17 | Anti-SNAP25                                                                               | SNAP25          | Mouse             | ICC 1:200           | Synaptic Systems   | 111011                |
| 18 | Anti-Calnexin                                                                             | Calnexin        | Rabbit polyclonal | ICC 1:200           | Abcam              | Ab22595               |
| 19 | Anti-GM130                                                                                | GM130           | Mouse monoclonal  | ICC 1:200           | BD Biosciences     | 610822 clone 35/GM130 |
|    | Anti-TOMM20                                                                               | TOMM20          | Mouse monoclonal  | ICC 1:200           | Proteintech        | 11802-1-AP            |
| 20 | Anti-Rabbit IgG Cy5                                                                       | Anti-Rabbit IgG | Donkey            | ICC1:500            | Dianova            | 711-175-152           |
| 21 | Anti-Mouse IgG Cy5                                                                        | Anti-Mouse IgG  | Donkey            | ICC 1:500           | Dianova            | SEC-183329            |
| 22 | Anti-Mouse IgG Star580                                                                    | Anti-Mouse IgG  | Goat              | ICC 1:100           | Abberior           | ST580-1001-500µg      |
| 23 | Anti-Rabbit IgG Star580                                                                   | Anti-Rabbit IgG | Goat              | ICC 1:100           | Abberior           | ST580-1002-500µg      |

**Supplementary Table 2. Information about antibodies used for target protein validation purposes.**  
The first nineteen antibodies listed are primary antibodies. Antibodies 20-23 are secondary antibodies. See Methods section for further information about the staining procedures.

|    | Protein            | Time window | Number of Images | Total number of sampled pixels |
|----|--------------------|-------------|------------------|--------------------------------|
| 01 | Blank (no protein) | N/A         | 24               | 2400000                        |
| 02 | Endobrevin (0111)  | overnight   | 10               | 10000                          |
|    |                    | 24h         | 12               | 10000                          |
|    |                    | 48h         | 10               | 10000                          |
|    |                    | 72h         | 5                | 10000                          |
| 03 | GalNacT (0110)     | overnight   | 9                | 3224                           |
|    |                    | 24h         | 12               | 10000                          |
|    |                    | 48h         | 10               | 2340                           |
|    |                    | 72h         | 5                | 10000                          |
| 04 | GFP (0100)         | overnight   | 10               | 10000                          |
|    |                    | 24h         | 12               | 10000                          |
|    |                    | 48h         | 5                | 10000                          |
|    |                    | 72h         | 5                | 10000                          |
| 05 | KDEL (1110)        | overnight   | 5                | 10000                          |
|    |                    | 24h         | 12               | 10000                          |
|    |                    | 48h         | 10               | 10000                          |
|    |                    | 72h         | 5                | 10000                          |
| 06 | Lifeact (1001)     | overnight   | 5                | 10000                          |
|    |                    | 24h         | 12               | 10000                          |
|    |                    | 48h         | 10               | 10000                          |
|    |                    | 72h         | 5                | 10000                          |
| 07 | NLS (1101)         | overnight   | 10               | 10000                          |
|    |                    | 24h         | 12               | 10000                          |
|    |                    | 48h         | 10               | 10000                          |
|    |                    | 72h         | 5                | 10000                          |
| 08 | SNAP25 (1100)      | overnight   | 5                | 5412                           |
|    |                    | 24h         | 12               | 10000                          |
|    |                    | 48h         | 10               | 10000                          |
|    |                    | 72h         | 5                | 10000                          |
| 09 | STX4 (0010)        | overnight   | 10               | 10000                          |
|    |                    | 24h         | 12               | 10000                          |

|    |              |           |    |       |
|----|--------------|-----------|----|-------|
|    |              | 48h       | 10 | 10000 |
|    |              | 72h       | 5  | 10000 |
| 10 | STX6 (0011)  | overnight | 10 | 10000 |
|    |              | 24h       | 12 | 10000 |
|    |              | 48h       | 11 | 10000 |
|    |              | 72h       | 5  | 10000 |
| 11 | TOM70 (1000) | overnight | 9  | 10000 |
|    |              | 24h       | 7  | 10000 |
|    |              | 48h       | 10 | 10000 |
|    |              | 72h       | 5  | 1000  |
| 12 | Vti1a (1000) | overnight | 9  | 1033  |
|    |              | 24h       | 12 | 10000 |
|    |              | 48h       | 10 | 10000 |
|    |              | 72h       | 5  | 3825  |

**Supplementary Table 3. Summary of the image data used for training and testing the deep network.**

Number of confocal images obtained for each protein (with the given nanobarcode) as well as number of pixels that have been sampled for the deep learning dataset. All images have the same dimensions of 512x512 pixels. With 72 h samples, each image contains 5 slices in a z-stack. Network training, validation, and testing are done only based on the sub-sampled pixels (with the numbers given in the last column), while the precision matrices in Supplementary Fig 20 are obtained on full-frame images.