Technical Journal Club

New tools to investigate transient and weak protein-protein interactions (PPIs)

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January 22nd, 2019

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1.) Introduction
2.) Some commonly used methods
3.) Paper 1 (Thakur & Movileanu 2018, Nature Biotechnology)
4.) Paper 2 (Liu et al. 2018, Nature Methods)
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Papers of todays' TJC

nature biotechnology

Real-time measurement of protein–protein interactions at single-molecule resolution using a biological nanopore

Avinash Kumar Thakur[1,2] & Liviu Movileanu[1–3]

10 December 2018

Papers of todays' TJC

nature biotechnology

Real-time measurement of protein–protein interactions at single-molecule resolution using a biological nanopore

Avinash Kumar Thakur[1,2] & Liviu Movileanu[1–3]

10 December 2018

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nature methods ARTICLES
https://doi.org/10.1038/s41592-018-0100-5

A proximity-tagging system to identify membrane protein–protein interactions

Qiang Liu[1,2,3,4], Jun Zheng[1,3,4], Weiping Sun[1,3], Yinbo Huo[1,2,3], Liye Zhang[1], Piliang Hao[1], Haopeng Wang[1*] and Min Zhuang[1*]

PUP-IT
(pupylation-based interaction tagging)

NATURE METHODS | VOL 15 | SEPTEMBER 2018

1.) Introduction

- Proteins facilitate most biological processes in a cell
- Including: gene expression, cell growth, proliferation, nutrient uptake, cell morphology, motility, intercellular communication and apoptosis
- Cells respond to diverse stimuli and protein expression and interaction is therefore a mostly dynamic process
- Proteins that are used to complete specific tasks may not always be expressed or activated and many proteins are expressed in a cell type–dependent manner
- This complexity leads to a challenge when it comes to the investigation of a protein function in a proper biological context

Kenji Muira, 2018, *Protein & Peptide Letters*
Phizicky EM, Fields S, 1995, *Microbiol Rev*

1.) Introduction

Full understanding of a proteins' functions requires knowledge of:

- Sequence and structure (e.g. motifs to predict function)
- Evolution and conserved sequence (e.g. regulatory residues)
- Expression profile and splicing (e.g. cell-type specificity)
- Post-translational modifications (e.g. P, Ac,Glyco, Ubiq)
- Compartment localization
- The interaction (hydrophobic bonds, vWF and salt bridges) with other molecules/proteins (function extrapolated by knowing interactions)

Kenji Muira, 2018, *Protein & Peptide Letters*
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1.) Introduction

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- The interaction (hydrophobic bonds, vWF and salt bridges) with other molecules/proteins (function extrapolated by knowing interactions)

→ Protein-Protein Interactions (PPIs)

Kenji Muira, 2018, *Protein & Peptide Letters*
Phizicky EM, Fields S, 1995, *Microbiol Rev*

1.) Introduction

- PPIs can be **transient** or stable
- PPIs are either **strong** (Kd in nanomolar range) or **weak** (Kd in micro- or milimolar range)
- Transient PPIs can also be fast and slow

- Transient interactions are dynamic and these interactions control the majority of cellular processes (protein modification, transport, folding, signaling, apoptosis and cell cycling)

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1.) Introduction

Important properties of methods **when investigating PPIs:**

- Possible to screen for protein interactors?
- Is a protein complex purification possible?
- Is the method ‚tag free'?
- Is the method matrix (solid phase)-free?
- Investigation of kinetics; (K_{off}, K_{on}) dissociation/association rate constant?
- Important to define and know affinity/equilibrium dissociation constant (Kd; $Kd=K_{on}/K_{off}$)
- Higher structure analysis possible with chosen method?

2.) Some commonly used methods

Methods	Detection of Protein Binding	Screening of Interactor Protein	Purification of Protein Complex	Tag Free Analysis	Matrix (Solid Phase)-Free Analysis	Affinity Analysis (KD)	Kinetic Analysis (Kon, Koff)	Higher Structure Analysis	Index to Confirm Protein-Protein Interaction
Pull down	+	+	+	-	-	-	-	-	Band/spot by SDS-PAGE
Two-hybrid	+	+	-	-	+	-	-	-	Colony formation
Gel Filtration	+	-	+	+	+	-	-	-	Retention time
ITC	+	-	-	+	+	+	-	-	Temperature
FRET	+	-	-	-	+	+	-	-	Fluorescence
LOCI	+	-	-	-	-	+	-	-	Luminescence
RIFS	+	-	-	±	-	+	+	-	Interference spectrum
SPR	+	-	-	±	-	+	+	-	Resonance angle
CD	±	-	-	+	+	-	-	±	Absorbance of circularly polarized light
ROA	±	-	-	+	+	-	-	±	Raman scattering by circularly polarized light
SAXS	±	-	-	+	+	-	-	±	Scattered X rays
NMR	±	-	-	+	+	-	-	±	Chemical shift
Cryo-EM	+	-	-	+	+	-	-	+	Images of proteins

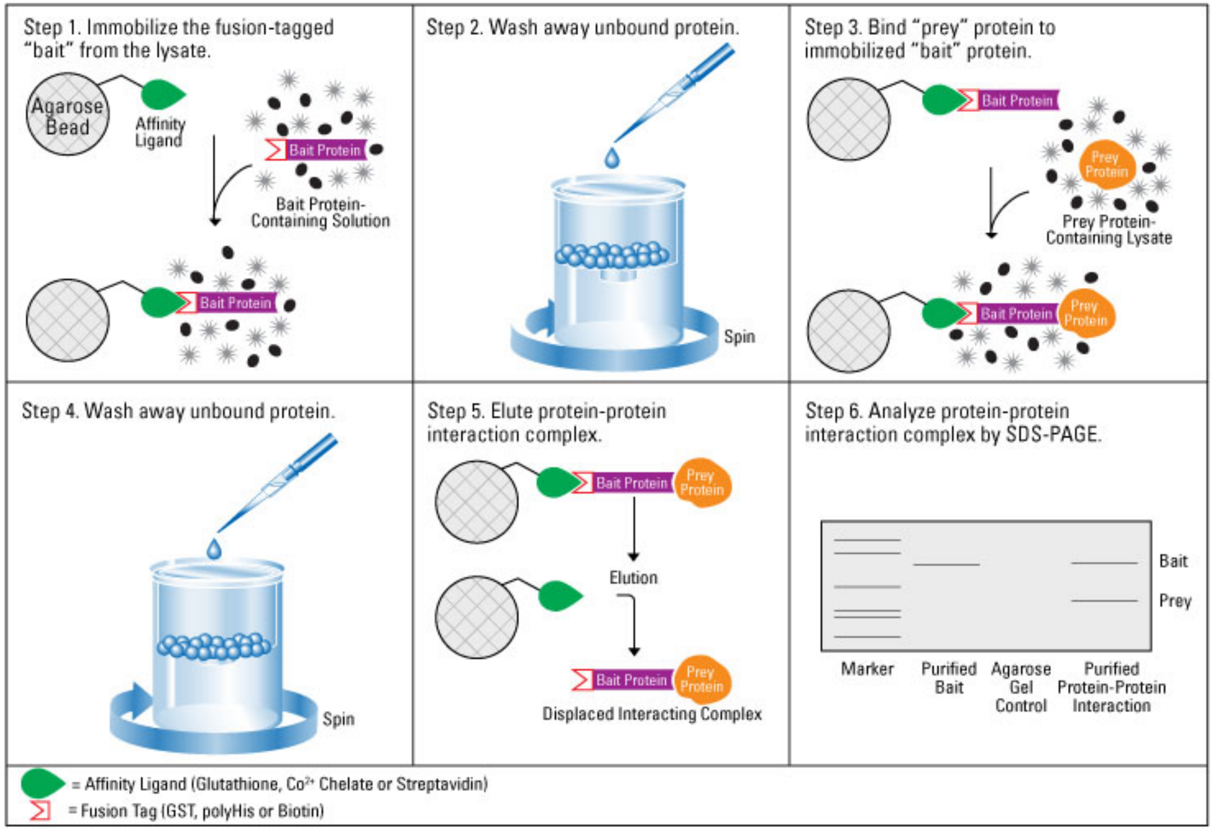
+: possible and suitable; ± : possible depending on conditions; -: rather unsuitable or impossible
Kon: association rate constant; Koff: dissociation rate constant; KD: dissociation constant

Kenji Muira, 2018, *Protein & Peptide Letters*

2.) Some commonly used methods

Pull down

- Tag (e.g. Biotin); on bait protein (interacts with prey protein)
- Matrix (e.g. Streptavidin)
- Protein complex subjected to SDS-PAGE
- Protein identified: Western blot, MS, sequencing



2.) Some commonly used methods

Among some others:

- Two hybrid assay
- Gel Filtration Chromatography
- Isothermal Titration Calorimetry (ITC)
- Surface Plasmon Resonance (SPR)
- Co-IP
- Circular Dichroism Spectroscopy (CD)
- Nuclear Magnetic Resonance Spectroscopy (NMR)
- Cryo-EM

2.) Some commonly used methods

Commonly used **methods are able to**:

- Detect protein binding
- Some allow tag-free analysis
- Some allow the performance of a screening for interactor proteins
- Some can purify protein complexes
- Methods perform only an average affinity (KD) analysis
- Describe a PPI with several parameters when combining them
- Functionality assays: Tango (beta-Arrestin) and PathHunter (beta-galactosidase)

2.) Some commonly used methods

Limitations and challenges **in commonly used methods:**

- Lack of structural protein information

- Real-Time measurements and kinetic analysis (K_{on}/K_{off})
- Single molecule resolution measurements of PPI
- Heterogeneous samples (patient/bovine plasma/serum)

- Research of PPIs along membrane proteins
- New orthogonal functionality assays (protein expression)
- Exogenously added compounds are often needed

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Paper 1

- Research of PPIs along membrane proteins
- New orthogonal functionality assays (protein expression)
- Exogenously added compounds are often needed

Paper 2

3.) Paper 1

nature biotechnology

Real-time measurement of protein–protein interactions at single-molecule resolution using a biological nanopore

Avinash Kumar Thakur[1,2] & Liviu Movileanu[1–3]

10 December 2018

Objectives:

- Measurement of transient PPIs at high-throughput level
- Real-time sampling at single-molecule resolution
- Measurement of PPIs in complex and heterogeneous biological fluids

Summary:

- Design of a nanopore-sensor: truncated outer membrane protein pore, flexible tether, protein receptor and peptide adaptor
- Reversible protein ligand; capture and release can be measured as current transitions; two open substates of the nanosensor

3.) Paper 1

Requirements to measure binding events between two folded proteins in solution using a protein nanopore:

1.) Reversible PPI must occur in aqueous phase

- Diameter of protein complex exceeds cross-sectional internal diameter of pore
- If interactions occur, they are only detected outside the nanopore lumen (useful for mammalian serum)

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Requirements to measure binding events between two folded proteins in solution using a protein nanopore:

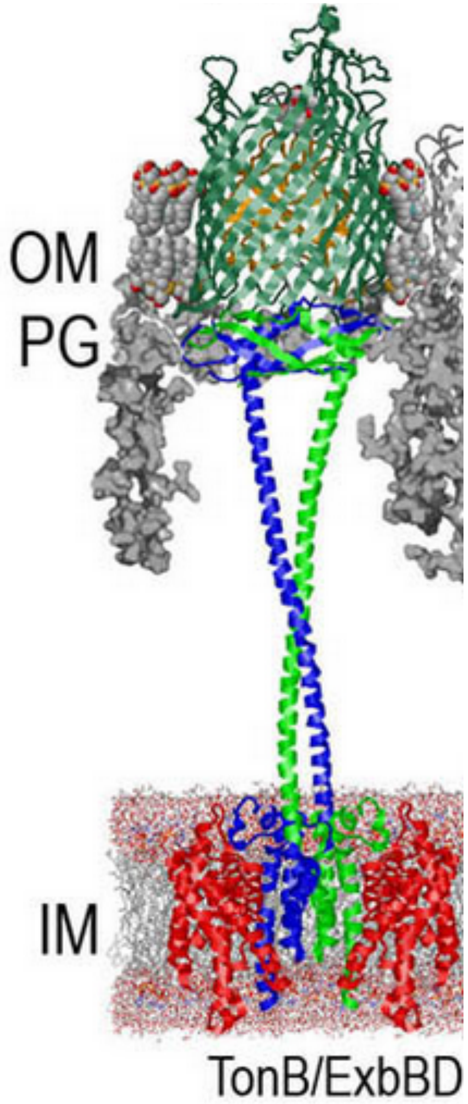
1.) Reversible PPI must occur in aqueous phase

- Diameter of protein complex exceeds cross-sectional internal diameter of pore
- If interactions occur, they are only detected outside the nanopore lumen (useful for mammalian serum)

2.) A transducing mechanism is required to convert reversible physical association and dissociation into a high-fidelity electrical signature of the sensor

3.) Paper 1

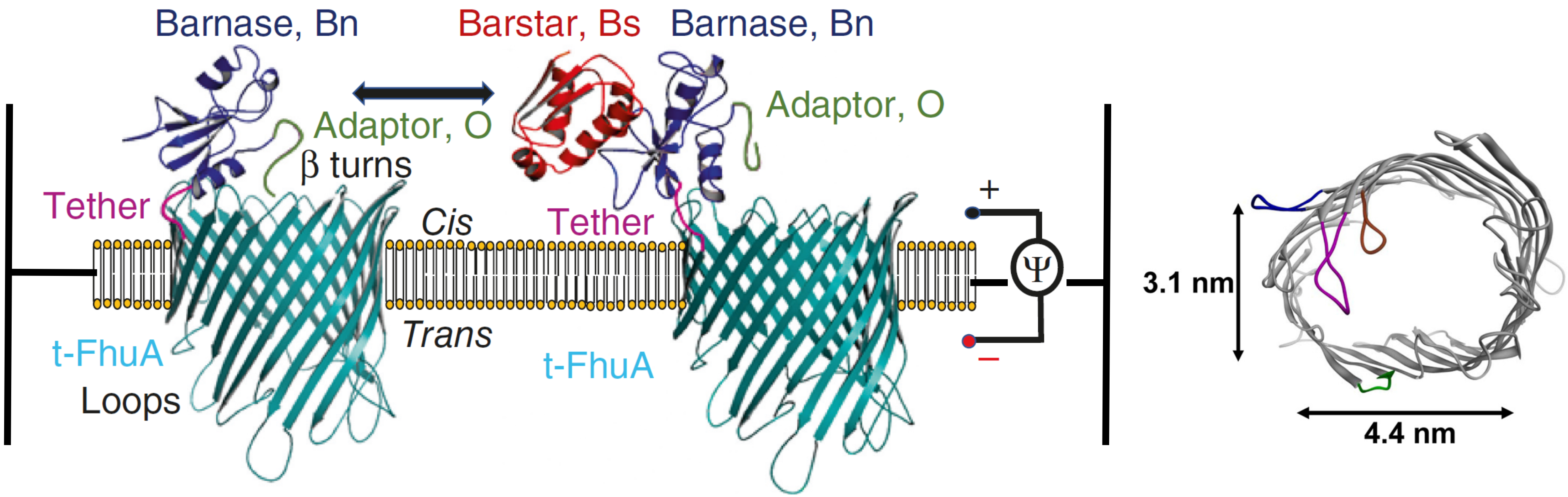
Ferric hydroxamate uptake component A (**FhuA**) from E.coli



- The OM is a crucial part for nutrient acquisition and protection of bacterial species (*Shigella (dysentery), Salmonella(typhoid fever), Vibrio(cholera), Neisseria(meningitis), Yersinia(plague)* and ***Escherichia****(food poisoning)*
- The metal ion Fe3+ is largely insoluble, so microbes secrete small organic compounds (siderophores) that solubilize Fe3+ by chelating it
- Ferric siderophores initiate the activity of iron in biological systems (e.g. Ferrichrome)
- Ferric siderophores cannot penetrate the trans-OM channels
- OM receptor proteins that recognize, bind and transport ferric siderophores into the periplasm with the help of TonB
- These OM iron receptors have structural porine-like features, class called ‘ligand-gated’ porins (LGP)
- Active transporter (accumulating iron against its concentration gradient)
- E.g. FhuA, the receptor for ferrichrome and transporter of antibiotics and bacteriophages

3.) Paper 1

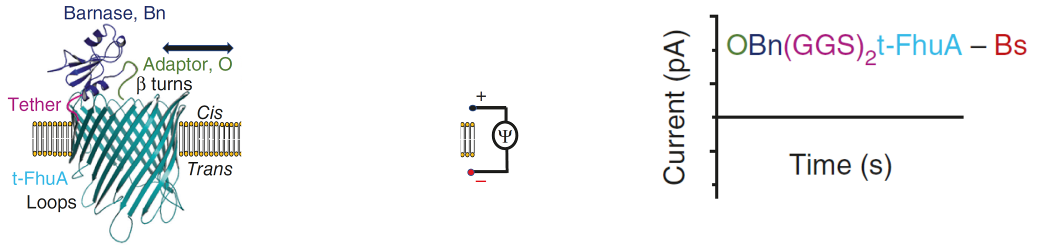
Buffer: 300 mM KCl, 10 nM TrisHCl pH8
Lipid bilayer: Glycerolphophaditylcholine



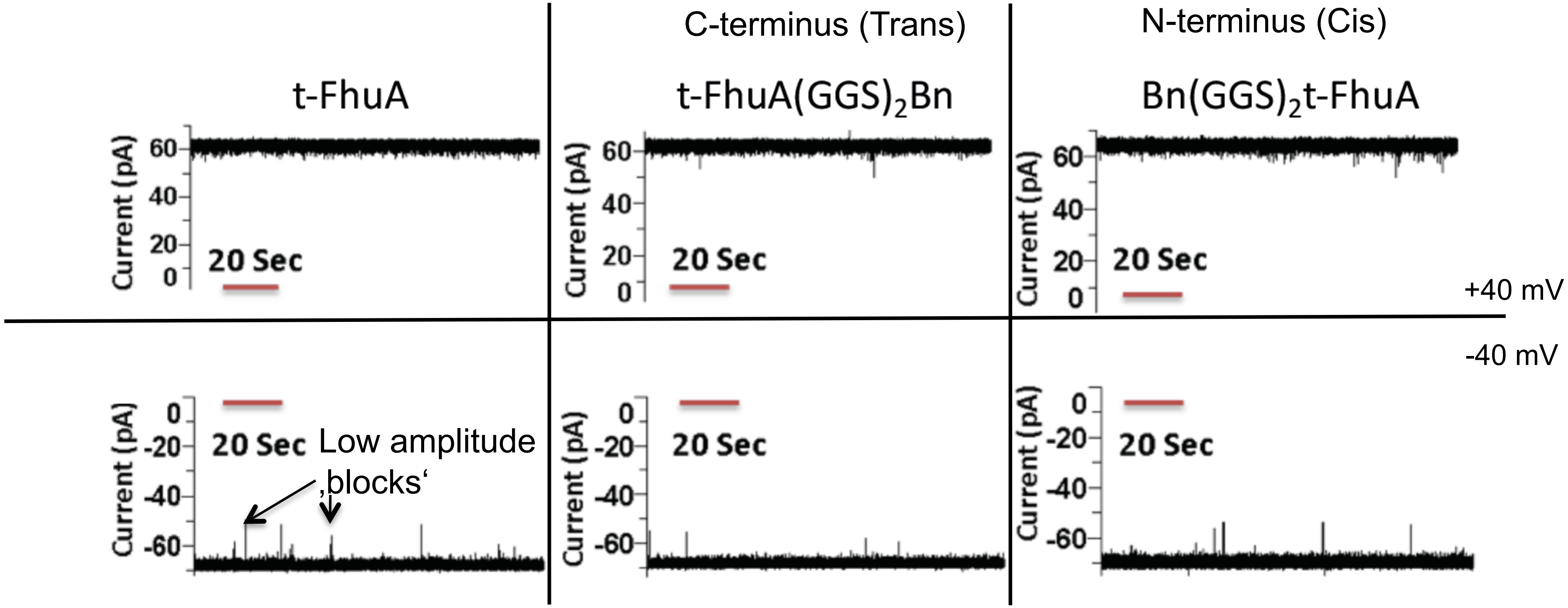
OBn(GGS)2-t-FhuA fusion protein

- Use of a **truncated version of FhuA** (monomeric 22-stranded beta-barrel)
- Extracellular loops do not fold back into the interior (unlike porins)
- (GGS)2-**Tether** on beta-turn side
- **Protein receptor**: barnase H102A (Bn) - RNase/110aa
- **Adaptor/O**: neg. charged, unstructured - 12aa
  (spans distance fron Bn-N-terminus and pore opening)
- Single molecule electrophysiology done in a planar lipid bilayer
- Single channel electrical currents were acquired using patch-clamp

3.) Paper 1

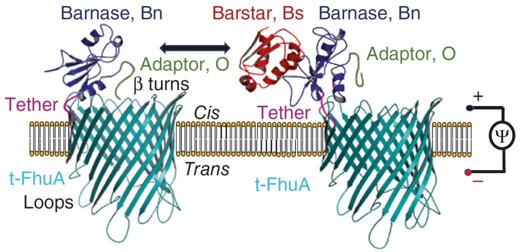


Channel conductance characteristics (without ligand)

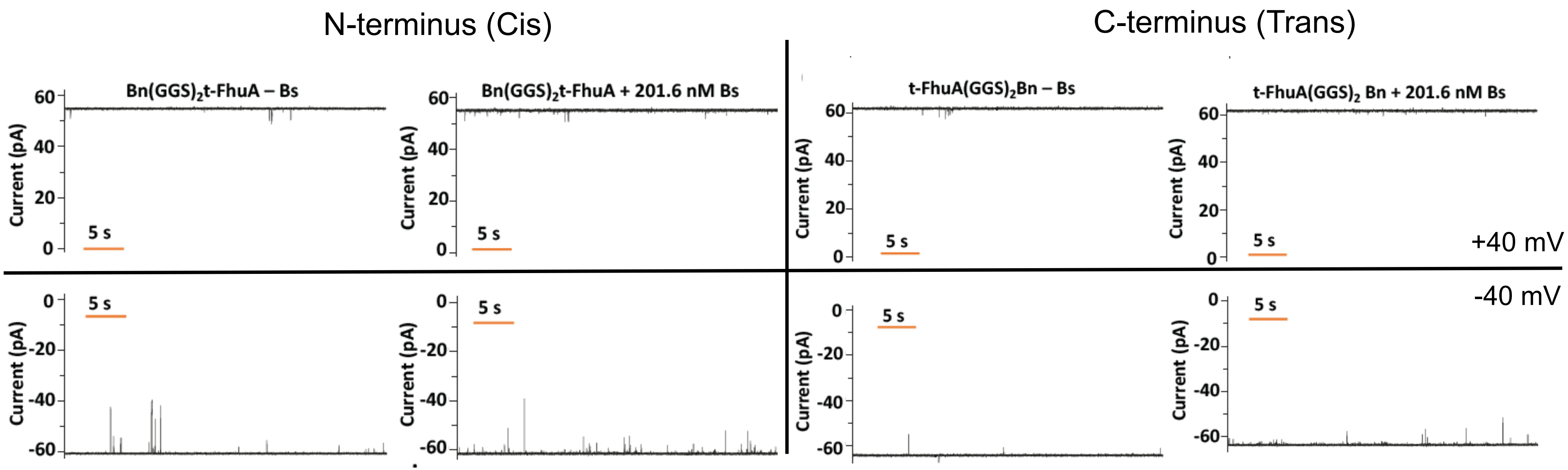


- Single-channel recordings: **t-FhuA** single channel **conductance 1.6 nS** (nanosiemens)
- **Fusion protein** conductance closely **similar to t-FhuA** alone
- N-or C-terminus fusion to t-FhuA did not deteriorate the SNR-> t-FhuA a **robust beta-barrel** scaffold and no distorting of open-state current for long periods
- Suggests that **Bn does not block pore lumen**
- Large polypeptide extensions at either terminus without affecting pore-forming ability

3.) Paper 1



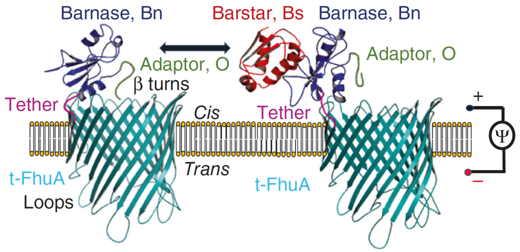
Channel conductance characteristics (with ligand)



- Protein receptor Bn fused on C-terminus (Trans) failed to produce reversible alterations in the electrical signal
- Explanations: Bn might changes conformation, Bs binding site not accessible any longer

Hypothesis: Transient Bn-Bs complex formation pulls Bn away from the pore opening

3.) Paper 1



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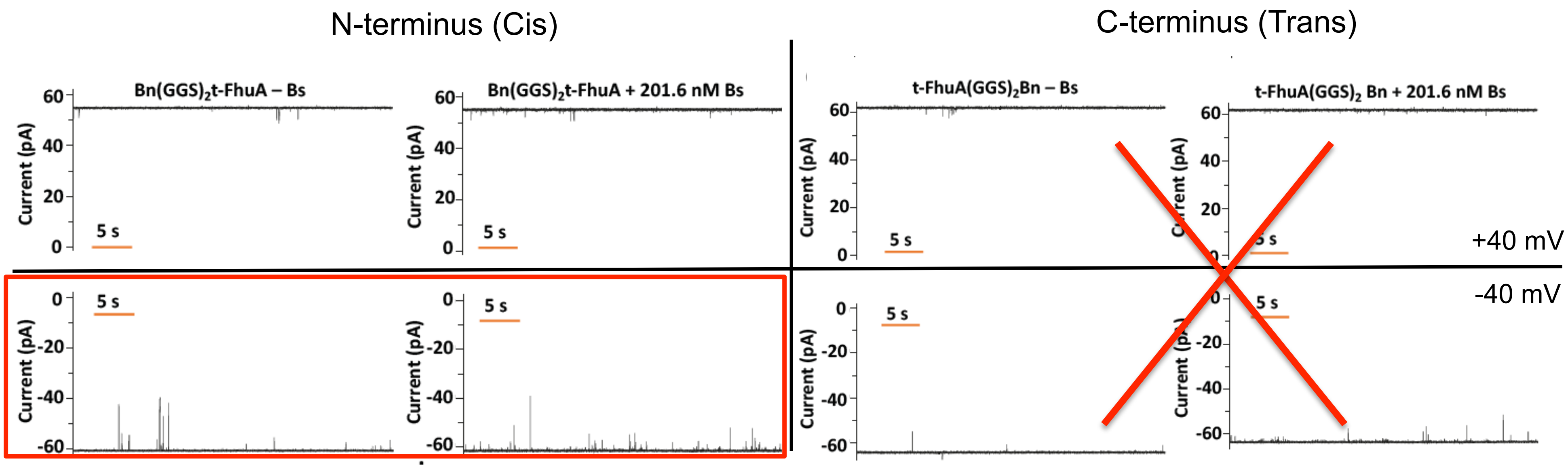

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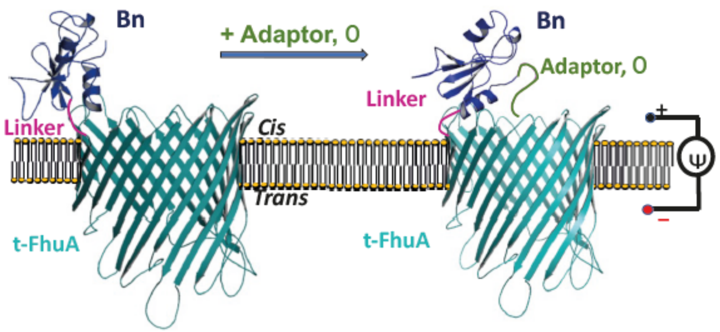
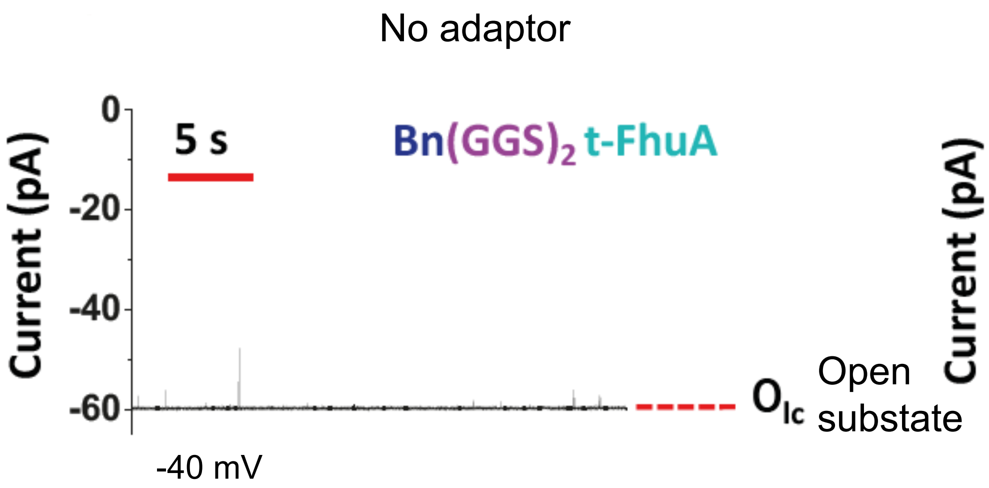
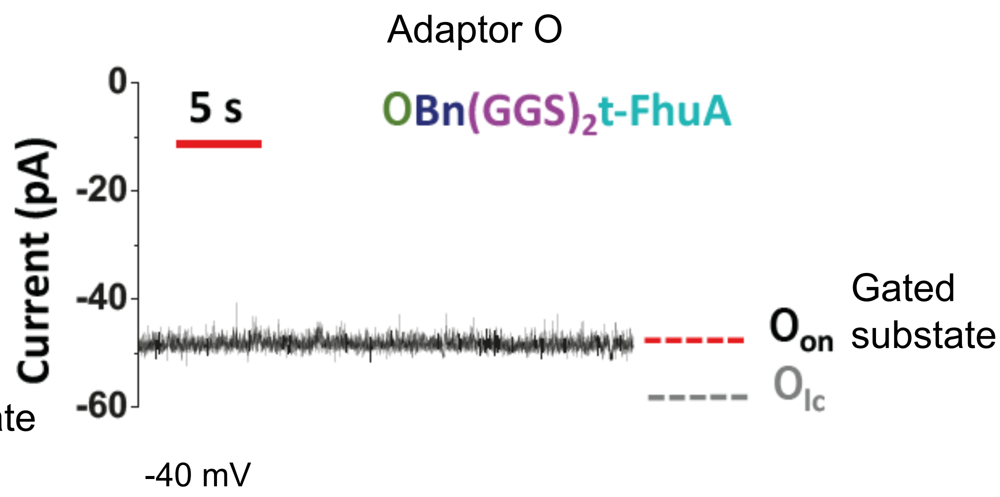


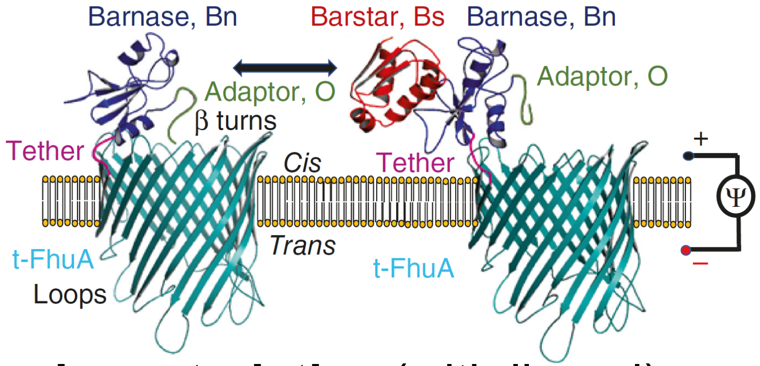
Engineering of a **peptide adaptor, O,** obstruction moiety to create a two substates



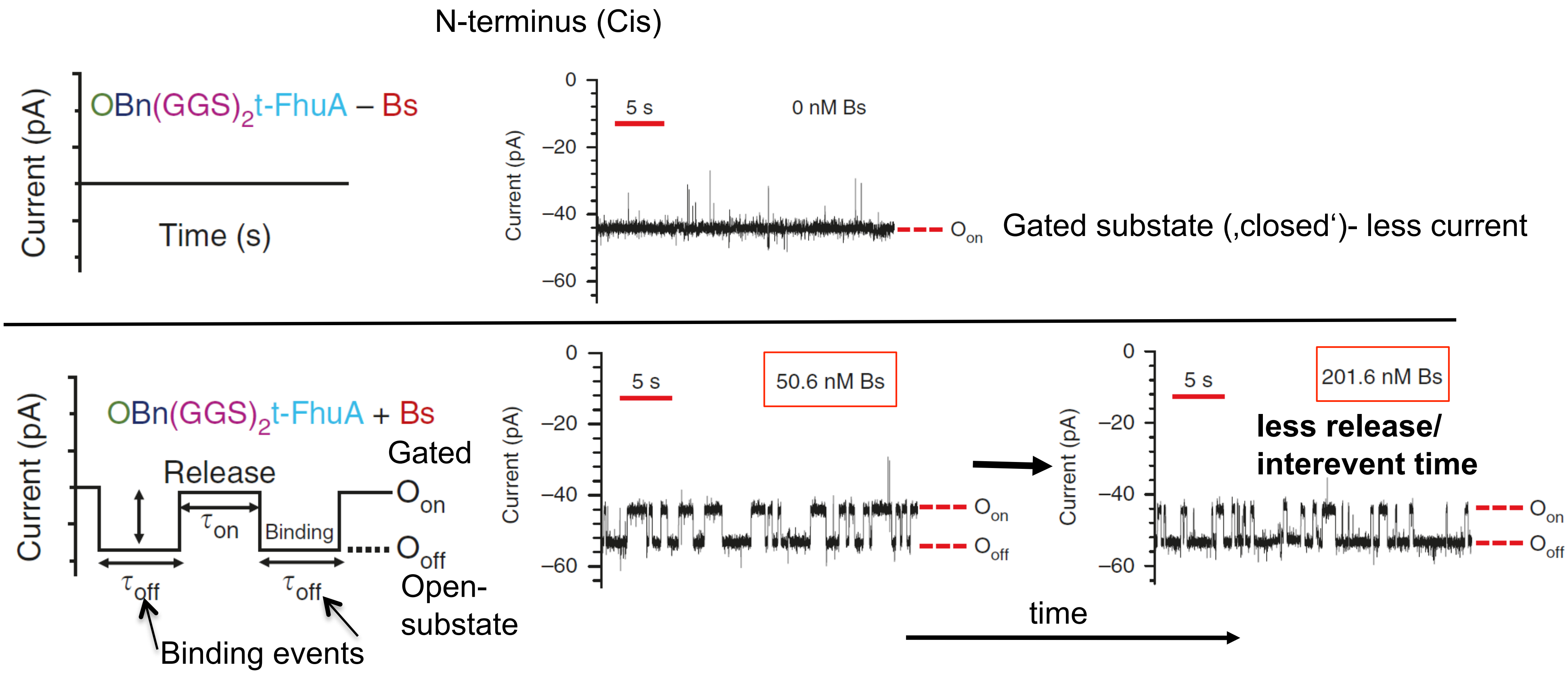


- **OBn(GGS)2t-FhuA: 1.23 nS** vs. t-FhuA: 1.6 nS
- Idea: to obtain an altered electrical signature that might be sensitive to Bn-Bs specific interactions and spans distance between N-term of Bn and pore opening
- **Open substate** O_{lc} (large-conductance)
- With adaptor O a new O_{on} **gated-substate** of the sensor
- Conductivity $I_{lc} > I_{on}$

3.) Paper 1

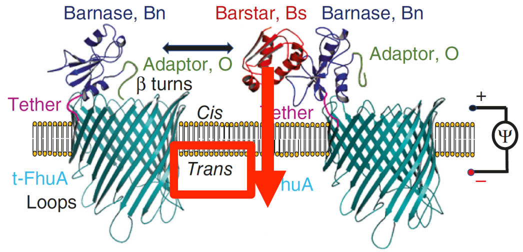


Channel conductance characteristics (with ligand)

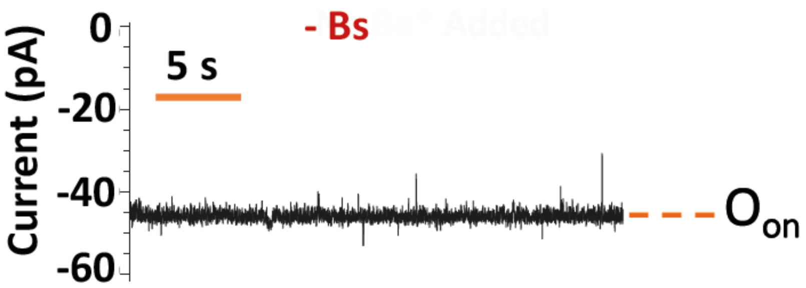


- Bn-Bs module: Bs is a inhibitory ligand of the RNase Bn
- **Bs applied on the cis side**
- Current transitions from O_{on} to O_{off} interpreted as capture and release events of Bs and Bn

3.) Paper 1

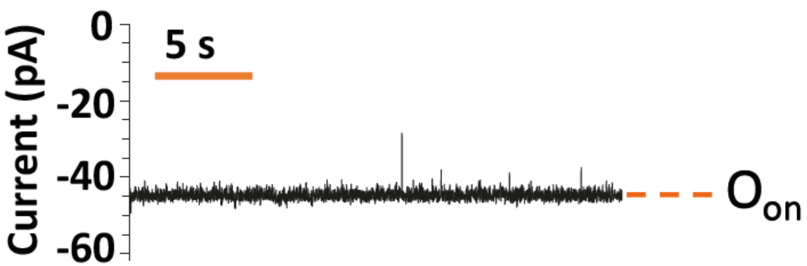


Channel conductance characteristics (with ligand)



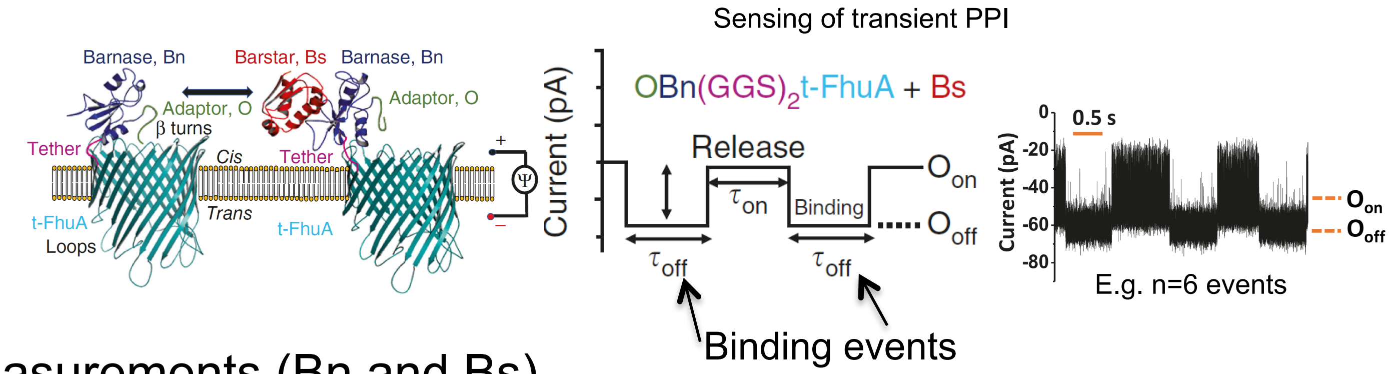
Adaptor on= more obstruction of pore on cis side

12.6 nM Bs added to the *trans* side

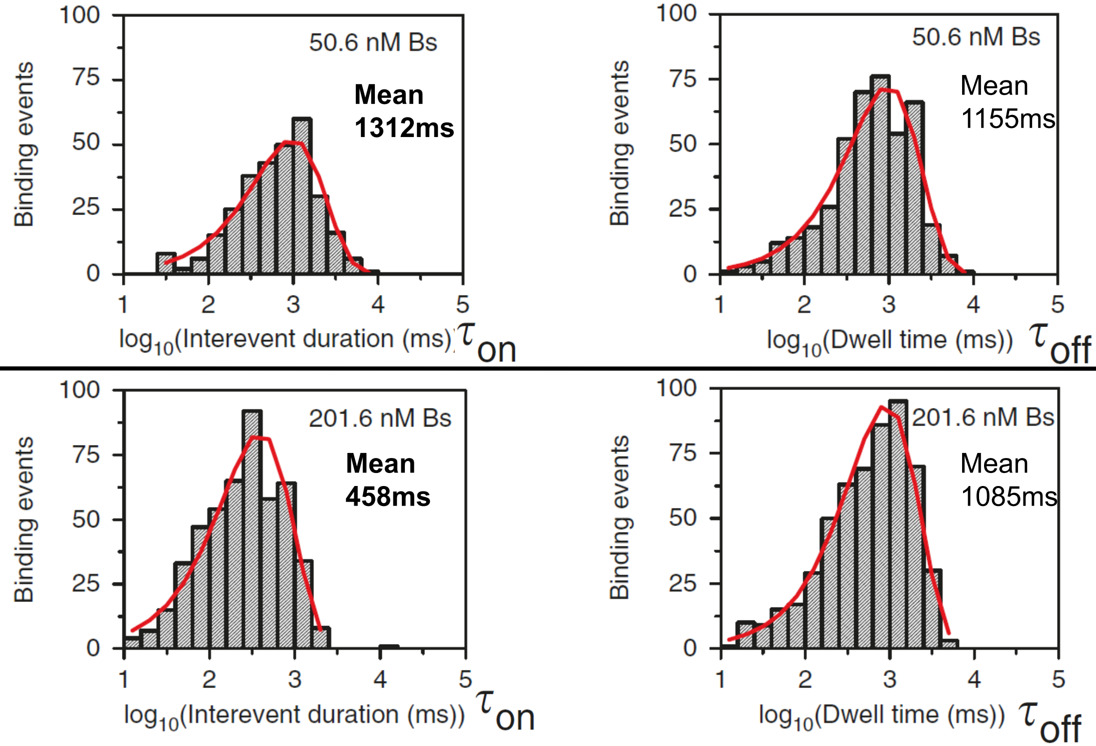


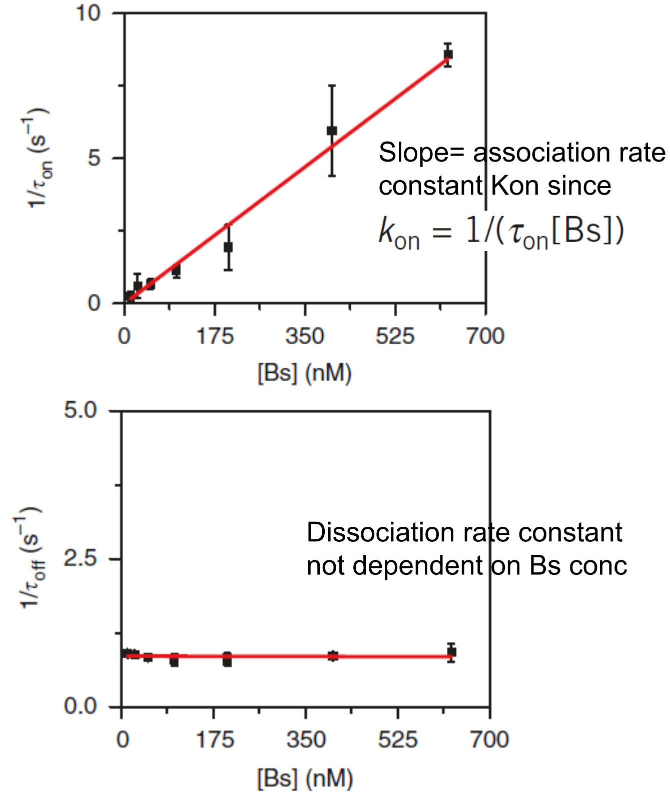
- No reversible current transitions when Bs was added to trans side to OBn(GGS)2-t-FhuA
- Insertion of OBn(GGS)2-t-FhuA nanopore into lipid bilayer happens with preferred orientation

3.) Paper 1



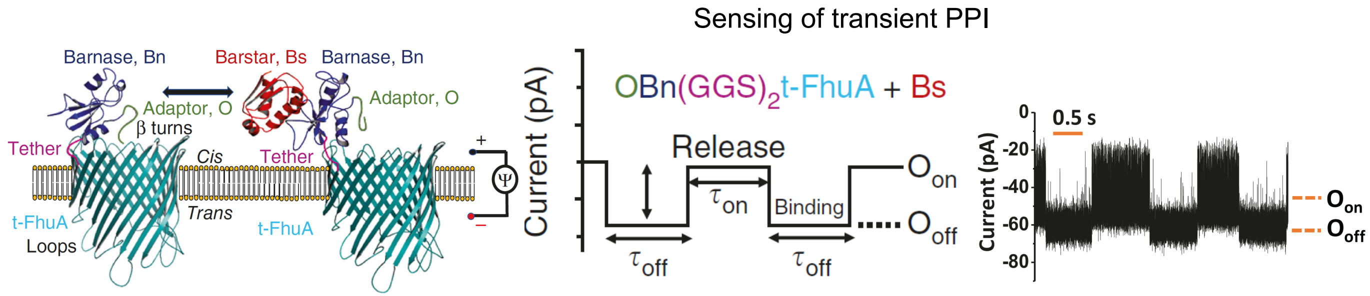
High-affinity PPI measurements (Bn and Bs)





- Two conductance substates: O_{on} (Bn alone, pore more tight) and O_{off} (Bn-Bs, open pore)
- Frequency of Bn-Bs binding events relatively increases with Bs concentration since τ_{on} decreases
- **Dissociation of Bs** from Bn (τ_{off}) is **independent of Bs concentration**
- **Linear** dependence confirms a **bimolecular association** process

3.) Paper 1



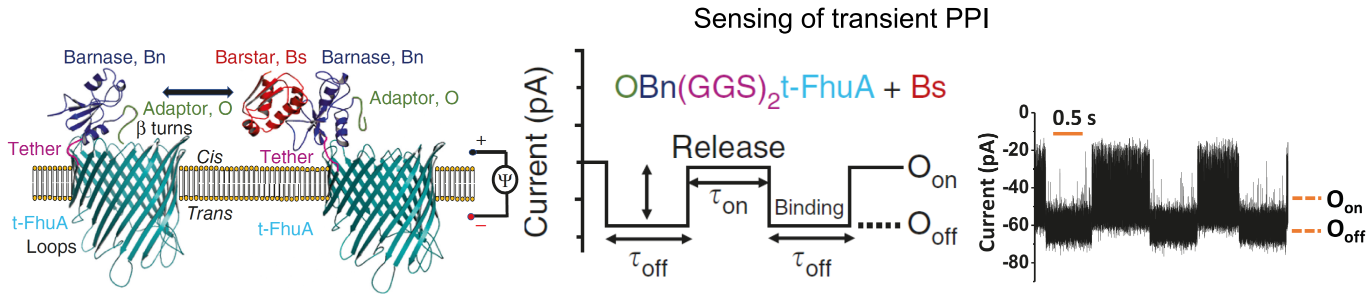
High-affinity PPI measurements (Bn and Bs)

Average values for the tansient Bn-Bs interactions

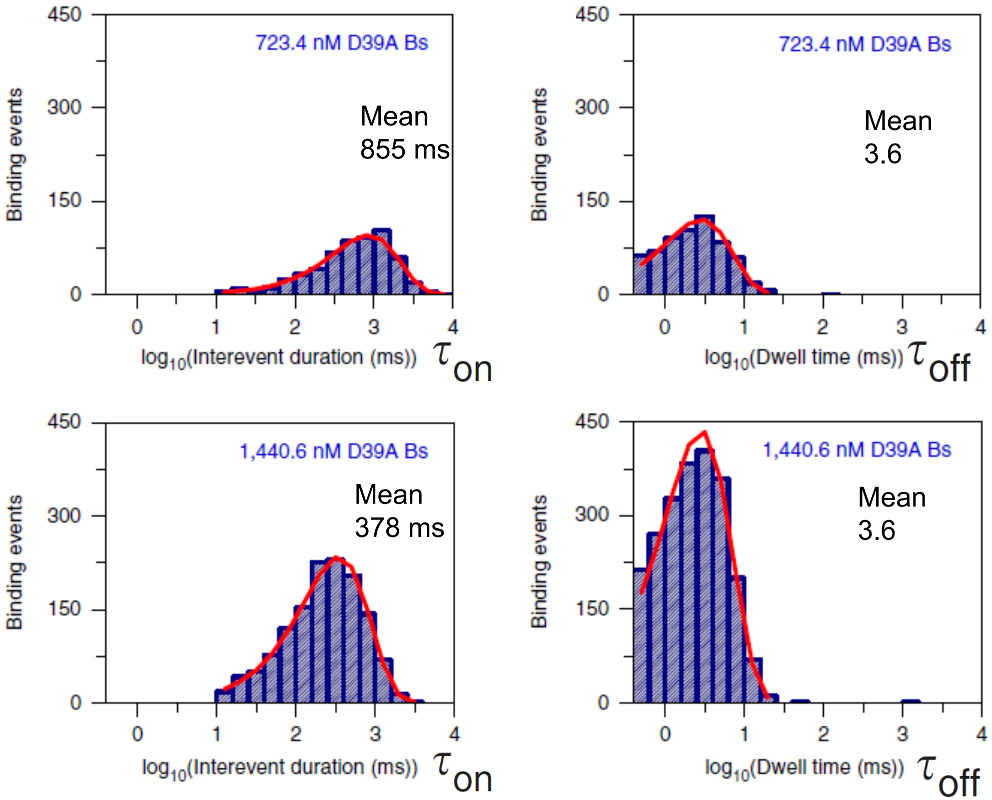
[Bs] (nM)	τ_{on} (ms)	τ_{off} (ms)	k_{on} ($M^{-1}s^{-1}$) × 10^{-7}	k_{off} (s^{-1})	K_d (nM)
12.6	5,054 ± 2,707	1,111 ± 46	1.57 ± 0.84	0.90 ± 0.04	64 ± 02
25.3	2,040 ± 997	1,126 ± 62	1.94 ± 0.95	0.89 ± 0.05	
50.5	1,595 ± 480	1,197 ± 36	1.24 ± 0.37	0.84 ± 0.03	
100.9	920 ± 190	1,263 ± 146	1.08 ± 0.22	0.80 ± 0.10	
201.6	595 ± 300	1,254 ± 147	0.83 ± 0.42	0.81 ± 0.10	
402.3	177 ± 50	1,166 ± 81	1.41 ± 0.39	0.86 ± 0.06	
627.2	117 ± 6	1,109 ± 173	1.37 ± 0.65	0.92 ± 0.16	

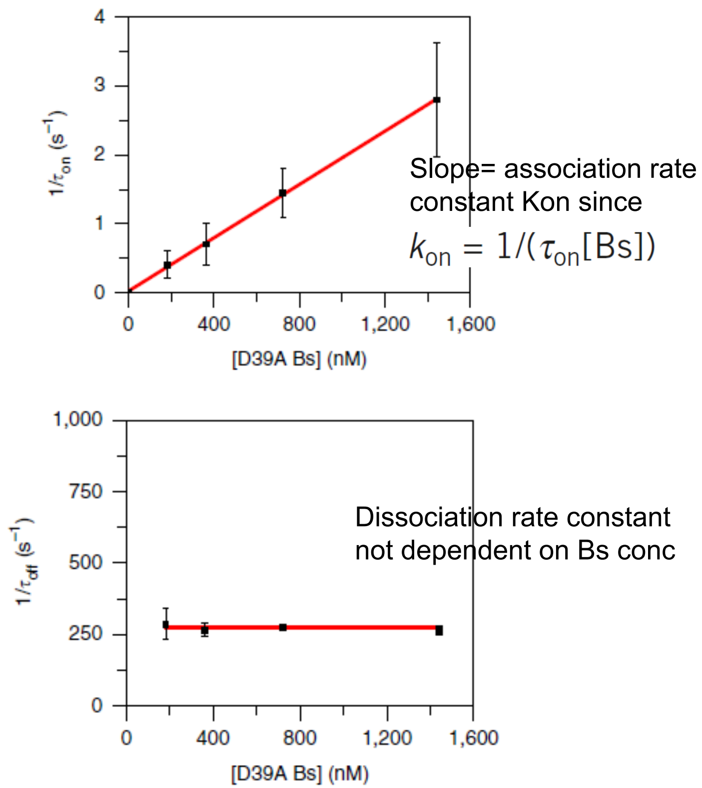
- Kon= 1.34 x 10^7 M^-1s-1 (in literature approx. 10^7-10^8 7 M^-1s-1)
- Koff= 0.86 s^-1
- Kd=64 nM→ high affinity PPI (agrees well with previous kinetic measurement of Bn-Bs interactions)

3.) Paper 1



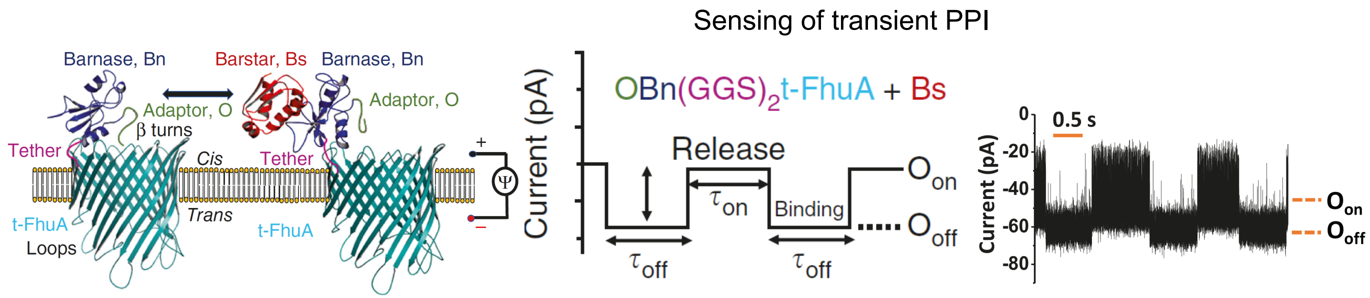
Low-affinity PPI measurement (Bn and D39A Bs)





- Main difficulty is: high-dissociation or low-association rate constants (or both)
- Still two conductance substates: O_{on} (Bn alone, pore more tight) and O_{off} (Bn-Bs, open pore)
- Dissociation of Bs from Bn (τ_{off}) is independent of Bs concentration

3.) Paper 1



Low-affinity PPI measurements (Bn and D39A Bs)

Average values for the tansient Bn-Bs interactions

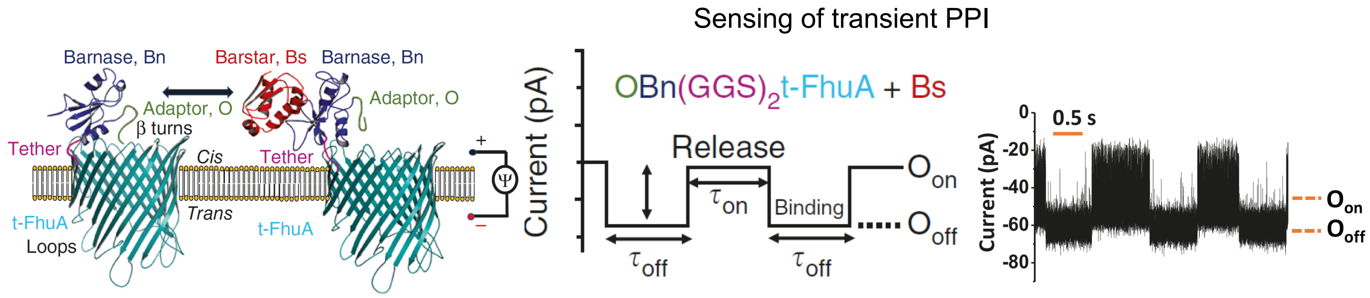
[D39A Bs] (nM)	τ_{on} (ms)	τ_{off} (ms)	k_{on} ($M^{-1}s^{-1}$) × 10^{-7}	k_{off} (s^{-1})	K_d (nM)
181.4	2,437 ± 1,216	3.6 ± 0.6	0.23 ± 0.11	287 ± 54	(146 ± 4) × 10^3
362.4	1,419 ± 614	3.8 ± 0.3	0.19 ± 0.08	267 ± 25	
723.4	690 ± 169	3.6 ± 0.1	0.20 ± 0.05	276 ± 5	
1,440.6	358 ± 105	3.8 ± 0.2	0.19 ± 0.06	266 ± 15	

- K_{on}= 0.193 x 10^7 M-1s-1
- K_{off}= 281 s^-1
- K_d=146 uM→ low affinity PPI (agrees well with previous kinetic measurement of Bn-Bs interactions)

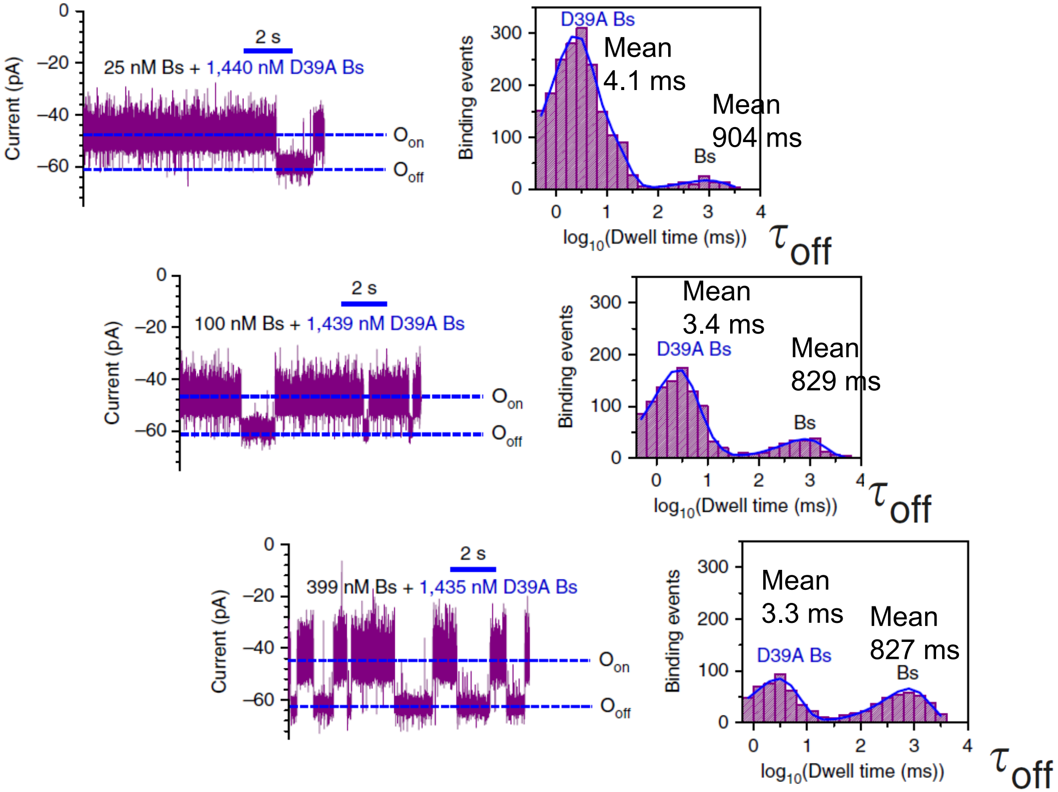
→ **This nanopore sensor can detect transient and weak PPIs at protein ligand concentrations several orders of magnitude below the measured Kd**

→ **Promise for detecting weak PPIs with high koff values**

3.) Paper 1

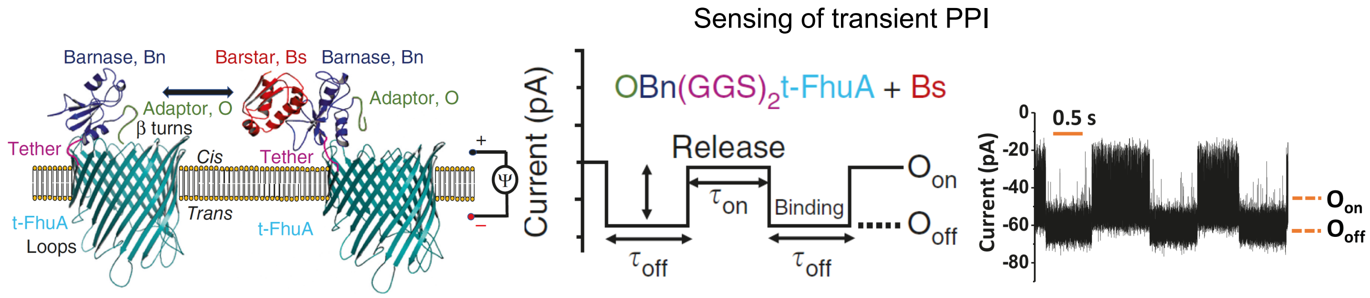


Concurrent detection of **weak and strong** PPIs (Bs and D39A Bs, cis)

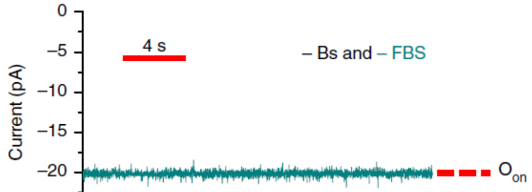


- Detection of long-lived and brief current transitions
- Increase of high-affinity Bs = increase in frequency of long-live current transitions
- Increase of high-affinity Bs= reduction in the frequency of brief binding events
- → discrimination of competitive interactions between two Bs variants for same binding site

3.) Paper 1

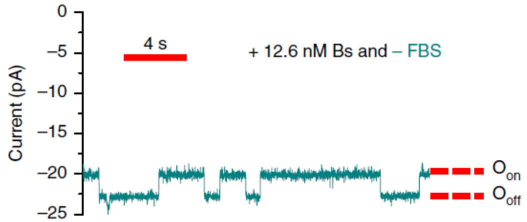


Single-molecule protein detection and observation of transient PPIs in FBS



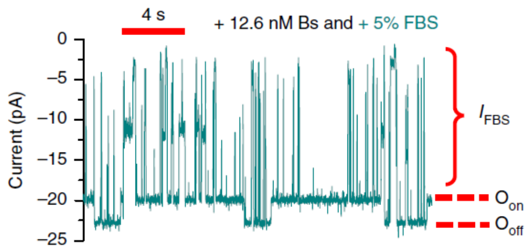
Gated substate (‚closed‘)

- Quiet unitary current at transmembrane potential of -15mV (shorter current blockades with FBS than at -40mV)



Gated substate (‚closed‘)
Open substate (‚no obstruction moiety‘)

- Reversible low-current amplitude transitions between O_{on} and O_{off}



- Serum constituents into pore lumen and blocks it (‚close‘ substate=large current blocks)
- But PPIs-induced (Bs) current transitions distinguishable from large amplitude current blockades

- K_{on} and K_{off} values of transient PPI in absence or presence FBS were similar
- Sensor sensitivity: determination of (known,12.6nM) Bs concentration in using $C_{Bs} = 1/(\tau_{on} k_{on})$ → Bs concentration was 13.3 nM; nanopore **sensor can detect, quantify and obtain detailed kinetics of a protein analyte in a comlex biological fluid**

3.) Paper 1

Conclusions:

- Basis for a nanoproteomics platform or HTS of small-molecules drugs/peptide inhibitors
- Tool for protein profiling and biomarker discovery
- Low amount of protein needed
- Promise for the identification of rare and brief binding events
- Examination of competitive protein interactions with the same binding sites
- K_{off} in the range of 10^2 to 10^3 s^-1 (very short PPIs events like in the cell signaling)
- Genetically encoded: combinatorial sensor library of different protein receptors
- Specific PPIs in a complex biological fluid→ single molecule protein detection in cell lysate, biopsies or blood

4.) Paper 2

nature methods ARTICLES
https://doi.org/10.1038/s41592-018-0100-5

A proximity-tagging system to identify membrane protein–protein interactions

Qiang Liu[1,2,3,4], Jun Zheng[1,3,4], Weiping Sun[1,3], Yinbo Huo[1,2,3], Liye Zhang[1], Piliang Hao[1], Haopeng Wang[1*] and Min Zhuang[1*]

NATURE METHODS | VOL 15 | SEPTEMBER 2018

PUP-IT
(pupylation-based interaction tagging)

Objectives:

- Introduce a new method to study membrane PPIs that are transient an weak in nature (which most of them are)
- Find a method to reveal membrane assisted PPIs that are largely missed in affinity pull down assays

Summary:

- A newly designed orthogonal (to mammalian cells) proximity-based tagging system to study membrane PPIs other than the previous published NEDDylator or BioID system

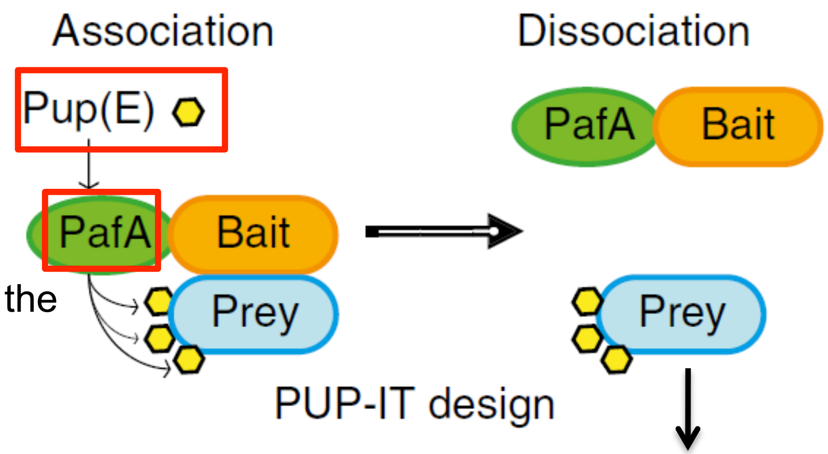
4.) Paper 2

Rational- Pupylation-based interaction tagging

PUP-IT system

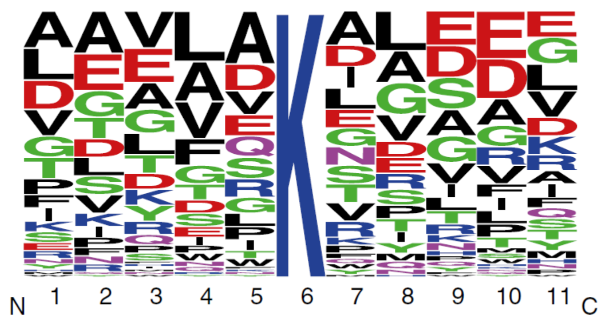
Pup: small bacterial 64aa protein with C-Gly-Gly-Gln
Pup(E): deaminated to Glu

PafA: Pup ligase (ATP) phosphorylation of Glu and consecutive conjugation to the lysine epsylon-aminogroup



Analysis of PUPylated proteins via MS

Sequence alignment of PafA modification sites

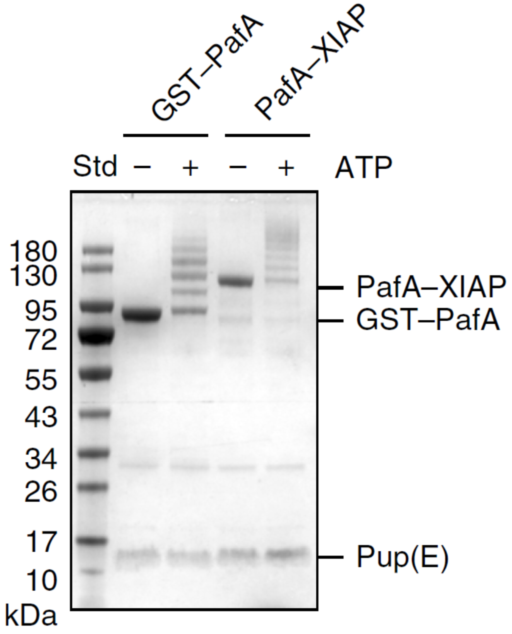


- Genetically **fusion of PafA** (Pup ligase) **to bait** protein
- Assumption: **Lysine is universal** in human proteins and suitable for a tagging system
- Any proteins (prey) that interact with the bait and contain lysine within the radius of PafA will be PUPylated

- Demonstrate **PUP-IT(CD28)** system on the **CD28 costimulatory signaling pathway**
- Application of **PUP-IT(IL-2)** on the extracellular protein **IL-2** to demonstrate ligand-mediated receptor labeling

4.) Paper 2

Biochemical characterization of PUP-IT system



Test promiscuity of PafA:
At the residues around target lysine- fusion of PafA to non-substrate proteins: PafA-GST and XIAP-GST

→ Modification with multiple Pup(E) in the presence of ATP

4.) Paper 2

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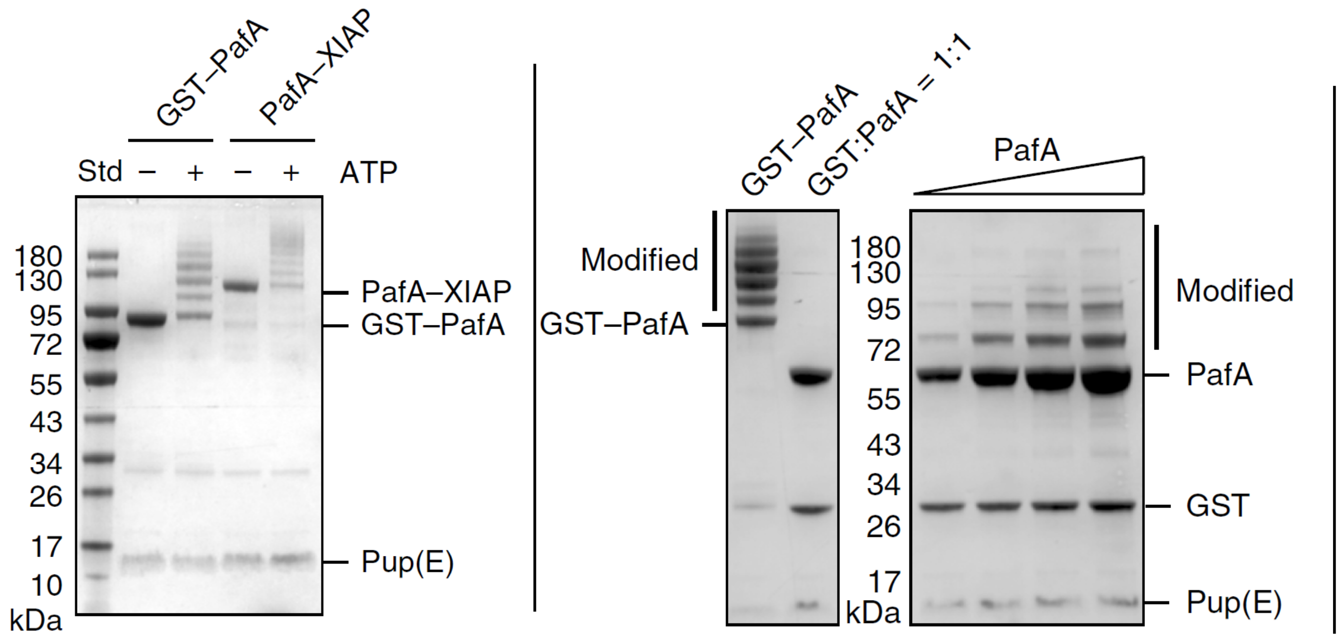

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Test for proximity-labeling:
In vitro pupylation in recombinant GST-PafA or GST with free PafA

→ GST alone is not modified
→ PafA is self-modified in higher PafA concentrations
→ PafA as good proximity tagging system

4.) Paper 2

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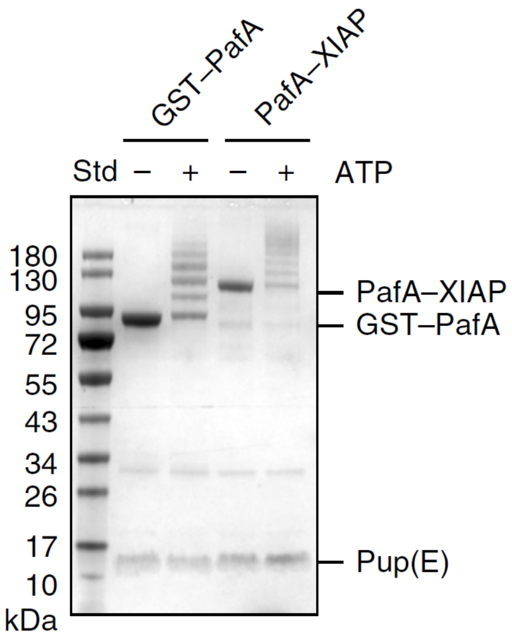

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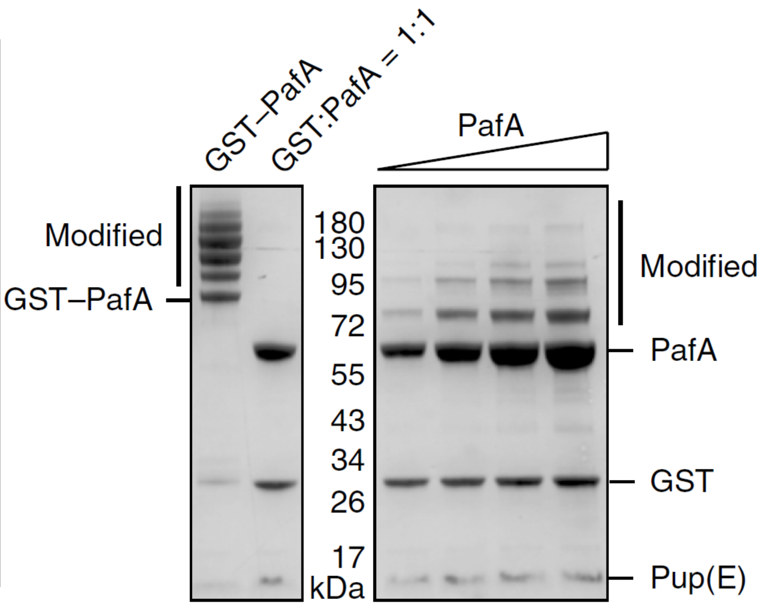

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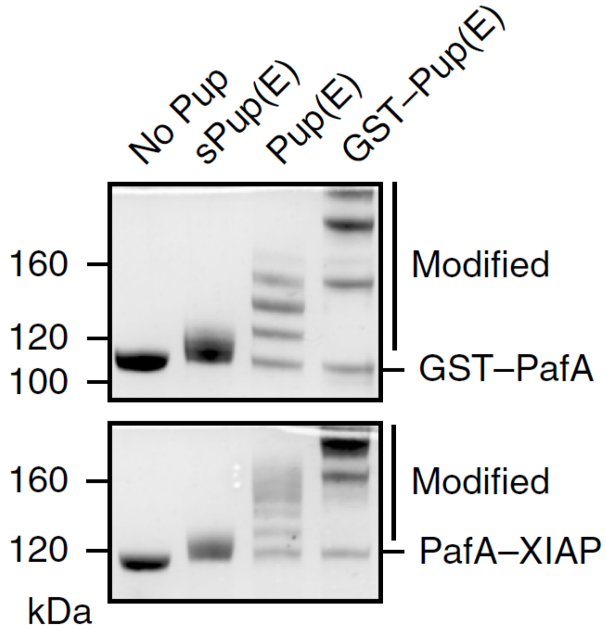

Test substrate specificity:
Different forms of Pup as substrate

- → Truncated version sPup(E) can still be conjugated to K
- → Specific since the activated Pup(E) intermediate cannot diffuse from the enzyme

4.) Paper 2

Characterization: PUP-IT labels weak PPIs

Bait	K_d (μM)
pep1	3.7
pep2	76
pep3	266
pep1(mut)	>1,000

→ Kd in micromolar range considered as weak PPI interactions

4.) Paper 2

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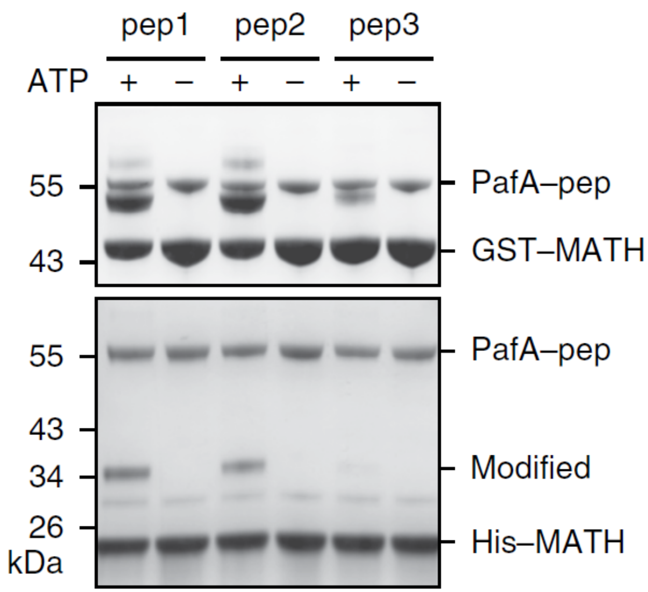

→**In vitro**

→ E.g. GST-tagged MATH was pupylated with all three low affinity PUP-IT peptides 1-3 in the presence of ATP

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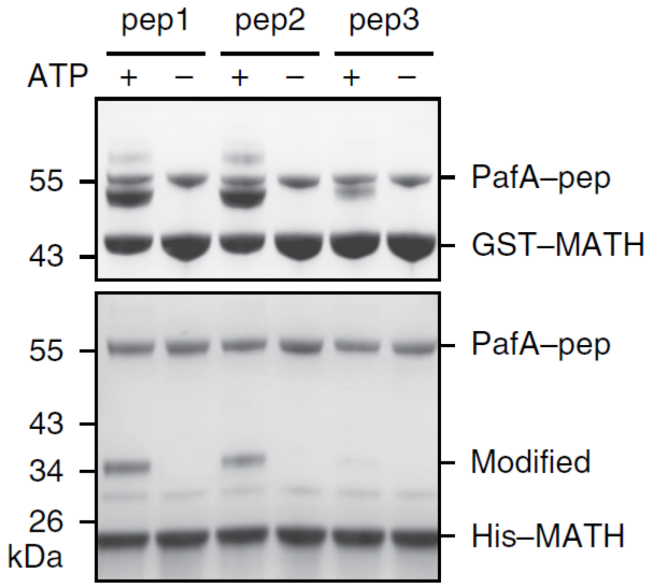

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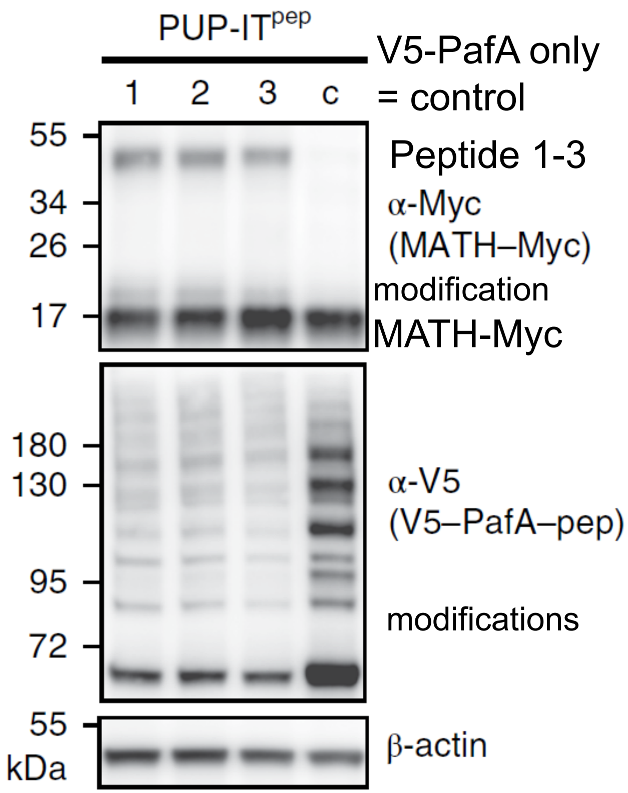


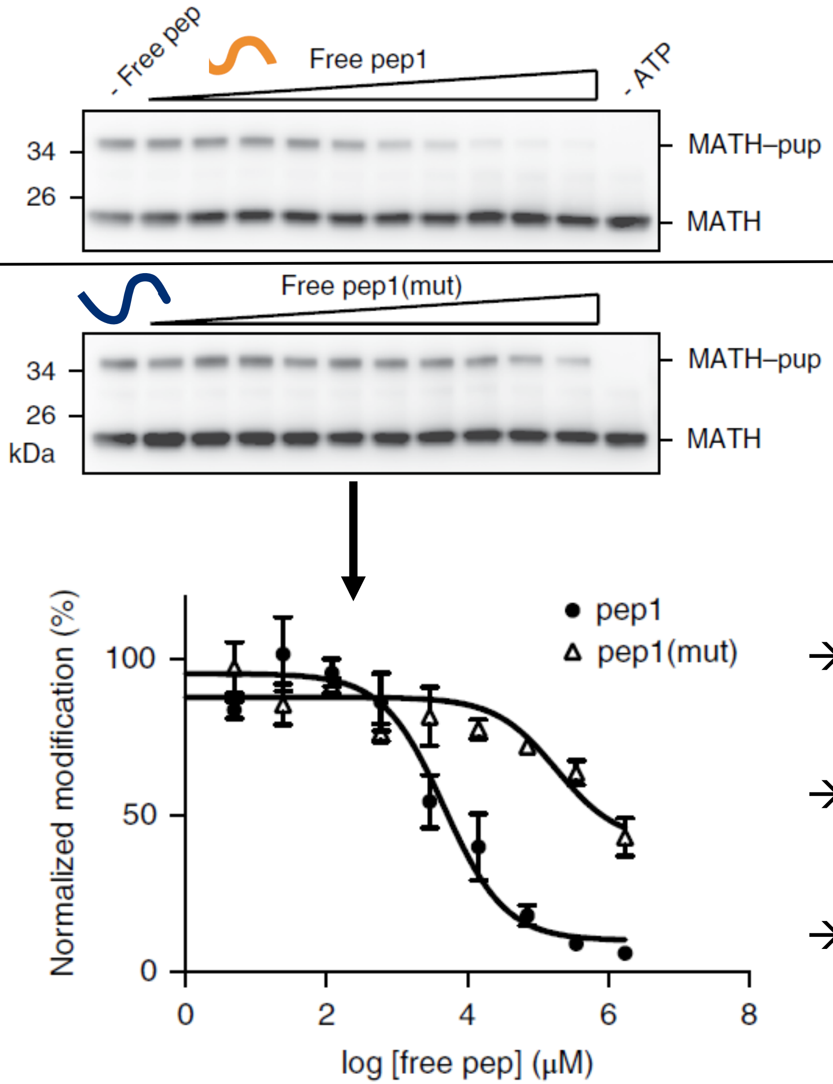
→Suitable for weak PPIs intracellularly?

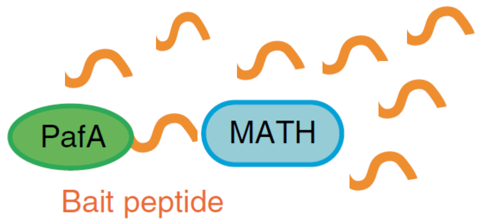
→**In vivo** co-transfection of PUP-IT peptides(PafA-pep), Pup(E) and MATH domain (SPOP/Cul3 ubiquitin ligase)

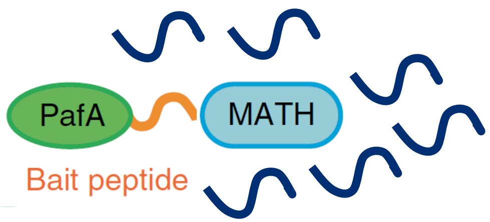
→ PUP-IT suitable for cellular studies

4.) Paper 2

Characterization: Dose dependent inhibition of MATH domain





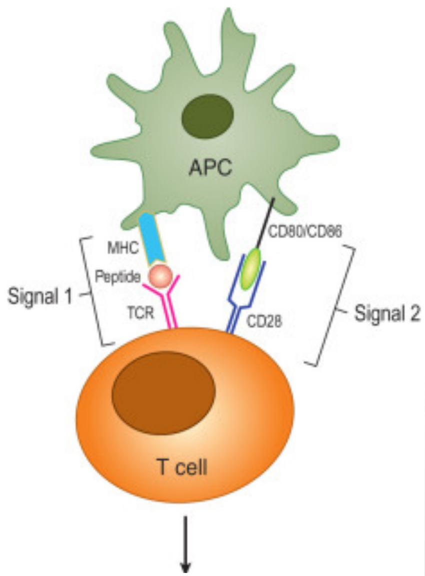


- → Free added pep1 to Paf1-pep1 MATH inhibited the pupylation (modification)
- → Addition of mutated pep1 (mut) to Paf1-pep1 MATH could not fully inhibit reaction
- → Specific interaction between MATH and pep1 is required for Pup(E) labeling

4.) Paper 2

PUP-IT for identification of **cytosolic binding proteins** of membrane proteins

T-cell activation

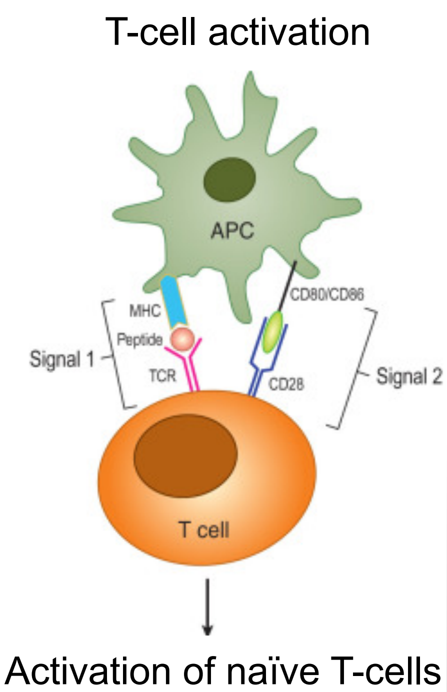


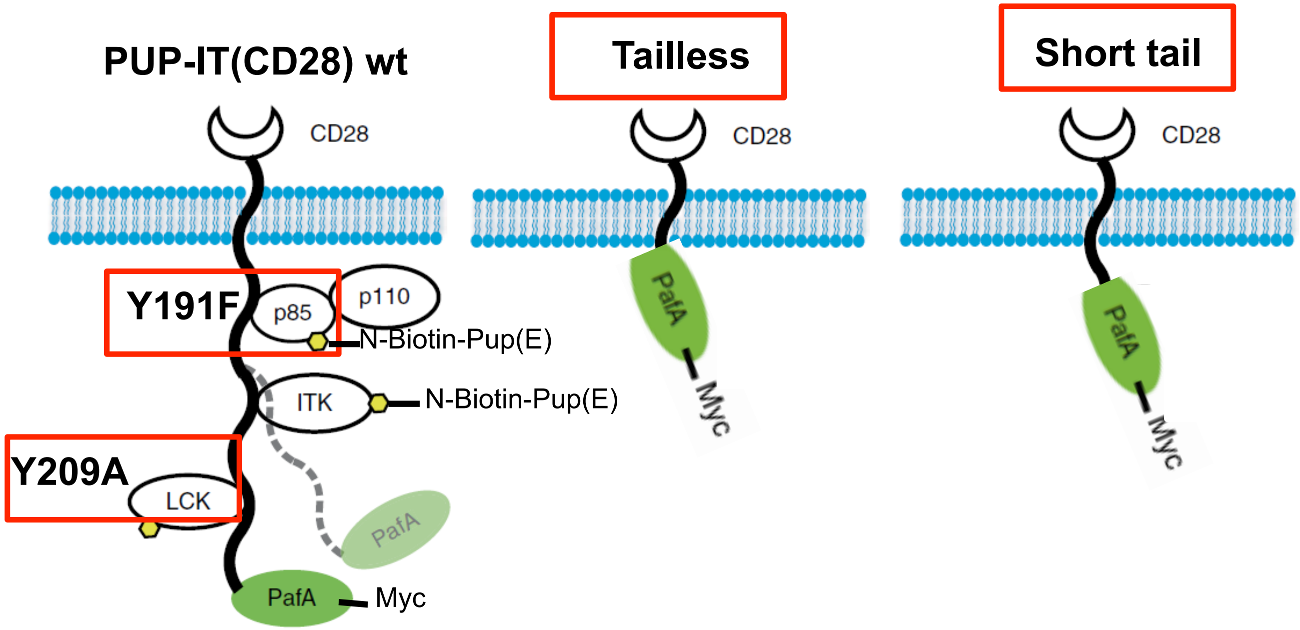
Activation of naïve T-cells

- Studying the interactome of membrane costimulatory receptor CD28 in T-cell activation

4.) Paper 2

PUP-IT for identification of **cytosolic binding proteins** of membrane proteins

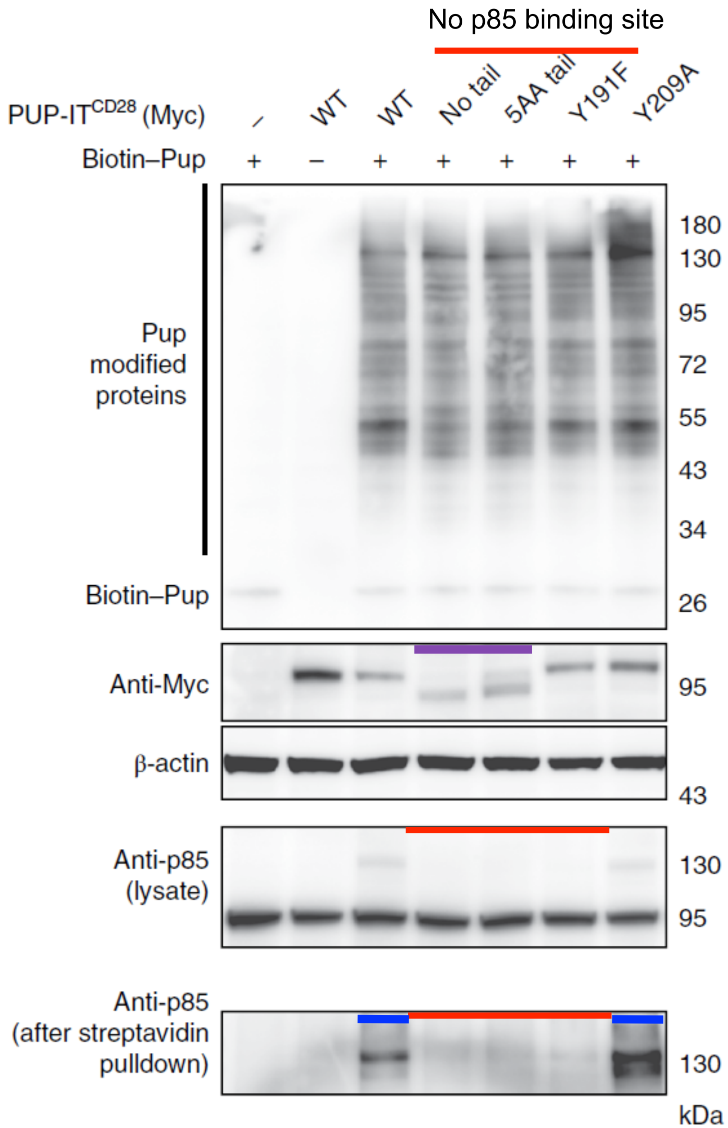


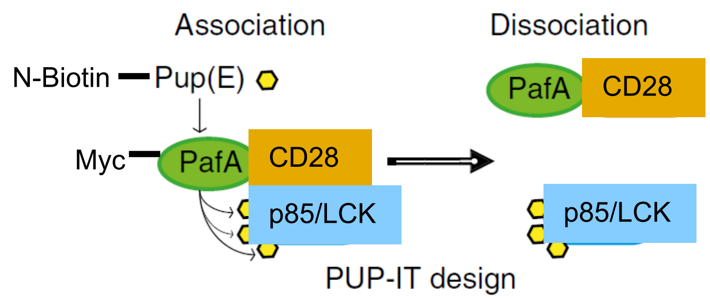


- Studying the interactome of membrane costimulatory receptor CD28 in T-cell activation
- p85, LCK have been shown to interact with the cytosolic tail of CD28
- **PUP-IT(CD28)wt**: C-terminal PafA fusion (Wt)
- Controls: - PUP-IT(CD28) with **p85-binding deficient** mutant **(Y191F)**
  - PUP-IT(CD28) with **LCK-binding deficient** mutant **(Y209A)**
  - PUP-IT(CD28) **tailless** CD 28 **and short-tailed** CD28 (5aa)

4.) Paper 2

PUP-IT for identification of **cytosolic binding proteins** of membrane proteins

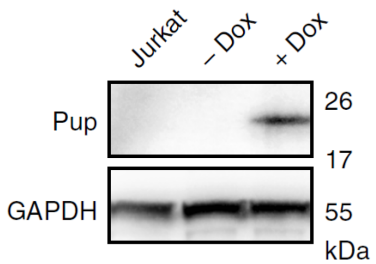




- No tail (tailless), short-tailed and Y191F PUP-IT(CD28) variants lack p85 binding site → no modification of p85 **(red bar)**
- WT and Y209A (only LCK binding deficient) could biot-pupylate p85 → shown with SA pull-down **(blue bar)**
- Anti-Myc antibodies show that 'no tail' and '5AA tail' CD28-PafA-Myc are present and shorter in length **(violet bar)**

4.) Paper 2

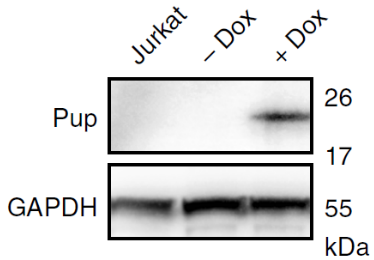
PUP-IT for identification of **cytosolic binding proteins** of membrane proteins



- Stable Jurkat cell line with stable PUP-IT(CD28) expression
- Doxycyclin induced (TET-ON) Bio-PupE to initiate labeling process→ tight control of Bio-PupE reduces background

4.) Paper 2

PUP-IT for identification of **cytosolic binding proteins** of membrane proteins



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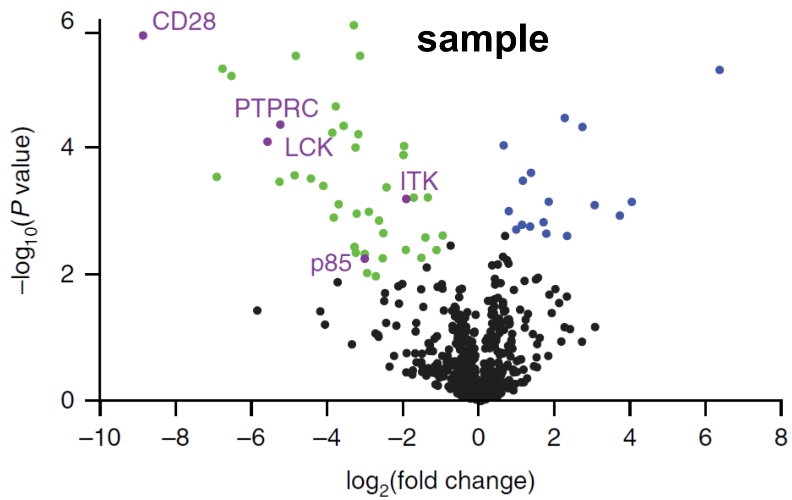
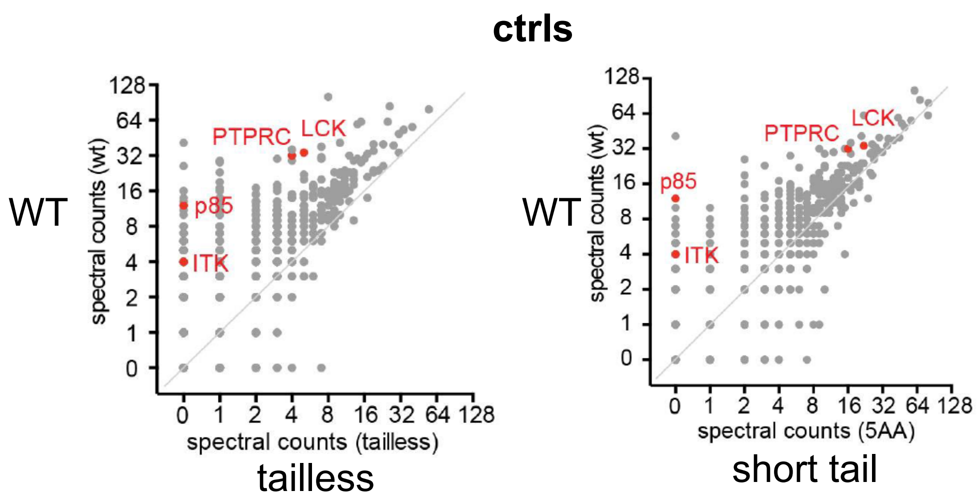
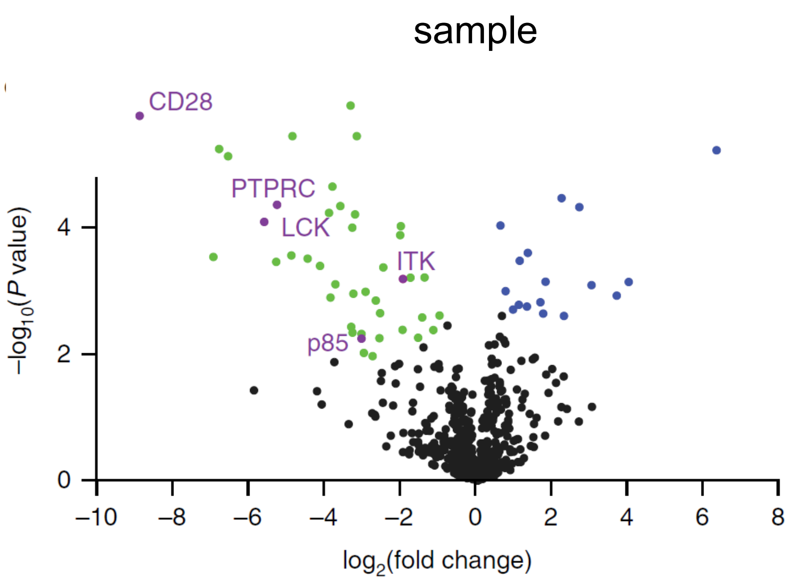


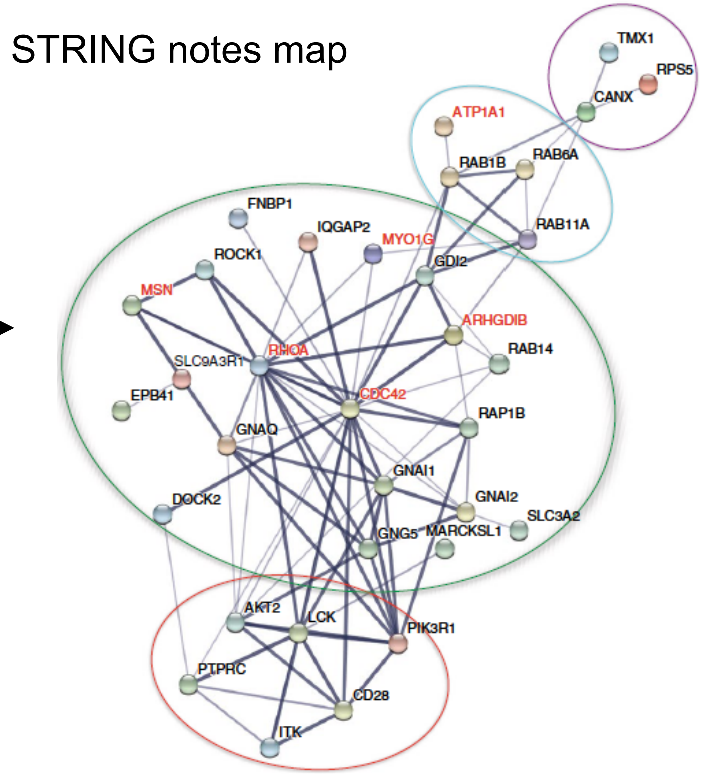


- Also co-transfection of PUP-IT(CD28) and Biotin-Pup(E) in Jurkat cells with cnsecutive SA pulldown of interactor proteins and LC-MS/MS characterization of Pup(E)-modified proteins
- Known CD28-tail interactors (e.g. p85, ITK, LCK) were highly enriched in PUP-IT(CD28) but not in PUP-IT(CD28_no tail/5AA tail)
- Total >41 proteins identified as potential CD28-tail-binding partners

4.) Paper 2

PUP-IT for identification of **cytosolic binding proteins** of membrane proteins



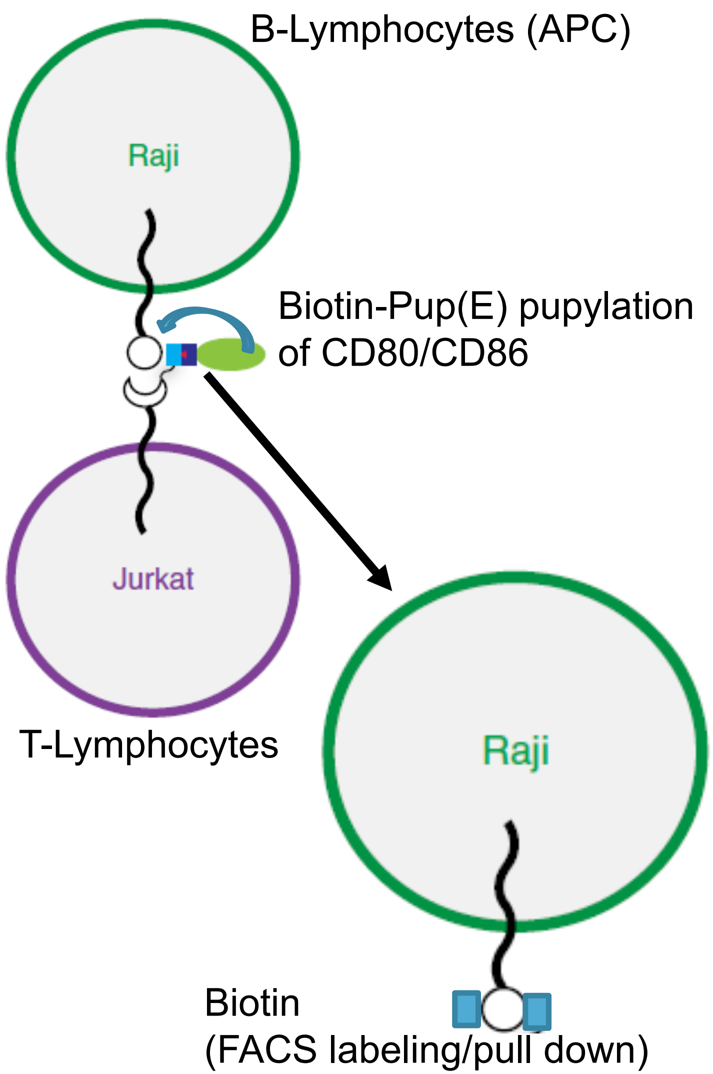


- All connected with the CD28 (colored rings):
- Signaling pathway
- Cytoskeletal remodeling
- Protein folding and processing
- Vehicle transport

- Modified lysine sites were all located on protein surface (in line with Pup modification of GST)

4.) Paper 2

PUP-IT for labeling of **cell-surface proteins**



4.) Paper 2

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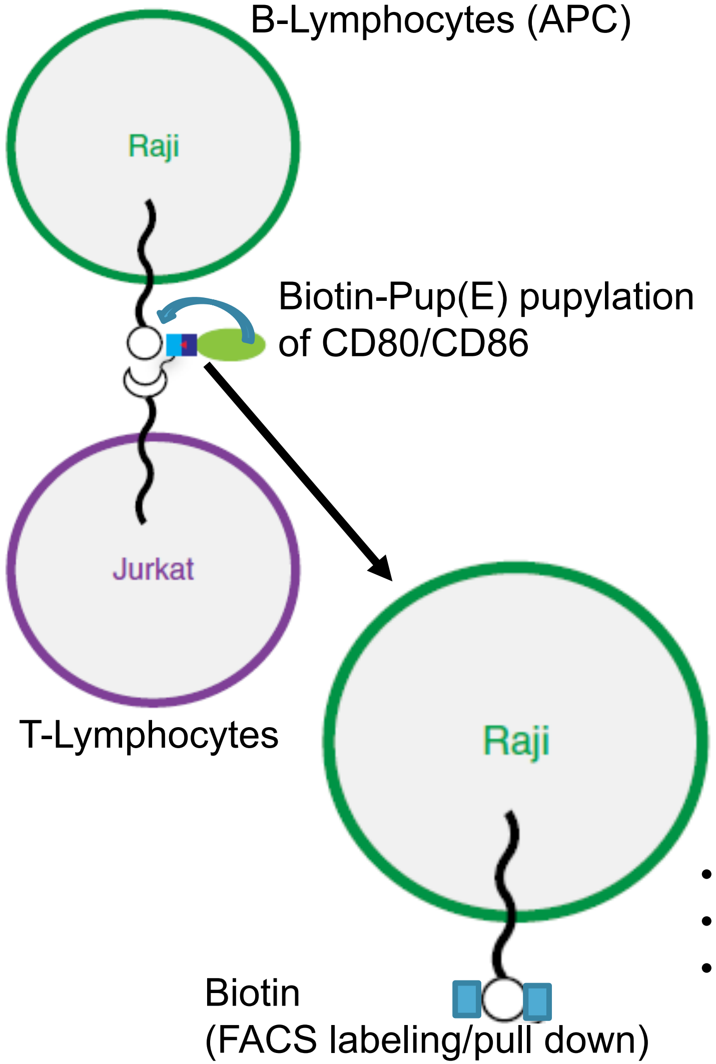
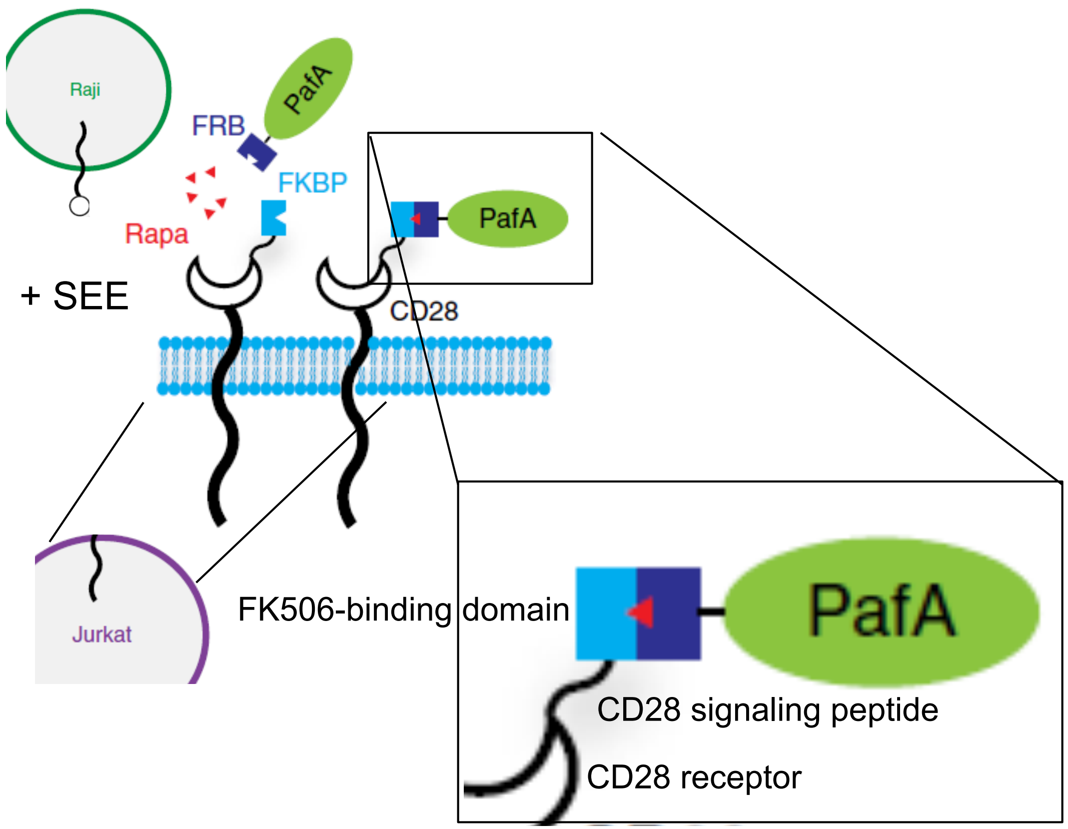
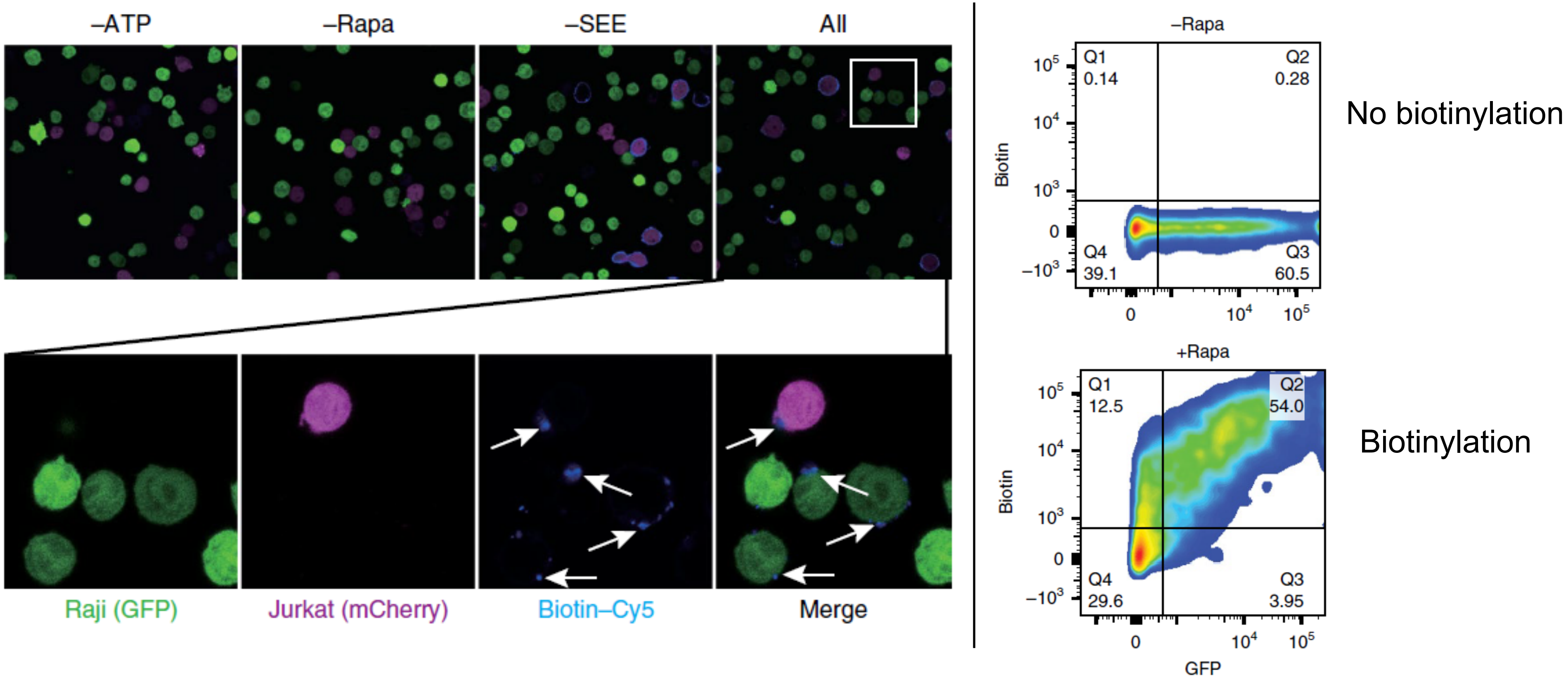




- Can PUP-IT perform ligand labeling (CD80/CD86) on APCs ?
- **Heterodimerization of FKBP and FRB** upon Rapamycin addition
- SEE (antigen) peptide required for engagement or Raji MHC and Jurkat T-cell receptor

4.) Paper 2

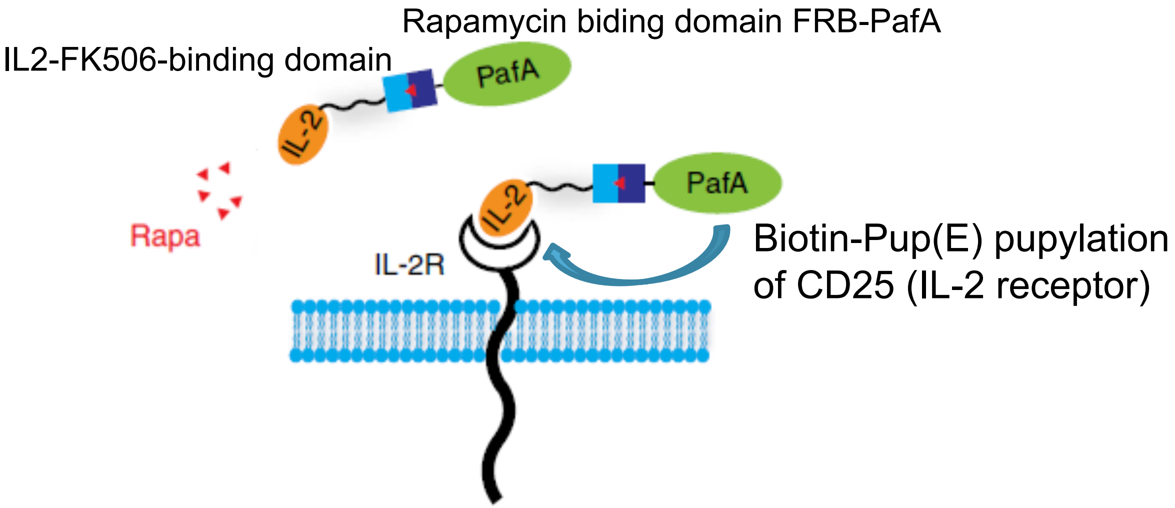
PUP-IT for labeling of **cell-surface proteins**



- Whole cell surface covered with biotin (antiBiotin-Cy-5) in FACS
- Merged: punctate modification sites on Raji cell surfaces consistent with partial direct contact of T-and B-cells
- No biotin labeling if GFP+**T-cells** and mCherry+/FKBP-CD28 expressing **T-cells** were co-cultured with addition of ATP, Rapa, FRB-PafA and SEE→ labeling requires direct interaction between B- and T-cells

4.) Paper 2

PUP-IT (IL-2) mediates **receptor labeling**



- Identification of receptor-ligand pairs: Does PUP-IT (Ligand) label its receptor(s)?
- IL2-FKBP: mammalian expression/FRB-PafA: prokaryotic expression
- Stimulation of T-cells and then addition IL-2-FKBP, FRB-PafA, w/wo Rapamycin

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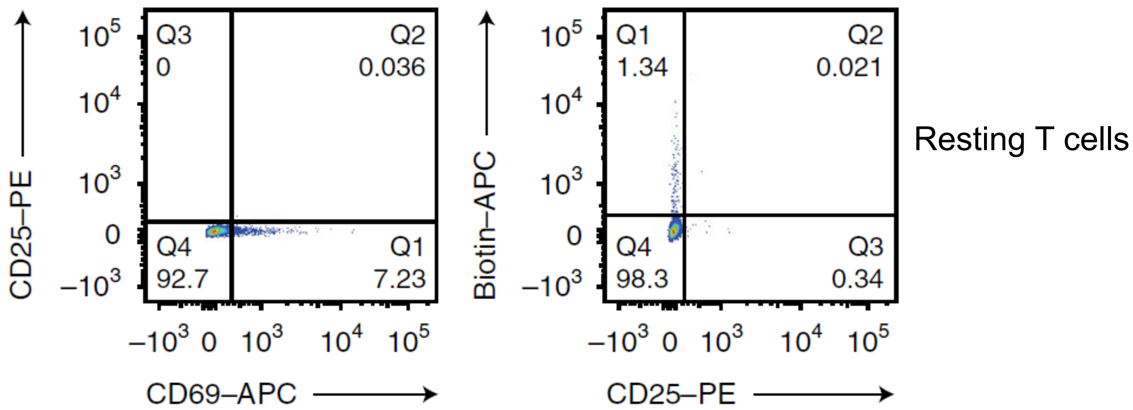

- Only activated T-cells (CD69+) could be labeled with biotin on the cell surface
- CD25+ (T-cell receptor) could no longer be detected: CD25 was labeled with Biotin-Pup(E) at lysine sites and modification blocks epitope for CD25 antibody for FACS analysis

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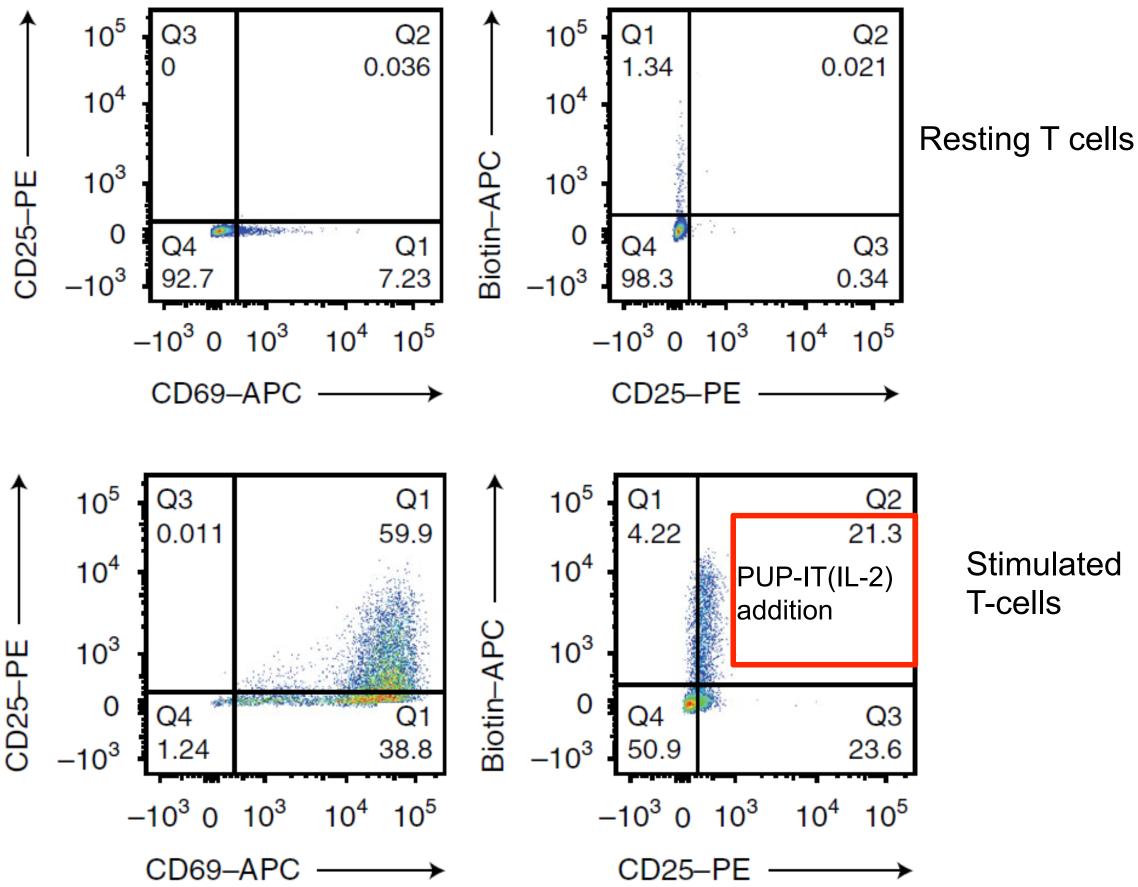

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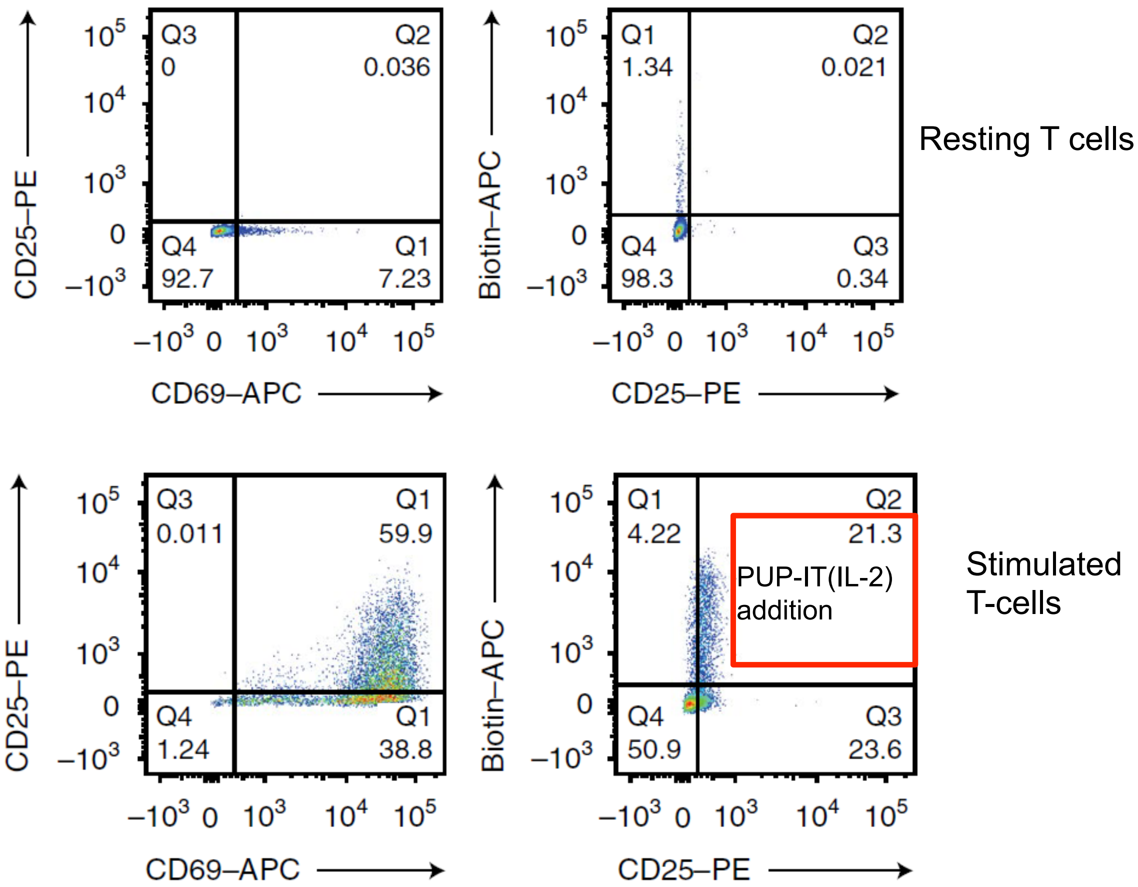

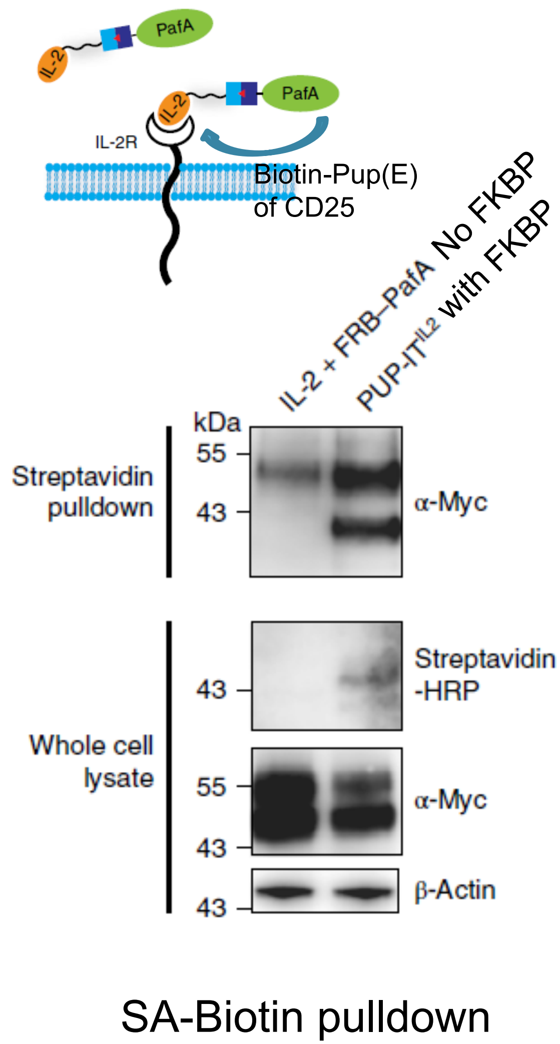

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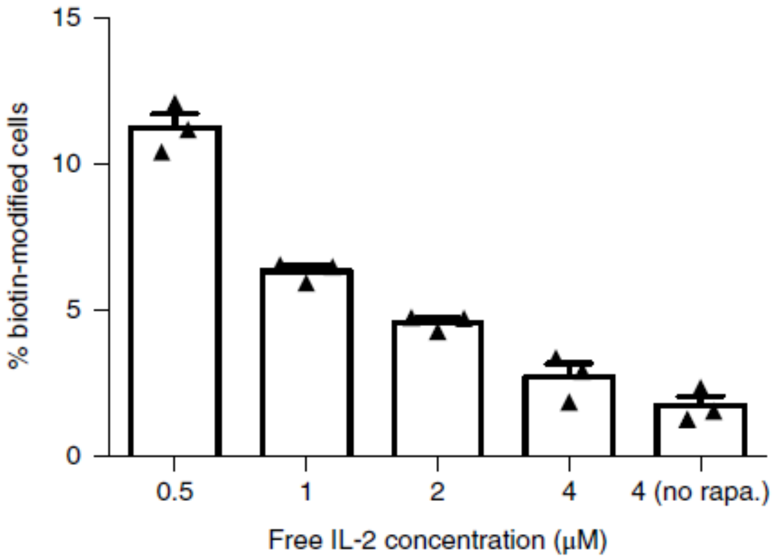
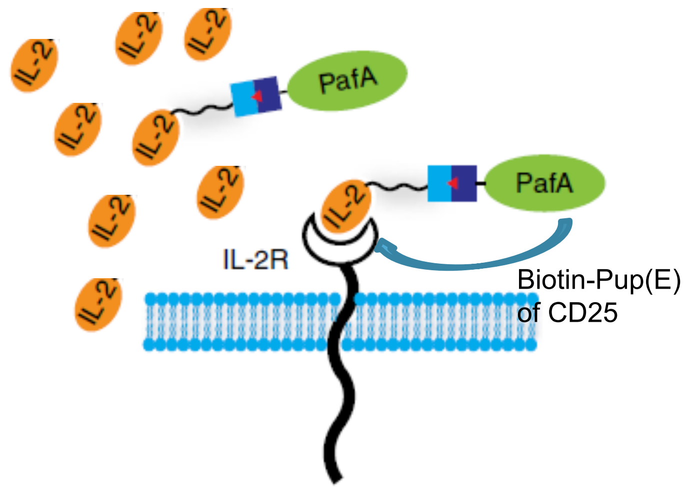




- Free IL-2 competes with IL-2-FKBP for CD25 binding: higher free IL-2= less cell-surface modifications
- To confirm that cell-surface labeling was induced by interaction between IL-2 and CD25 they titrated IL-2-FKBP in increasing concentration and this induced higher grade of modifications
- **Summary: PafA fused to a peptide cytokine can effectively label corresponding receptor**

4.) Paper 2

Conclusions:

- Flexible linker (15-20aa) between PafA and the bait protein allows a radius of 60-80 Å (60kDa globular protein)
- Larger proteins need larger linker
- Self-modification is inevitable with PUP-IT
  (potentially inactivate enzyme, deplete substrates and background signal)
- On-site ligation by PafA does not guarantee that labeled proteins interact directly with the bait→ indirect proteins within tagging radius could be modified
- Good tool to study membrane proteins
- Since PUP-IT is from prokaryotic systems minimized risk of interference with normal cellular events (orthogonal)
- PUP-IT keeps the activated Pup(E) bound to the enzyme and operates with more restricted labeling radius
- PUP-IT seems to be the most specific tagging system available
- They do not talk about the velocity of the system
- Biotin-phenol or H2O2 is not required and PUP-IT does not cause cell stress
- Pup(E) is a rather large substrate and cannot diffuse across membranes! Method not suitable for studying interactions between organelles
- Pup(E) modification retain motif of lysine and can be identified by MS
- Potentially powerful in animal models since no chemical compounds must be delivered into cells→ All components of PUP-IT can be expressed in cells

5.) General conclusions

- The **nanopore sensor**:

A valuable instrument for real-time measurement of single, weak and transient molecule/protein interactions and their kinetics in complex fluids like mammalian serum

- **PUP-IT system**:

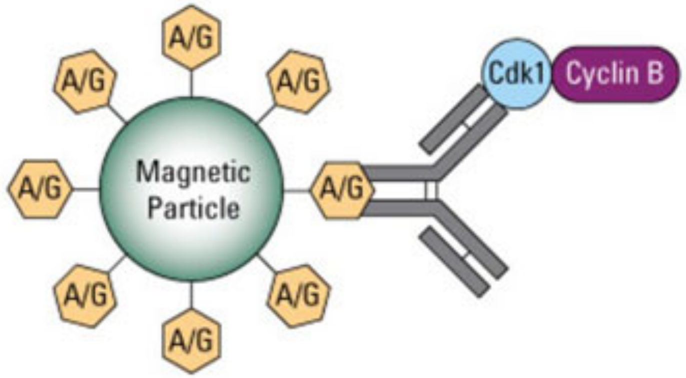
A potentially valuable orthogonal (to mammalian cells) instrument to investigate weak and transient membrane protein interactoms

Thank you for your attention.

Addendum Some commonly used methods

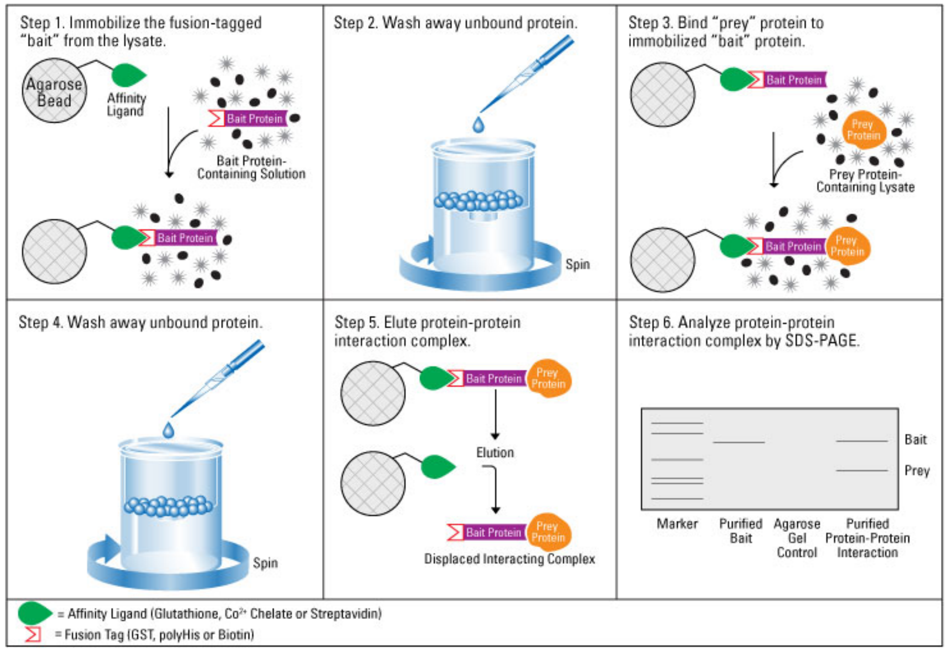
Co-IP

- Popular for protein interaction discovery
- Antigen-Ab (bait) and interacting protein (prey)
- Protein identified: Western, SDS-PAGE



Pull down

- Tag (GST, Biotin…)
- Matrix (GSH,..)
- Protein complex subjected to SDS-PAGE
- Protein identified: Western, MS, Sequencing

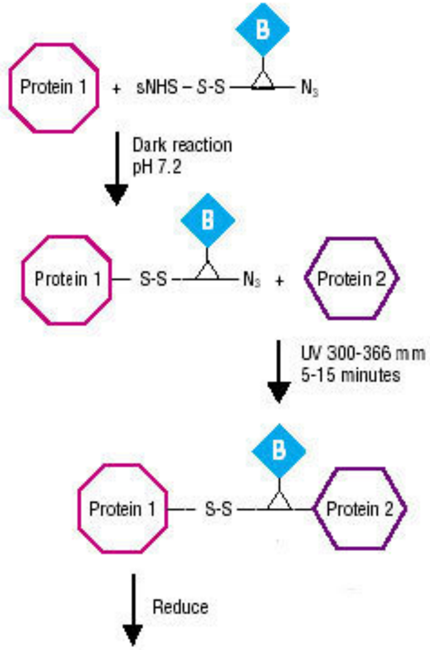


Addendum Some commonly used methods

Label transfer protein interaction analysis

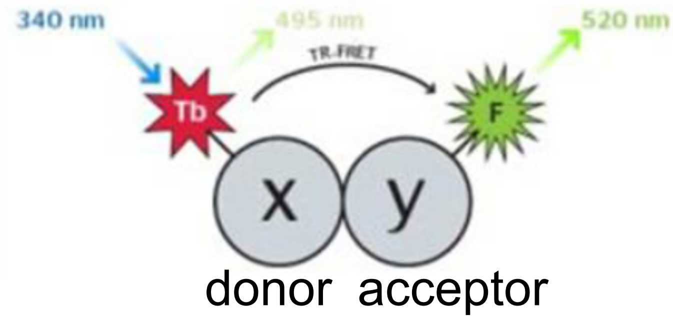
(crosslinking protein interaction analysis)

- For weak or transient interactions



FRET

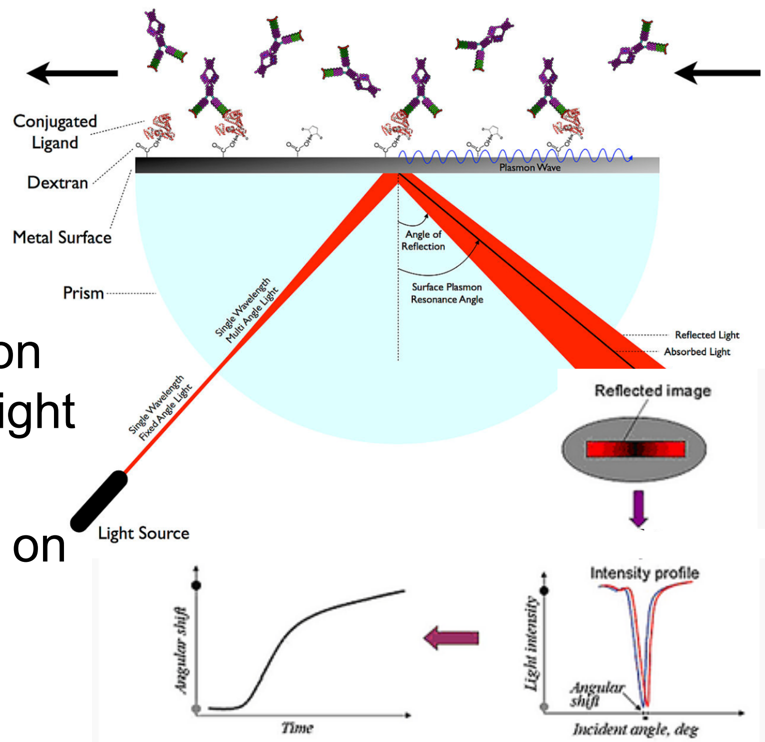
- Förster Resonance Energy Transfer
- Energy transfer from donor fluorescent dye to an acceptor fluorescent dye when one of two neighboring fluorescent dyes is excited
- If FRET occurs: fluorescence of donor decreases and acceptor increases



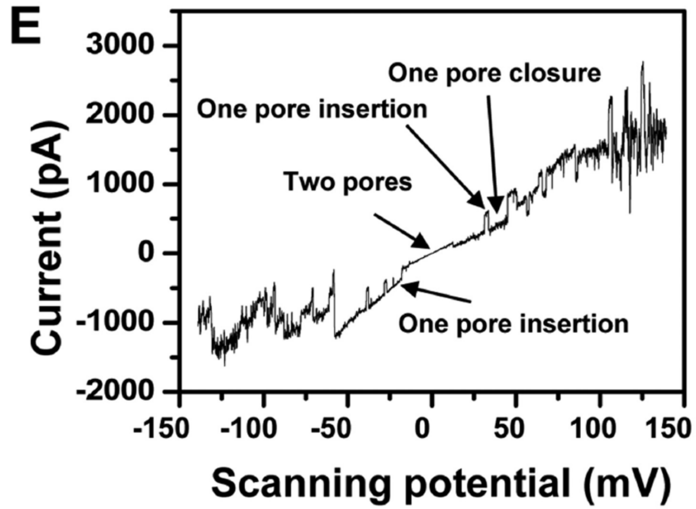
Addendum Some commonly used methods

Surface Plasmon Resonance Spectroscopy (SPR)

- Protein immobilization on gold/dextran surface
- Longitudinal waves when prism irradiated with light =surface plasmon
- Angle larger than critical angle, surface plasmon is resonant with the vibration of the irradiated light =surface plasmon resonance
- The light of resonated wavelength is absorbed on metal surface
- not absorbed wavelength is totally reflected
- Dark line in the spectrum when SPR is excited
- PPI increases density on metal surface and changes incident angle= resonance angle causing SPR (absorption changes, dark line detection changes)



Addendum Suppl, nanopore insertion



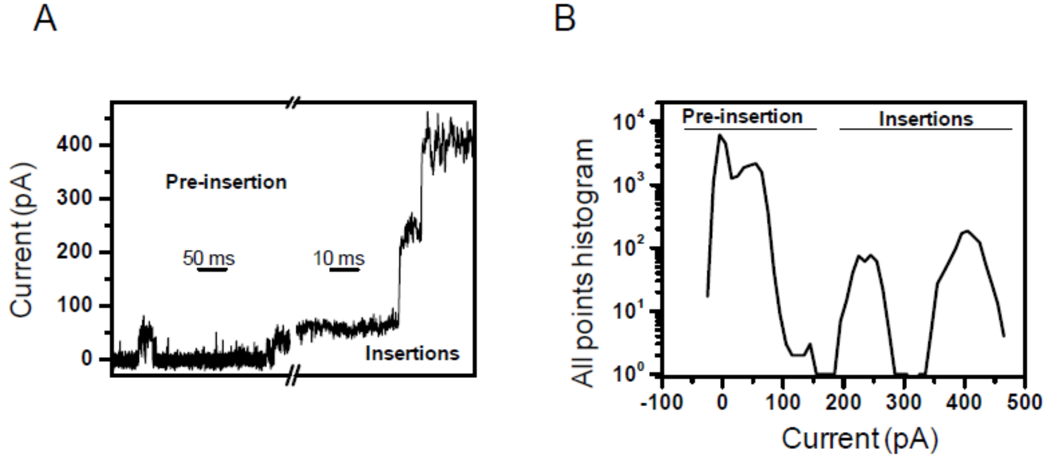


Figure S7. Single-channel electrical recordings of the membrane extracted FhuA ΔC/Δ4L protein (mFhuA ΔC/Δ4L). (A) A stepwise increase of the electrical current recordings showing pre-insertion activity of the mFhuA ΔC/Δ4L protein proceeded by single-channel insertions into the lipid bilayer. Protein was added to the *cis* side of the lipid bilayer. The transmembrane potential was +40 mV. Break was made in the X axis in panel (A) to compress the long trace, two different time scales are shown to the left and the right of the break. The pre-insertion conductance is 1.2 nS. The conductances of the first and second insertions are 5.4 and 4.2 nS, respectively. (B) All-points current amplitude Gaussian histograms that show the most probable current sub-states of the channel.