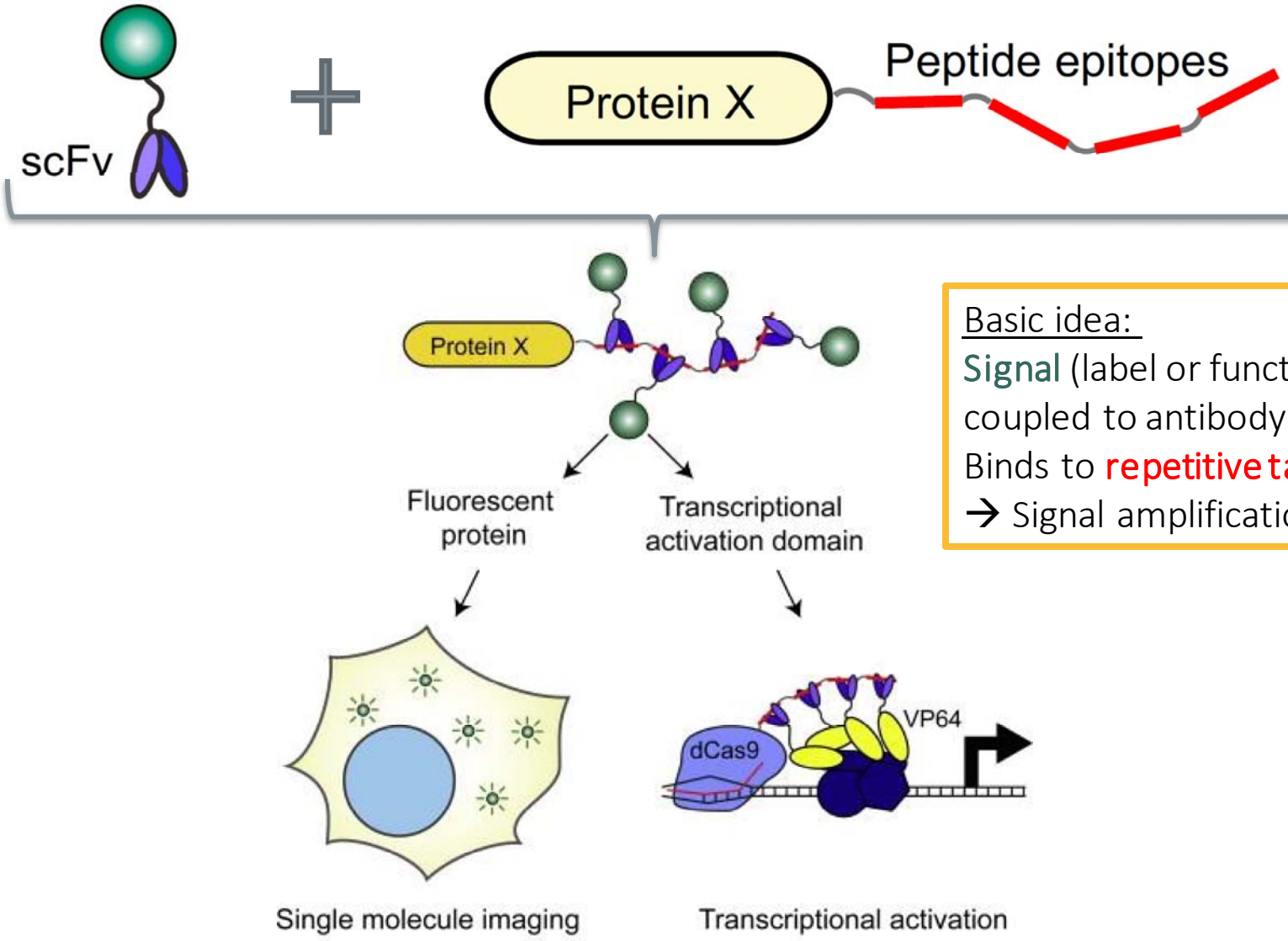
Sun, Moon and SUperNova

Protein-Tagging Systems for Signal Amplification

08.10.2019
Alexandra Bentrup
Technical Journal Club



Basic idea:
Signal (label or function) coupled to antibody fragment
Binds to repetitive tag
→ Signal amplification

Multi-Color Single-Molecule Imaging Uncovers Extensive Heterogeneity in mRNA Decoding

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[2]Janelia Research Campus, Howard Hughes Medical Institute, Ashburn, VA, USA
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https://doi.org/10.1016/j.cell.2019.05.001

A Protein-Tagging System for Signal Amplification in Gene Expression and Fluorescence Imaging

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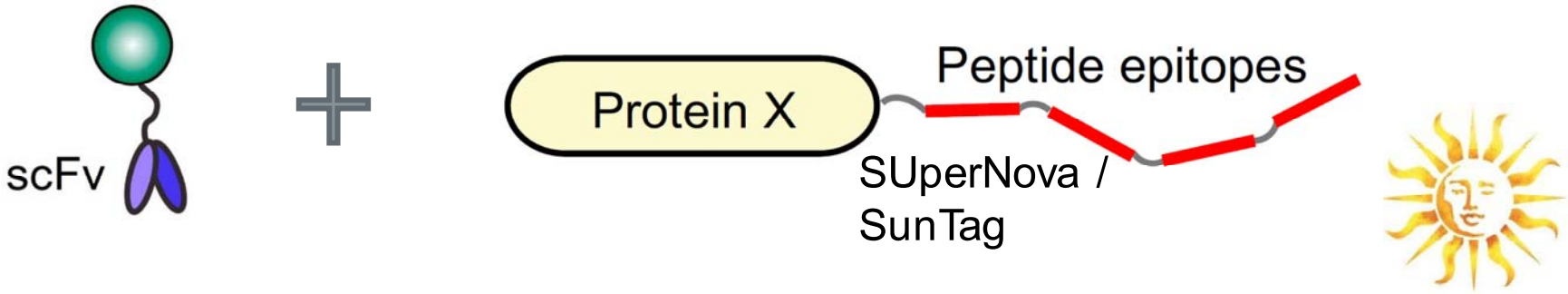
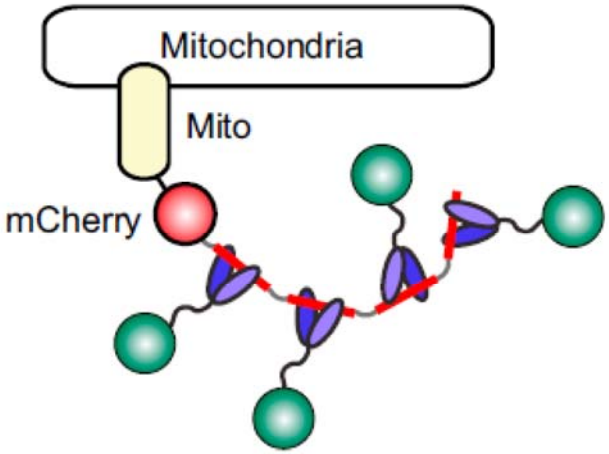


1. GFP for fluorescence imaging of very brightly labelled single molecules



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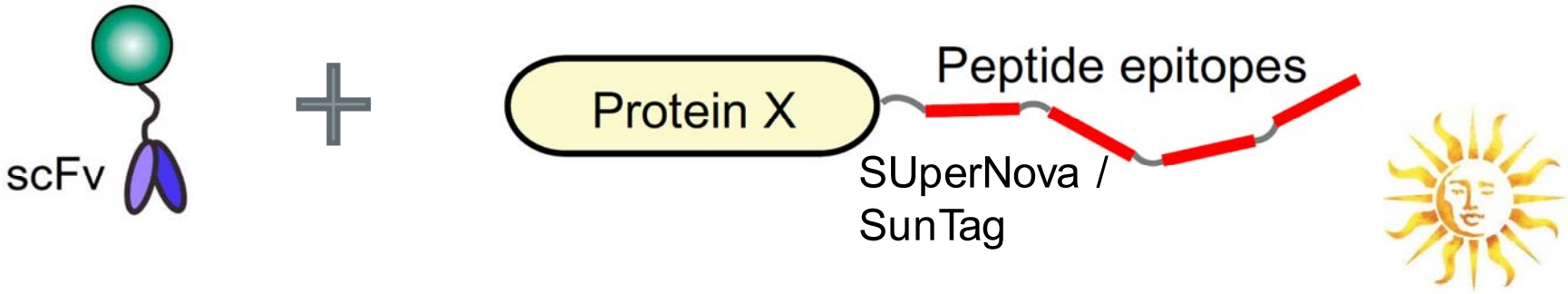

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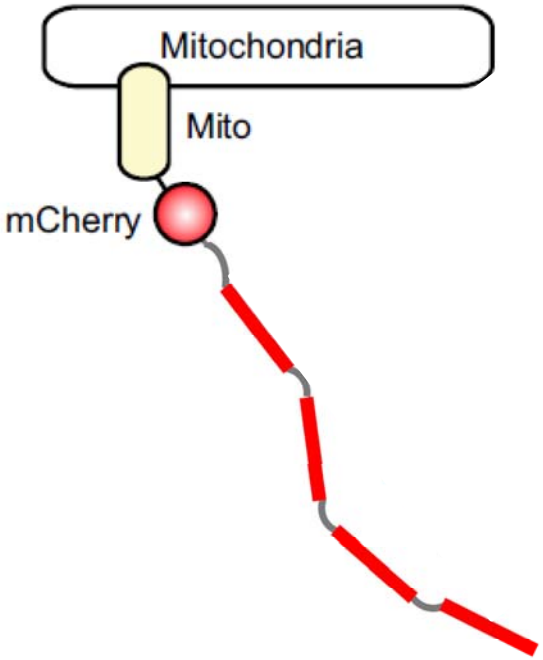
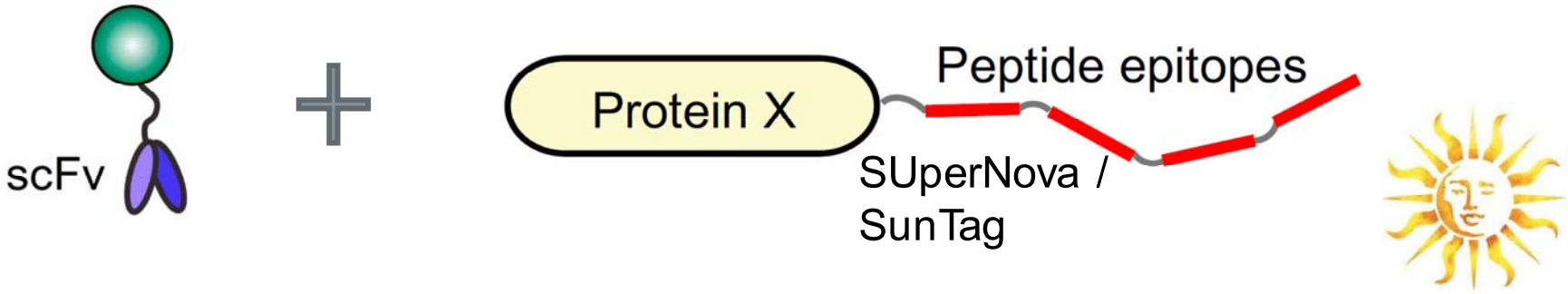


Strategy:

- Find short unstructured artificial epitope that differs from naturally occuring ones
- Use scFv for better folding in cytoplasm

→ mCherry tagged Mito
→ If scFv binds specifically
= co-localization of red & green



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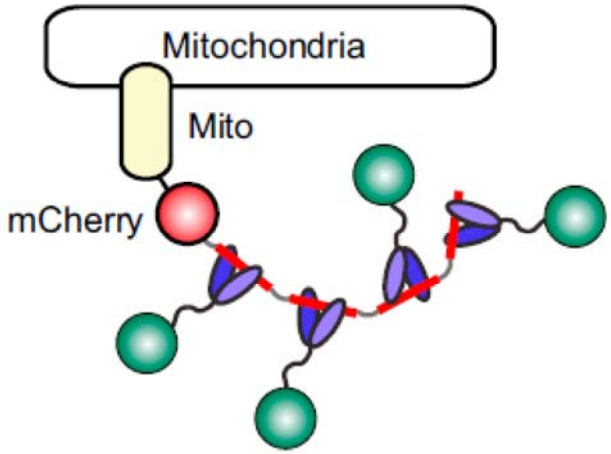

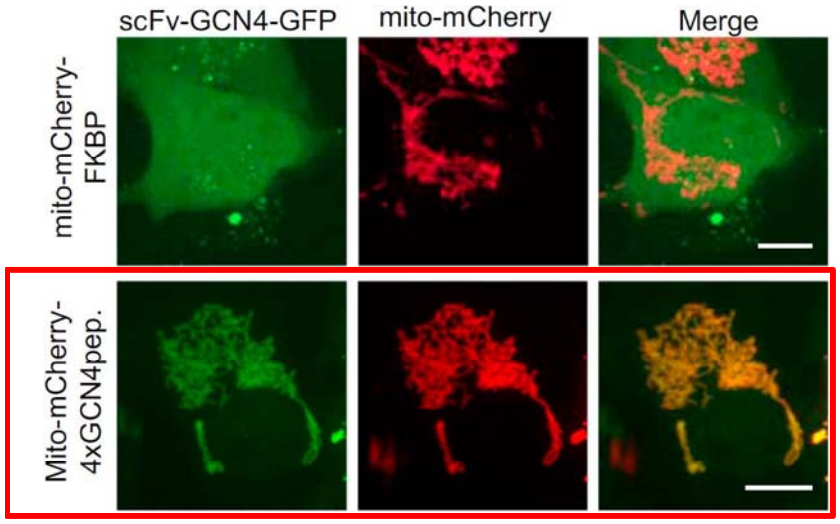

Strategy:

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- Use scFv for better folding in cytoplasm

→ Used scFv/epitope pair has been previously published: **GCN4** (from yeast)

→ Optimizations for eukaryotic expression
  - Superfolder GFP (sfGFP)
  - Solubility tag GB1

scFv-GCN4-sfGFP-GB1

→ Optimizations of linker length

→ Optimizations of epitope repeats

SunTag

Single Molecule Fluorescence Live Cell Imaging

- Mitochondria (mitoNEET)
- Histones (H2B)
- Plasma membrane targeted (CAAX-domain)
- Motor protein Kinesin-1
  (truncated w/o cargo-binding domain, K560)

Single Molecule Fluorescence Live Cell Imaging

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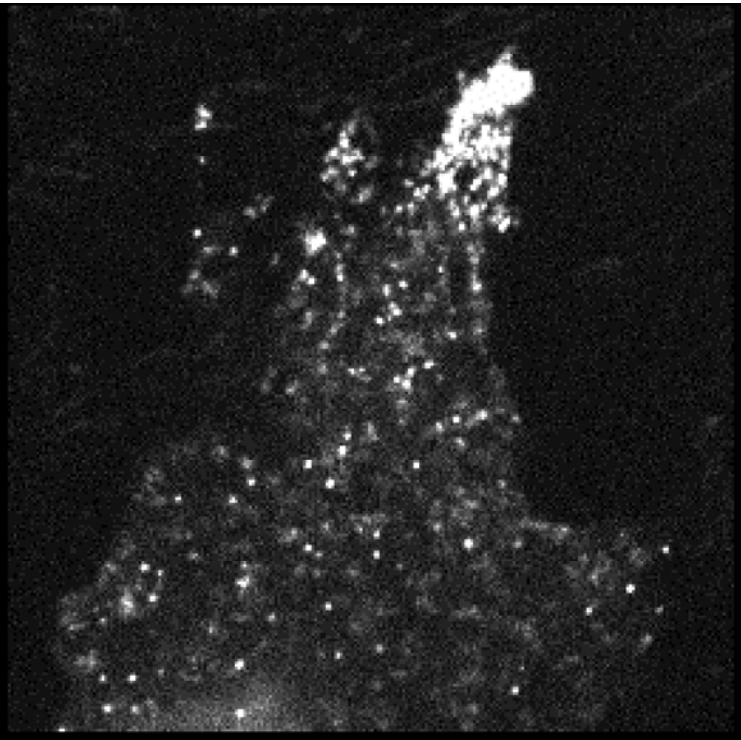
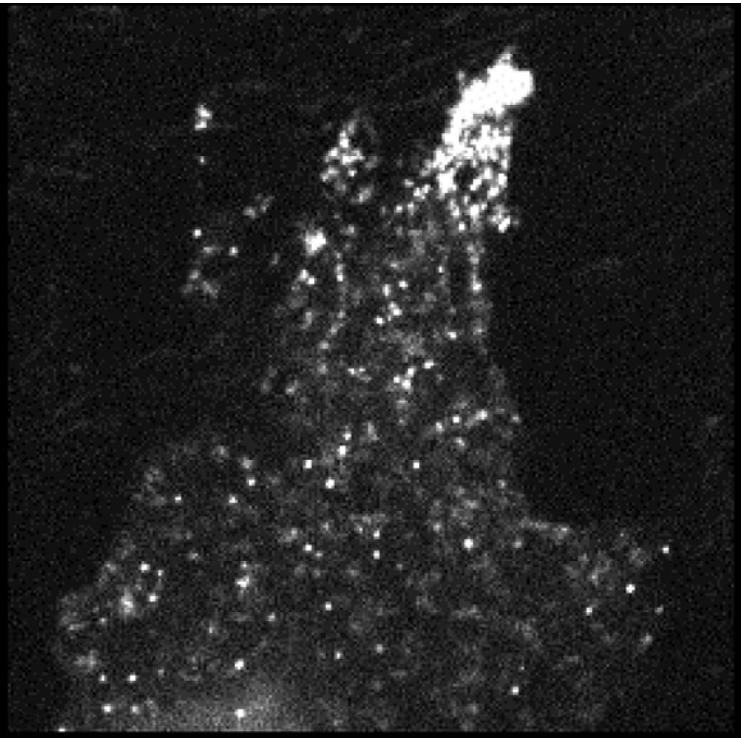


K560-SunTag$_{24x}$-GFP molecules in U2OS cells, spinning-disk confocal microscopy, 24 h post-transfection → continuous illumination for 30 s with 200 ms integration time per image

Single Molecule Fluorescence Live Cell Imaging

- Mitochondria (mitoNEET)
- Histones (H2B)
- Plasma membrane targeted (CAAX-domain)
- Motor protein Kinesin-1 (truncated w/o cargo-binding domain, K560)
- Run length comparable to previously reported lengths



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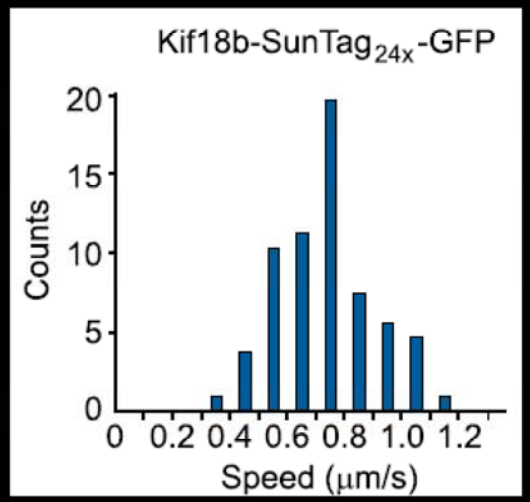

↑
Characterization of less well known motor proteins

Single Molecule Fluorescence Live Cell Imaging

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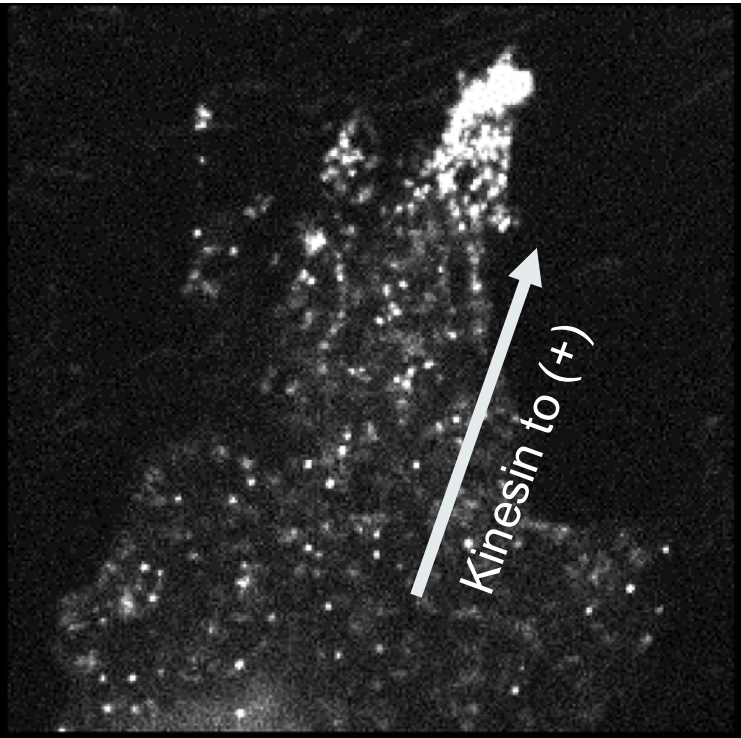
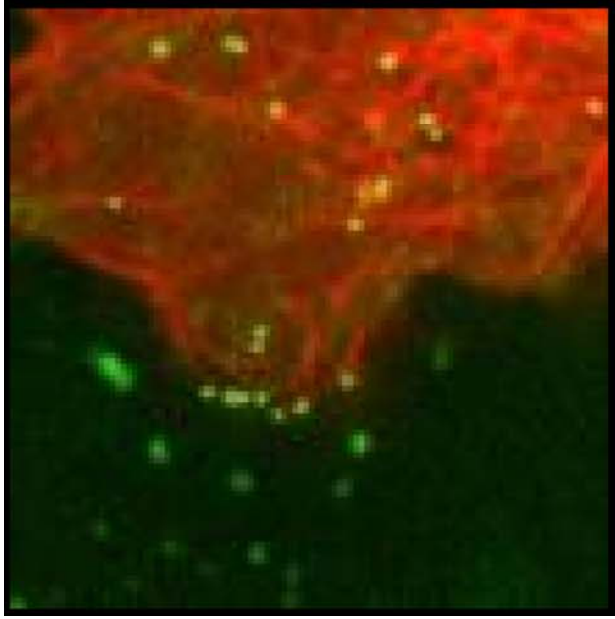


K560-SunTag$_{24x}$-GFP molecules in U2OS cells, spinning-disk confocal microscopy, 24 h post-transfection → continuous illumination for 30 s with 200 ms integration time per image



→ fusing the microtubule minus-end binding protein Camsap2 to SunTag

← Camsap2-SunTag24x-GFP molecules in U2OS cells, spinning-disk confocal microscopy, 24 h post-transfection continuous illumination for 25 s with 500 ms integration time per image

Single Molecule Fluorescence Live Cell Imaging

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- Plasma membrane targeted (CAAX-domain)
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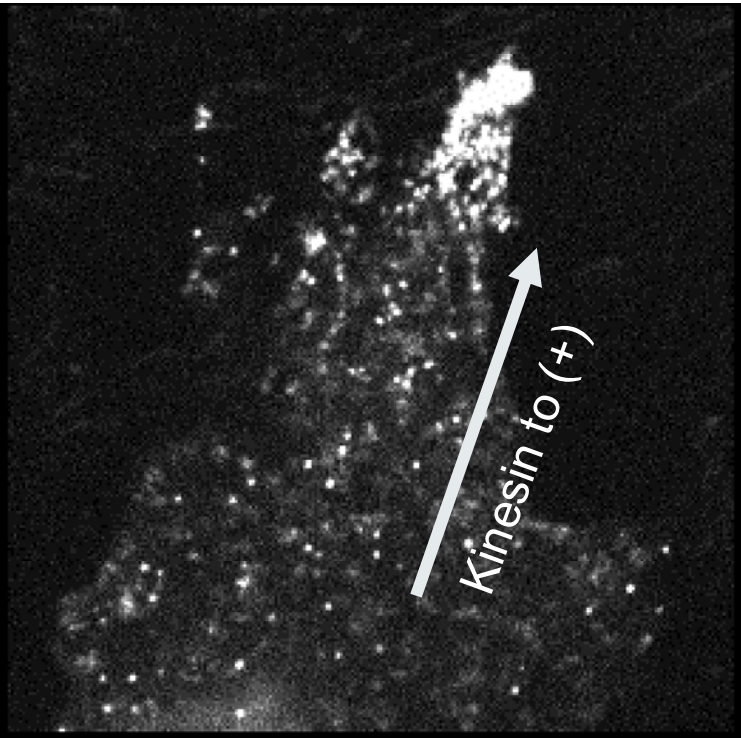
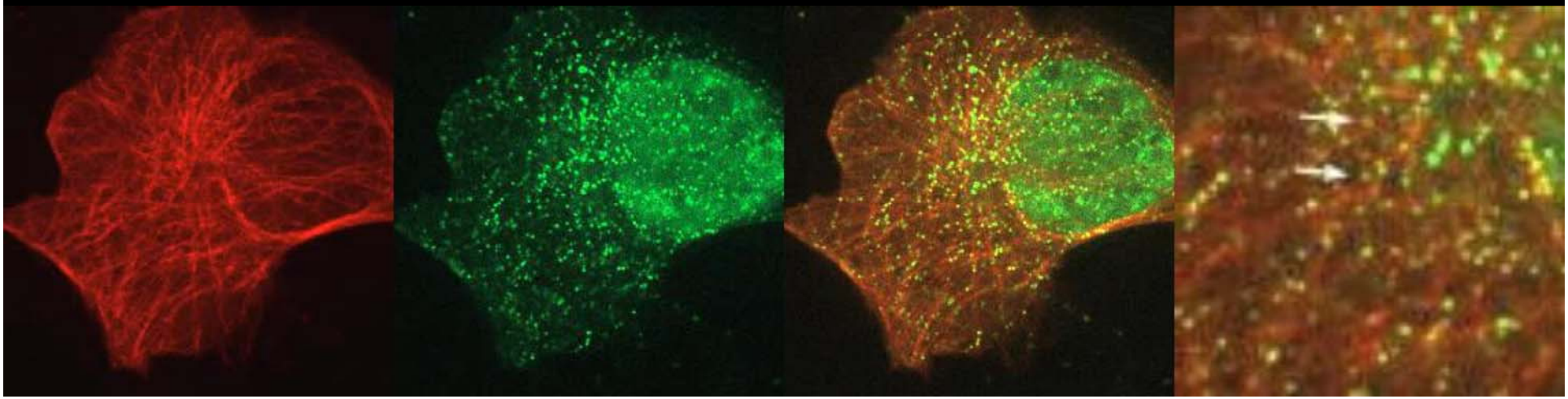


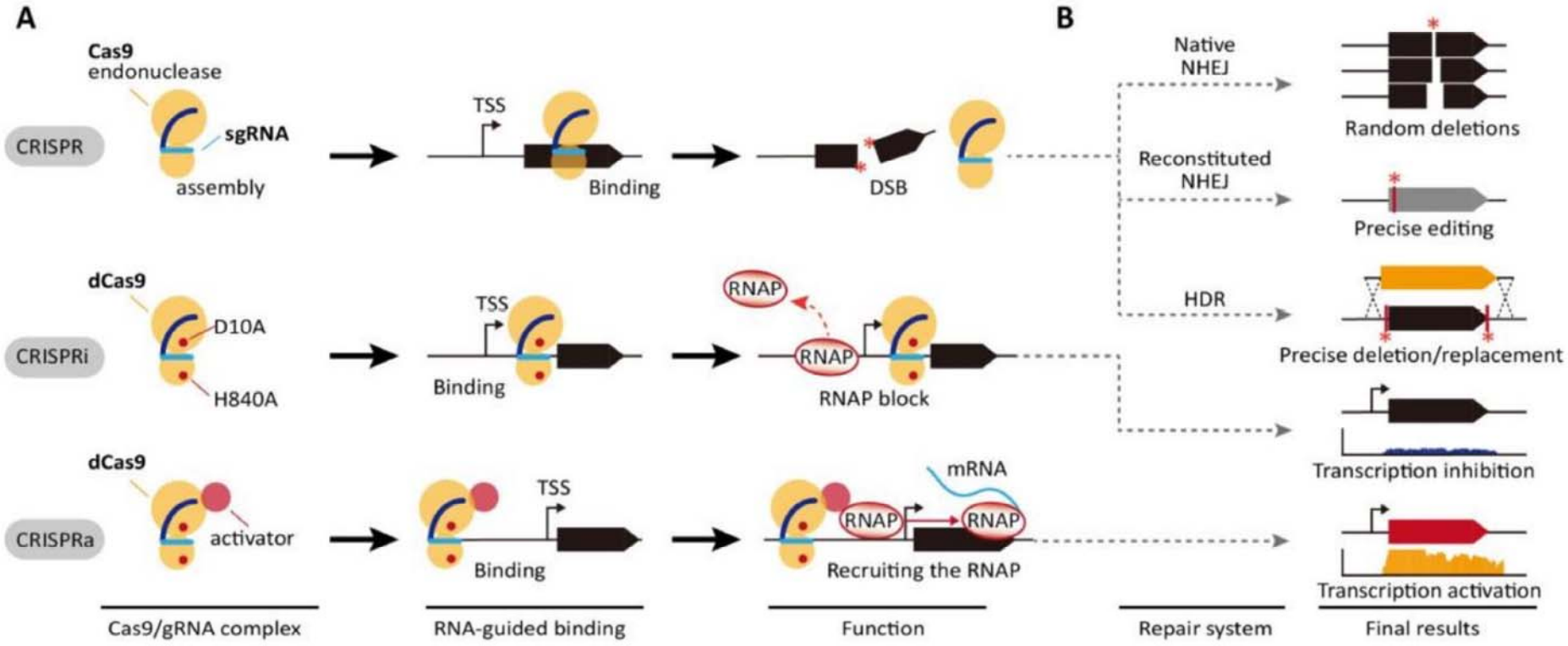
- Single filament dynamics:
  movements of individual microtubules
  ↓

K560rig-SunTag$_{24x}$-GFP (=hydrolysis defective, positional marker) + mCherry-α-tubulin,
U2OS cells, spinning-disk confocal microscopy, 24 h post-transfection
continuous illumination for 60 s with 600 ms integration time per image↓



1. GFP for fluorescence imaging
2. VP64 for dCas9 activation and protein upregulation

CRISPR / CRISPRi / CRISPRa



Suhyung Cho, Jongoh Shin and Byung-Kwan Cho
Int. J. Mol. Sci. **2018**, 19, 1089; doi:10.3390/ijms19041089

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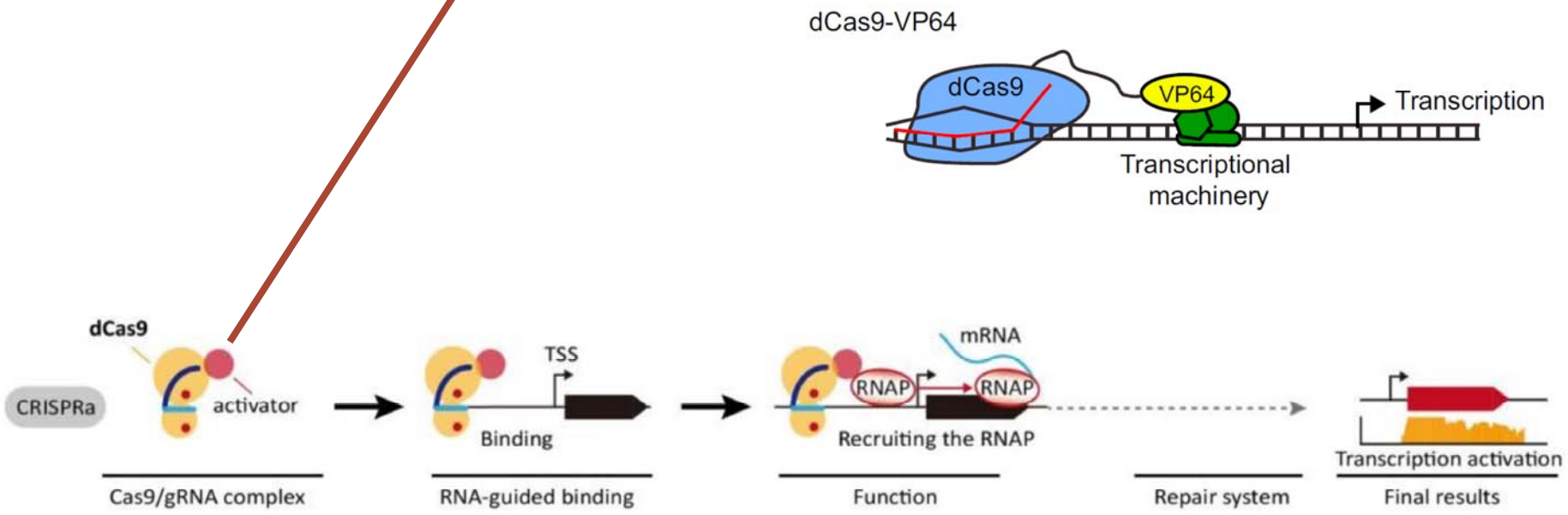
CRISPRa

VP64 = transcriptional activator
= 4x herpes virus transcriptional activation domain VP16
(previously published)



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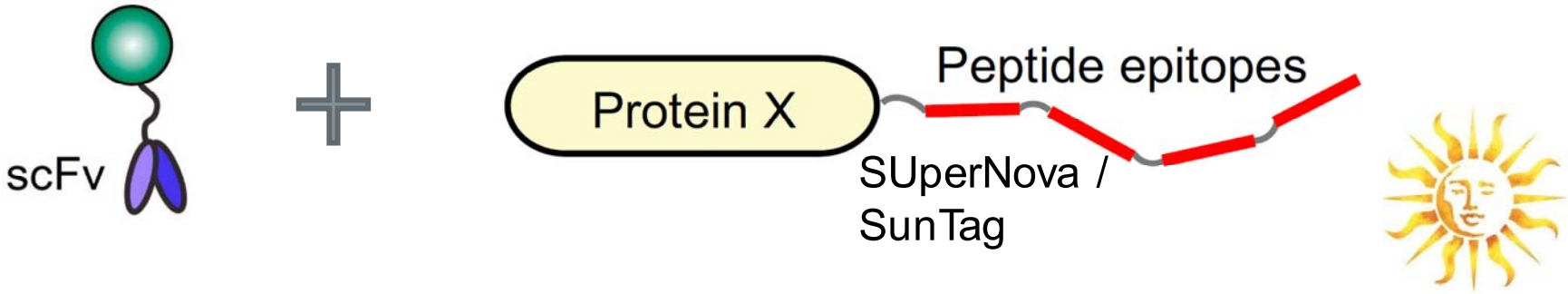

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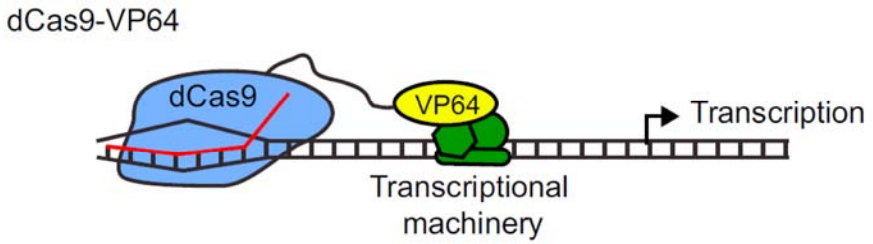

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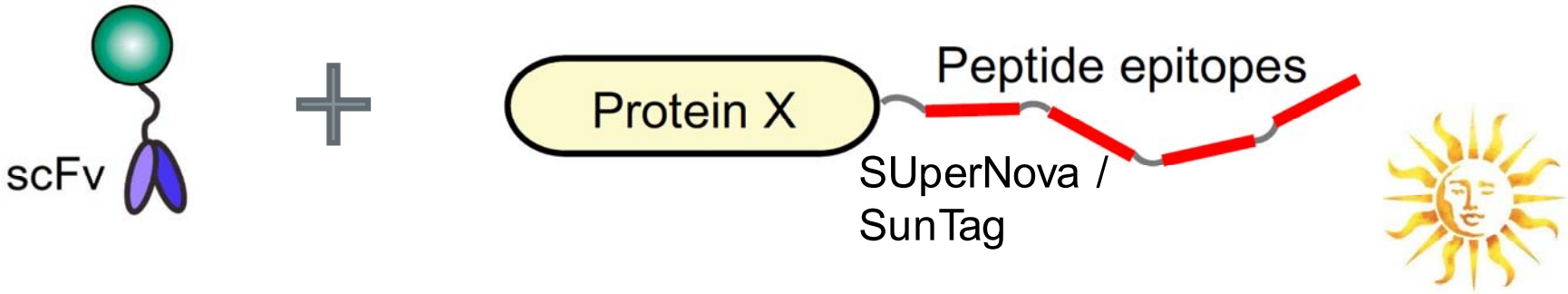

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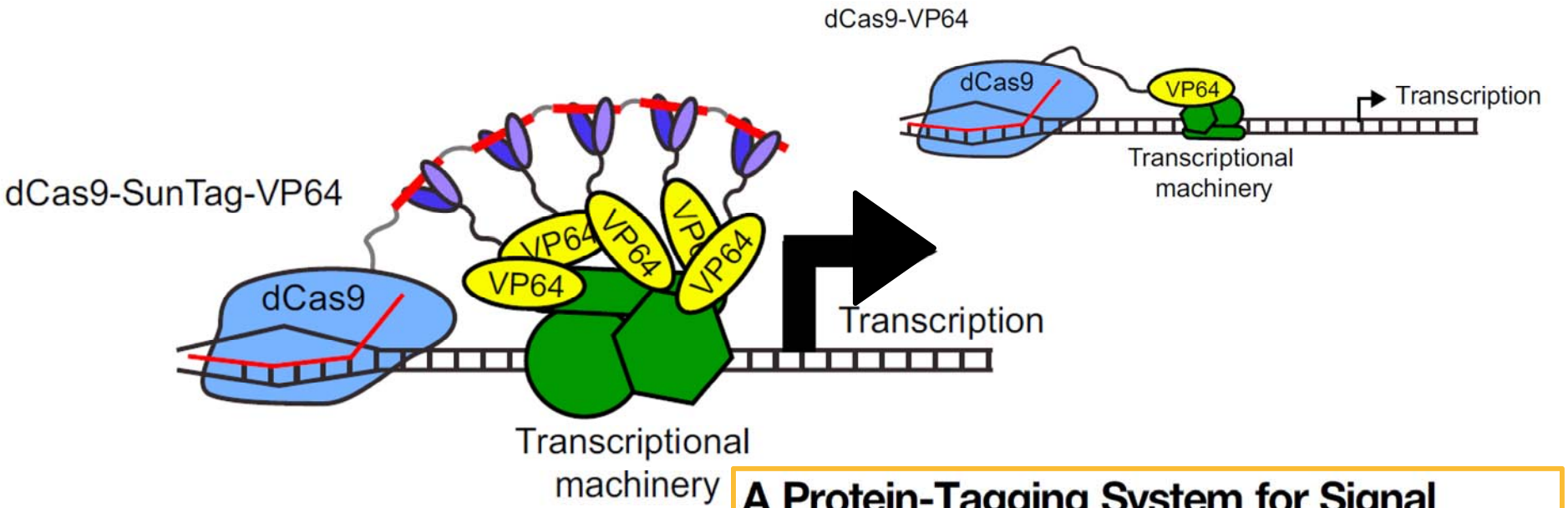

Hypothesis:

By recruiting multiple activators,
Transcript number can be increased

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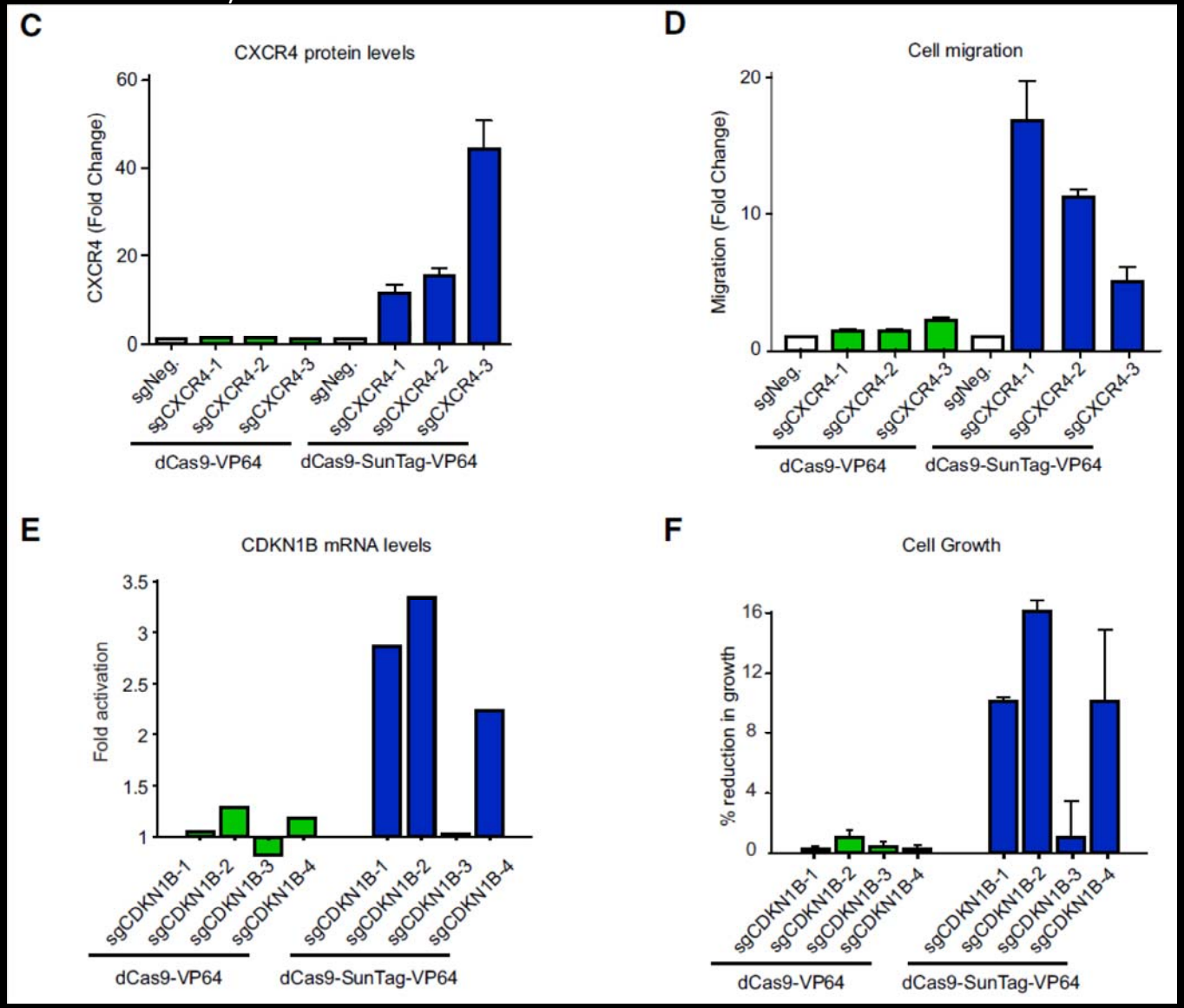
Transcriptional Activaction of Endogenous Genes

Protein/mRNA Level

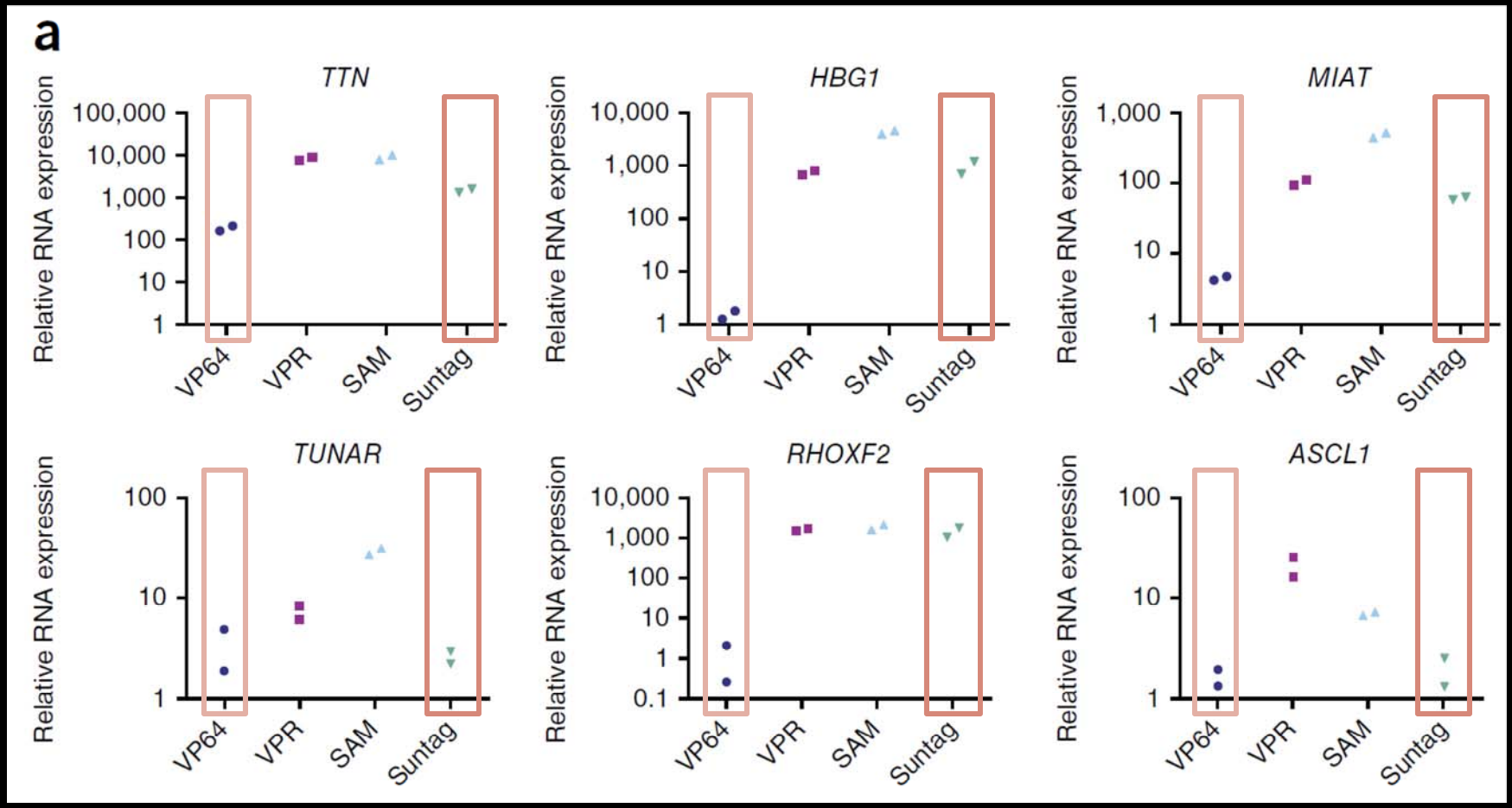
Functional Readout



CXCR4: chemokine receptor stimulating cellular migration in response to activation by SDF1a

CDKN1B (=p27kip1): cell-cycle inhibitor
→ physiologically high levels expressed
→ further up-regulation possible

Transcriptional Activaction of Endogenous Genes



→ Validation that VP64 activation is less effective than Suntag (= multiple VP64)

Review on CRISPR activators

by Chavez et al. nature methods | VOL.13 NO.7 | JULY 2016

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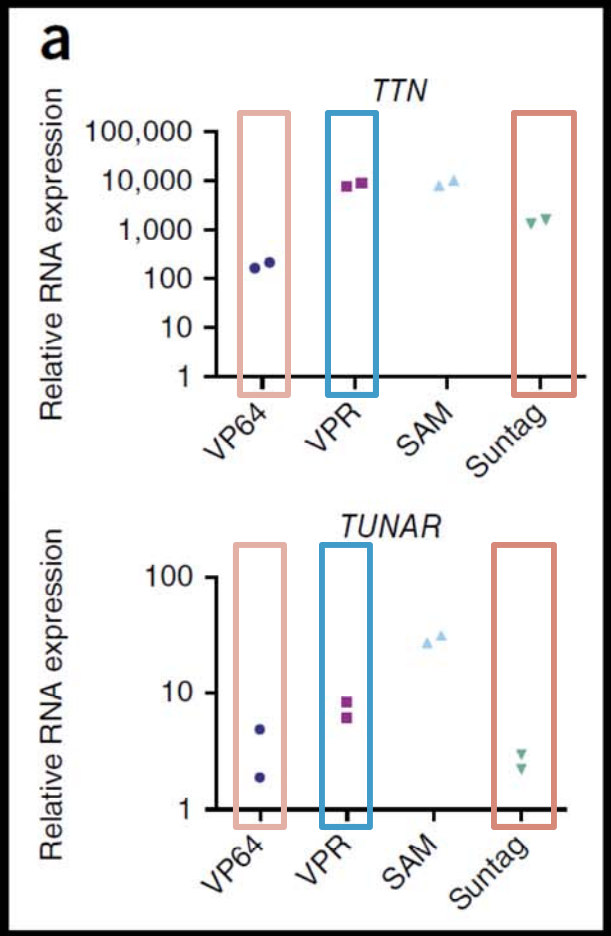

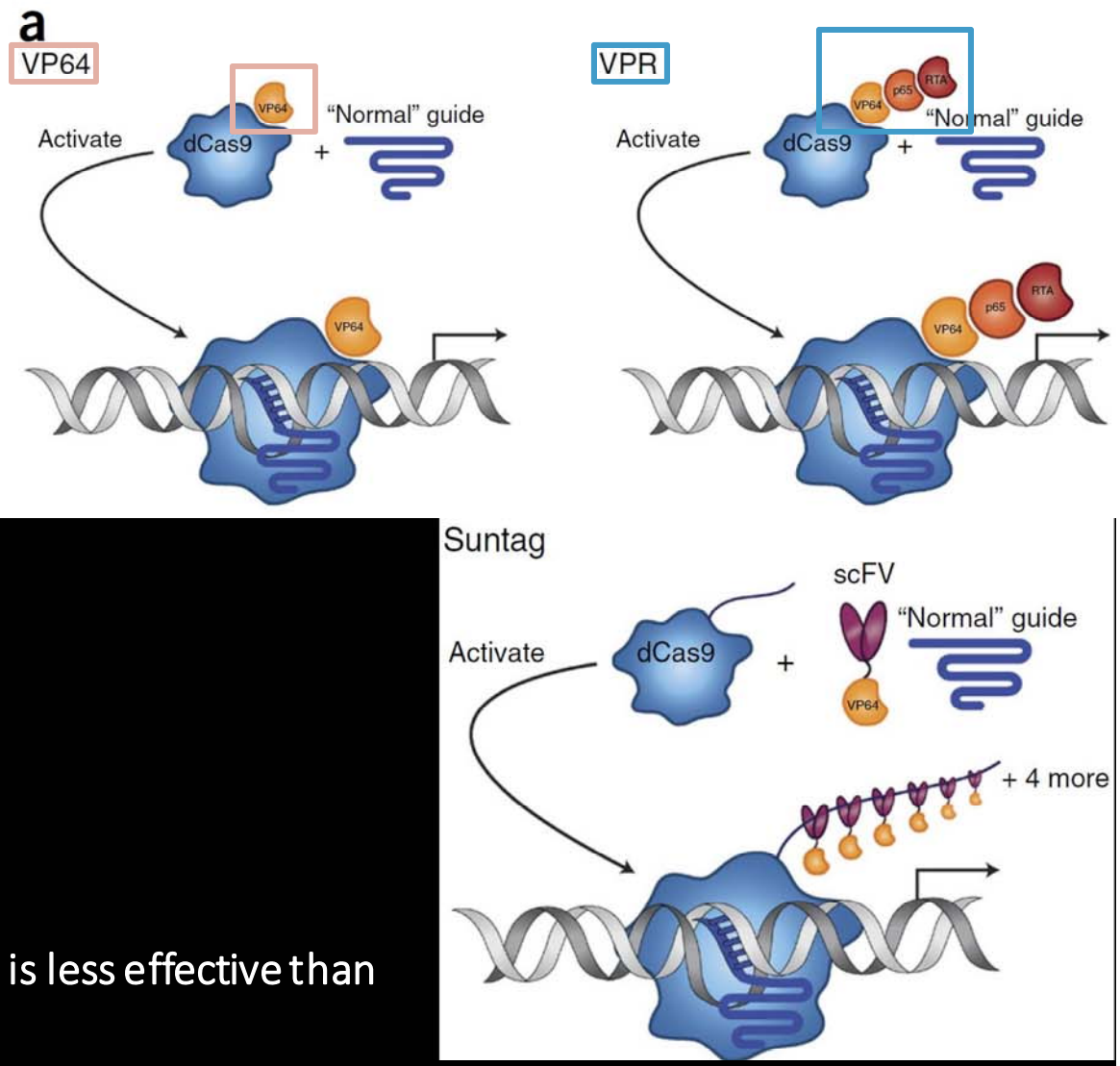

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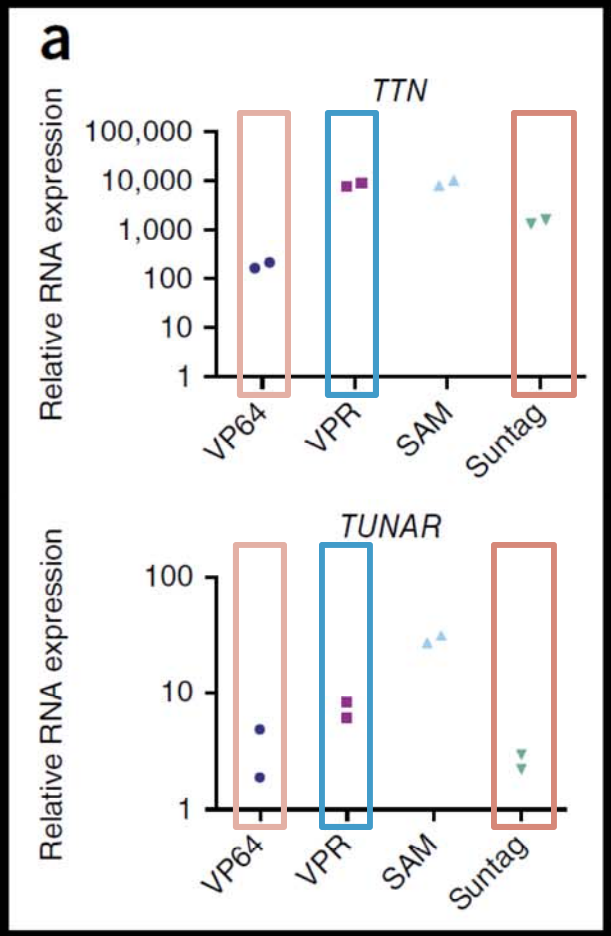
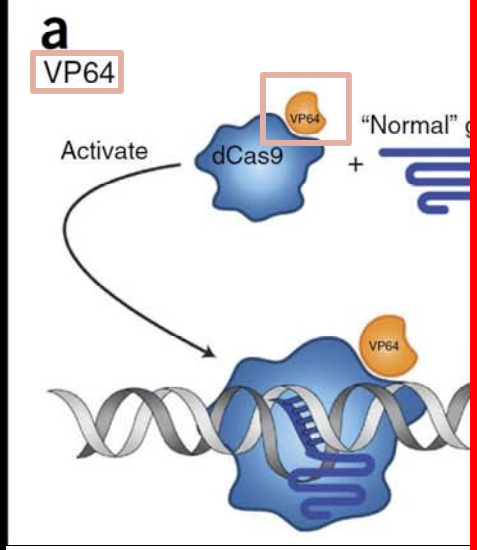
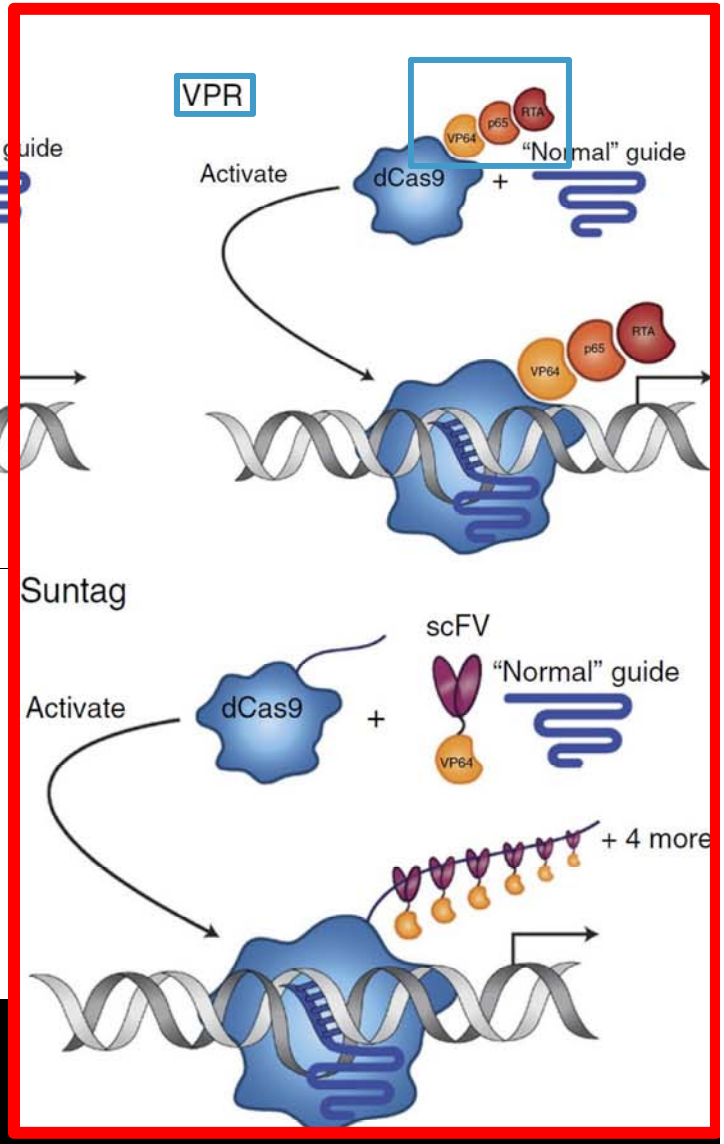




Even more activation by combining Suntag and VPR?



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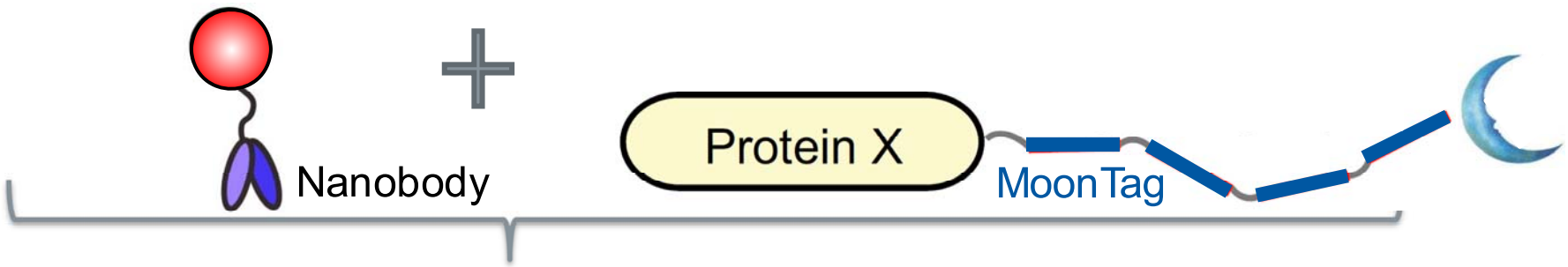

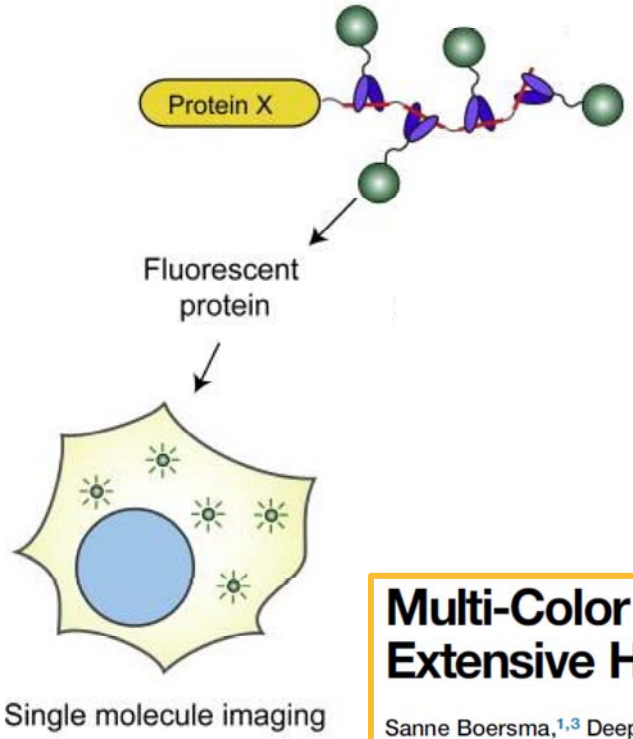

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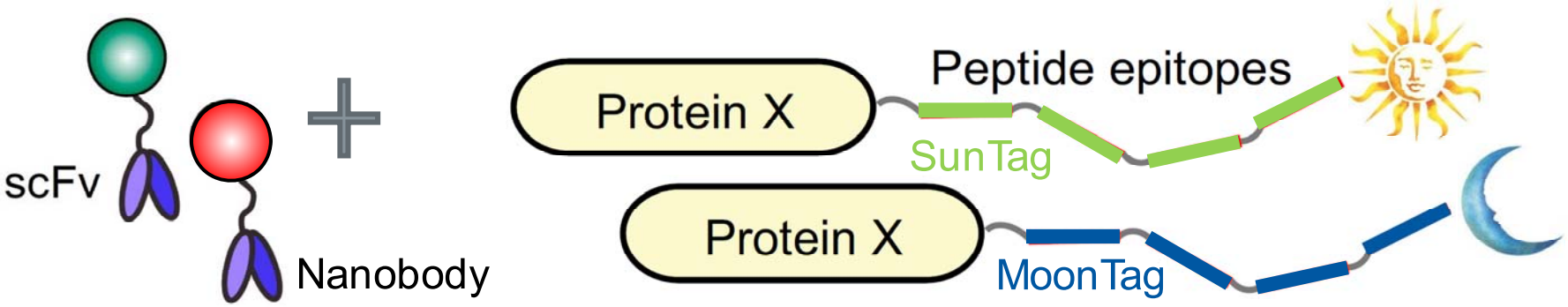

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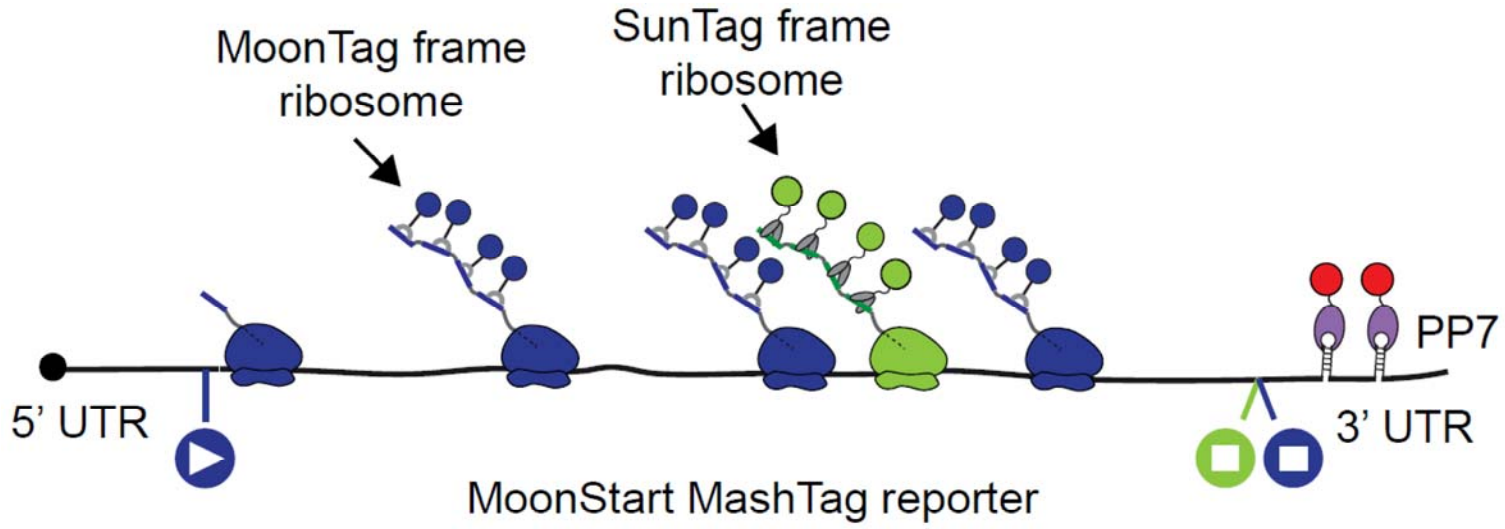
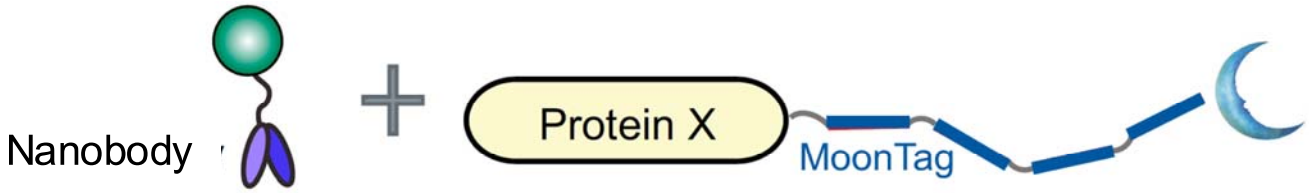
2. VP64 for dCas9 activation and protein upregulation
3. two fluorophores indicating reading frame of ribosome



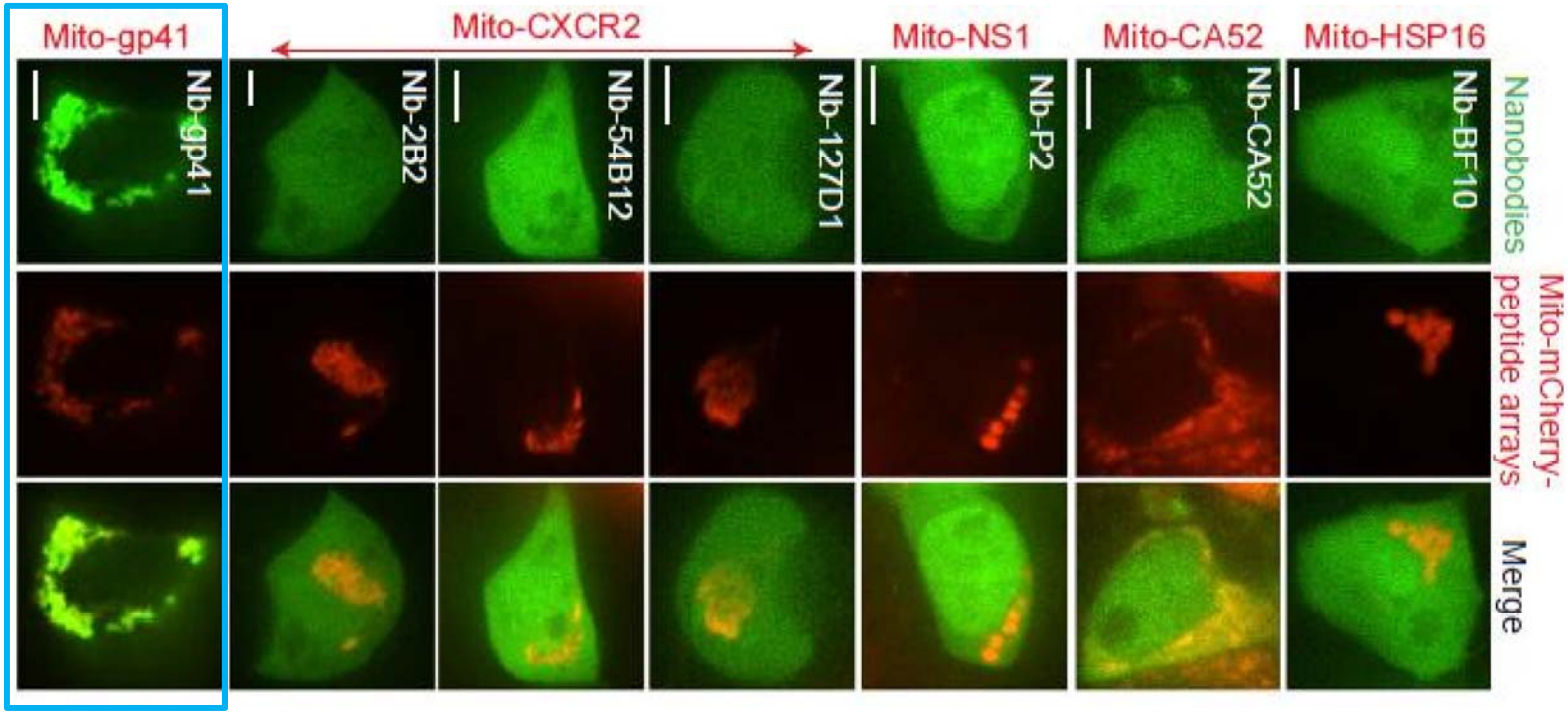


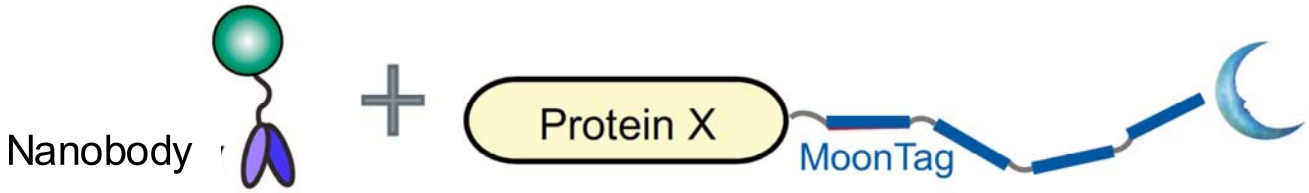
MoonTag:

Same idea as SunTag: peptide sequence acting as epitope for a small antibody (Nanobody), repeated several times for recruitment of multiple NBs fused to e.g. fluorescent protein.

→ Testing of nanobodies binding to linear epitopes with high affinity in vitro found in literature

→ **Visualization** of Proteins / Organelles





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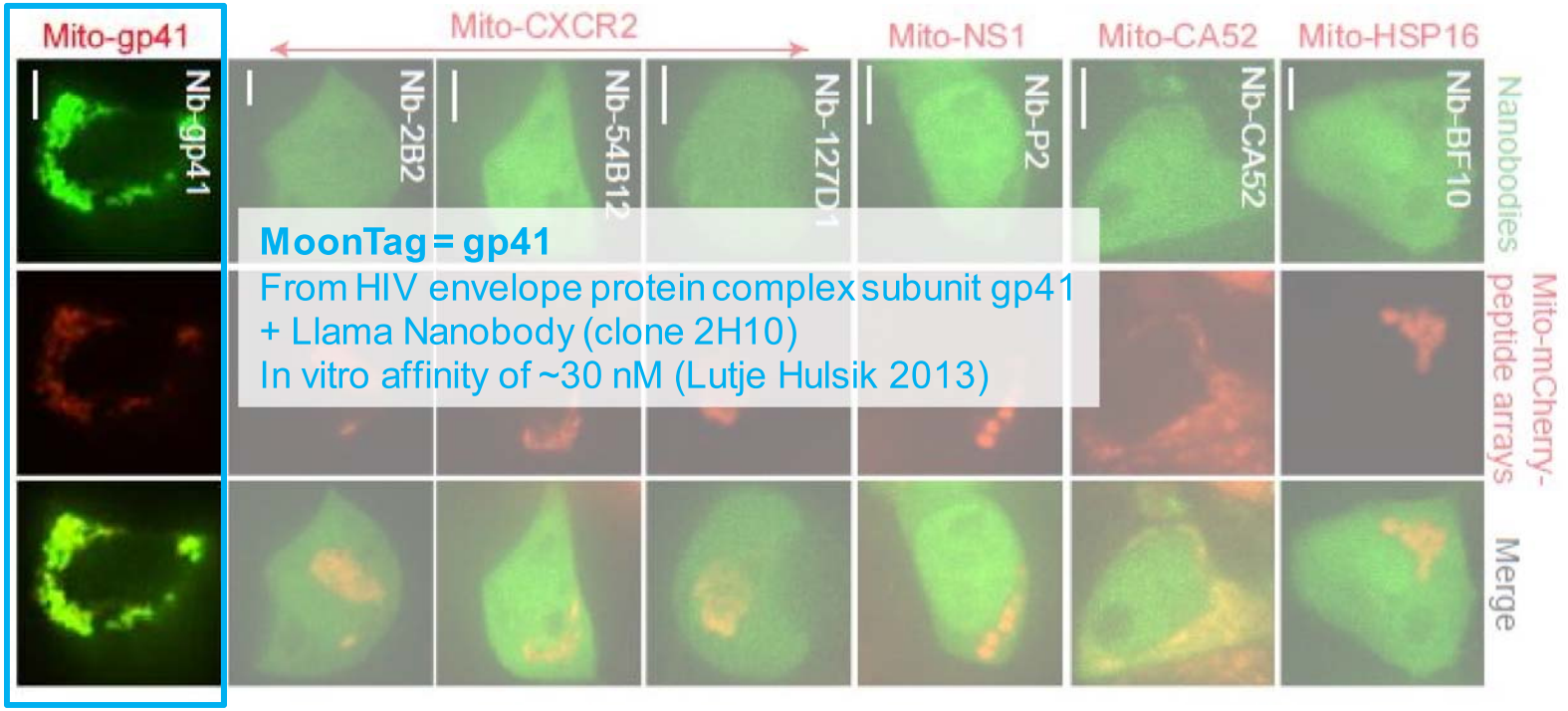
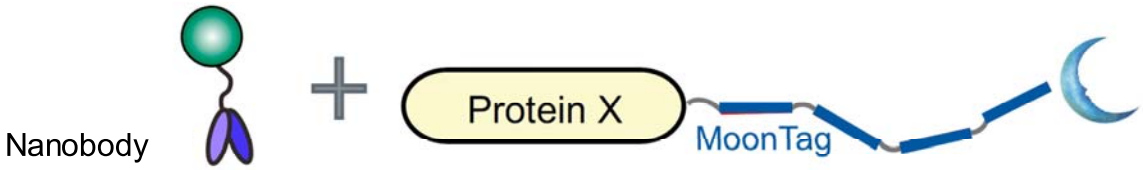
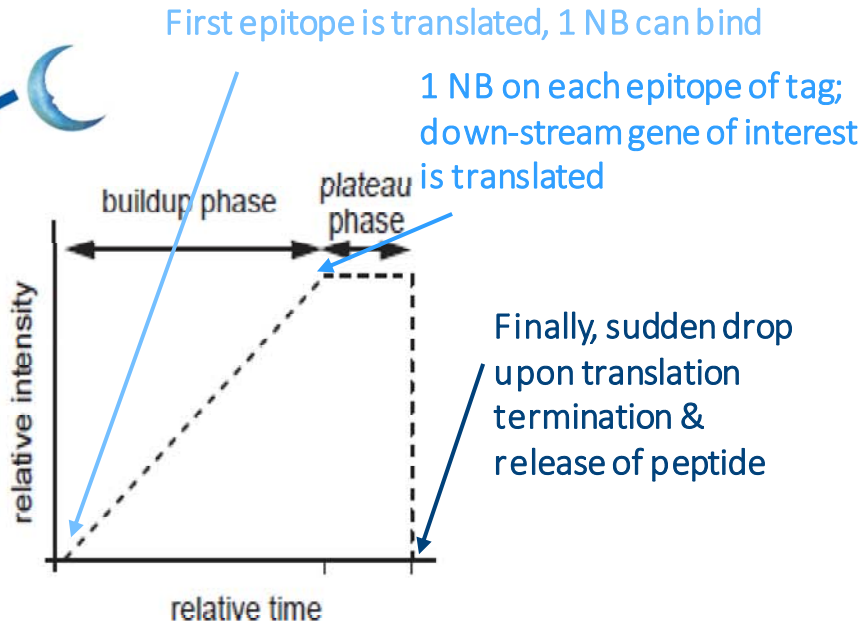
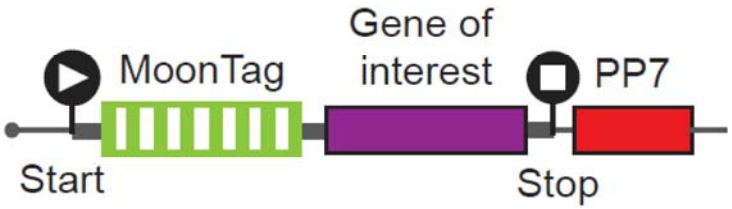


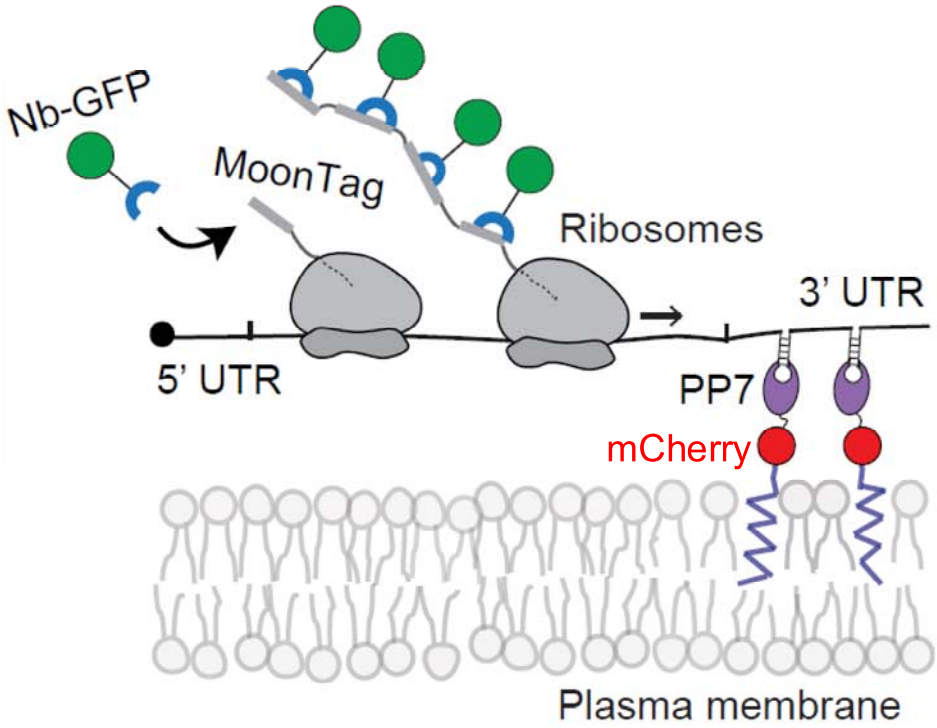


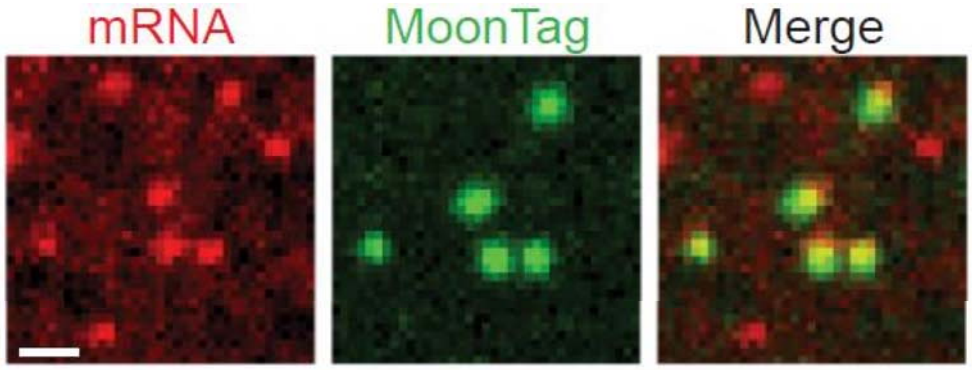


→ Translation imaging reporter

- MoonTag peptides are synthesized before the protein of interest
- MoonTag NB binds rapidly co-translationally
  = direct readout of translation of single mRNA molecules
- PP7: labelled by mCherry & tethers complex to PM to increase signal-to-noise ratio







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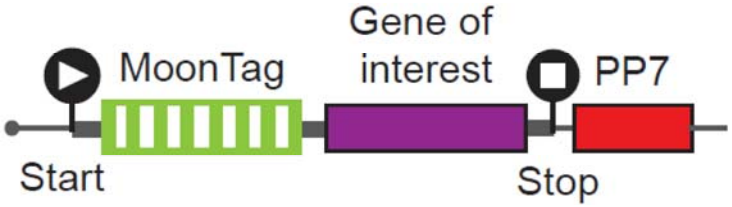
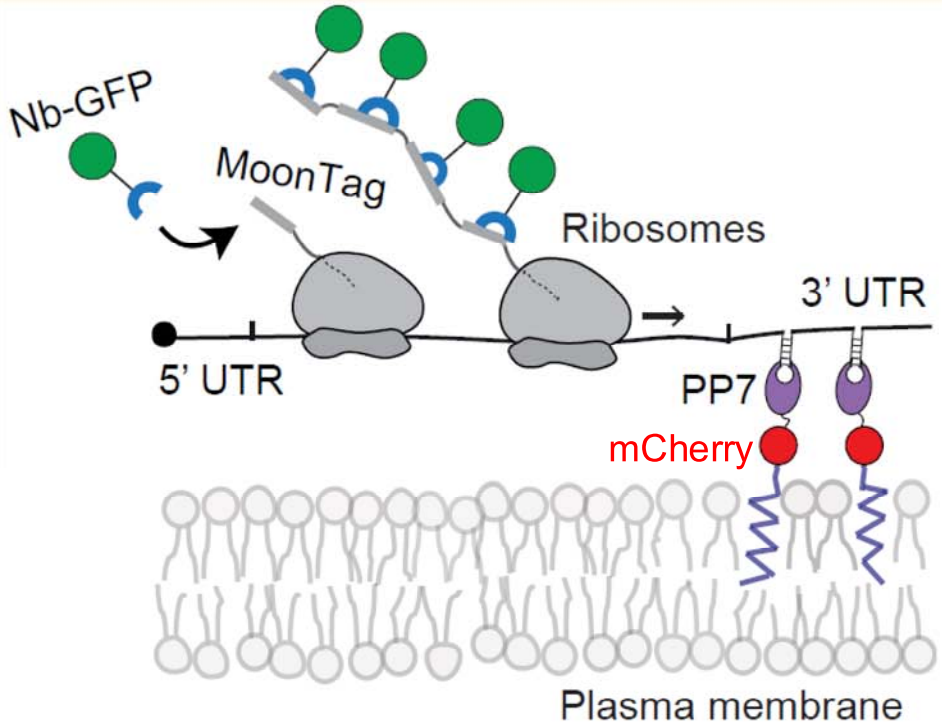
→ Testing of nanobodies binding to linear epitopes with high affinity in vitro found in literature

→ Visualization of Proteins / Organelles

→ Translation imaging reporter

- MoonTag peptides are synthesized before the protein of interest
- MoonTag NB binds rapidly co-translationally
  = direct readout of translation of single mRNA molecules
- PP7: labelled by mCherry & tethers complex to PM to increase signal-to-noise ratio





Single mRNA molecule visualization has been previously established for SunTag

In vivo Translation Imaging using SunTag

- mRNAs are tethered to the membrane and fluorescently labeled with PP7-mCherry.
- Translation is visualized by labeling nascent peptide epitopes with antibody-GFP.

Dynamics of Translation of Single mRNA Molecules In Vivo

Xiaowei Yan,[2] Tim A. Hoek,[1] Ronald D. Vale,[2] and Marvin E. Tanenbaum[1,*]
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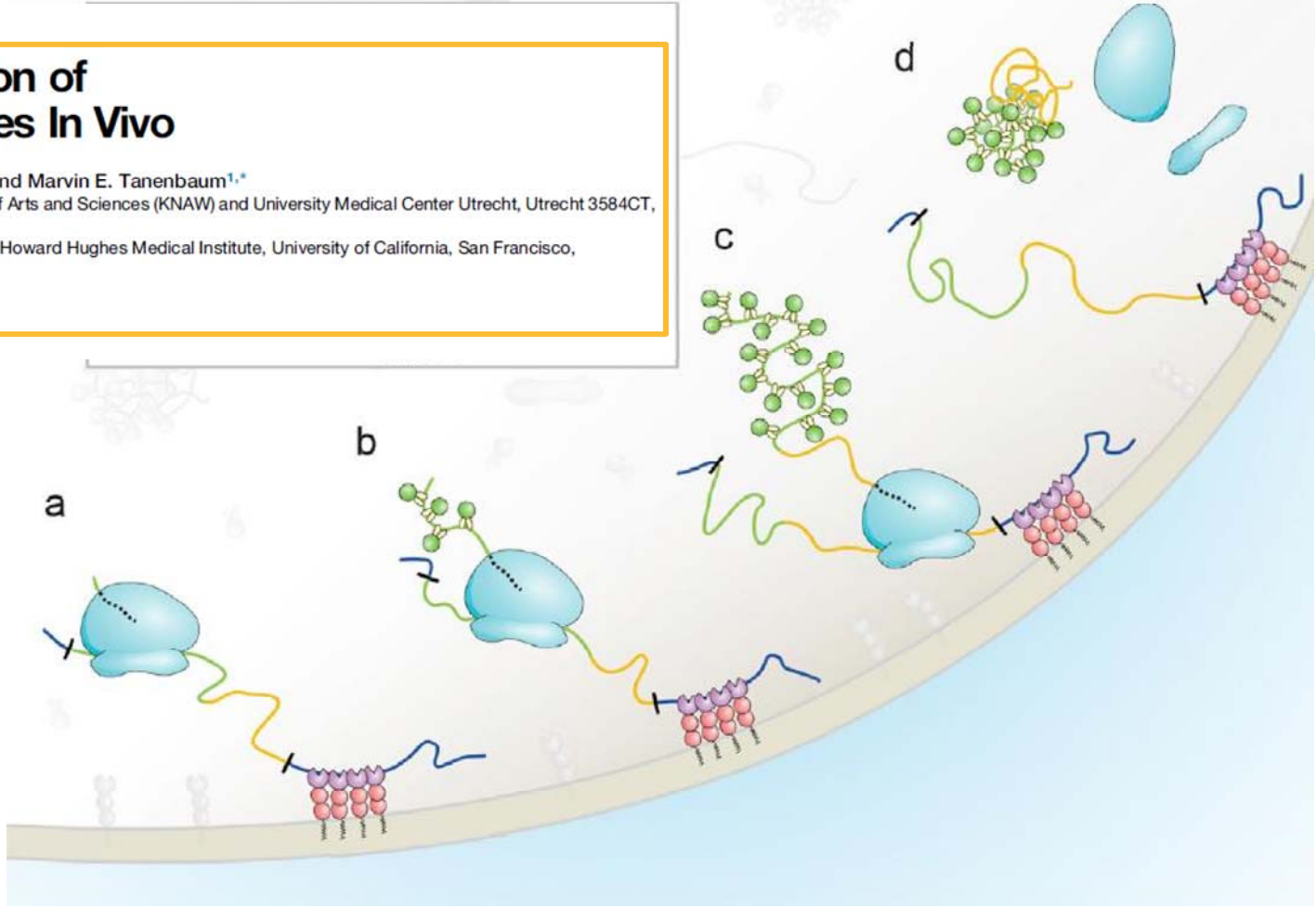
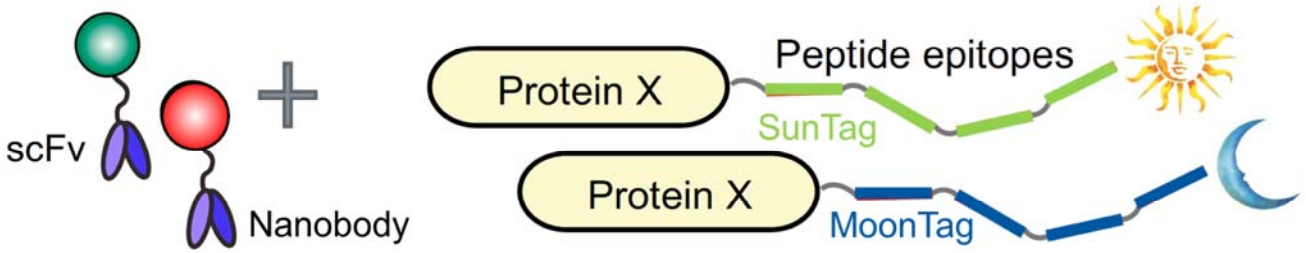
[2]Department of Cellular and Molecular Pharmacology, Howard Hughes Medical Institute, University of California, San Francisco, San Francisco, CA 94158-2517, USA
*Correspondence: m.tanenbaum@hubrecht.eu
http://dx.doi.org/10.1016/j.cell.2016.04.034





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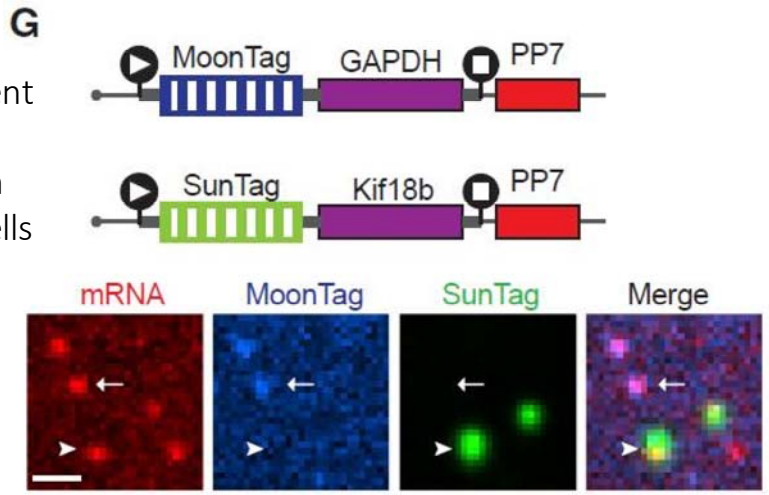
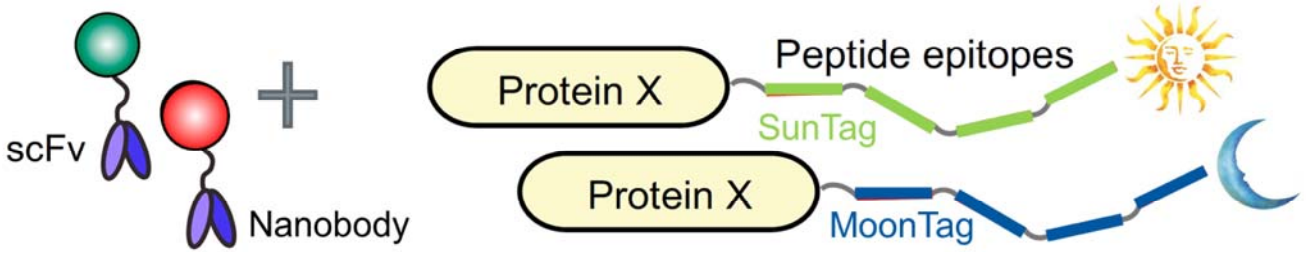
→ Combination of Moon- and SunTag allows **multiplexing**

2 different types of mRNA in single cells





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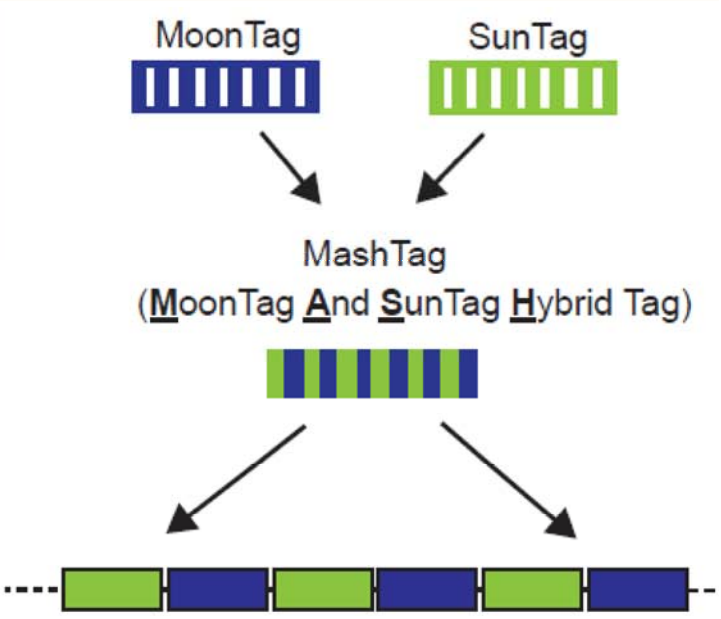
→ **Moon- and SunTag Hybrid = MashTag**



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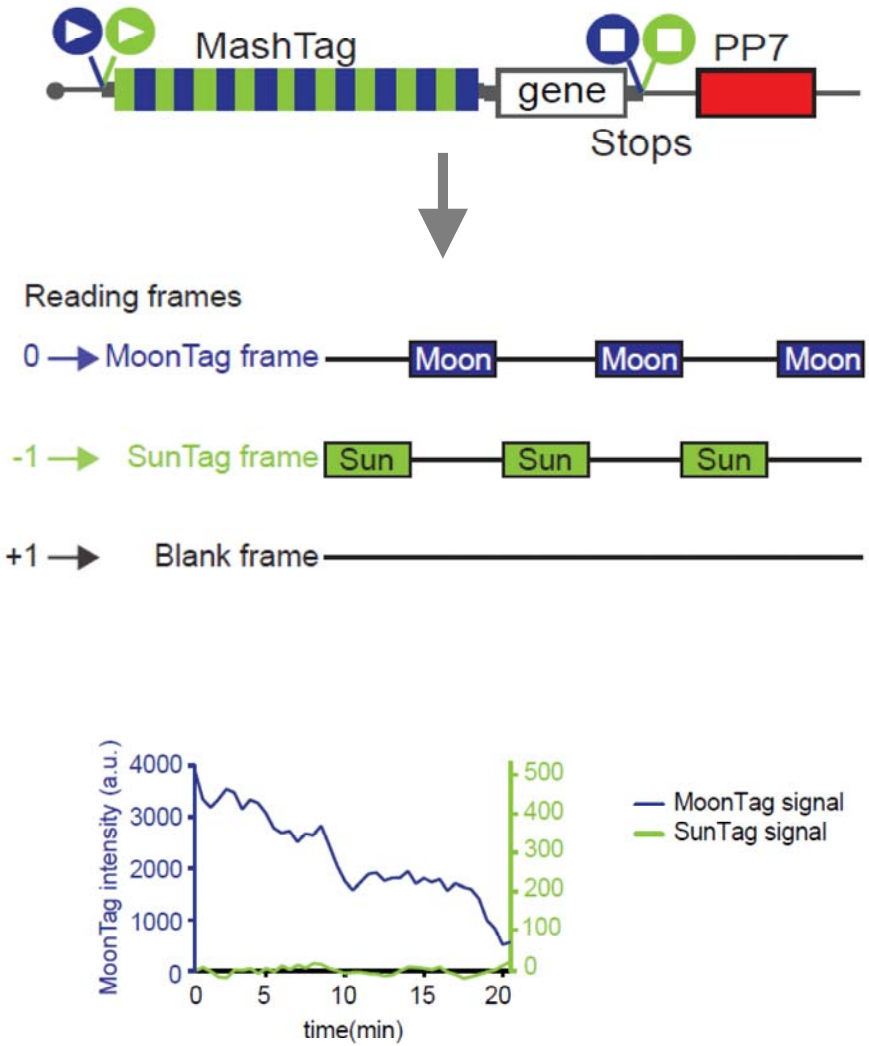
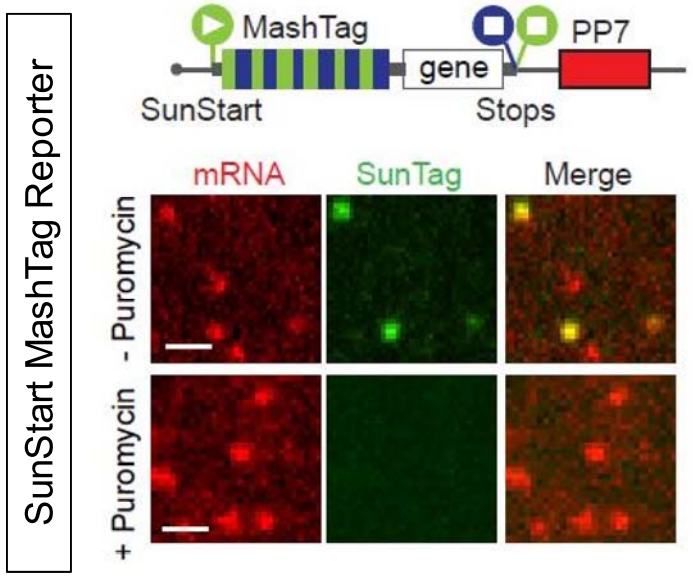
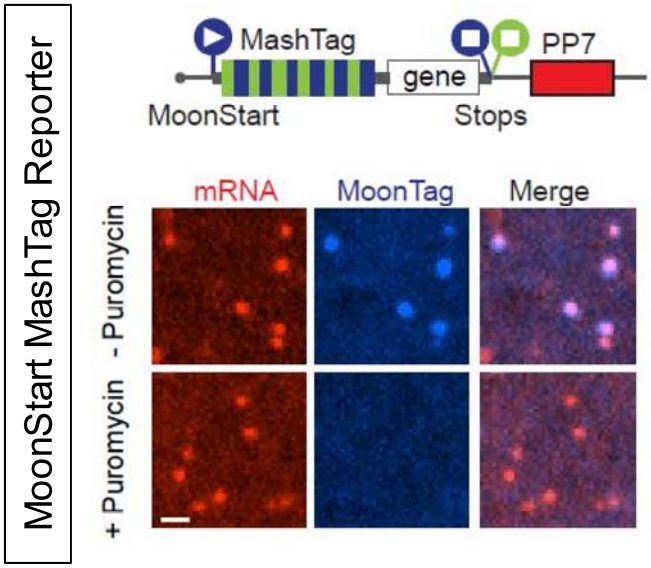
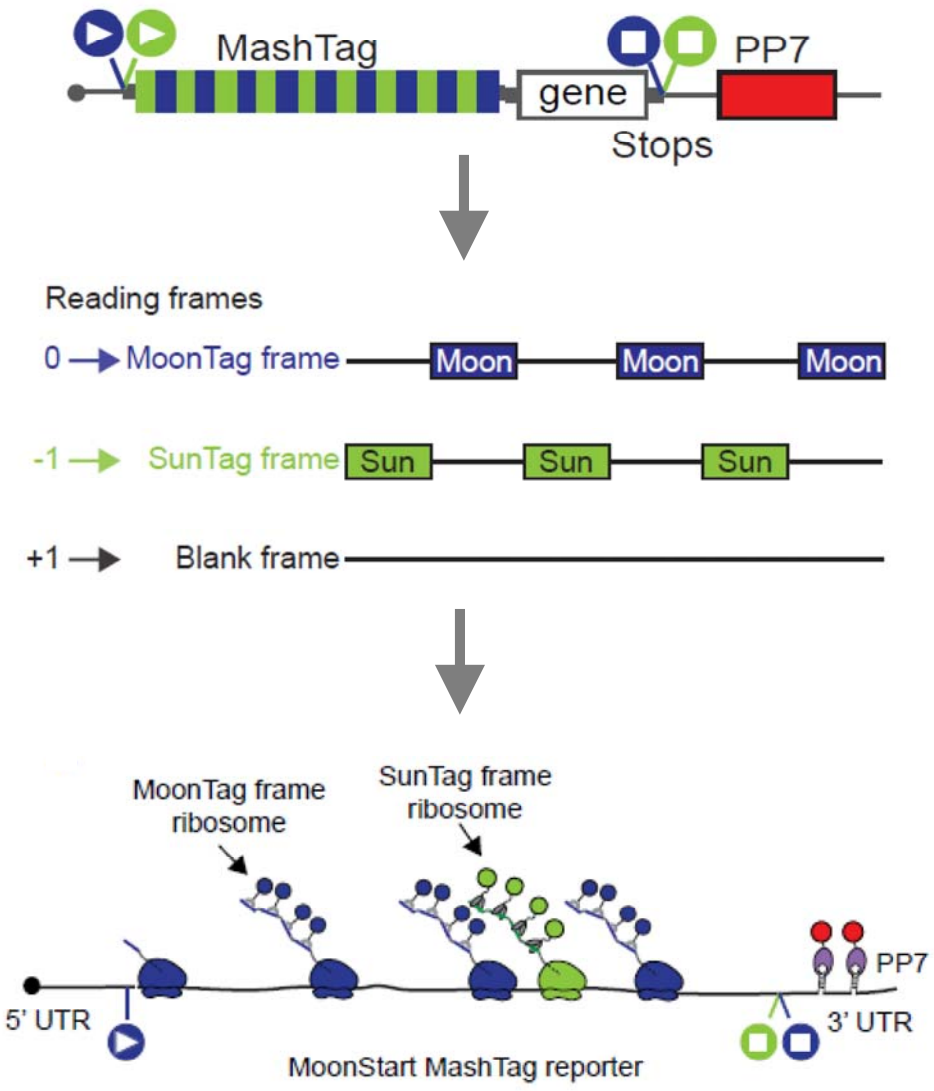


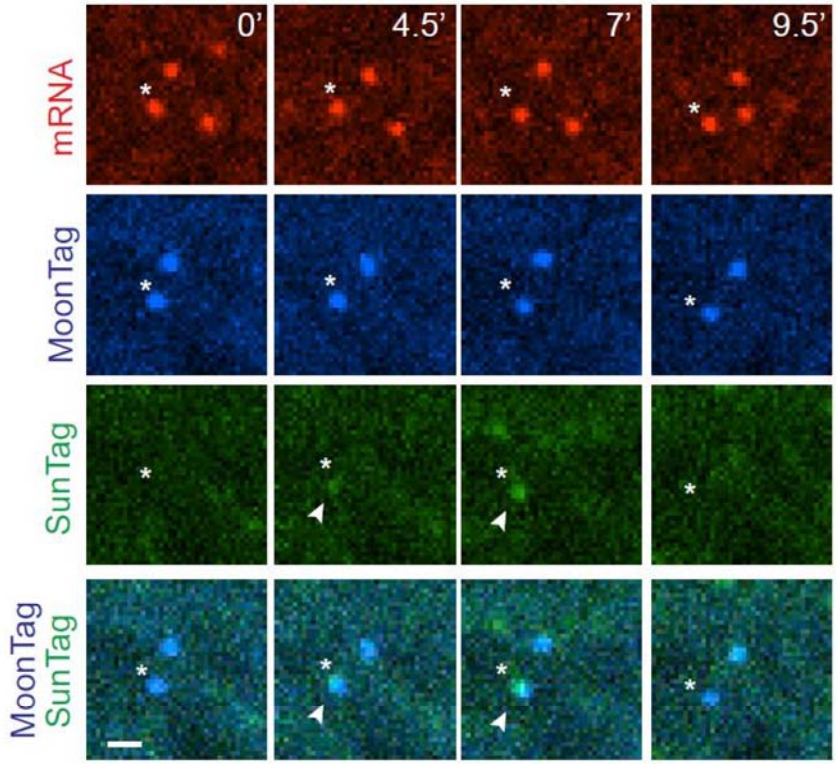




- Depending on start site, reading frame is shifted & only one tag is translated
- Puromycin = translation inhibitor
  → Moon/Sun signal reflects translation

Moon- and SunTag Hybrid = MashTag





- SunTag «pulses» in MoonStart MashTag Reporter
- mCherry intensities stay the same
  = no mRNA multimers
- No such signal in MoonTag Reporter
  = no bleed-through

⇒ out of frame translation (OOF)

1. Alternative start site selection?
2. Ribosome frameshifting?

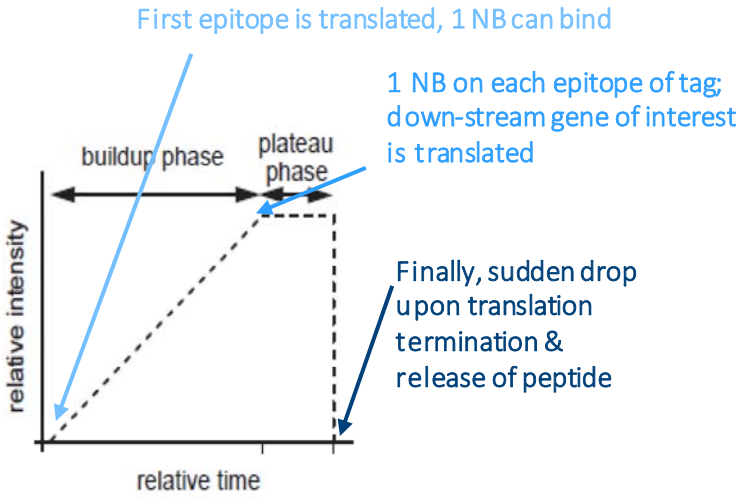
MashTag Reading Frame

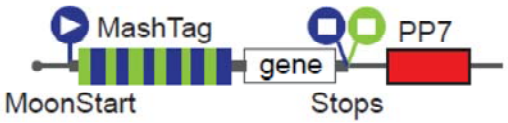
⇒ **OOF:** Alternative start site or ribosome frameshifting?

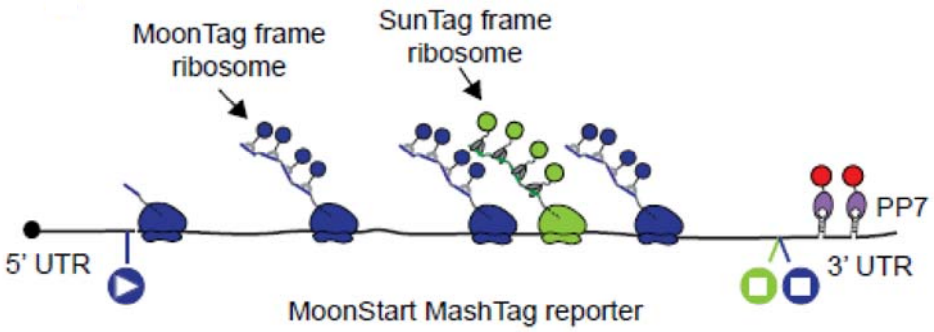
⇒ evaluation of intensity trace

- Start site selection happens close to 5' end
  = all epitopes are SunTag = max. intensity reached
- Ribosome frameshifting is also likely in the middle of mRNA = intensity would be reduced
  (frameshifting *only* at 5' is unlikely as sequence is very repetetive and slipping would then occur also further downstream)

⇒ Introduction of translation repressive 5'UTR sequence
= only 1 ribosome per mRNA







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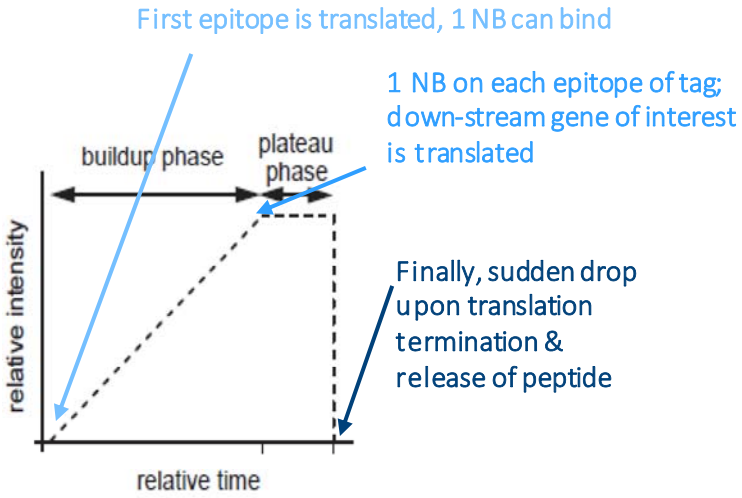

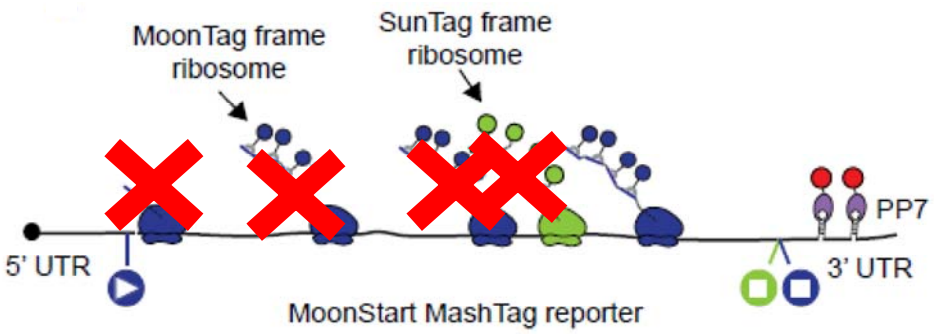

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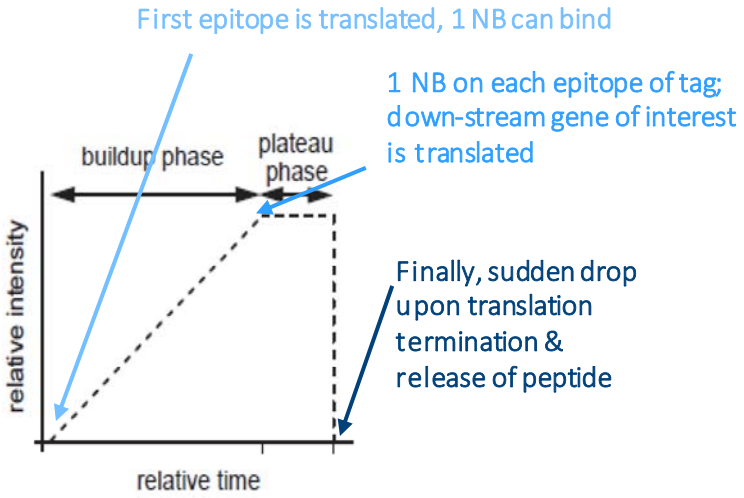
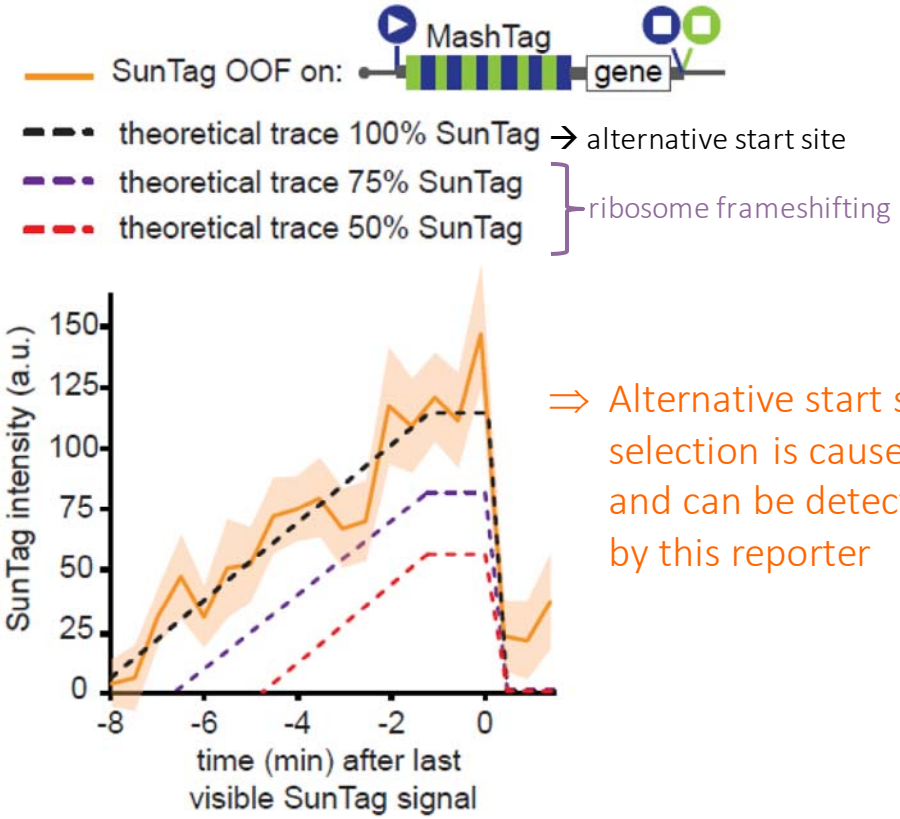




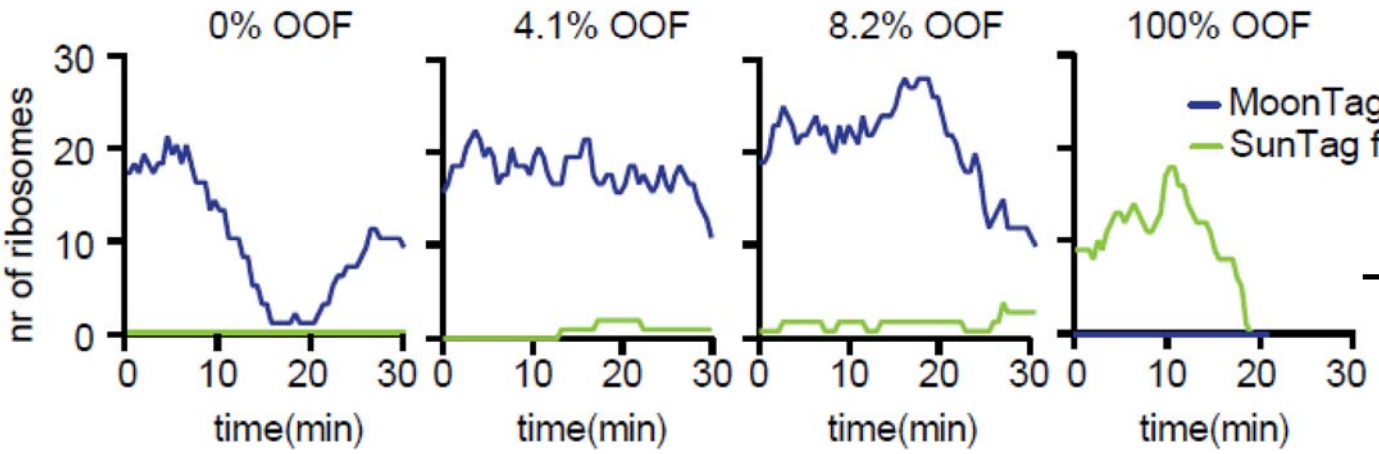
⇒ Alternative start sequence selection is cause for OOF and can be detected by this reporter

MashTag Reading Frame

→ High variability in frequency of OOF translation

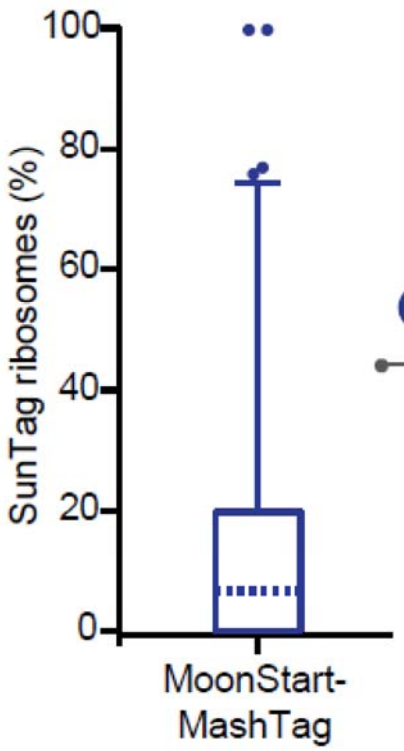
–

Example graphs of
↓ *representative single mRNAs*



→ Translation rate is not constant

MashTag Reading Frame



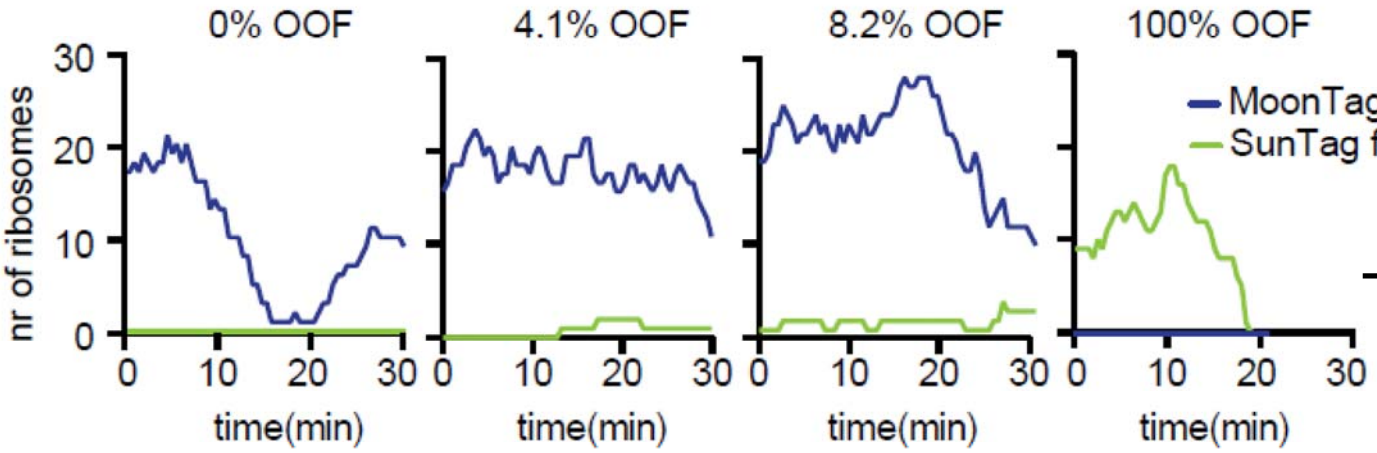
→ High variability in frequency of OOF translation

→ About 7% (median) of ribosomes show OOF translation

→ **Despite use of strong translation start site sequence context = in endogenous setting this value would be higher**

← *Several mRNAs from single genomic locus tested*

Example graphs of
↓ *representative single mRNAs*



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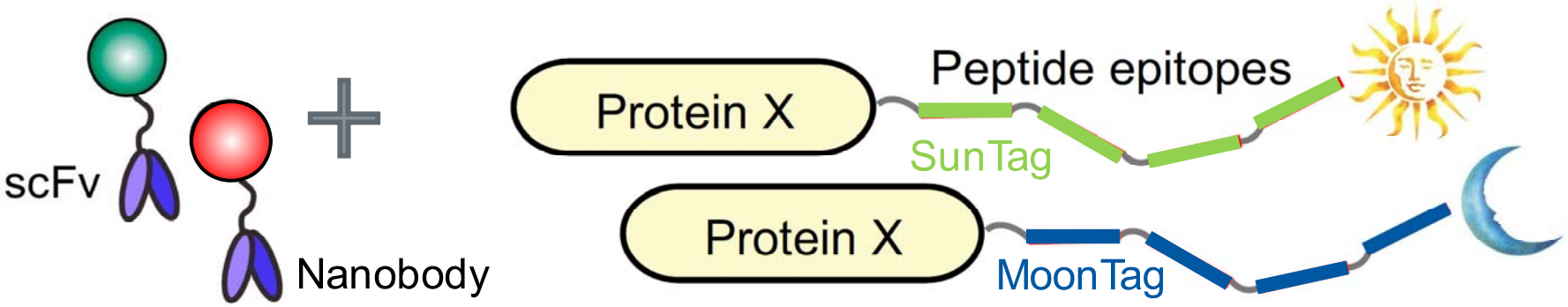
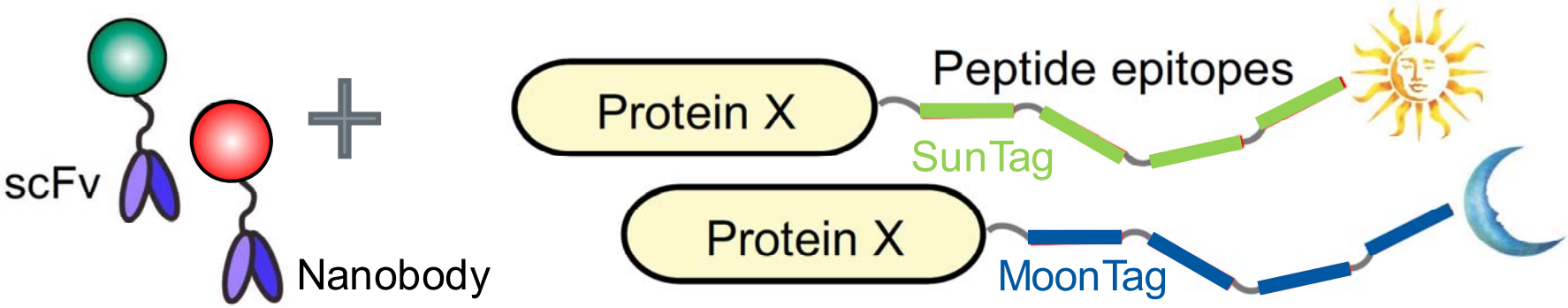


	Advantages	Disadvantages
Fluorescence Imaging		
CRISPRa		
mRNA Tracking		
General Aspects		



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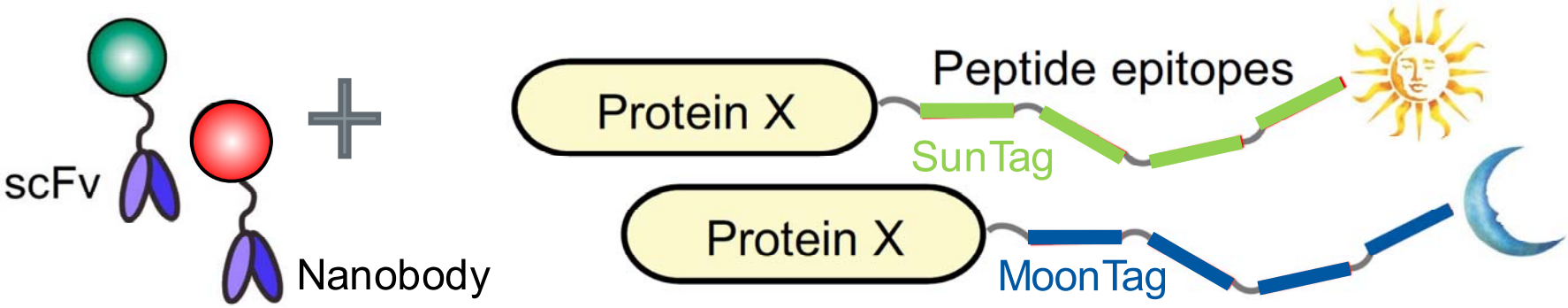

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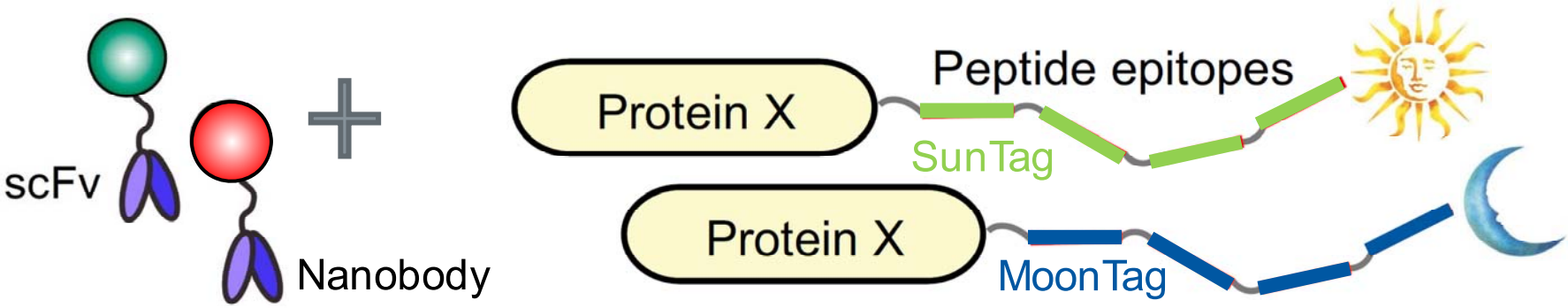

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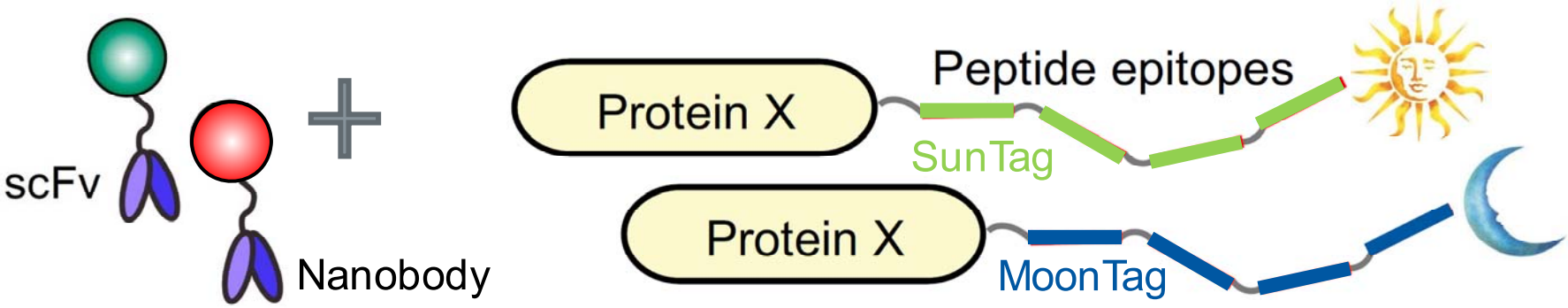

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General Aspects	• Smaller plasmids than if just fusing several copies of FP / activator • Very versatile	• Several plasmids needed to encode tag and antibody-fragment

Sun, Moon and SUperNova

Protein-Tagging Systems for Signal Amplification

Multi-Color Single-Molecule Imaging Uncovers Extensive Heterogeneity in mRNA Decoding

Sanne Boersma,[1,3] Deepak Khuperkar,[1,3] Bram M.P. Verhagen,[1] Stijn Sonneveld,[1] Jonathan B. Grimm,[2] Luke D. Lavis,[2] and Marvin E. Tanenbaum[1,4,*]
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[3]These authors contributed equally
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https://doi.org/10.1016/j.cell.2019.05.001

A Protein-Tagging System for Signal Amplification in Gene Expression and Fluorescence Imaging

Marvin E. Tanenbaum,[1,2] Luke A. Gilbert,[1,2,3,4] Lei S. Qi,[1,3,4] Jonathan S. Weissman,[1,2,3,4] and Ronald D. Vale[1,2,*]
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http://dx.doi.org/10.1016/j.cell.2014.09.039

08.10.2019
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