Harnessing nanopores for single-molecule enzymology and protein sequencing

Giovanni Maglia

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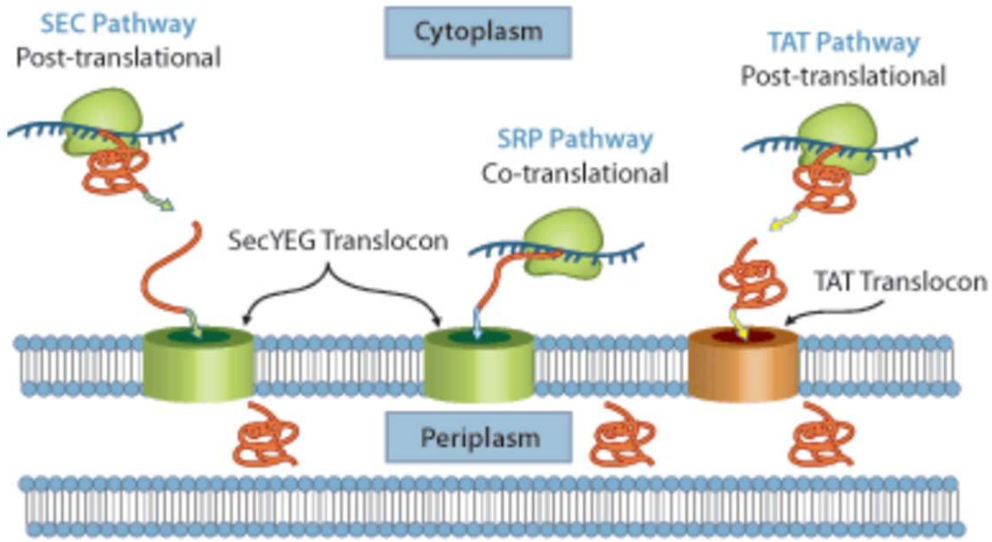
rijksuniversiteit groningen

Outline

- Nanopore technology
- Single-molecule nanopore enzymology and metabolite sensing
- Sequence identification of proteins and peptides
- *Control of transport across nanopores*

Protein transport across nanopores

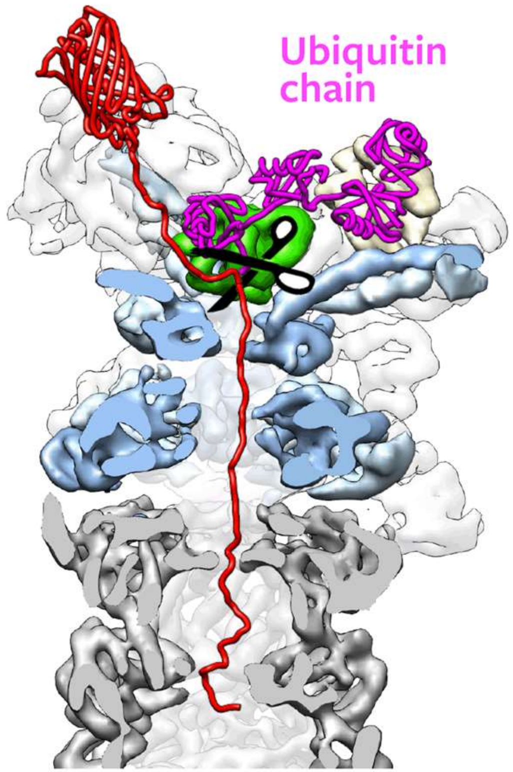
Secretion



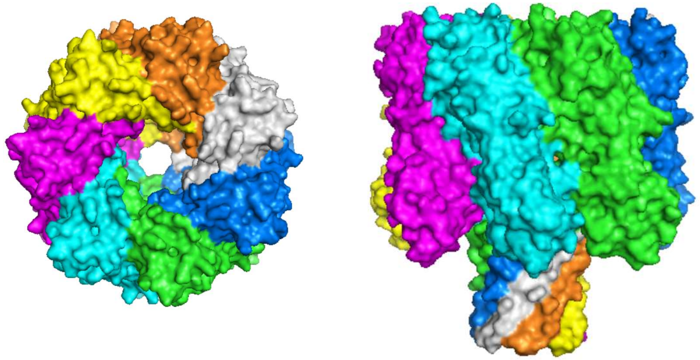
Un-folded

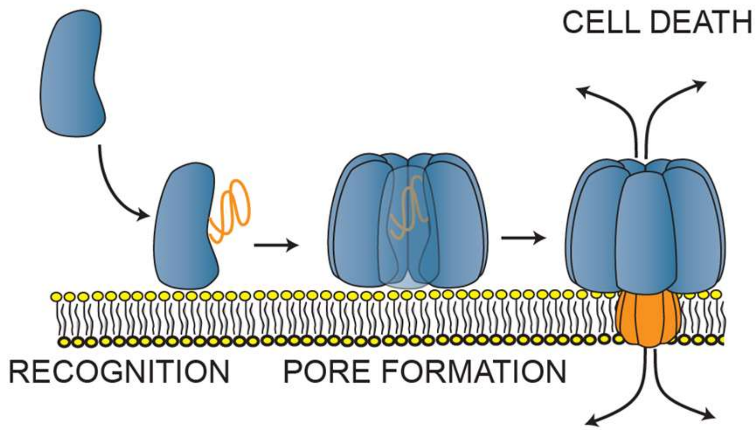
Folded

Degradation



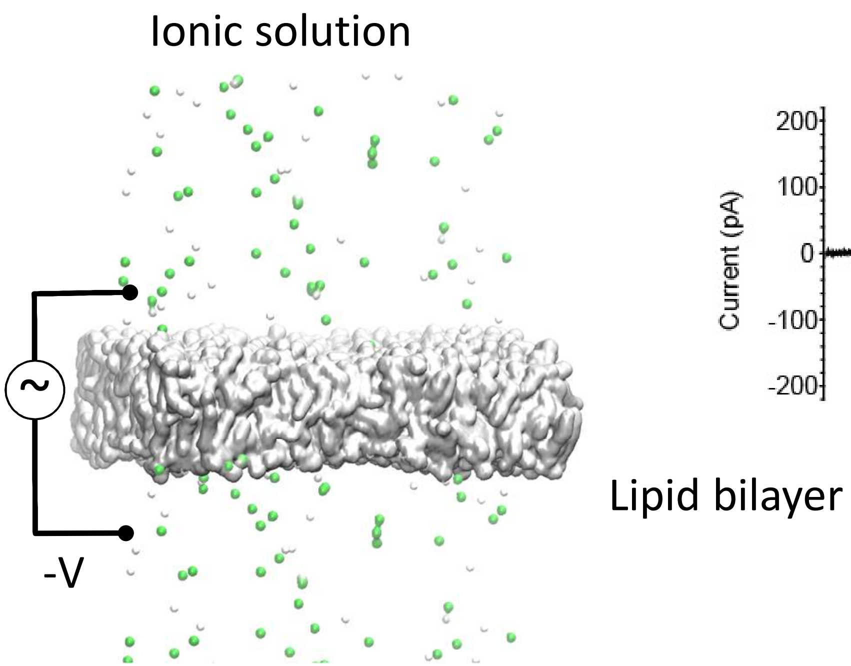
Biological nanopore

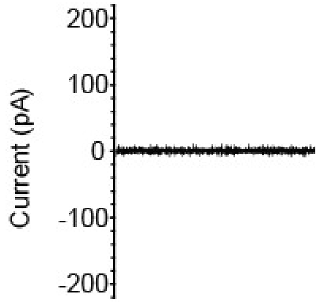




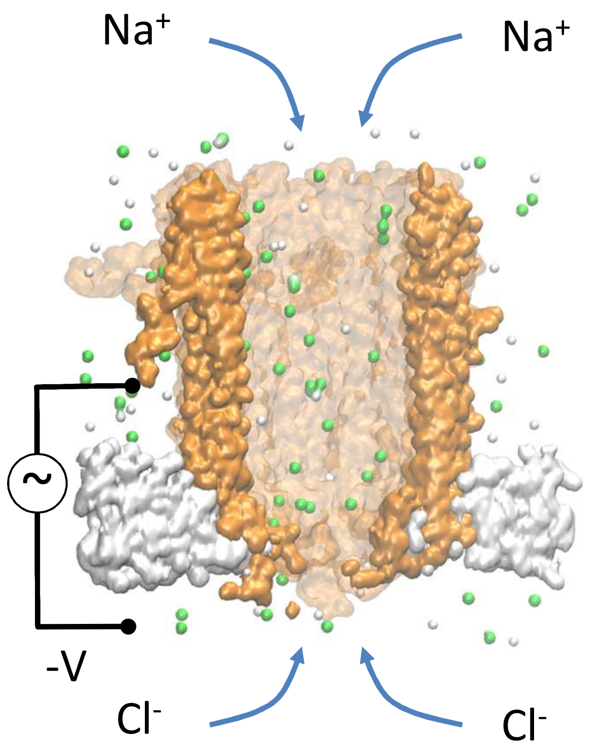
- Water soluble monomer that assembles on membrane
- Oligomers
- Toxins (cell army)
- Porins (control of membrane traffic)
- Highly stable
- **dsDNA** and **protein** analysis

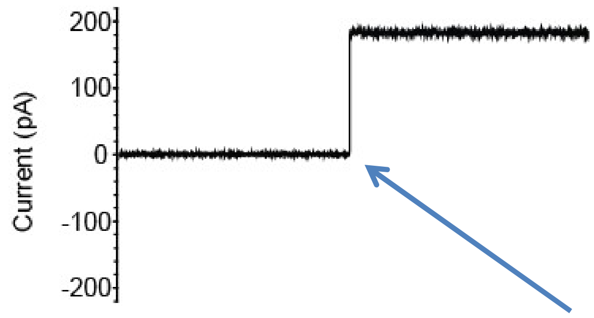
Biological nanopore technology





Nanopore technology

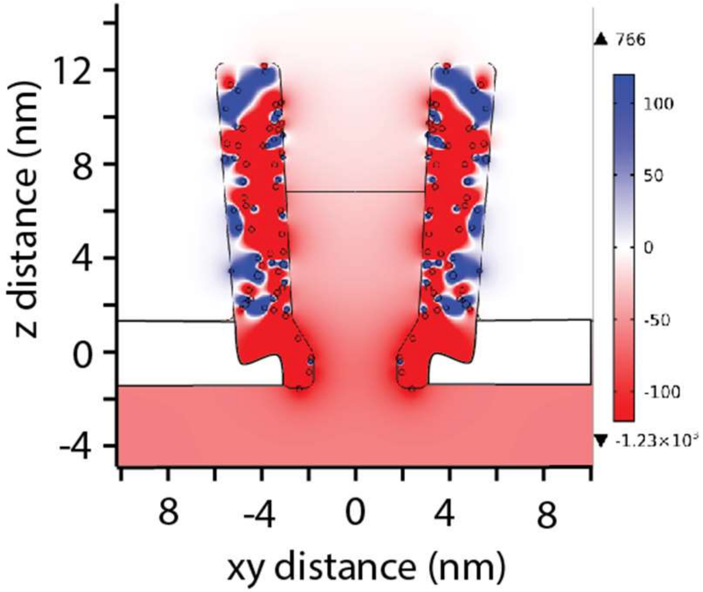


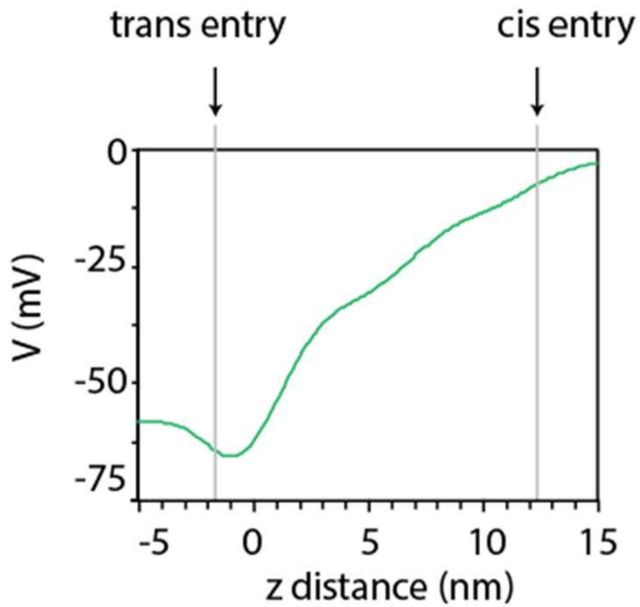


Single nanopore

- ~ 10^9 ions per seconds
- Single molecule (20^{21} molecules in a tablet of ibuprofen)

Trapping Forces : Electrophoresis

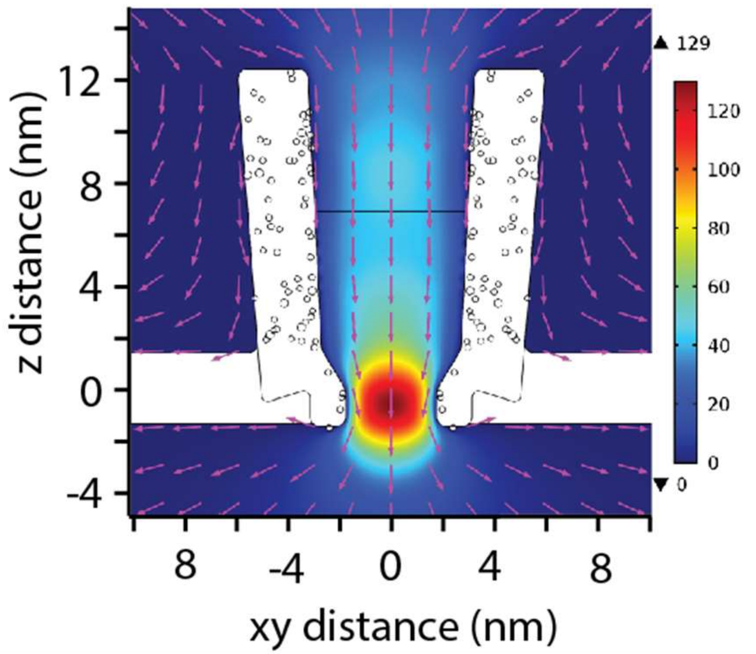


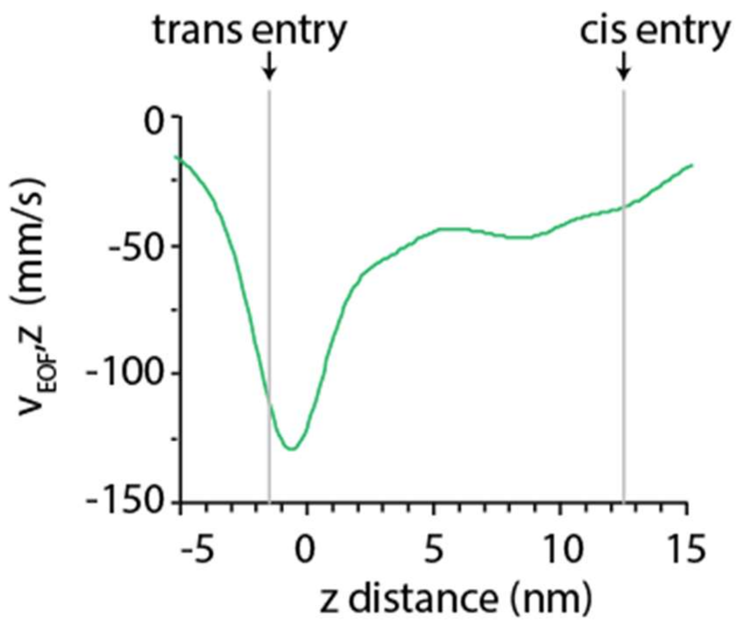


- F_{EP}=EQ
- F_{EP} is strong and extends outside the nanopore
- For DNA ~10 pN under +150 mV
- Proteins are usually not strongly charged

Electroosmotic flow

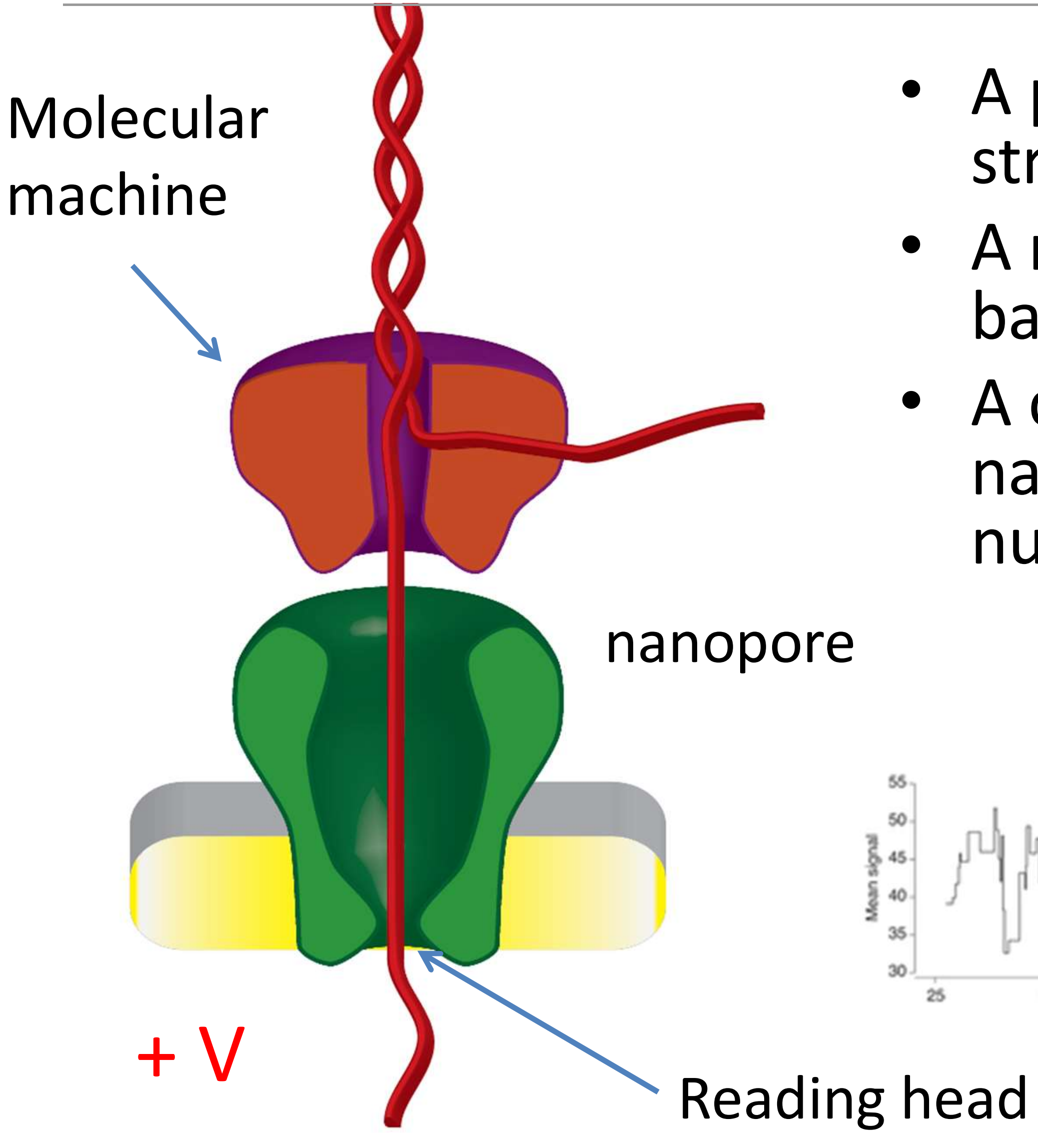
Willems, van Dorpe, IMEC



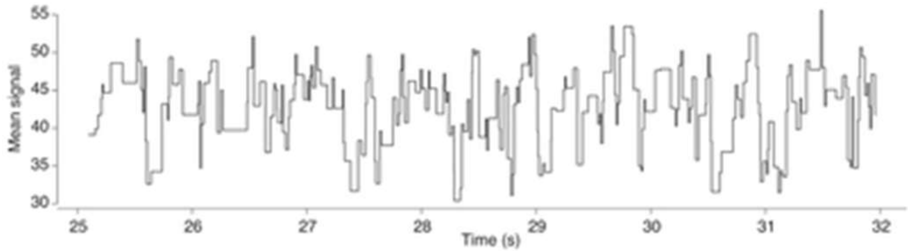


- Brownian force, a few pN
- Protein are weakly charged: Electro-osmosis dominates
- The field extends outside the nanopore

Single-molecule DNA sequencing



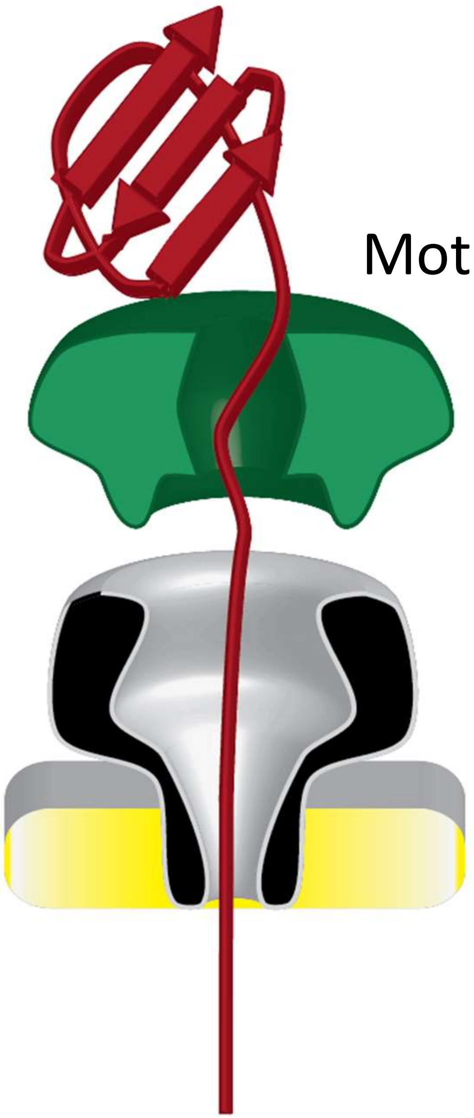
- A positive applied potential stretches the DNA
- A molecular machine steps base-by-base
- A constriction in the nanopore recognize nucleobase(s)



Protein analysis with nanopore: challenges

- Non-uniform charge. How amino acids with opposite charge can translocate at a fixed potential?
- How proteins enter the nanopore?
- Will proteins or amino acids can be recognized by nanopore currents?
- Will folded proteins interact / unfold inside a nanopore?

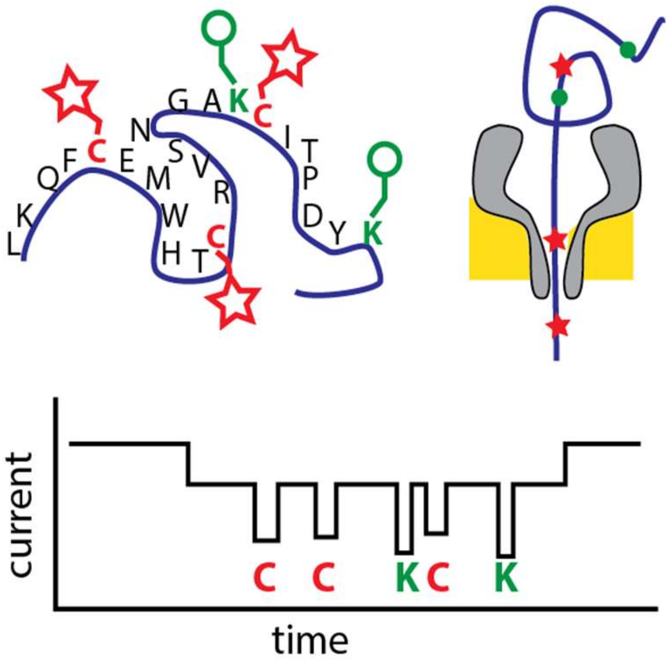
Applications: protein sequencing



- Unfold a protein
- Feed it through a nanopore amino acid-by-amino acid
- Single-molecule sequencing
  - low abundance proteins
  - Protein modifications

Protein Mapping

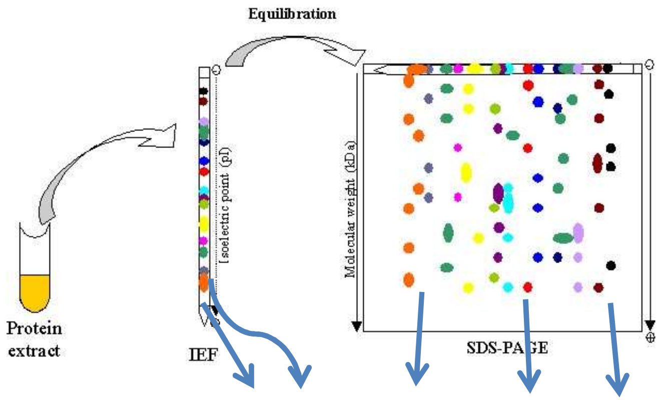
Labeling



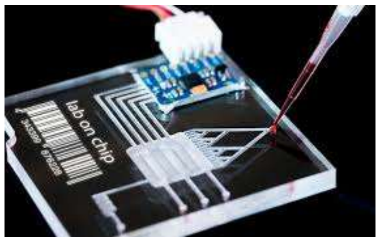
- 25,000 protein sequences of proteins in the human proteome
- Proteins can simply be identified for biomarker detection

Identification of Folded Proteins

Folded proteins



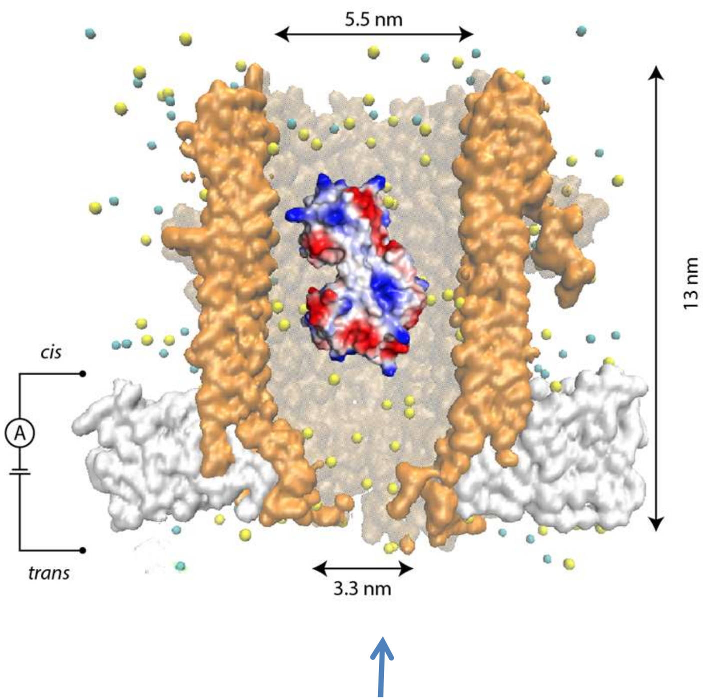
Nanopore analysis



- Proteins might not need unfolding
- Ionic current can be used to recognize sub-populations of proteins (e.g. different nanopores identify different size of proteins)
- Purification / Separation, can be integrated in a microfluidic device

Single-Molecule enzymology

Folded proteins



Biological sample

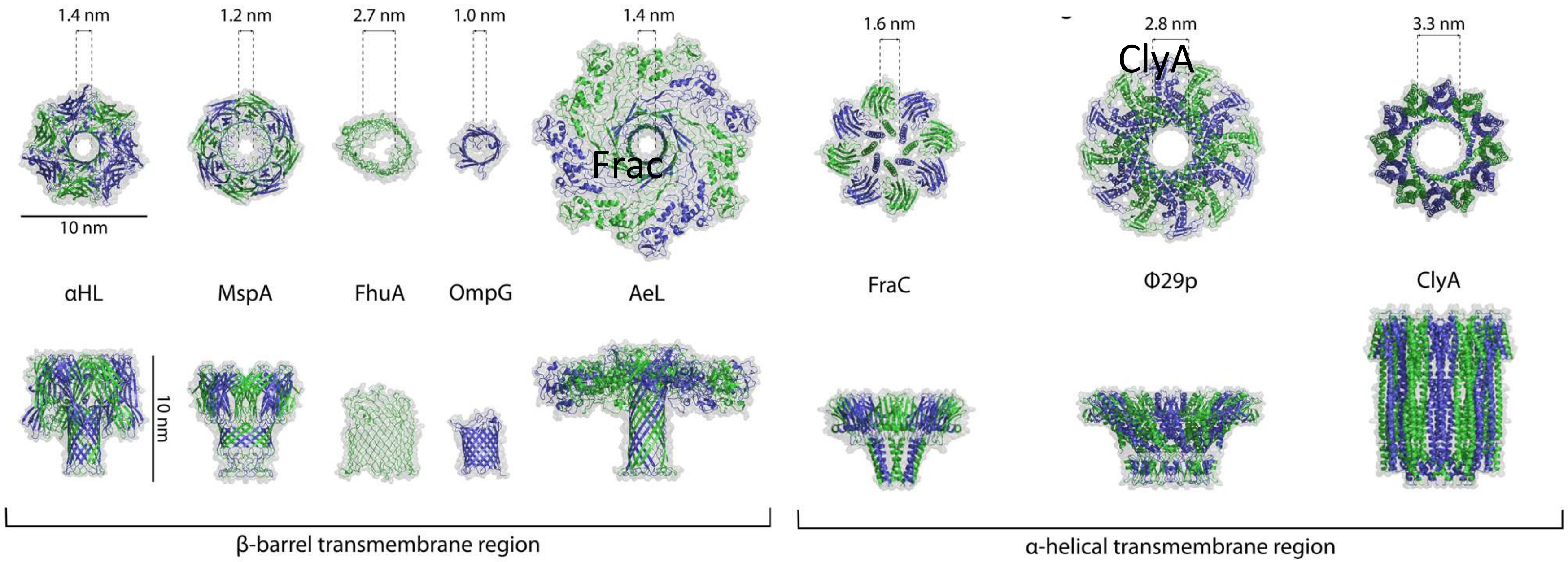
- Single-molecule enzymology
  - Native proteins

- Metabolite detection
  - multiplexing

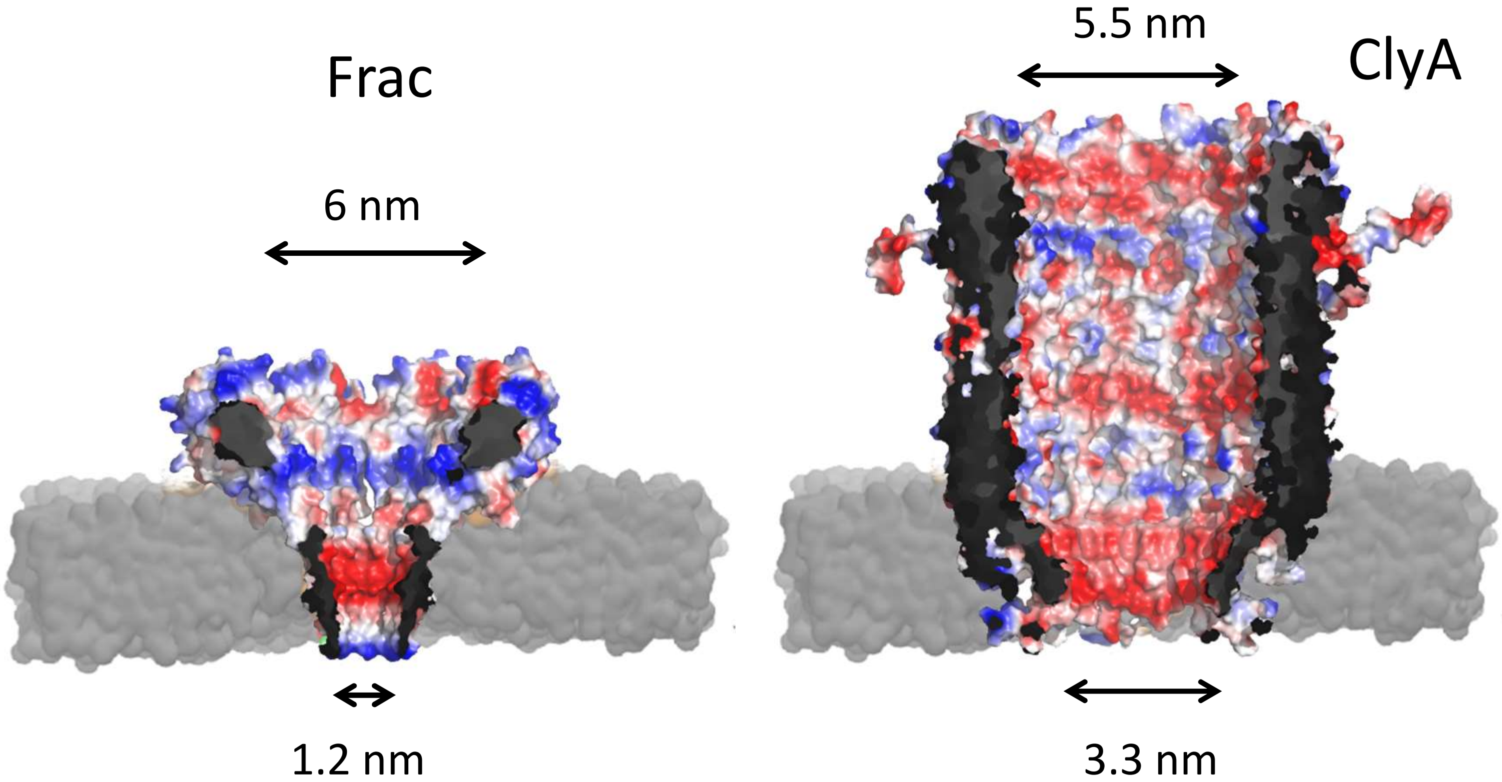
This talk

- Single-molecule enzymology and detection of metabolites
- Recognize of peptides during translocation across the nanopore in single-molecule protein sequencing

Nanopores

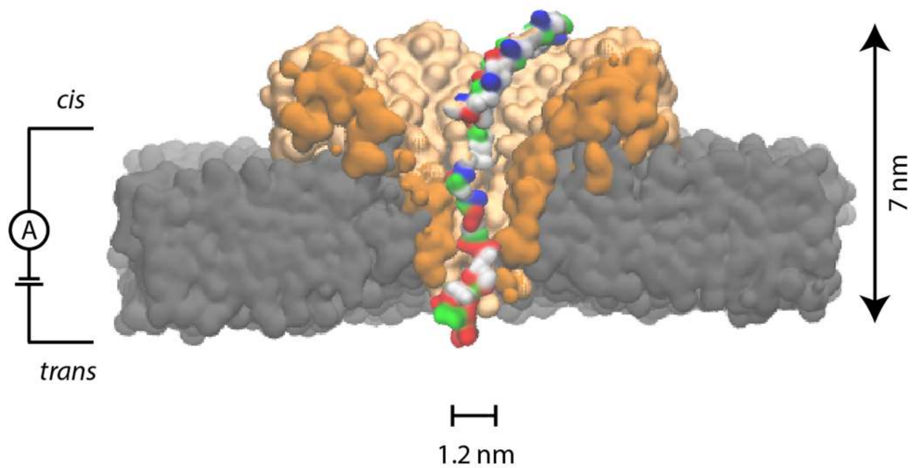

1.4 nm
1.2 nm
2.7 nm
1.0 nm
1.4 nm
1.6 nm
2.8 nm
3.3 nm
ClyA
Frac
10 nm
αHL
MspA
FhuA
OmpG
AeL
FraC
Φ29p
ClyA
10 nm
β-barrel transmembrane region
α-helical transmembrane region

Nanopores



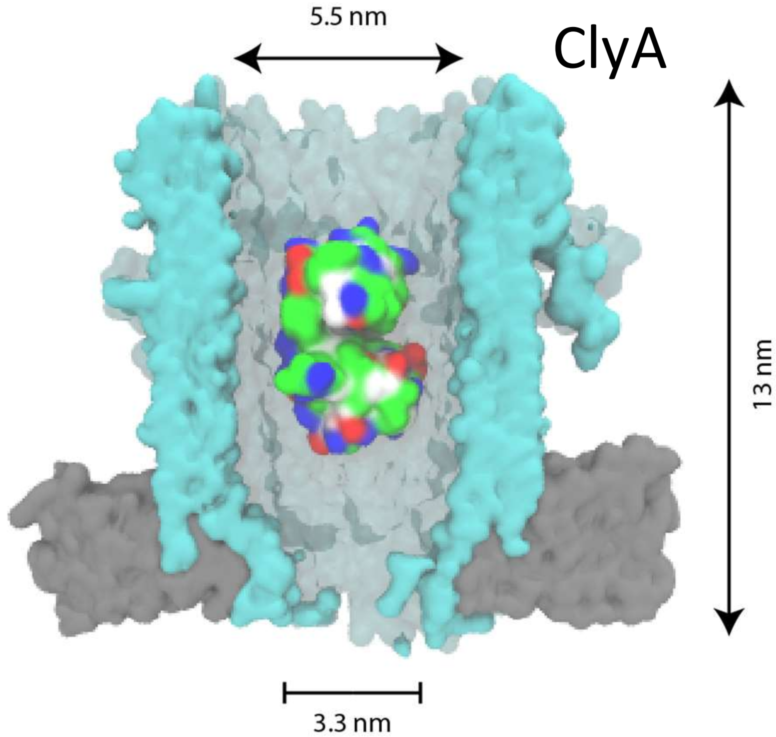
Applications

Frac



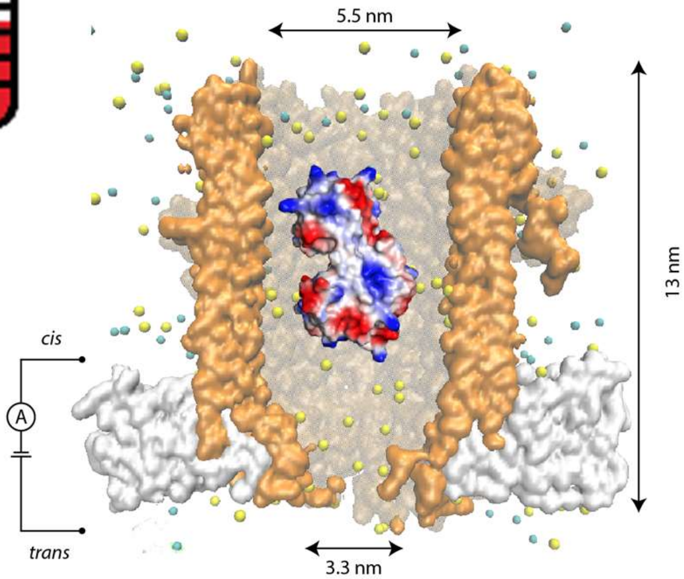
Unfolded-folded protein analysis

ClyA



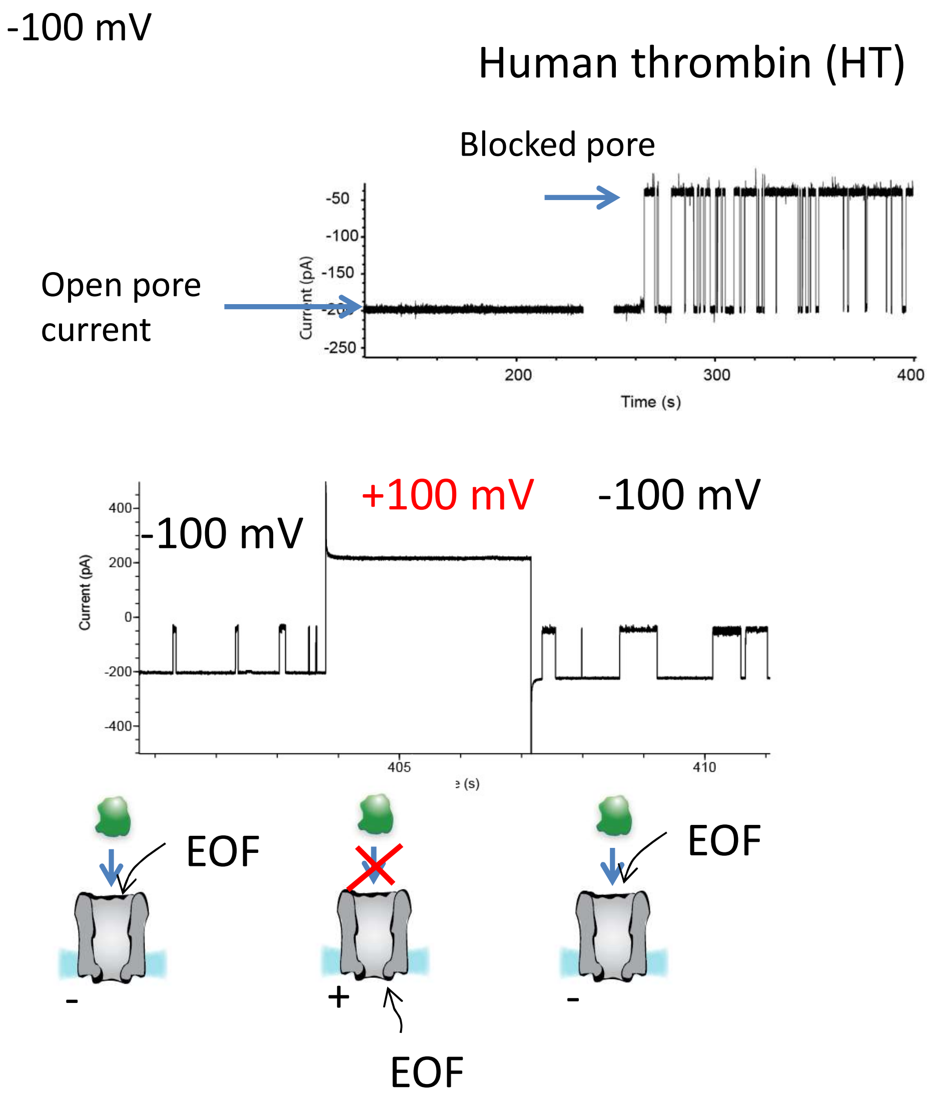
Folded protein analysis

A nanopore test-tube



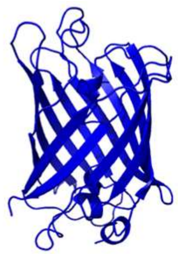
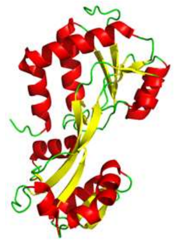
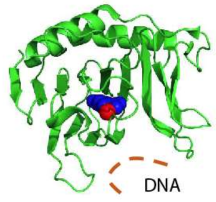
- Do protein enter the nanopore (they are not highly charged)
- Can the confinement of proteins inside the nanopore be controlled?
- Can we observe the binding of analytes inside the nanopore?
- Is confinement changing the properties of the proteins?
  - Protein surface effects
  - Is confined water the same as bulk water?

Protein entry



- At –V, proteins enter the nanopore (current block)
- Electro-osmosis is important
- Protein charge is not (very) important: Electroosmosis dominates
- Proteins enter and remain inside the nanopore for ms-hours

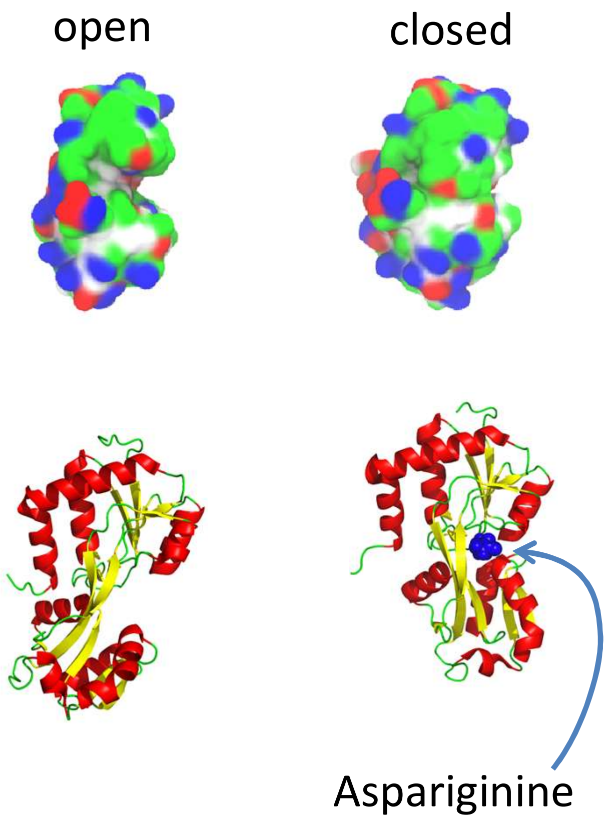
Proteins inside the nanopore

Thrombin (35 kDa)	GFP (27 kDa)	SBD (25 kDa)	AlkB (23 kDa)	DHFR (19 kDa)	Lysozyme (14 kDa)
					
hours	ms	minutes	minutes	ms	us

- Size charge and shape are important
- All give a disstinguished current signal
- Limits: ~35<protein<~14 kDa proteins

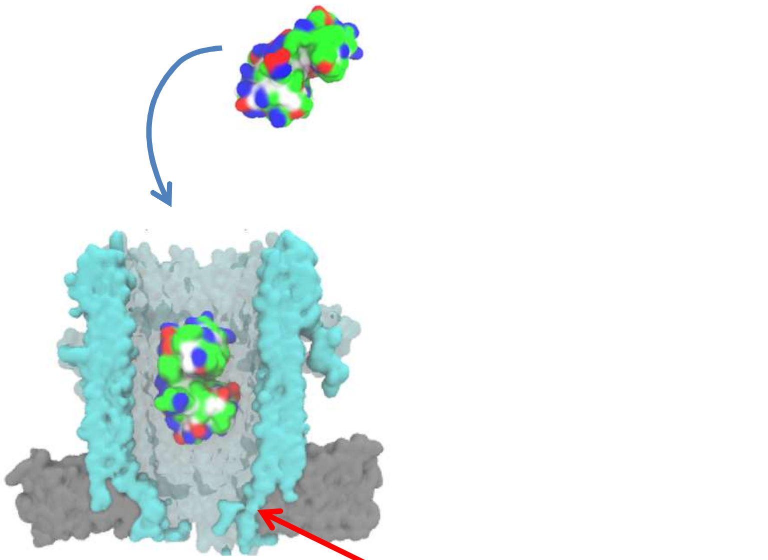
SBD proteins

25 kDa

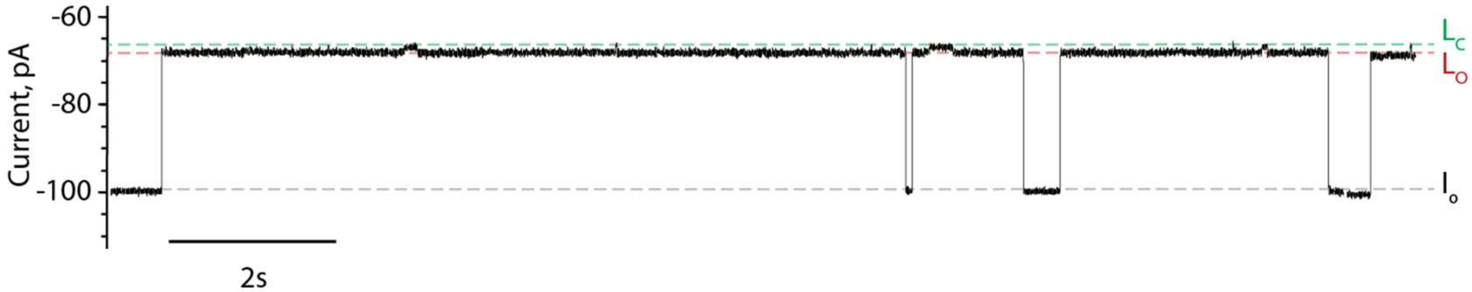


- Venus flytrap protein domains
- Large conformational changes
- Many, e.g glucose and glutamine

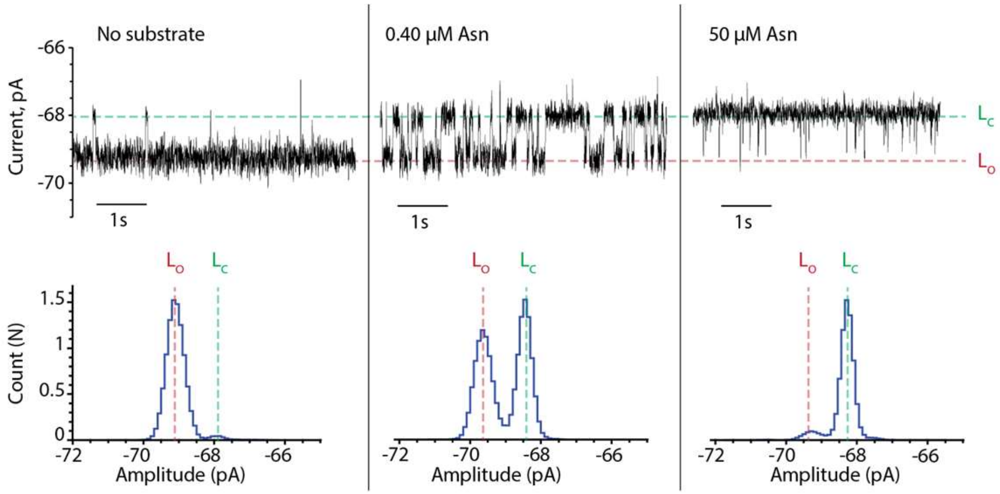
Capture

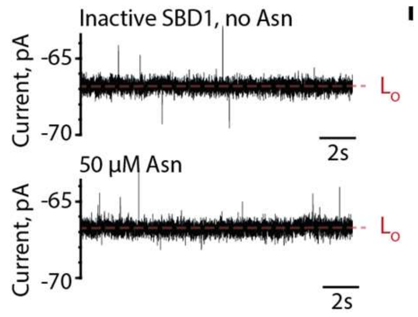


- Proteins spontaneously enter the nanopore but only at negative applied potentials
- Nanoscale trap
- Proteins are confinement depends on the protein



SBD

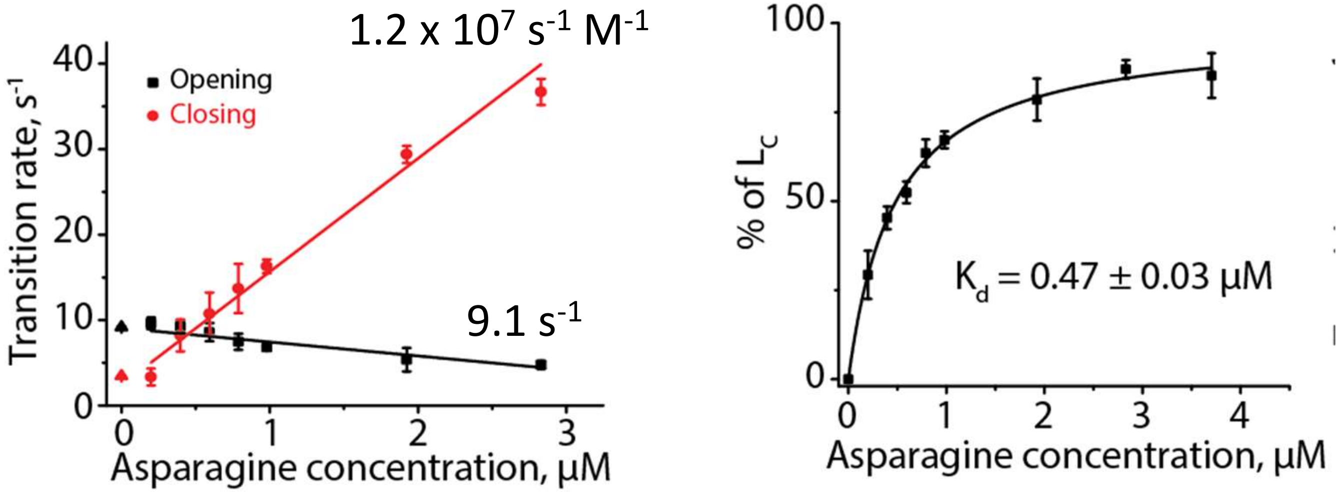




$\mathbf{K_D}$= 470 nM (350 nM with **FRET**)
Off rate = 9.1 s^{-1}, (4.2 s^{-1} from FRET)
On rate = 1.2 x 10^7 s^{-1} M^{-1} (2.2 × 10^7 s^{-1} M^{-1} from FRET)

- **Intrinsic dynamics** and **conformational changes**

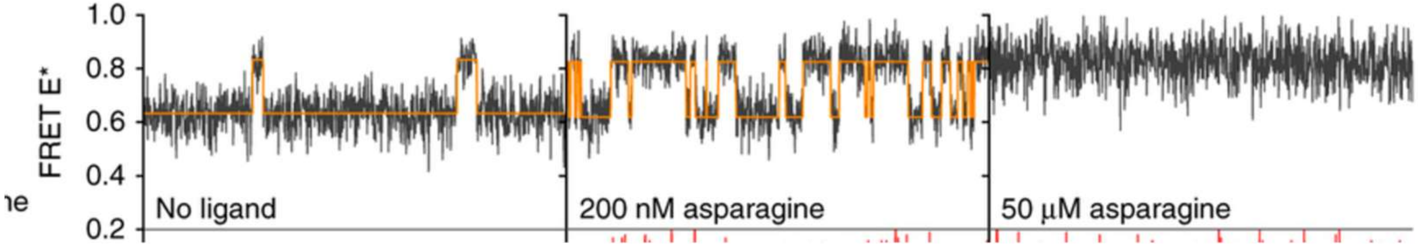
SBD

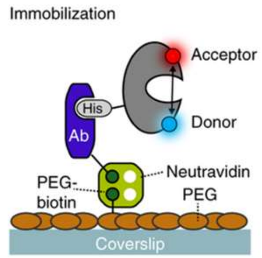


- **Intrinsic dynamics** and **conformational changes**

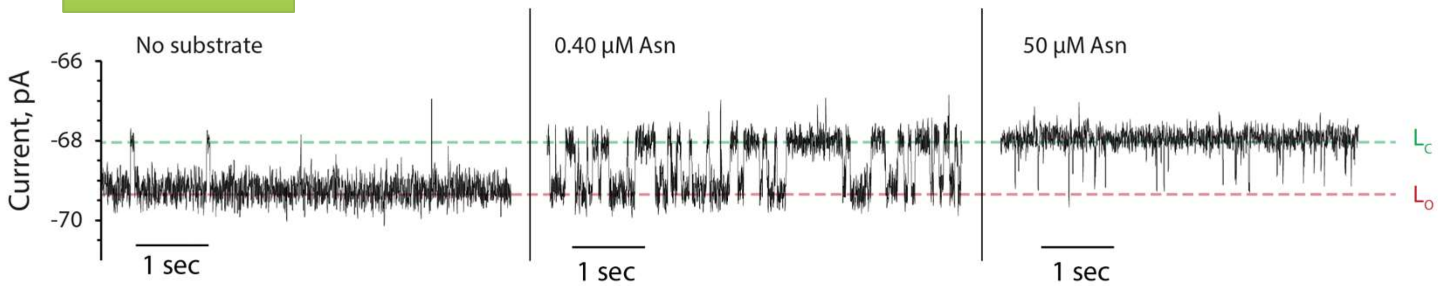
FRET Studies

SM-FRET





nanopore



- K_D= 350 nM from FRET, 470 nM nanopore (0.2 μM bulk experiment
- Off rate 4.2 s^{-1} from FRET, 9.1 s^{-1} from nanopore
- On rate = 2.4 × 10^7 s^{-1} M^{-1} from FRET, 2.2 x 10^7 s^{-1} M^{-1} nanopore
- Neutral analyte almost bulk/like concentration

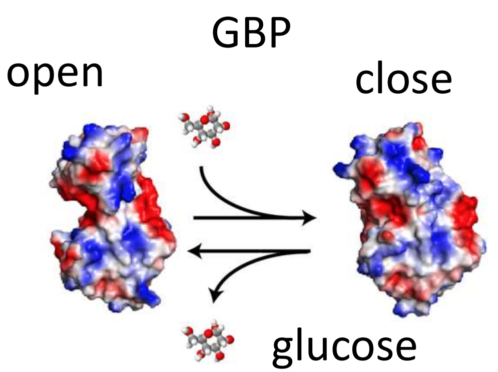
Confinement

- Intrinsic dynamics of proteins appear to be almost identical than in bulk
- The charge of molecules influence the diffusion through the nanopore (more to be done)
- The surface charge of the nanopore and confinement does not appear to be a big issue (more to be done)

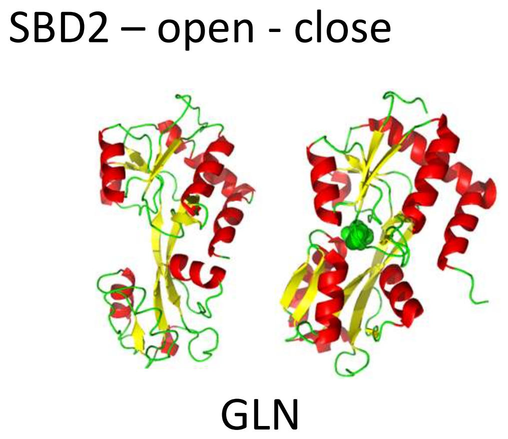
Why

- Nanopores can be interfaced with electronic devices
- If enzymatic reaction can be observed, any biological active molecule can be detected
- Analogue to digital converter
- Small volumes, high sensitivity, wearable sensors.

Ohther SBD protein tested:

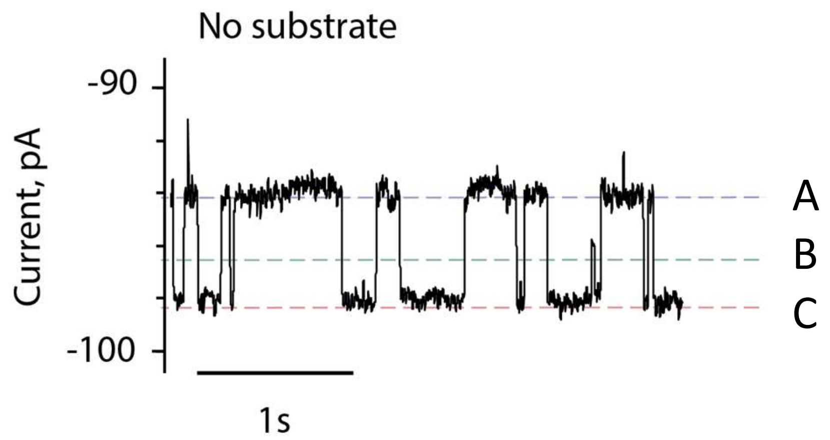


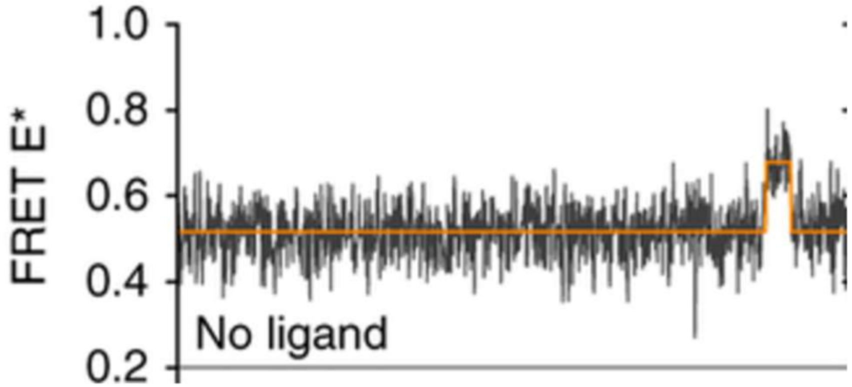
- Glucose binding protein behave the same as SBD1



- The case of SBD2 (binds glutamine)

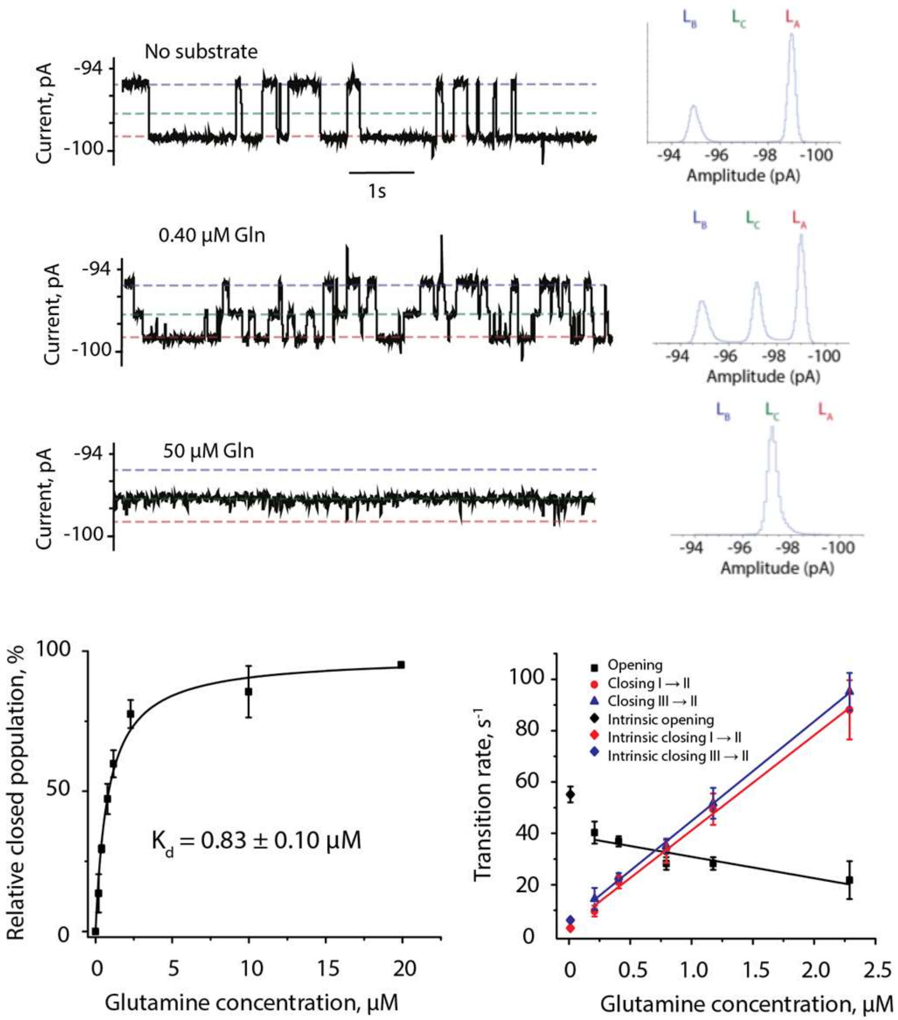
SBD2





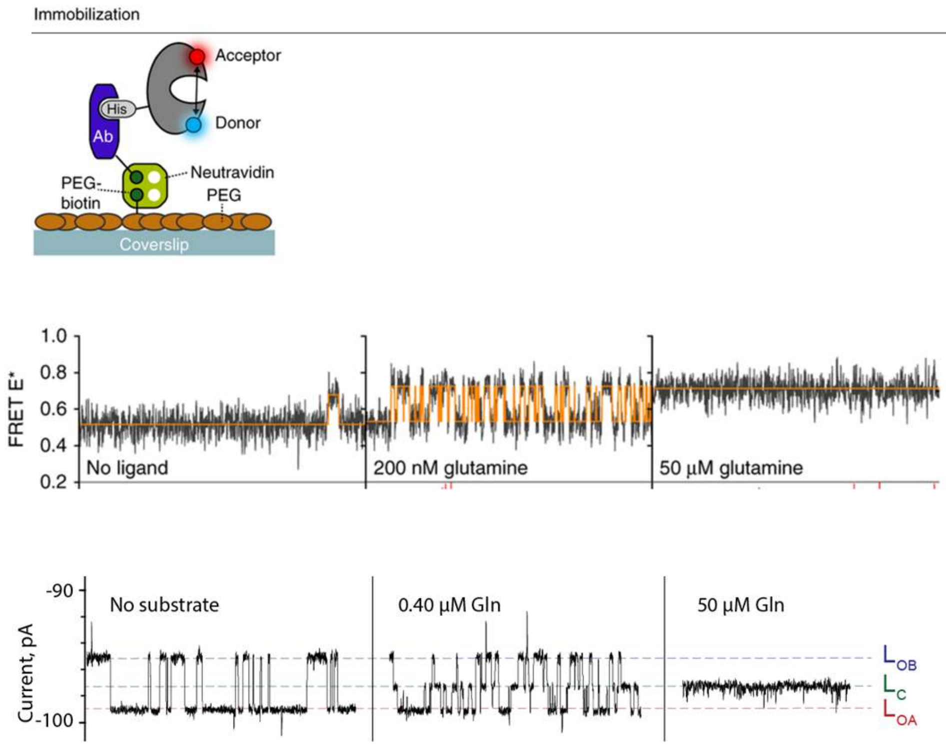
- The signal switches between two levels
- Conformational dynamics or different conformations inside the nanopore
- One conformation from FRET experiments

SBD2



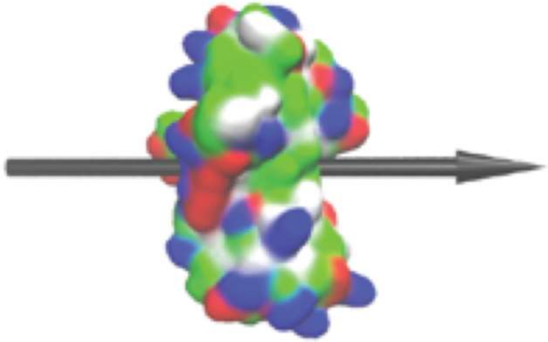
- Closed conformation is a different level
- The off rate is identical from both level
- Most likely two conformations inside the nanopore

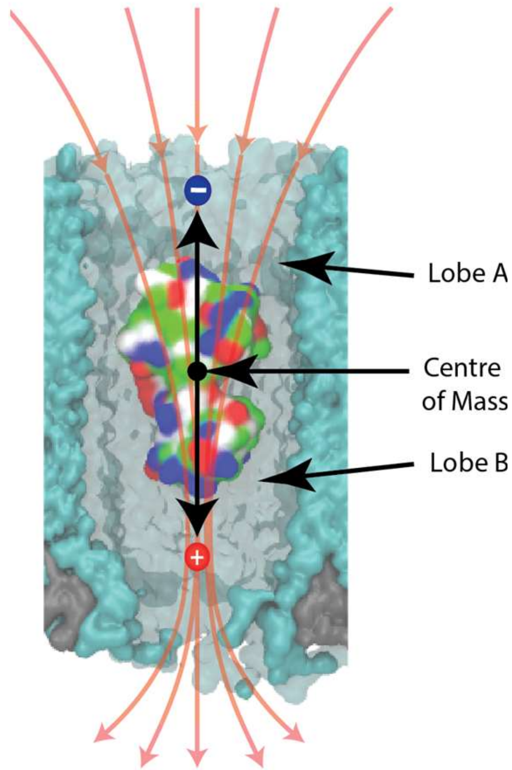
FRET Studies

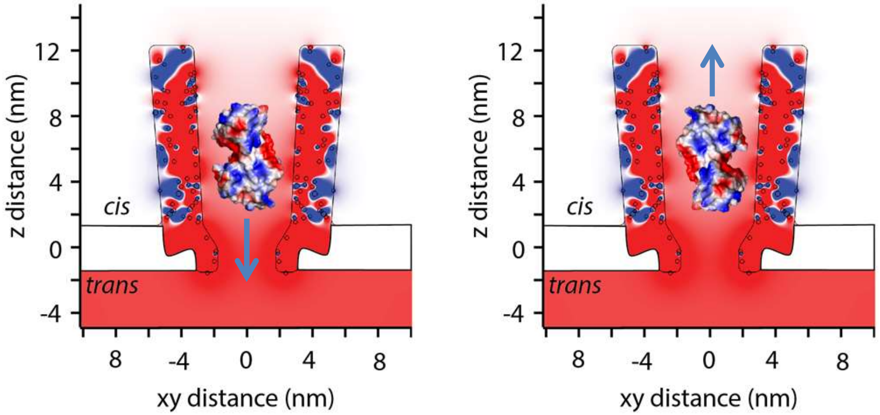


- Intrinsic dynamics
- SBD2 K_D^Q= 1.1 µM (0.9 µM with ITC) : 0.8 µM with nanopore
- On rate = 3.8×10^7 s^{-1} M^{-1} : 3.7×10^7 s^{-1} M^{-1} (level A) 3.8×10^7 s^{-1} M^{-1} (level C) with nanopore
- Off rate 17.2 s^{-1} : 39.8 s^{-1} with nanopore

explanation

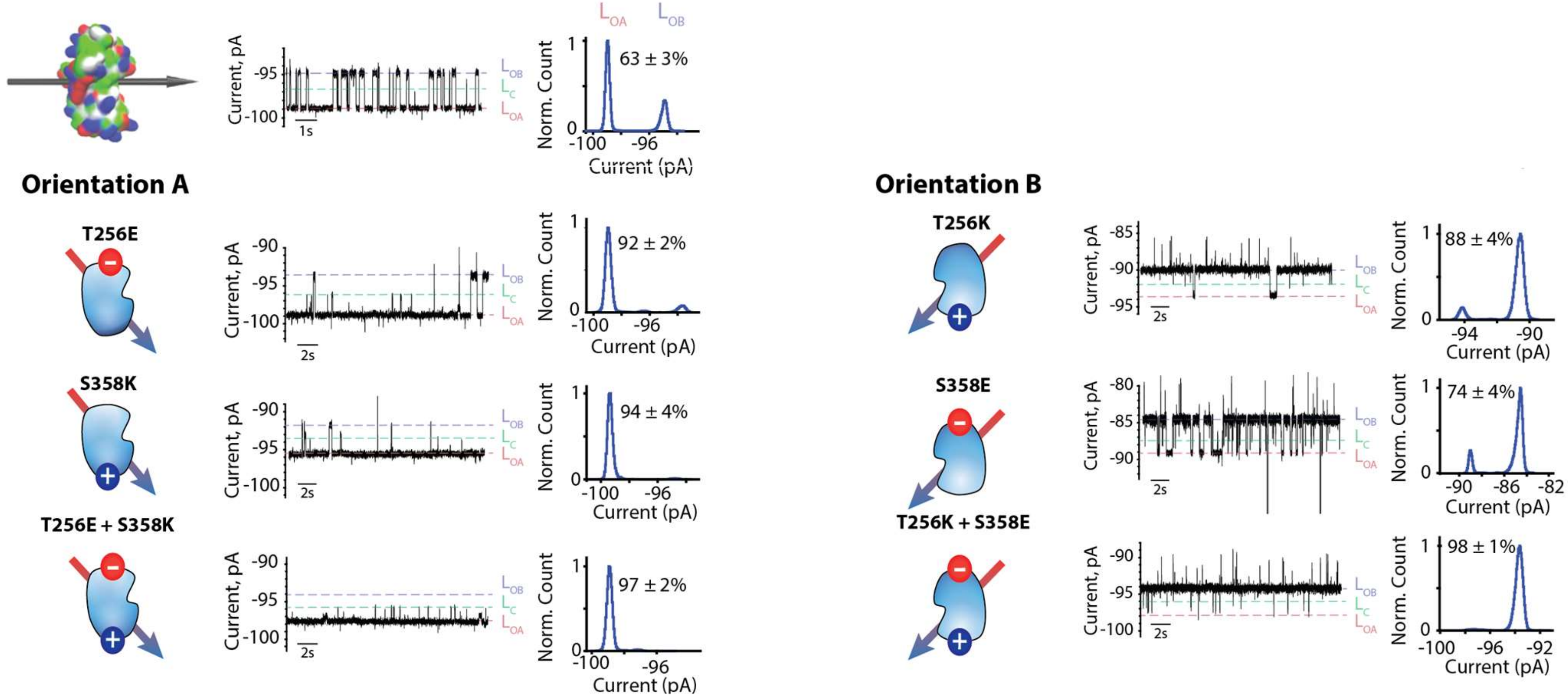




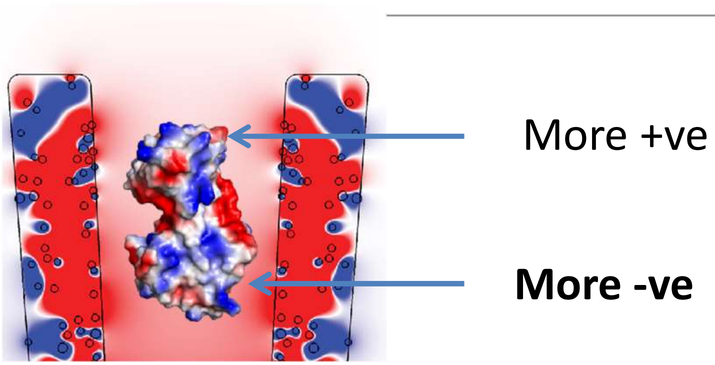


- Different orientations?

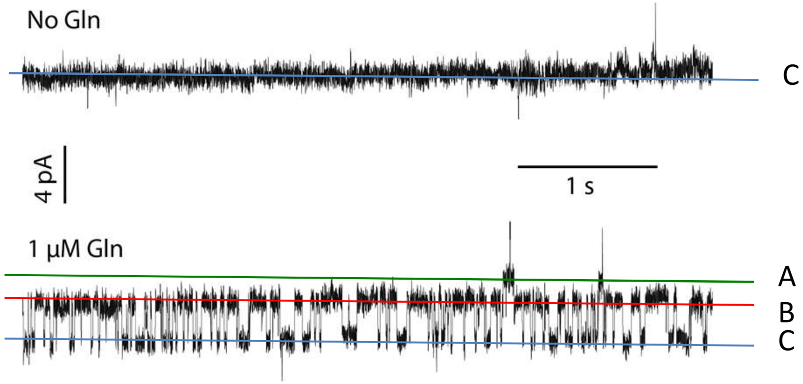
Orientation



SBD2 tumbles inside the pore

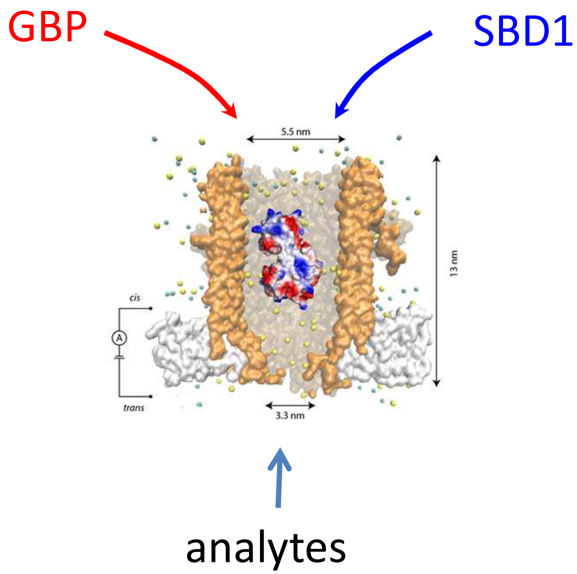


Orient the protein inside the nanopore



- Proteins inside the pore are oriented
- The EF and EOF most likely keep the protein in the middle of the nanopore
- The orientation of the protein can be selected

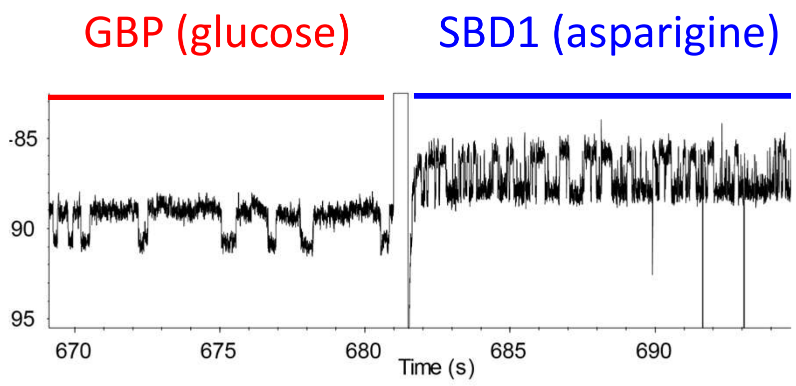
Detection from bodily fluids



- The nanopore serve as a natural filter for large molecules
- Wearable devices

Blood, sweat, urine…

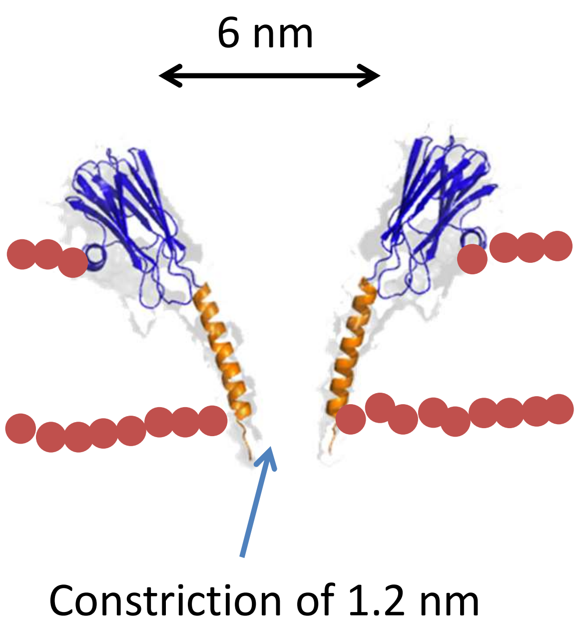
Detection from bodily fluids



	V	[Glucose] (mM) nanopore	[Glucose] (mM) spectroscopy
Sweat	2 µL	0.08 ± 0.004	0.12 ± 0.05
Saliva	8 µL	0.02 ± 0.003	0. 2 ± 0.003
Urine	2 µL	0.38 ± 0.01	0.38 ± 0.01
Human blood	5 pL	5.7 ± 0.4	5.6 ± 0.6

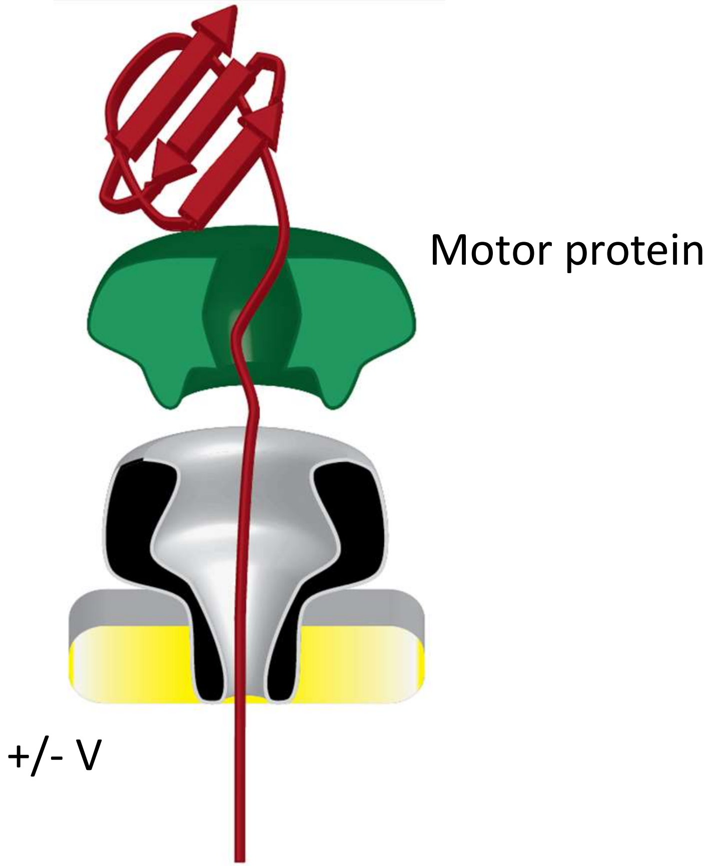
- Glucose can be detected directly from sweat, urine and blood
- Good comparison with to other methods
- Multiple analytes can be detected simultaneously
- Wearable devices?

Peptide analysis and sequencing



- FraC nanopores
- Ideal shape to study peptide

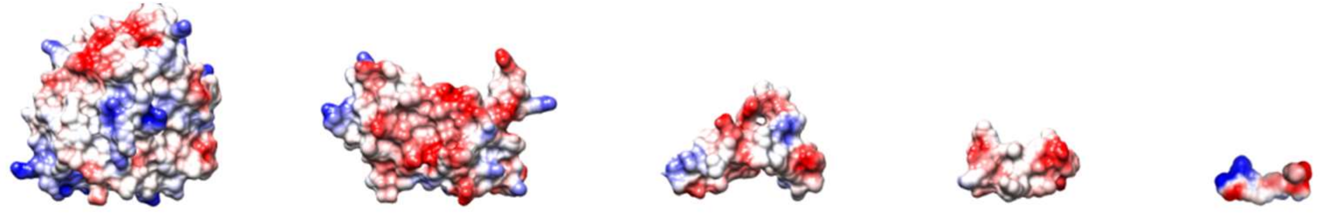
Peptide analysis and sequencing



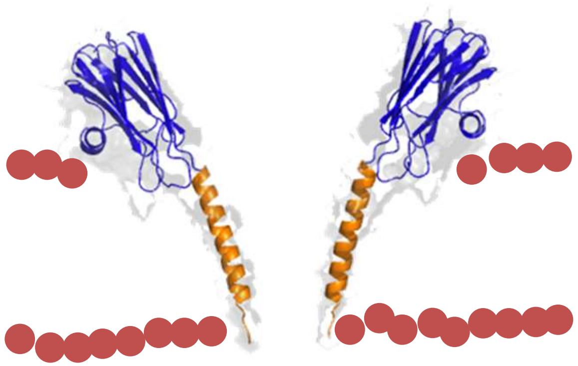
- FraC nanopores
- Ideal shape to study peptide
- Small constriction, ideal to sequence proteins
- **Issue**: can peptide be **translocated** at a fixed applied potential? Can a EOF >> EF be engineered?

Biomarker analysis

25 kD, pI 8.8 | 11.6 kDa, pI 5.6 | 6.2 kDa, pI 4.2 | 2.5 kDa, 4.1 | 1.3 kDa, -2



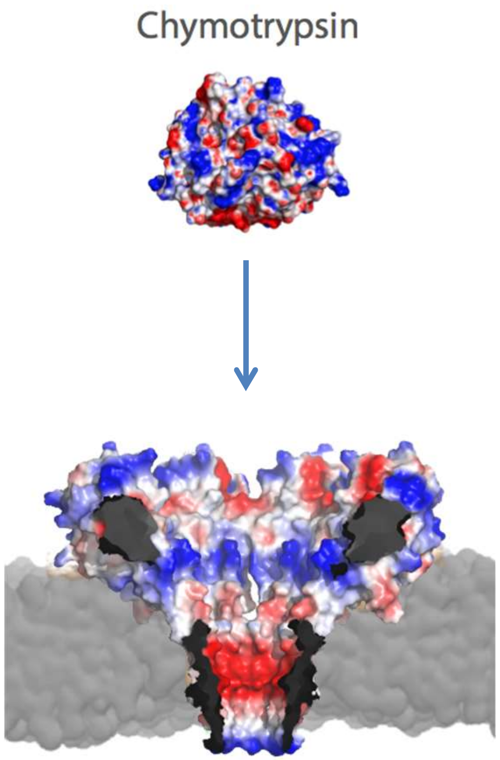
Chymotrypsin | β-microglobulin | EGF | Endothelin 1 | Angiotensin I

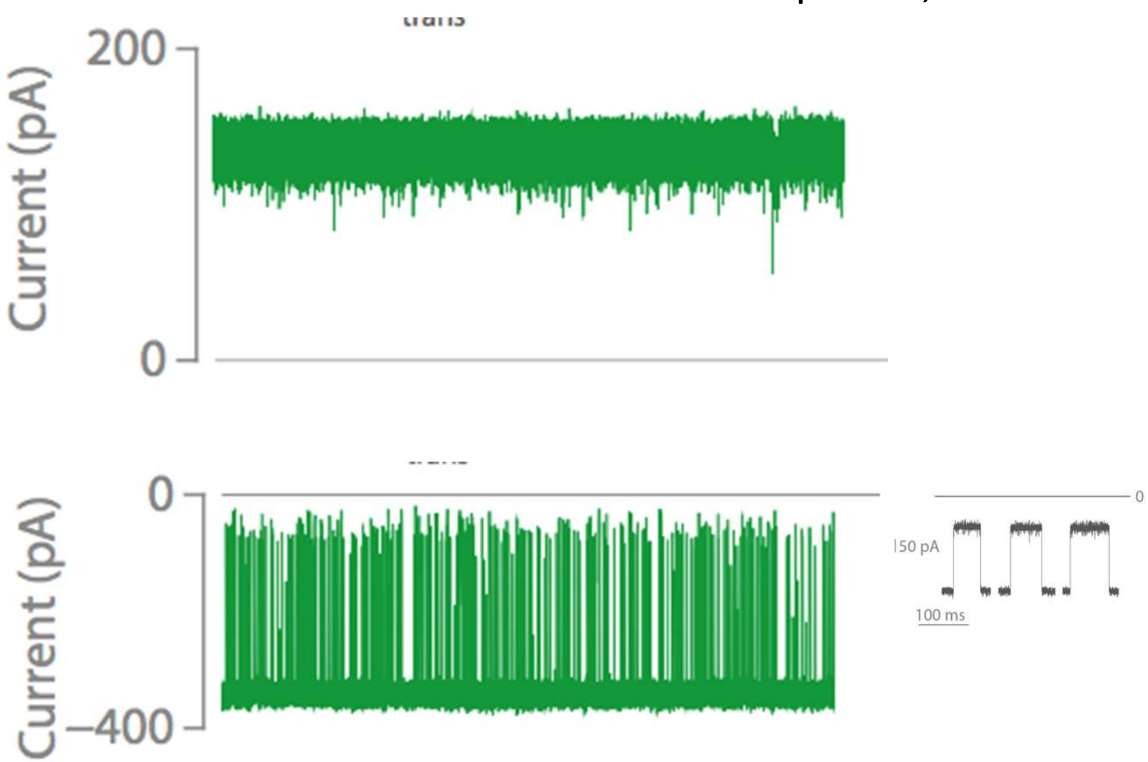


- 5 protein-peptide biomarkers
- Different size, shape and folds

Protein Capture

pH 7.5, 1 M KCl





Capture only at -V

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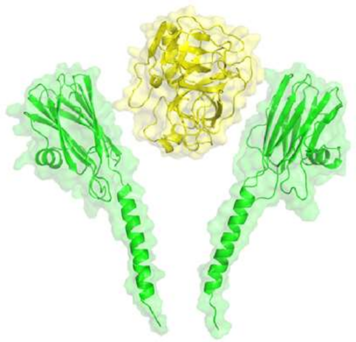
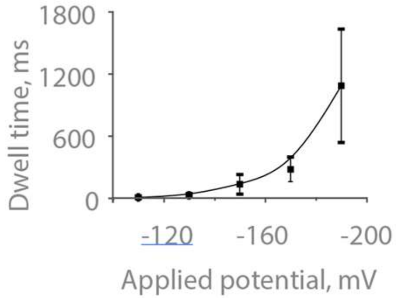
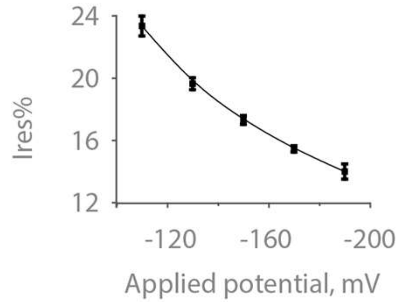
No translocation



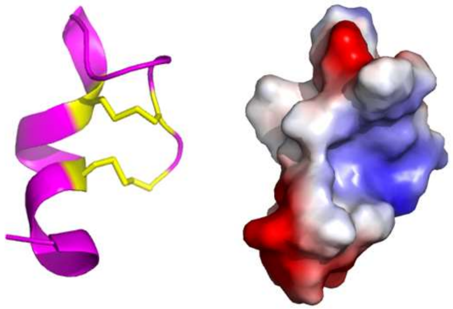




- Electro-osmosis is important
- Protein can be trapped for a few seconds
- Protein do not translocate across the nanopore
- Proteins are ‘pressed’ against the constriction

Peptide Capture

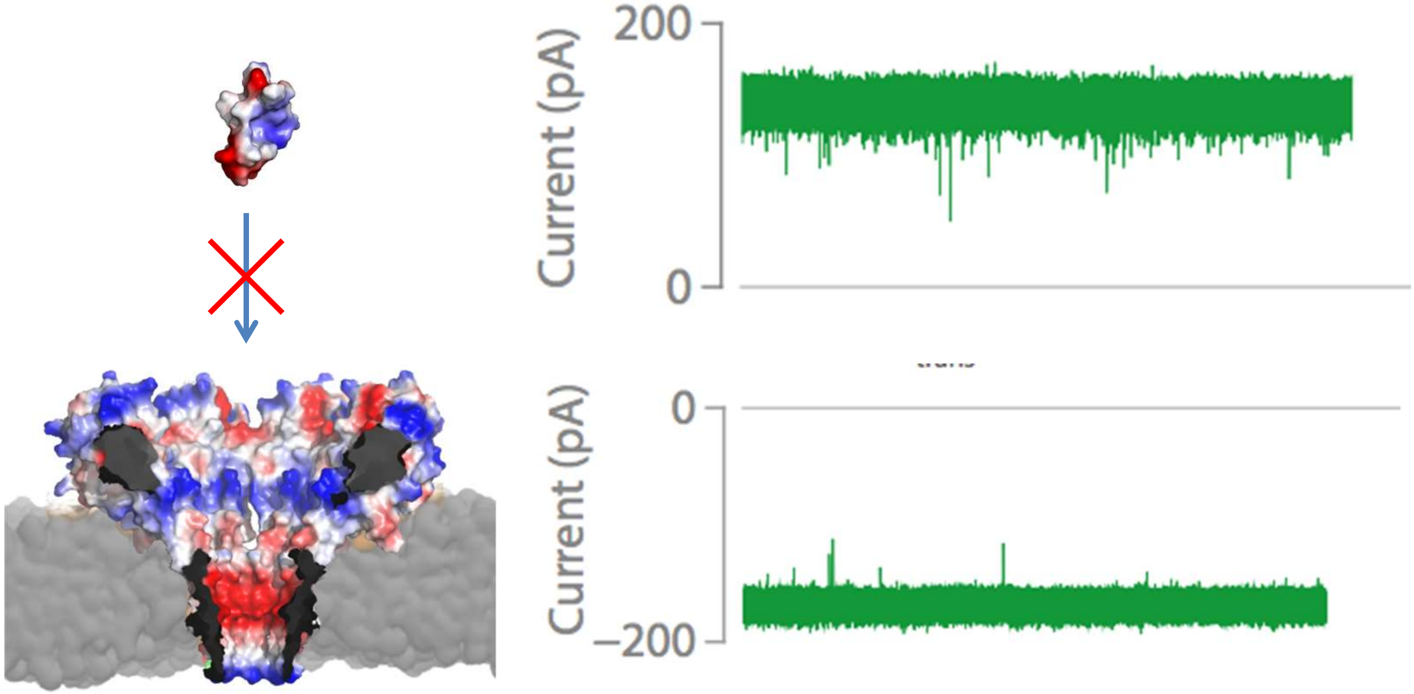
Endothelin 1



- An helix kept folded by disulfide bridges
- Formal charge -2

Peptide Capture

pH 7.5, 1 M KCl

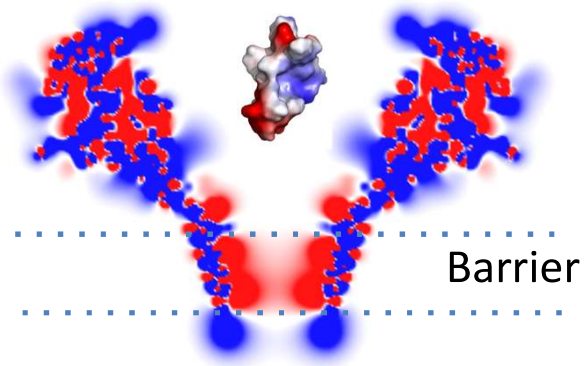


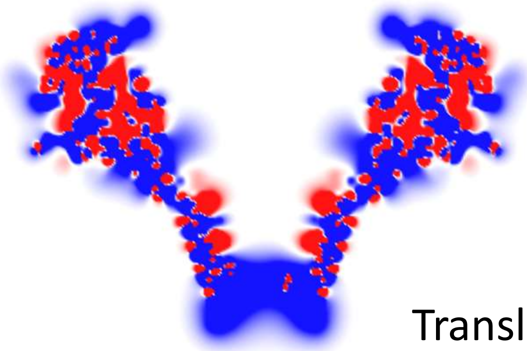
No Capture

-

Why?

WT Frac

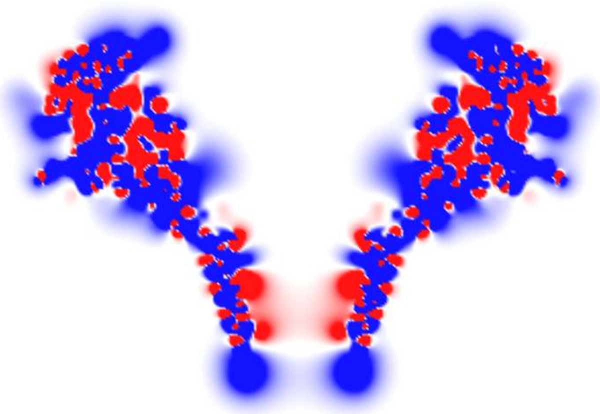




Translocation is observed

- The constriction prevents the translocation of negatively charged peptides
- Translocation is observed when the constriction is positive
- However, a charged constriction is necessary to induce an electro-osmotic flow
- SOLUTIONS?

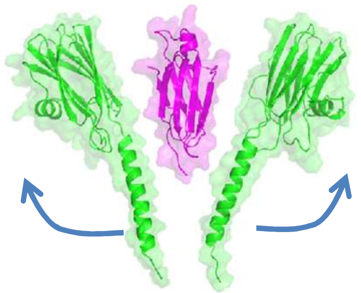
Magic pH 4.5

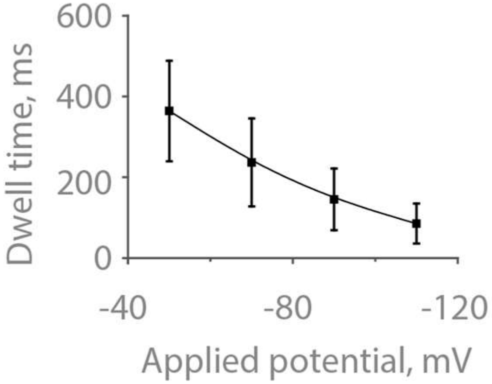


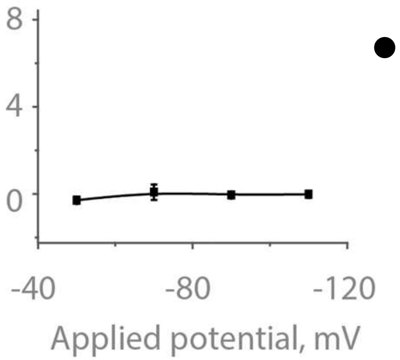
- All peptides are captured
- Important for protein sequencing applications

β2-Microglobulin

11.6 kD, pI=5.6



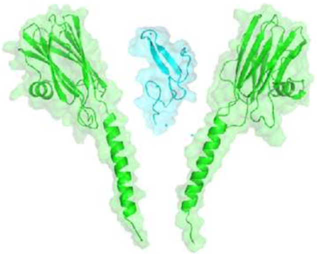


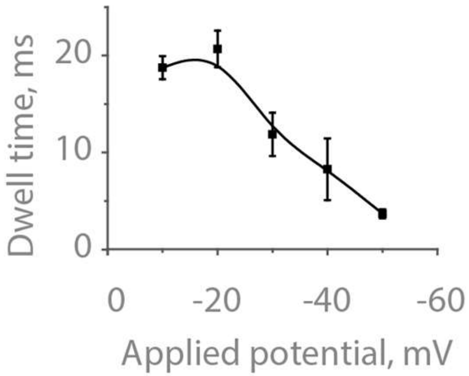


- The protein translocate across the nanopore
- It cannot be captured at pH 7.5
- The alpha helices probably move to let the protein pass

EGF human
Endothelin 1

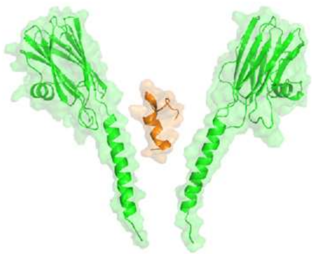
6.2 kD, pI=4.5

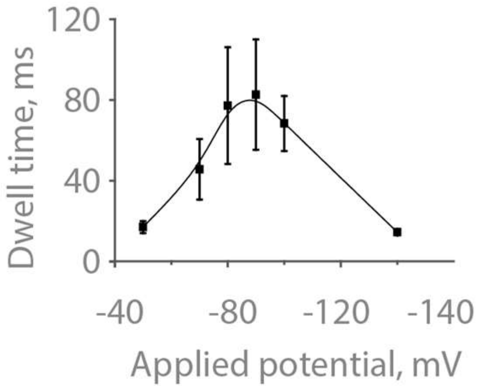




- Translocation is above a threshold potential

2.5 kD, pI=4.1

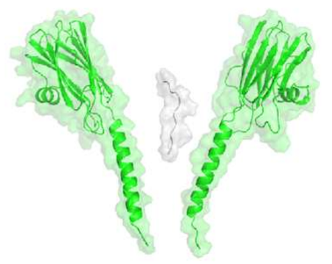


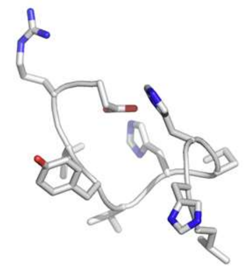


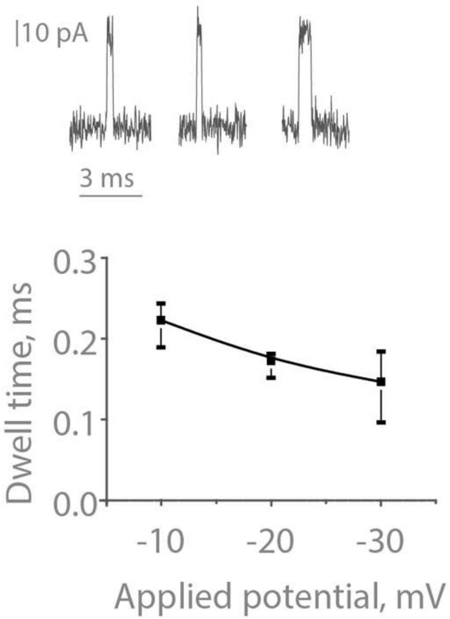
Angiotensin I

1.3 kD, pI=7.9,

12 AA

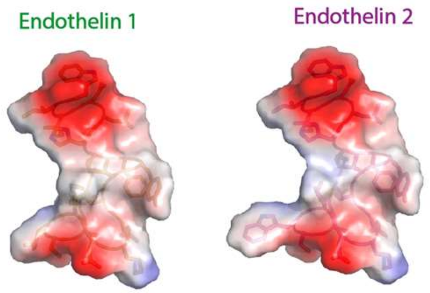


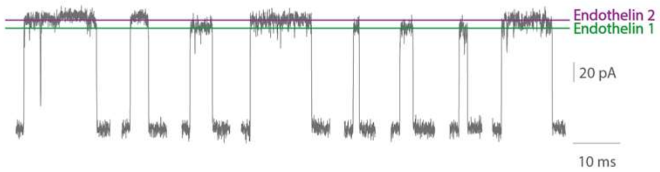


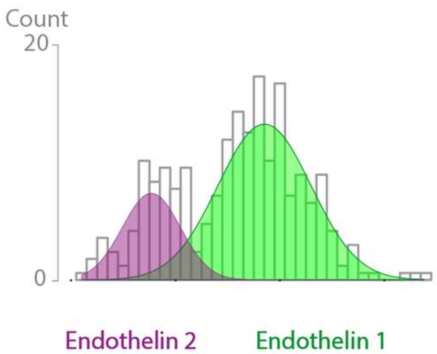


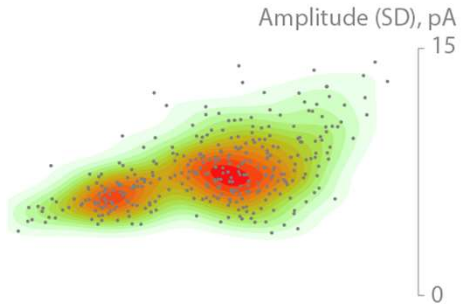
- The blockades are very short
- Probably the limit of detection with FraC nanopores

Polypeptide recognition









- Small differences can be observed

Conclusion

- Ionic current through nanopores can be used to study proteins
- Single-molecule enzymology
    - Native proteins
    - High-bandwidth
    - Conformational dynamics
    - Binding Constants
    - Protein confinement is not an issue
- Biomarker detection
    – Protein: will require a purification step
    – Metabolites with protein adaptor: no purification
    – Sensing device for metabolyte and glucose
- Multiplexing
    – Multiple nanopores to detect many proteins
    – Panel of hundreds metabolites
- Wearable devices

Aknoweledgments

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Misha Soskine
Carsten Wloka
Veerle Van Meervelt
Annemie Biesemans
Kevin Huang
Kherim Willems
Mariem Bayoumi
Nicole Galenkamp
Natalie Mutter

Collaborators:

Bert Poolman
Gea K. Schuurman-Wolters
(Groningen)

Pol Van dorp
Tim Stakenborg
(IMEC)

PhD and Post-doc positions available