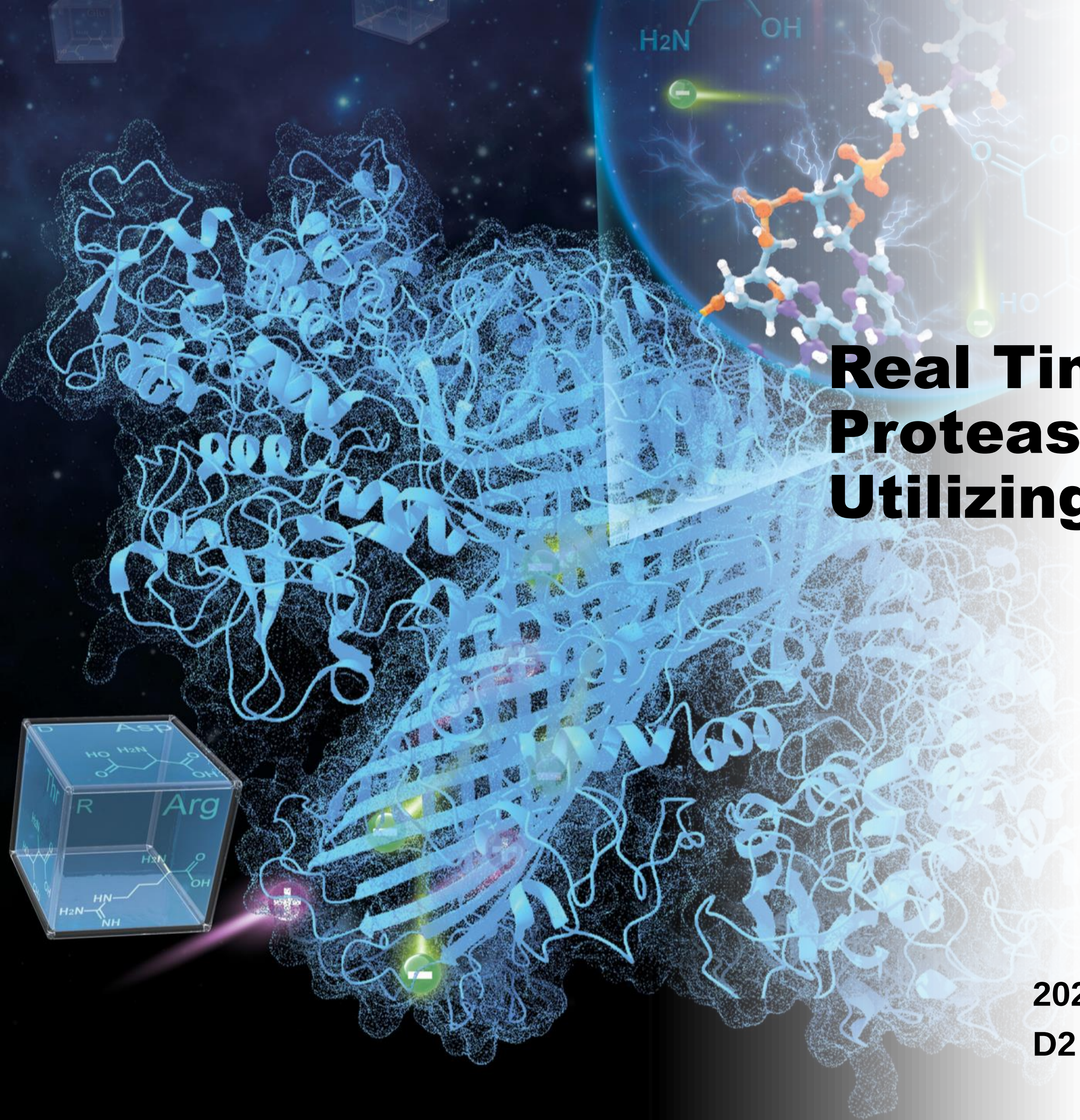


Real Time Analysis of Protease Reaction Utilizing Nanopore

2023. 4. 22. Literature Session

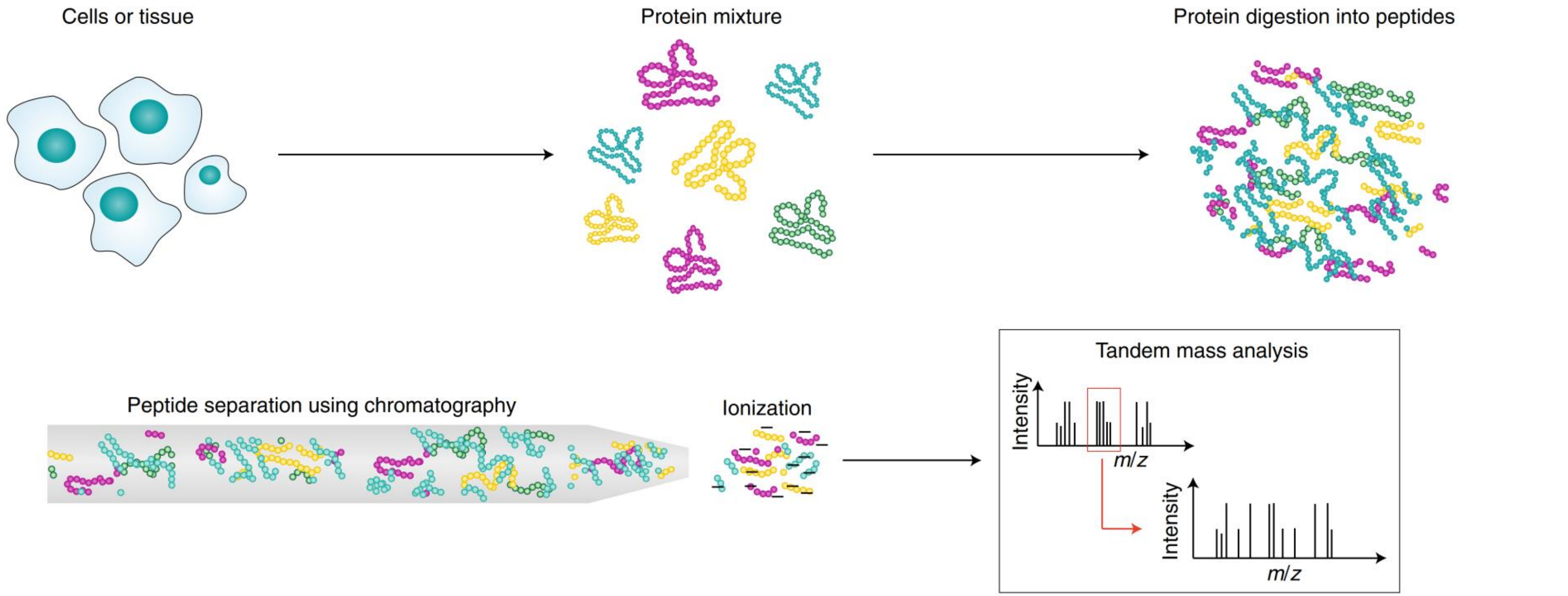
D2 Takeuchi Aoi

Contents

Protein Sequencing

identification and quantification of proteins: essential for understanding of biological processes and diseases

mass spectrometry: gold standard for proteome analysis



drawbacks of mass spectrometry

- **dynamic range (10^4-10^5): insufficient to cover the range of protein concentration (~10^9)**
- **detection limit (0.1-10 fmol): difficulty in detecting proteins in low-copy numbers (<100 molecules/cell)**
- **ensemble measurement: limited ability to provide dynamic information**

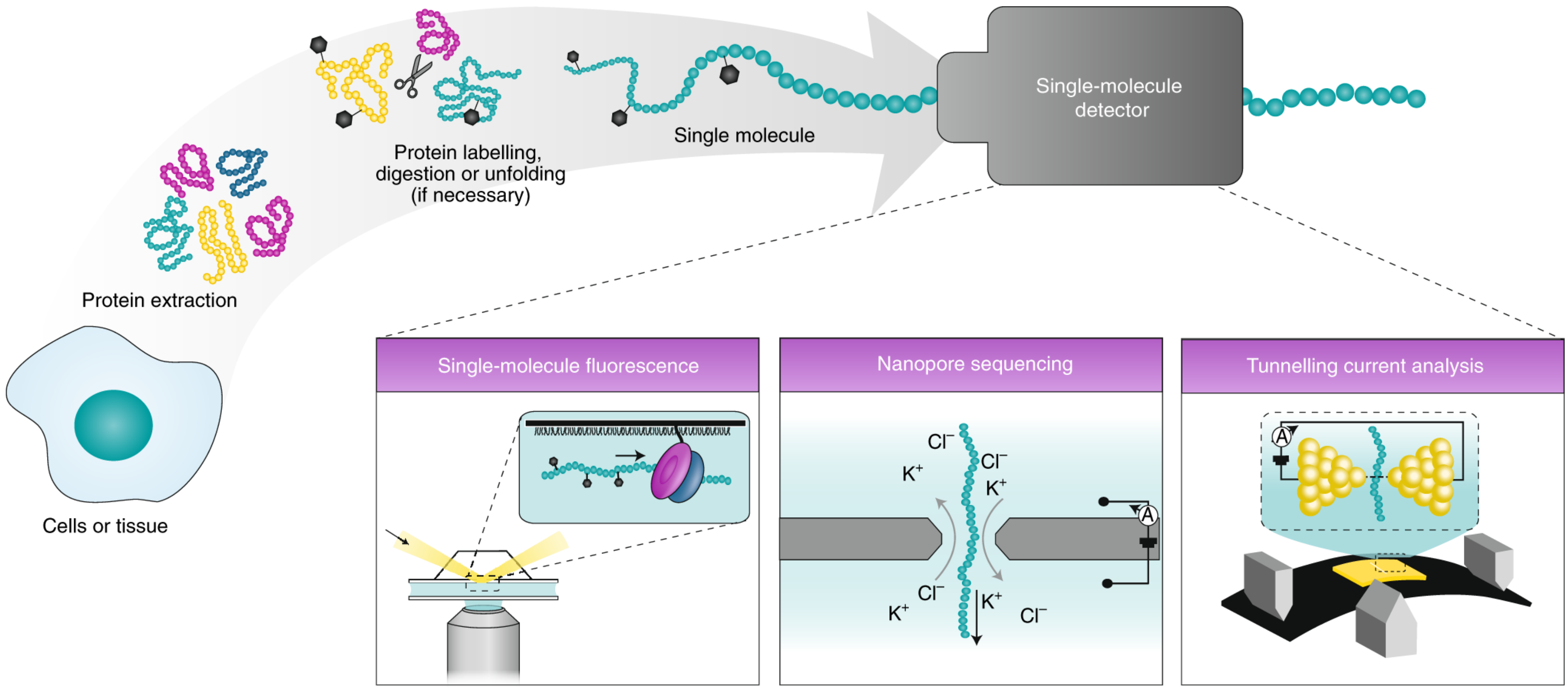
Single-molecule sensing have been pursued for more comprehensive analysis.

Restrepo-Pérez, L.; Joo, C.; Dekker, C. *Nat. Nanotech*. **2018**, *13*, 786.

Single-Molecule Sequencing of Protein

properties of biopolymers for sequencing	nucleic acid	protein
types	4 nucleobase	20 amino acids
amplification	PCR	unable

The development of highly sensitive, high-throughput protein sequencing techniques is a great challenge.

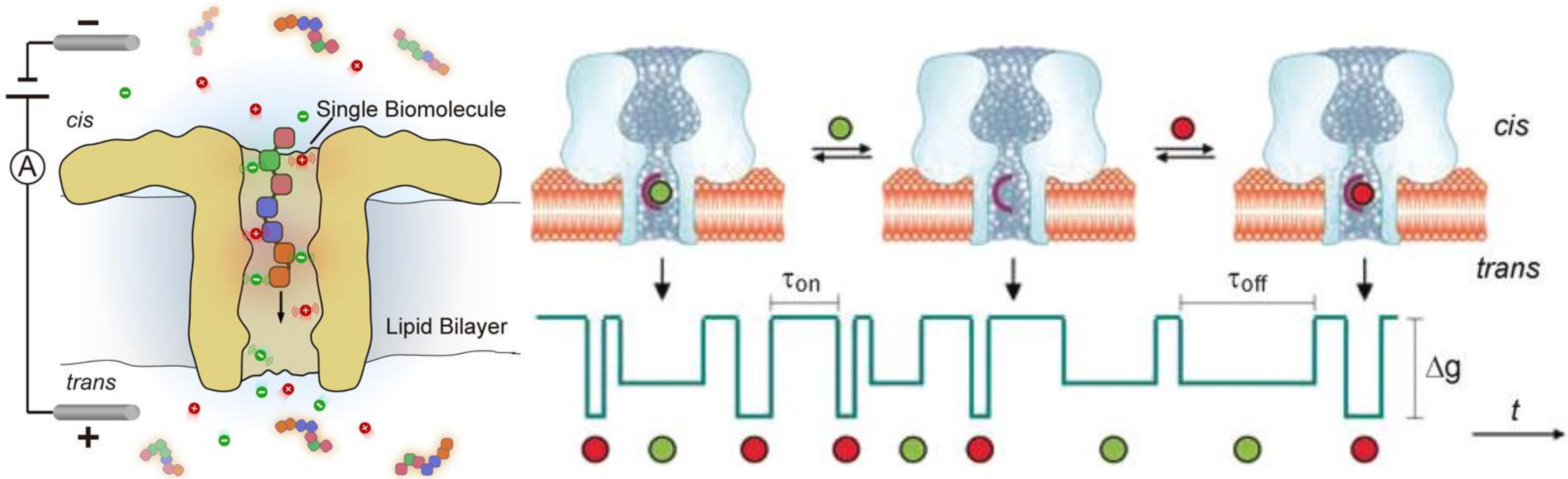


Restrepo-Pérez, L.; Joo, C.; Dekker, C. *Nat. Nanotech*. **2018**, *13*, 786.

Principle of Nanopore Sensing

- Translocation of the analyte induces the characteristic current event.
- The magnitude of the associated resistance pulses reflects the nature of the analyte, thus allowing differentiation between different analytes.

  ✓ single-molecule detection
  ✓ high-throughput
  ✓ label-free



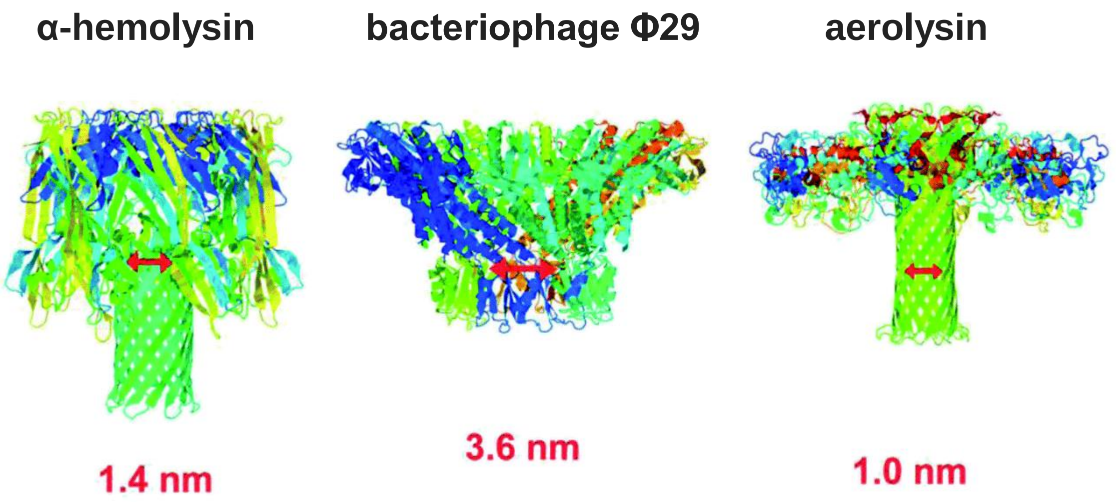
Nanopore sensing can

- identify the properties without losing information for low-abundance molecules.
- monitor the dynamic transformation processes of a series of biomolecules in a complex system.

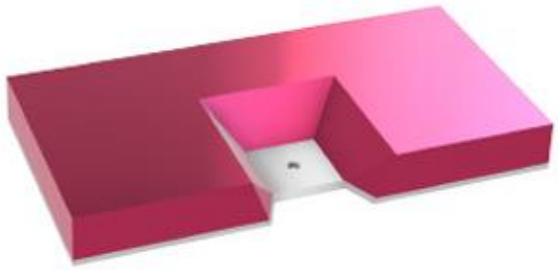
Gooding, J.; Gaus, K. *Angew. Chem. Int. Ed.* **2016,** *55*, 11354. Ying, Y.-L.; Long, Y.-T. *J. Am. Chem. Soc.* **2019**, *141*, 15720.

Types of Nanopores

- **Biological nanopore**
  - **porin in phospholipid membrane**
  - **natural interaction with bio-analyte**
  - **engineering by site-directed mutagenesis**



- **Solid-state nanopore**
  - **inorganic material (SiNx or SiO_2, graphene etc.)**
  - **pore fabrication by focused ion or electron beams.**
  - **easy tuning of the size and geometry of the pore**
  - **mechanical and chemical stability**



Bhatti, H.; Jawed, R.; Ali, I.; Iqbal, K.; Han, Y.; Lu, Z.; Liu, Q. *RSC Adv.* **2021**, *11*, 28996.

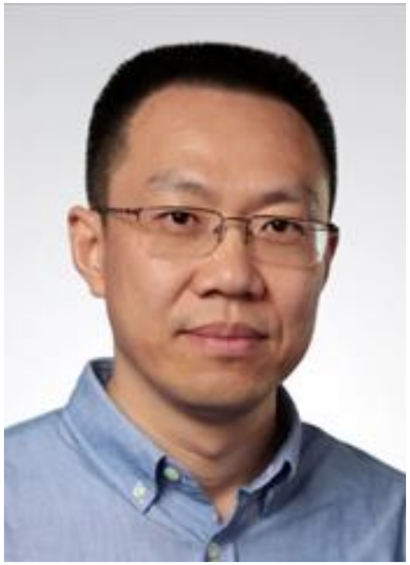
Contents

- Introduction

- **Protein nanopore reveals the renin–angiotensin system crosstalk with single-amino-acid resolution**

Jiang, J.; Li, M.-Y.; Wu, X.-Y.; Ying, Y.-L.; Han, H.-X.; Long, Y.-T. *Nat. Chem.* **2023**, *15*, 578.

Author's Profile



Yi-Tao Long

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- Ph.D.: Bioelectrochemistry, Nanjing University (1998)

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  Prof. Michael Grunze
- Research Associate: University of Saskatchewan (2001-06)

  Prof. Jeremy S. Lee
- Senior Research Scientist: University of Calfornia, Berkeley (2006-07)
- Distinguished Professor: East China University of Science and Technology (ECUST) (2007-18)
- Professor: Nanjing University (2019-)

Research interests

Nanopore Single Molecule Analysis

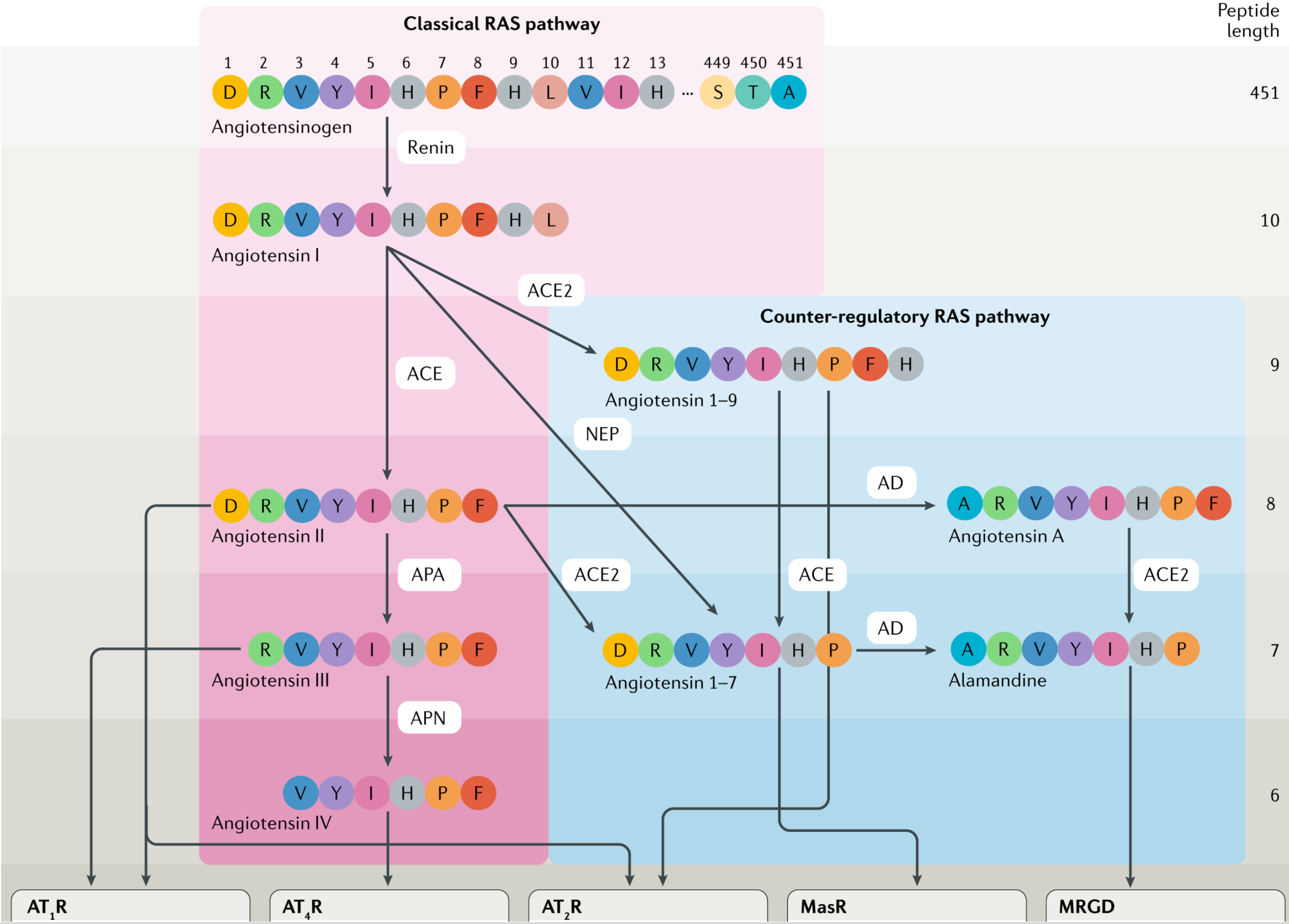
Nanoelectrochemistry

in-situ Spectroelectrochemistry

纳米孔道
Nanopore

Renin–Angiotensin System (RAS)

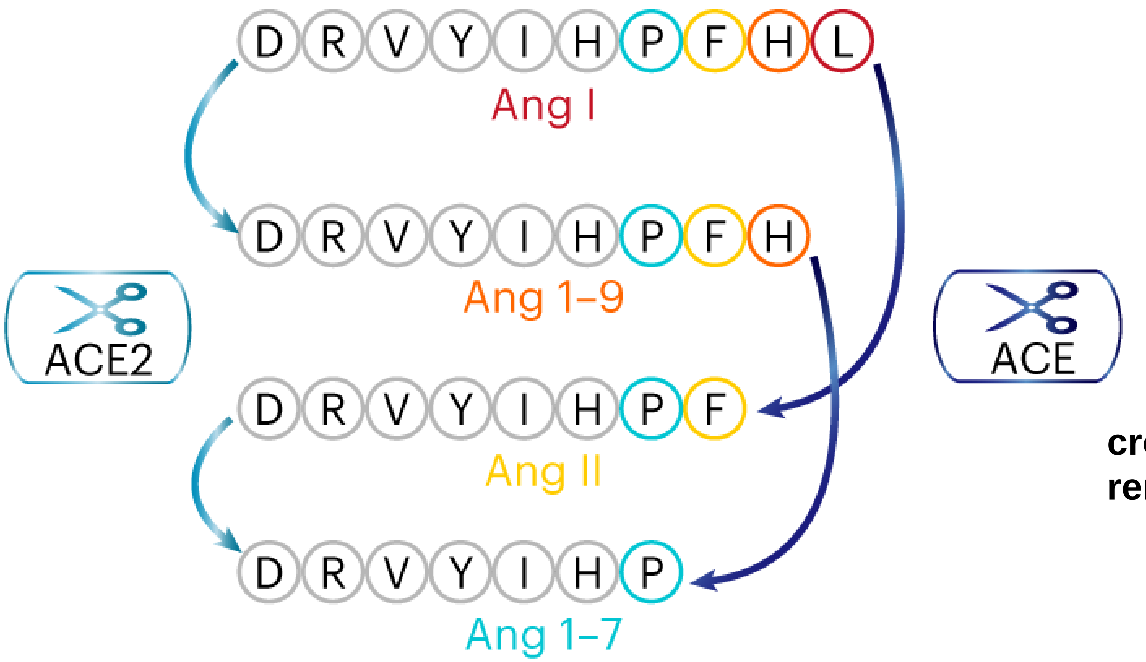
renin-angiotensin system: hormone system that regulates blood pressure and electrolyte balance[1]



1. Ocaranza, M. P.; Riquelme, J. A.; Garcia, L.; Jalil, J. E.; Choing, M; Santos, R. A.; Lavandero, S. *Nat. Rev. Cardiol.* **2019**, *17*, 116.

Crosstalk in the Renin–Angiotensin System

Renin-angiotensin system involving ACE and ACE2



crosstalk effects between ACE and ACE2 remains undetermined.

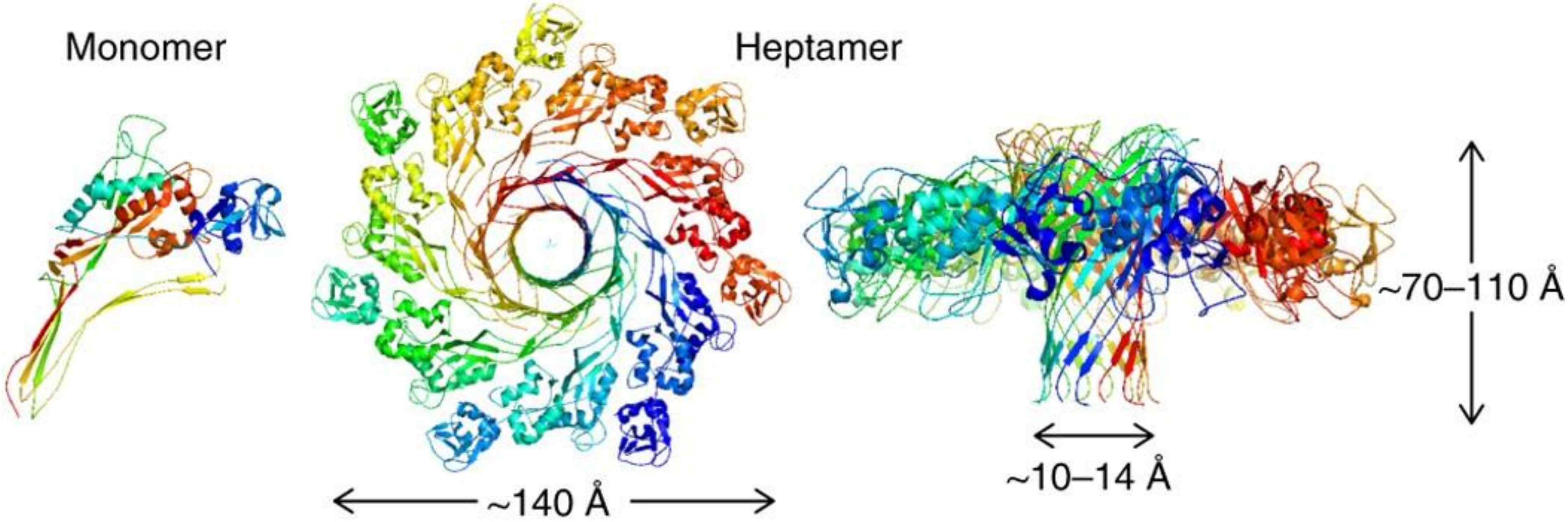
direct reading a number of individual molecules of multiple Ang peptides during the enzymatic process
→ **nanopore framework to reveal crosstalk effects**

requirement:
(1) sufficient resolution to identify peptides with one-amino-acid difference
(2) high capture efficiency to ensure real-time analysis
(3) peptide detection that are compatible with the enzyme reaction environment

Aerolysin

aerolysin: β-barrel pore-forming toxins produced by *Aeromonas hydrophila*[1]

structure of aerolysin (PDB ID: 5JZT)[2]

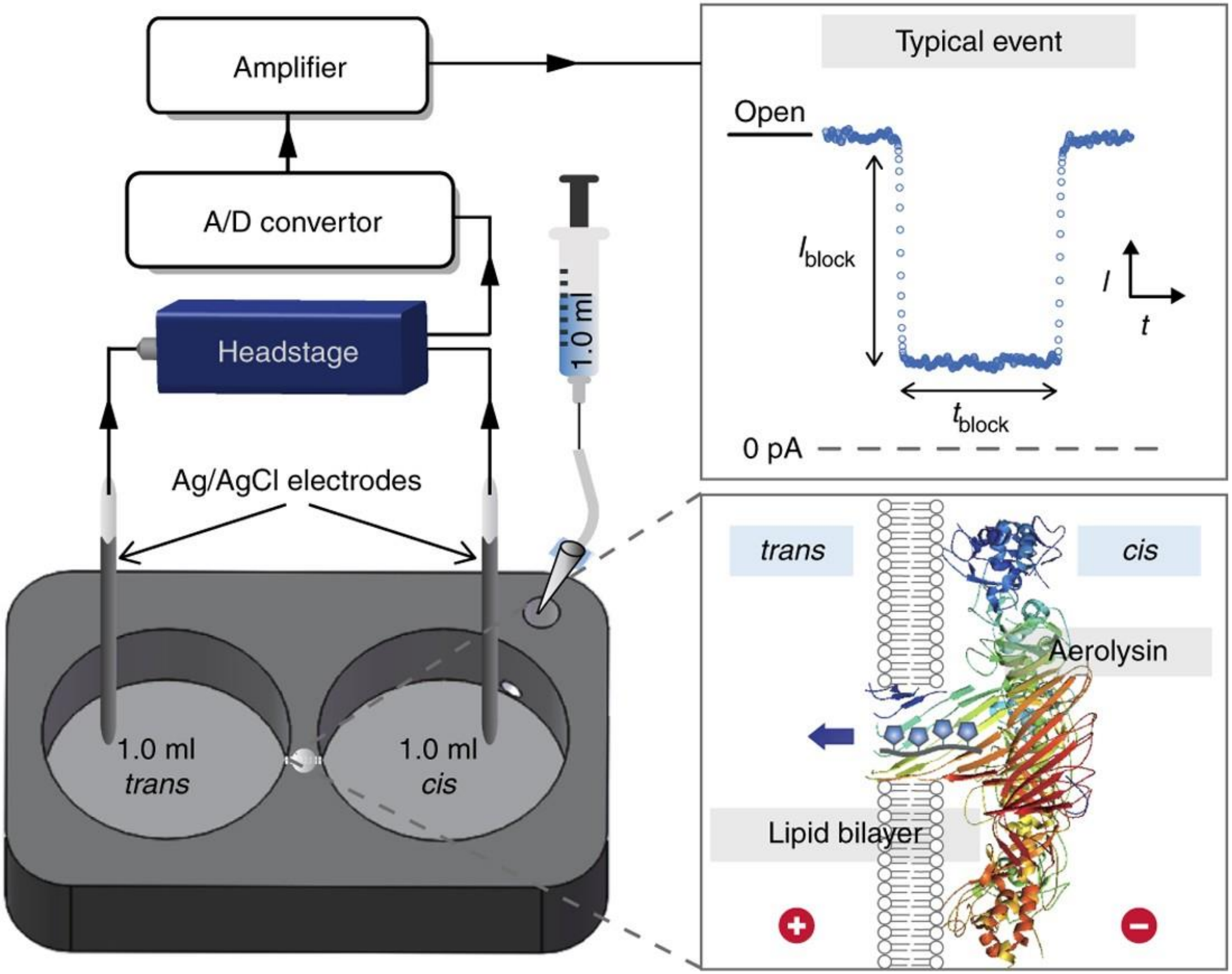


- **long retention of analytes**
- **applicable in wide range of pH (4.0~10.1)**
- **stable under denaturing agents**

Aerolysin has been utilized as a suitable nanopore for precise analysis of single molecules.

1. Parker, M. W.; Buckley, J. T.; Postma, J. P. M.; Tucker, A. D.; Leonard, K.; Pattus, F.; Tsernoglou, D. *Nature* **1994**, *367*, 292.
2. Iacovache, I.; Carlo, S. D.; Cirauqui, N.; Peraro, M. D.; van der Goot, F. G.; Zuber, B. *Nat. Commun*. **2016**, *7*, 12062.

Experimental Setup



monitored parameters
I_{block} : blockade current
t_{open} : duration
f : frequency

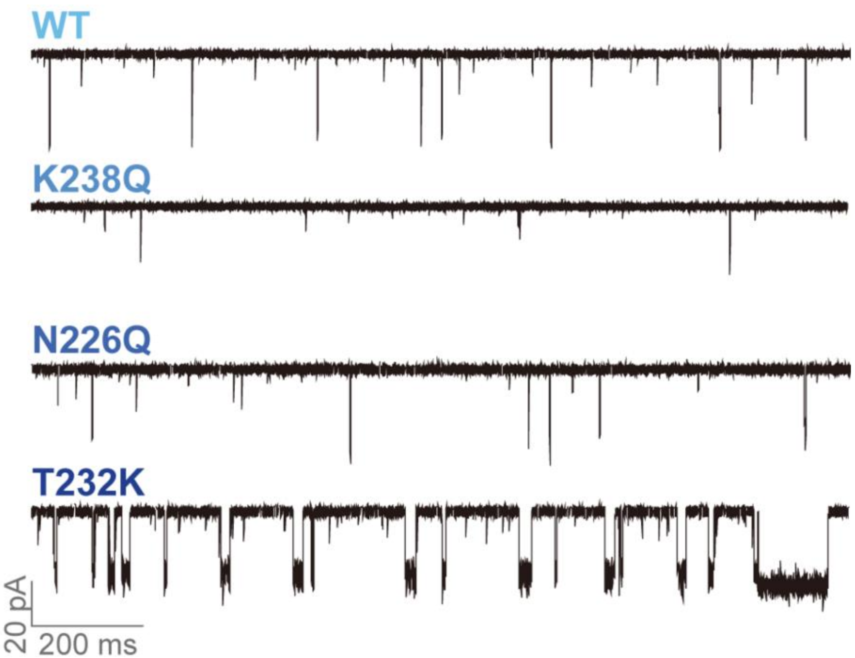
translocation event is characterized by $I/I_0 = I_{block}/I_{open}$
I_{open}: open pore ionic current

Cao, C.; Liao, D.-F; Yu, J.; Tian, H.; Long, Y.-T. *Nat. Protocols* **2017**, *12*, 1901.

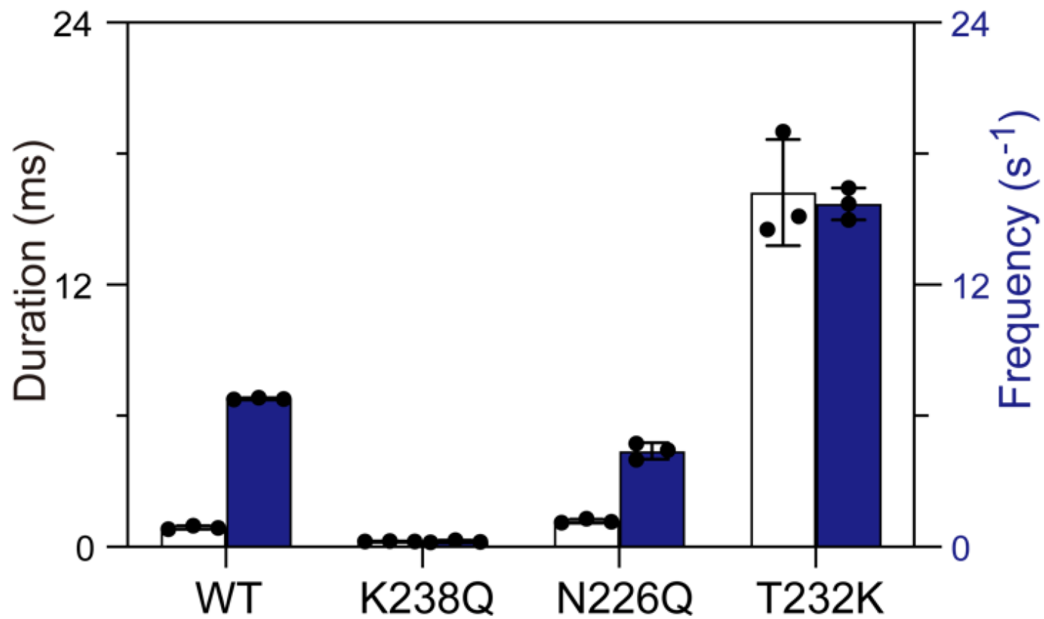
Establishment of a Nanopore Framework

For the discrimination of Ang peptides in a single amino acid resolution, mutant aerolysin candidates[1,2] were investigated to introduce electroosmotic flow and enhance the interactions with target peptides.

Detection of Ang I



Duration (white) and capture rate (blue) of Ang I



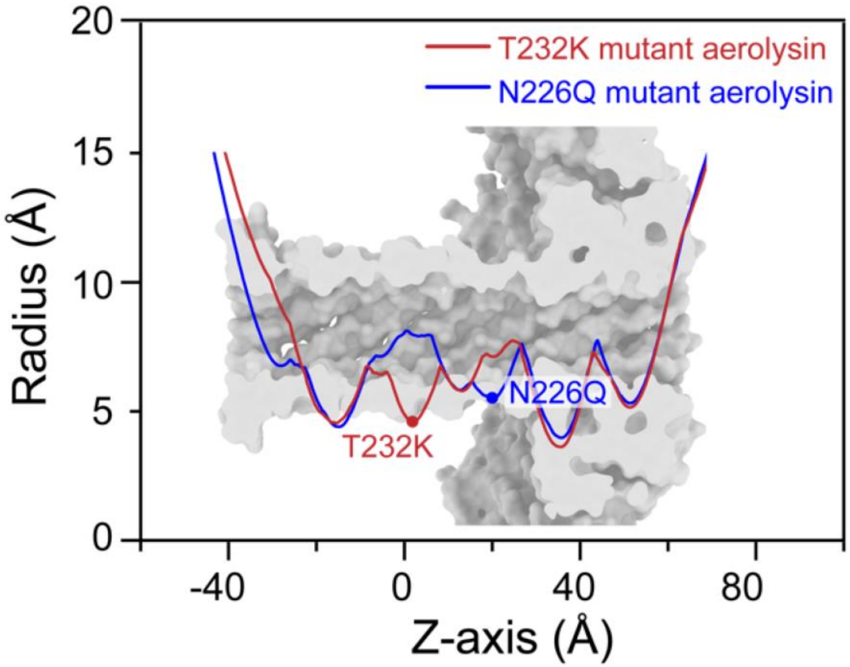
T232K aerolysin exhibited the longest duration and highest capture efficiency for Ang I

1. Wang, Y.-Q.; Cao, C.; Ying, Y.-L.; Li, S.; Wang, M.-B.; Huang, J.; Long, Y.-T. *ACS Sens.* **2018**, *3*, 779.
2. Wu, X.-Y.; Wang, M.-B.; Wang, Y.-Q.; Li, M.-Y.; Ying, Y.-L.; Huang, J.; Long, Y.-T. *CCS Chem*. **2019**, *1*, 304.

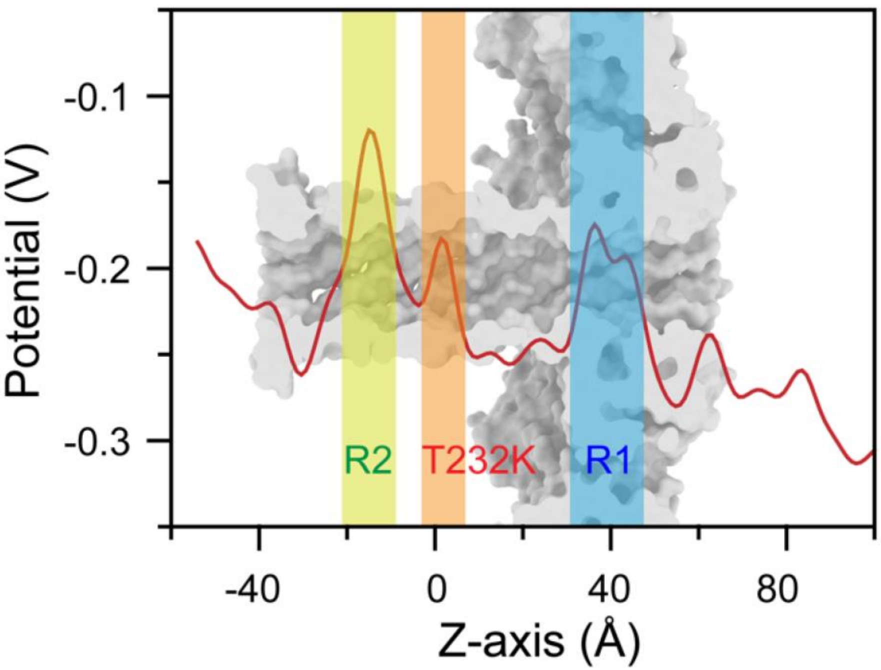
MD Simulation of T232K Mutant Aerolysin

For the discrimination of Ang peptides in a single amino acid resolution, mutant aerolysin candidates[1,2] were investigated to introduce electroosmotic flow and enhance the interactions with target peptides.

Pore radius of T232K and N226Q mutant



Potential distribution of T232K mutant



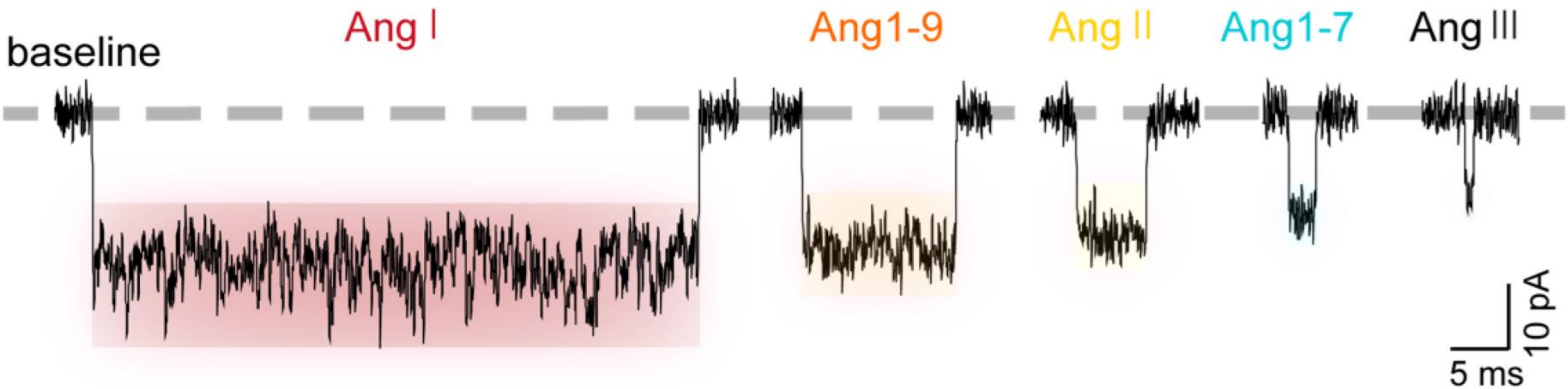
- **narrow radius and the longer side chain of lysine**
- **enhancement of electroosmotic flow by the positive charges of lysine**
- **introduction of an extra potential fluctuation region inside the T232K aerolysin**

T232K aerolysin exhibited the longest duration and highest capture efficiency for Ang I

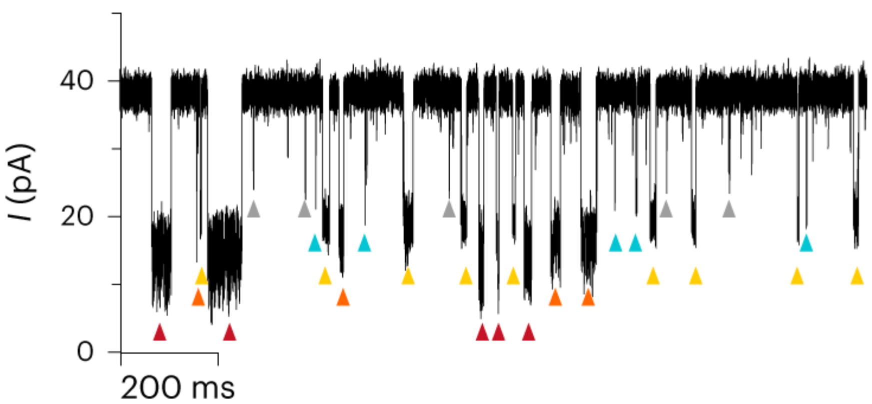
1. Wang, Y.-Q.; Cao, C.; Ying, Y.-L.; Li, S.; Wang, M.-B.; Huang, J.; Long, Y.-T. *ACS Sens.* **2018**, *3*, 779.
2. Wu, X.-Y.; Wang, M.-B.; Wang, Y.-Q.; Li, M.-Y.; Ying, Y.-L.; Huang, J.; Long, Y.-T. *CCS Chem*. **2019**, *1*, 304.

Distinguishing Ang Peptides in the Mixture

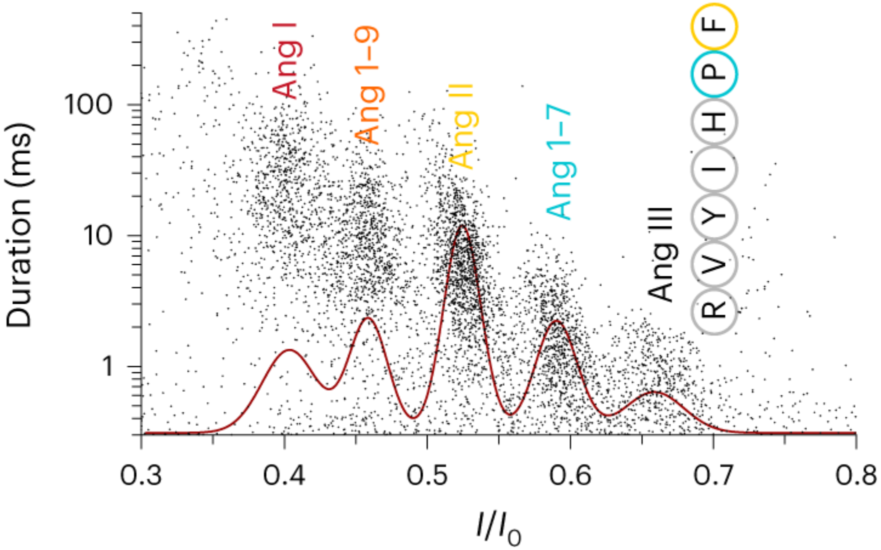
Current fluctuation corresponding to each Ang peptide



Raw current traces for Ang peptides mixture



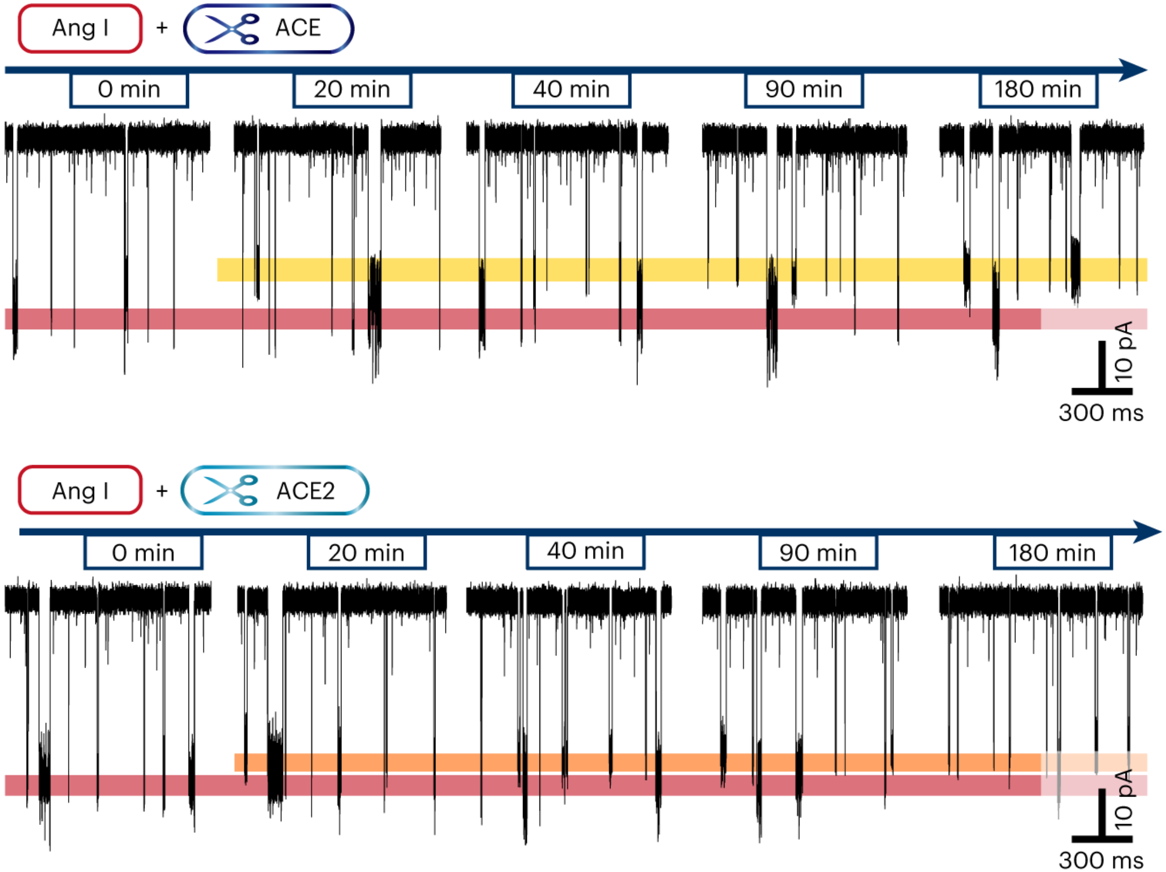
Scatter plots and gaussian fitting of I/I_0 histogram



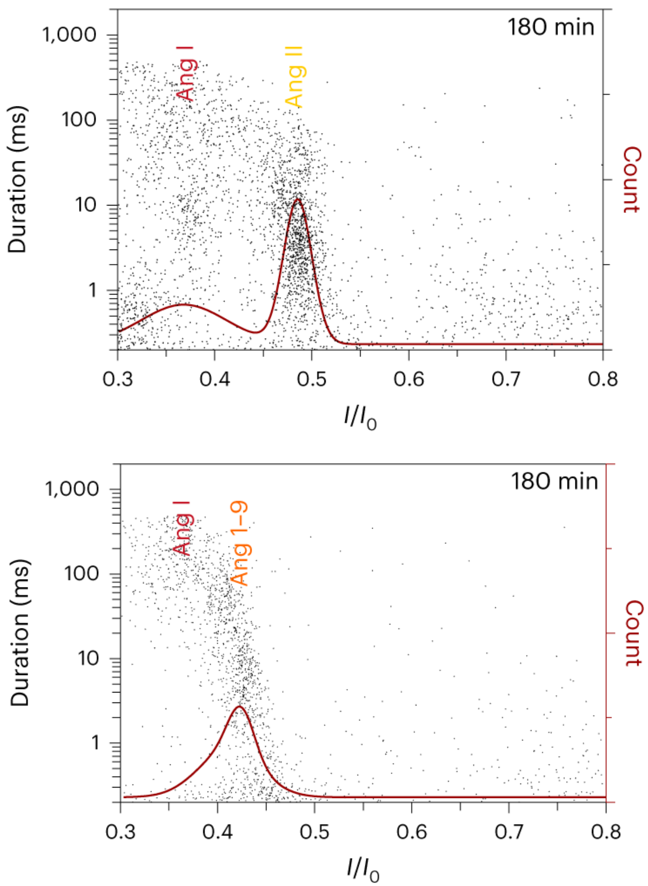
T232K aerolysin could identify all the five Ang peptides in the mixture in single-amino acid resolution.

Ang I Cleavage Recording by Nanopore

Raw current traces for Ang I cleaved by ACE or ACE2



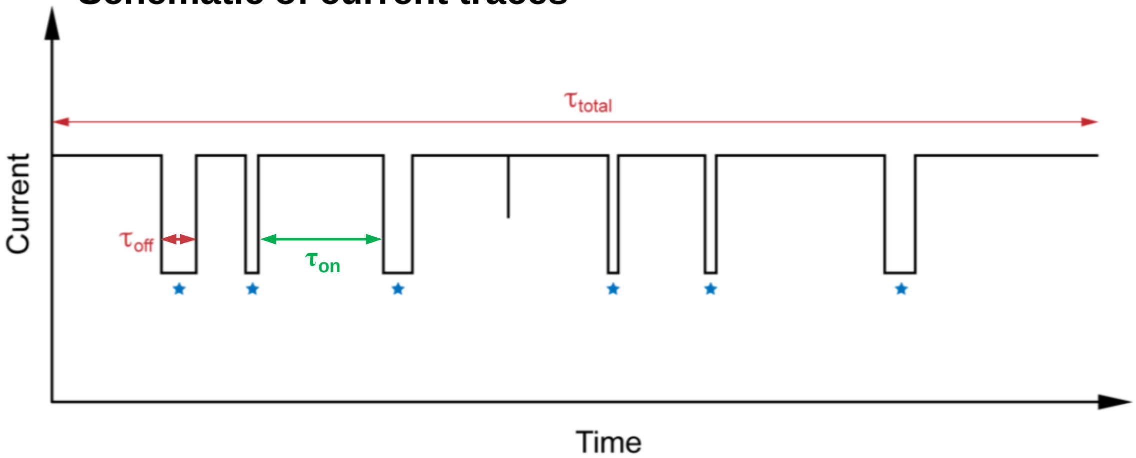
Scatter plots and gaussian fitting of I/I_0 histogram



Enzymatic reactions of ACE and ACE2 were observed by nanopore sensing.

Redefinition of Capture Efficiency

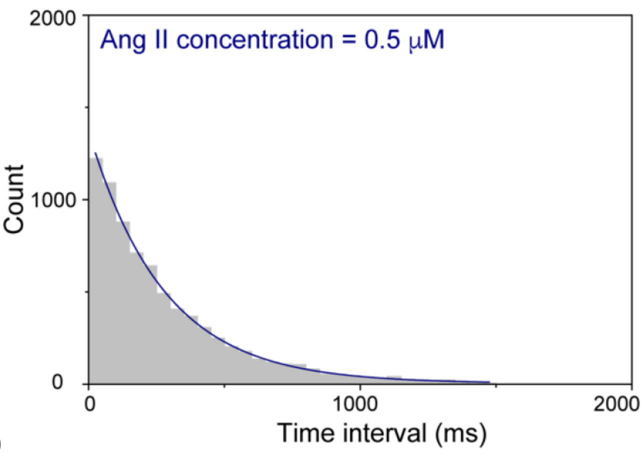
Schematic of current traces



τ_{on} : inter-event interval
τ_{off} : dwell time of a peptide
τ_{total} : current recording time

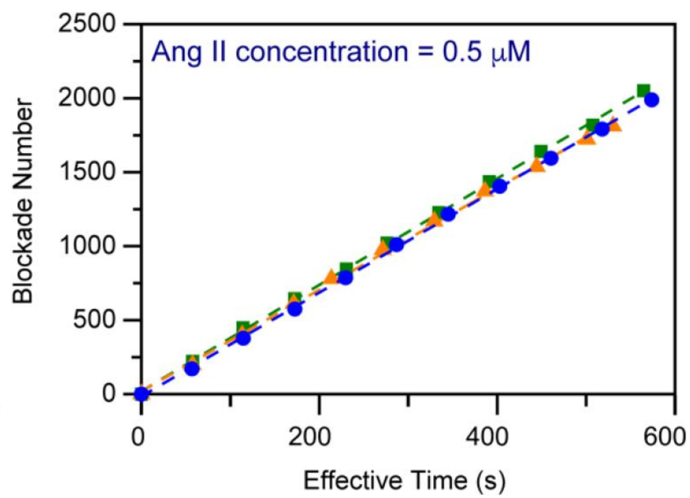
Capture efficiency of a peptide

$f = 1/\tau_{on}$



long recording time required (> 500 counts)

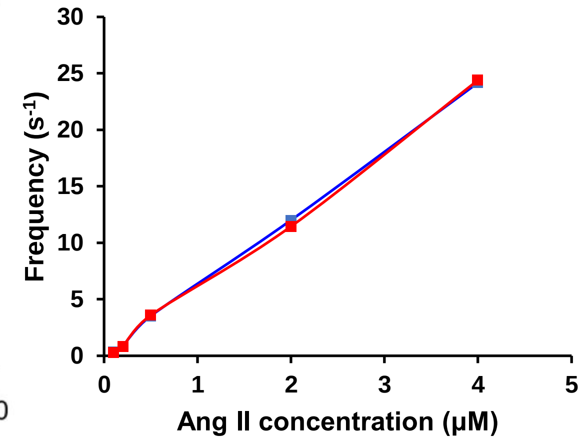
$f = n_{peptide}/\tau_{effective}$
$(\tau_{effective} = \tau_{total} - \Sigma\tau_{off})$



derivable in real-time analysis

Detection of Ang II

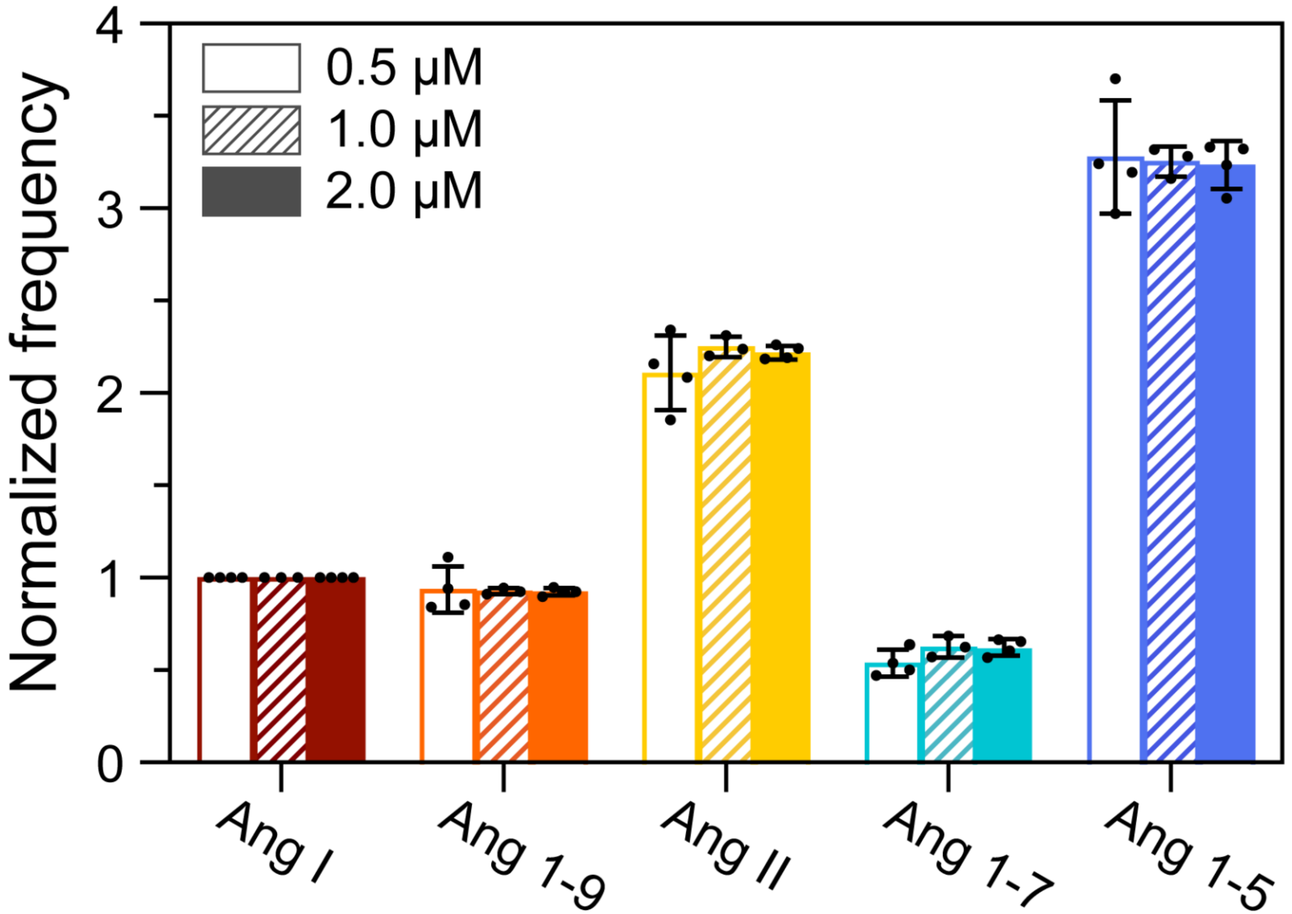
— $f = 1/\tau_{on}$
— $f = n_{peptide}/\tau_{effective}$



$n_{peptide}/\tau_{effective}$ is a comparative index for capture efficiency applicable in real-time analysis.

Capture Efficiency Normalization

Capture efficiency of T232K mutant aerolysin for Ang peptides in their mixture



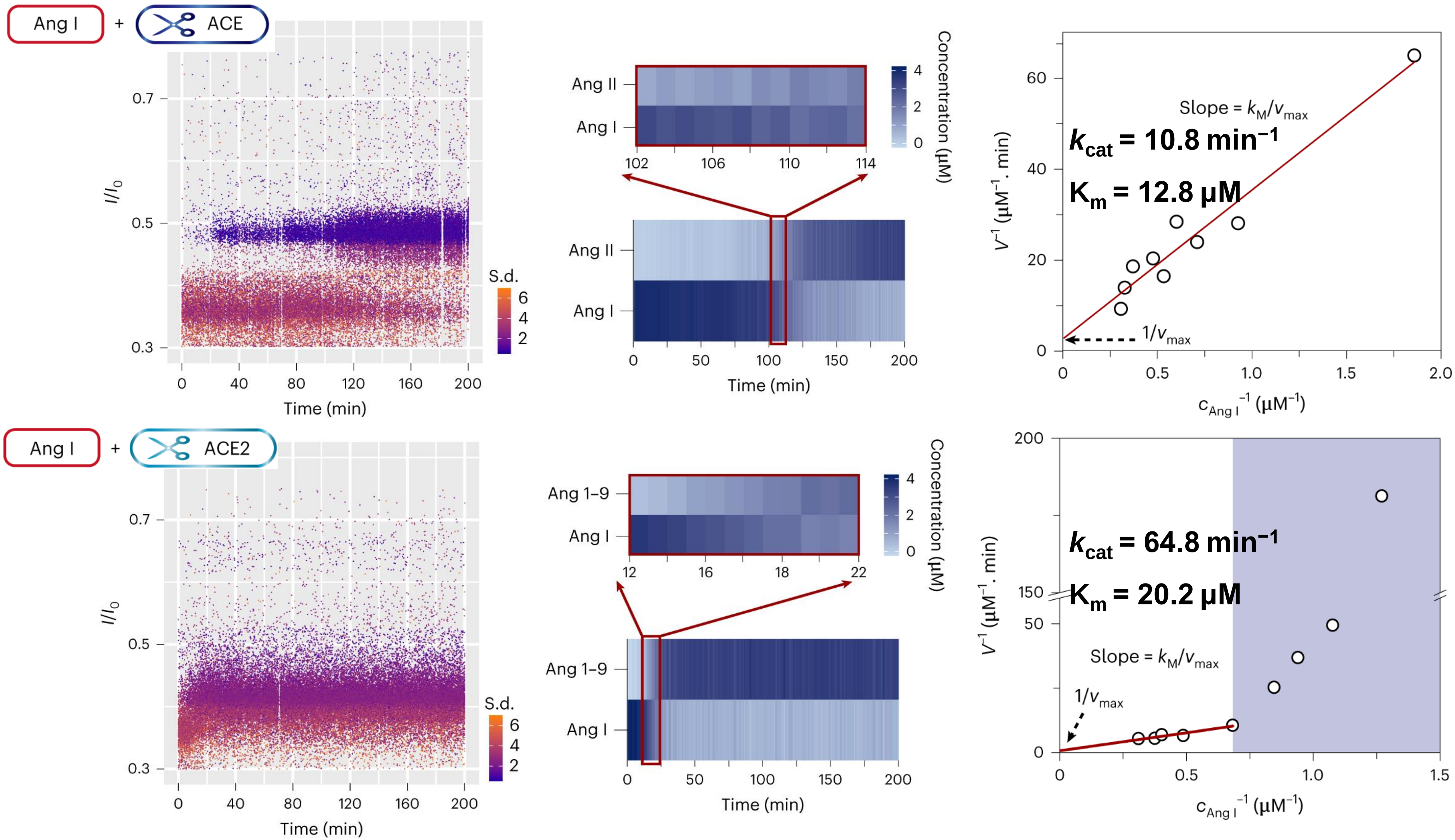
No detectable concentration dependency was confirmed in frequency (capture efficiency).

Quantification is possible by combining the number of detected signals and the capture efficiency.

Real-Time Analysis of Enzymatic Reactions

capture efficiency normalization → **Michaelis-Menten equation** $v = k_{cat}[E]_0[S]/(K_m+[S])$

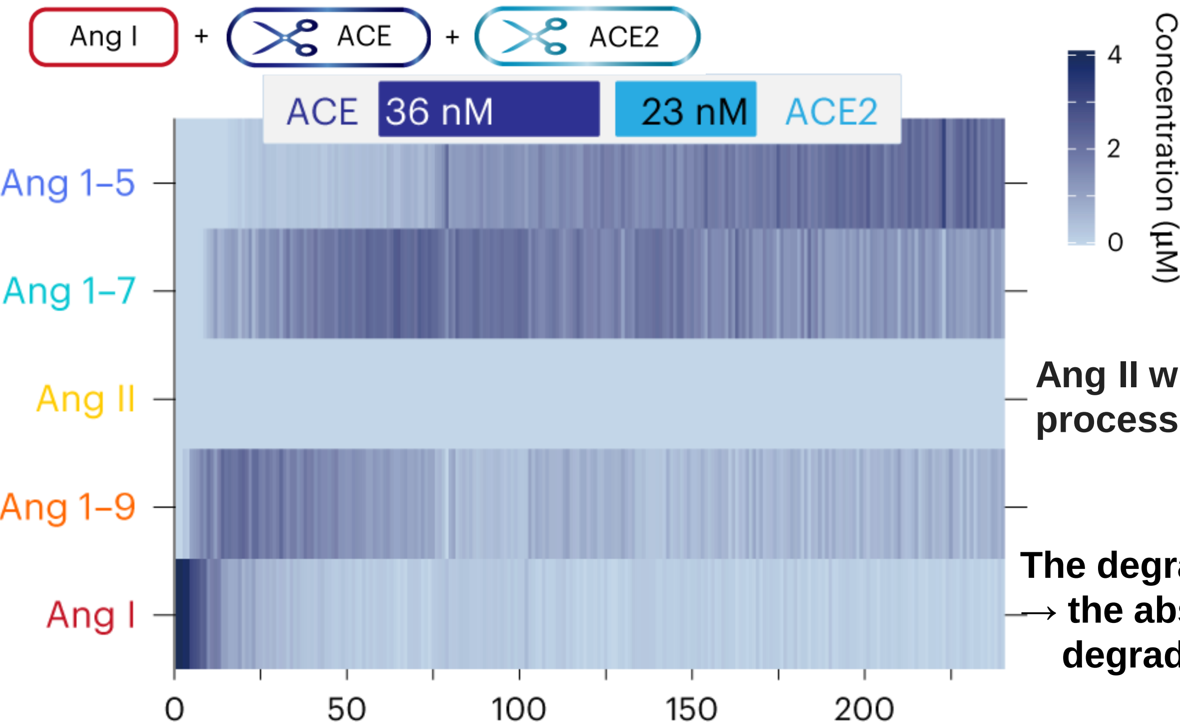
I/I_0 versus reaction time | **Evolution of Ang peptides** | **Lineweaver-Burk plot**



Nanopore sensing achieved real-time monitoring of the evolution processes.

Crosstalk Between ACE and ACE2

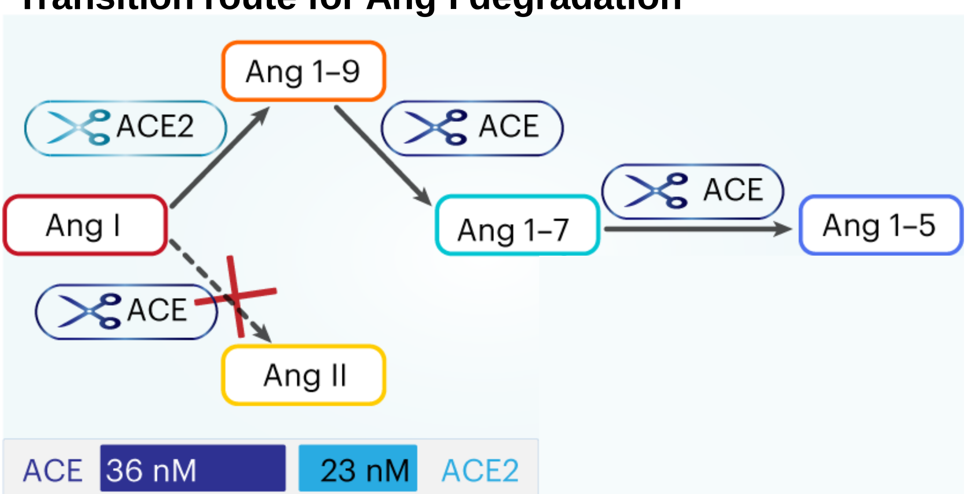
Real-time monitoring of the enzymatic degradation of Ang I



Ang II was not observed at all throughout the process.

The degradation velocity of Ang I was unchanged.
→ the absence of Ang II was not caused by rapid degradation by ACE2.

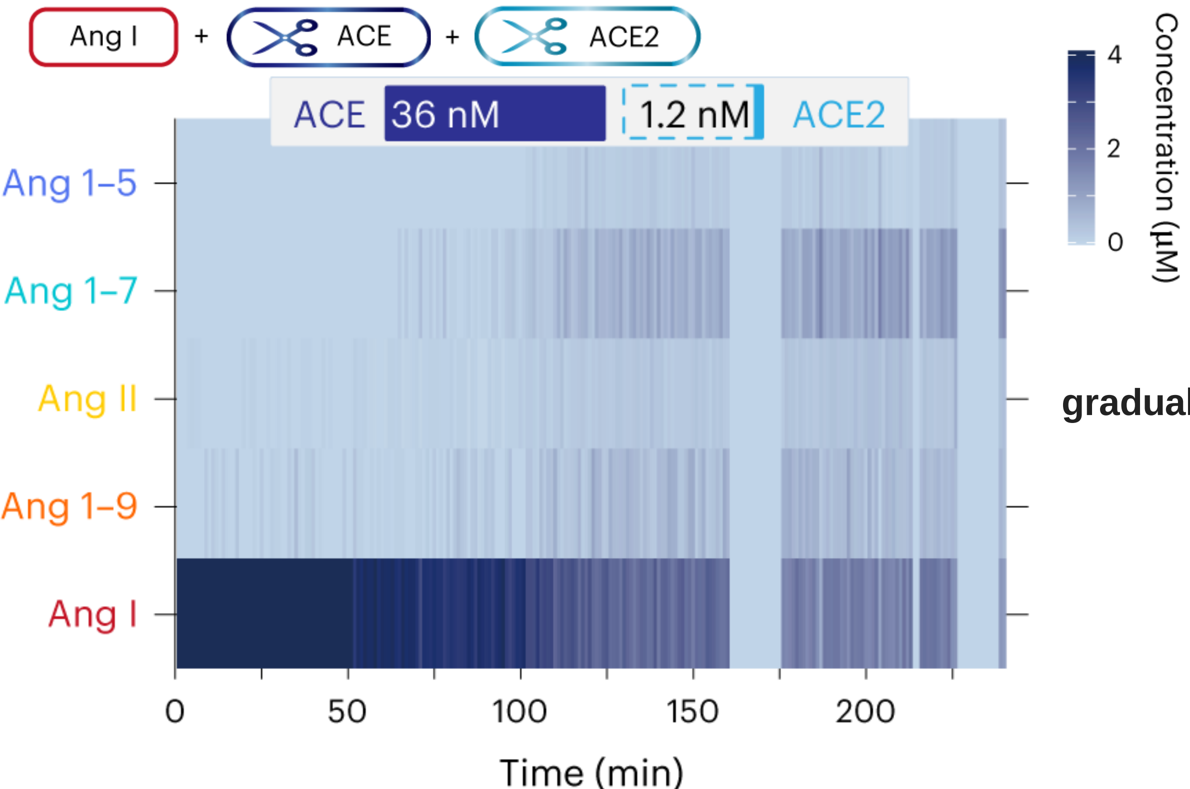
Transition route for Ang I degradation



ACE is severely and selectively suppressed by ACE2 only on the production of Ang II.

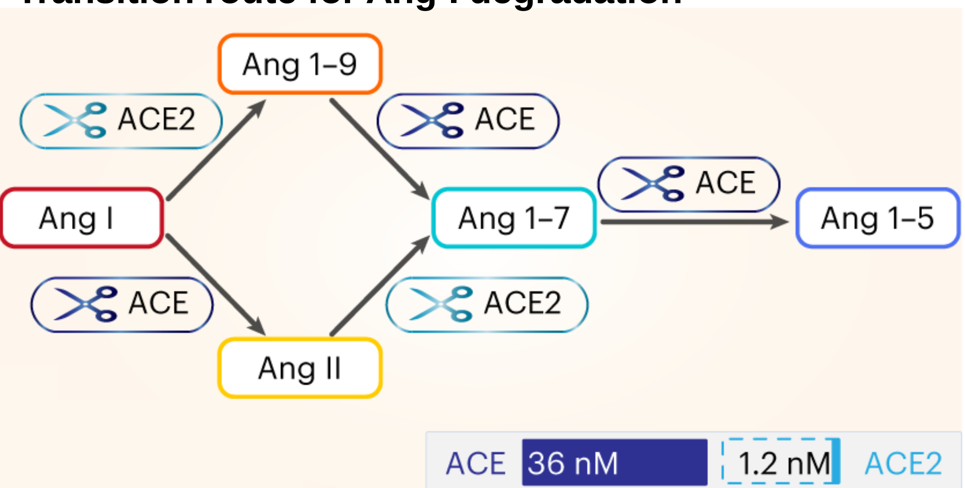
Effect of the Ratio of ACE/ACE2 to the Crosstalk

Real-time monitoring of the enzymatic degradation of Ang I



gradual generation of Ang II was observed.

Transition route for Ang I degradation

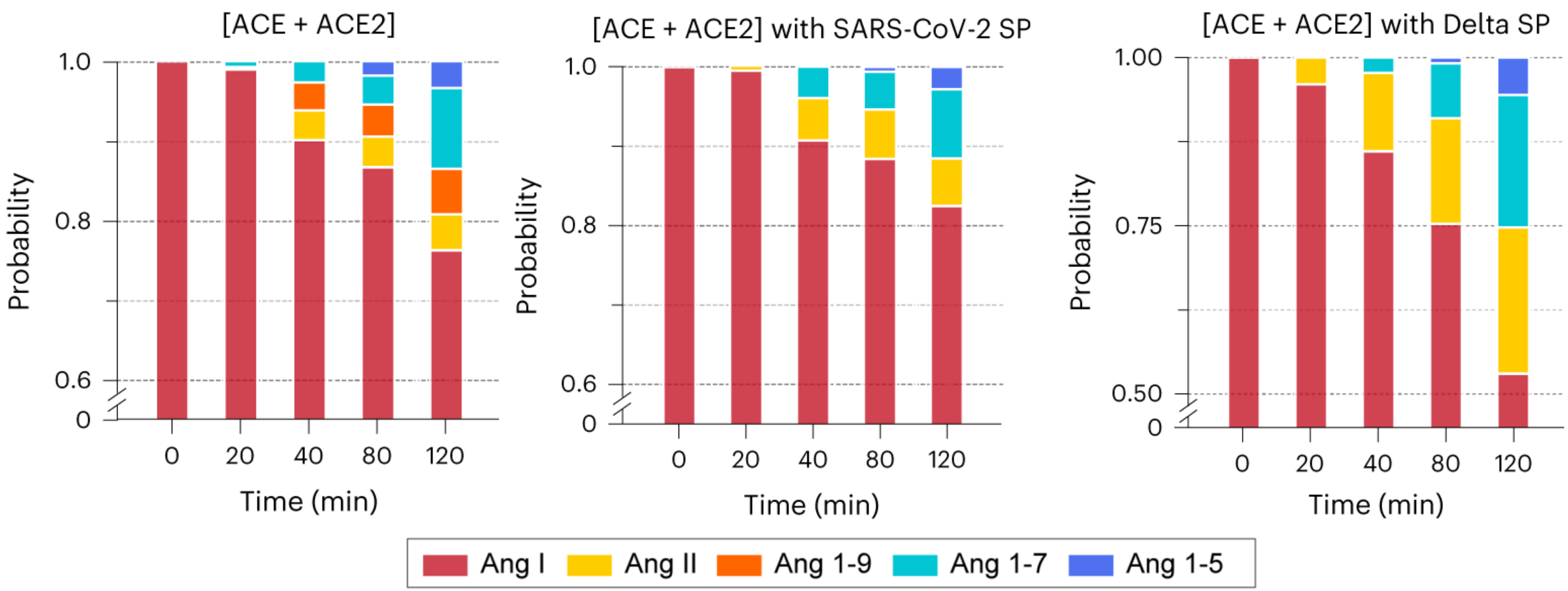


Crosstalk between ACE and ACE2 was alleviated by reducing ACE2.

Influence of the SARS-CoV-2 Spike Protein

ACE2 has been regarded as the cellular receptor of SARS-CoV-2.[1]

Time-dependent probability during the cleavage of Ang I by ACE and ACE2 (ACE:ACE2 = 1:0.03)



- **decrease in Ang 1-9**
- **increase in Ang II**

➡

- **inhibition of ACE2 by the spike protein of SARS-CoV-2**
- **disappearance of inhibition of ACE2 to ACE for Ang I cleavage**

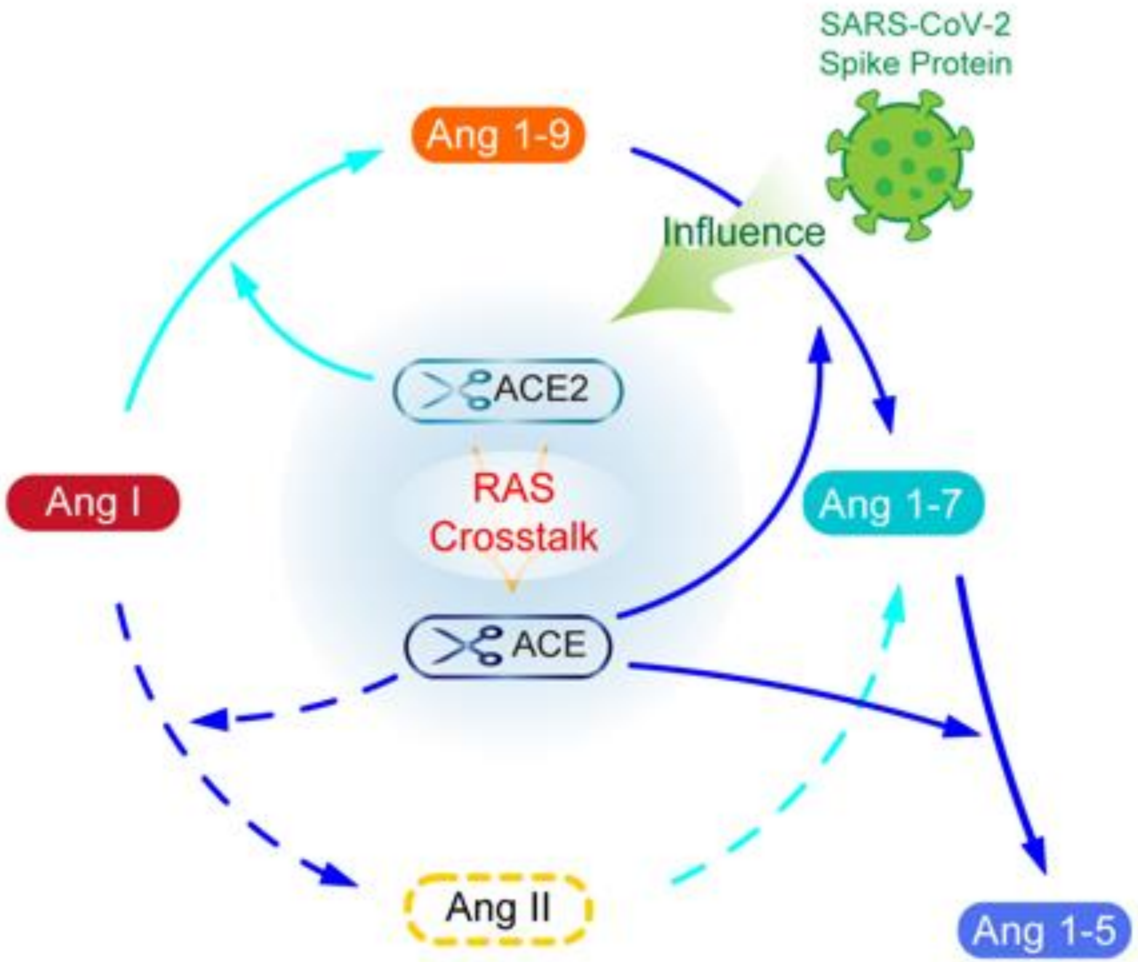
- **escalation in Delta variant**

➡

- **Over-accumulation of Ang II[2] could be associated with aggravation risk.**

1. Walls, A. C.; Park, Y.-J.; Tortorici, M. A.; Wall, A.; McGuire, A. T.; Veesler, D. *Cell* **2020**, *181*, 281.
2. Ocaranza, M. P.; Riquelme, J. A.; Garcia, L.; Jalil, J. E.; Choing, M; Santos, R. A.; Lavandero, S. *Nat. Rev. Cardiol.* **2019**, *17*, 116.

Summary

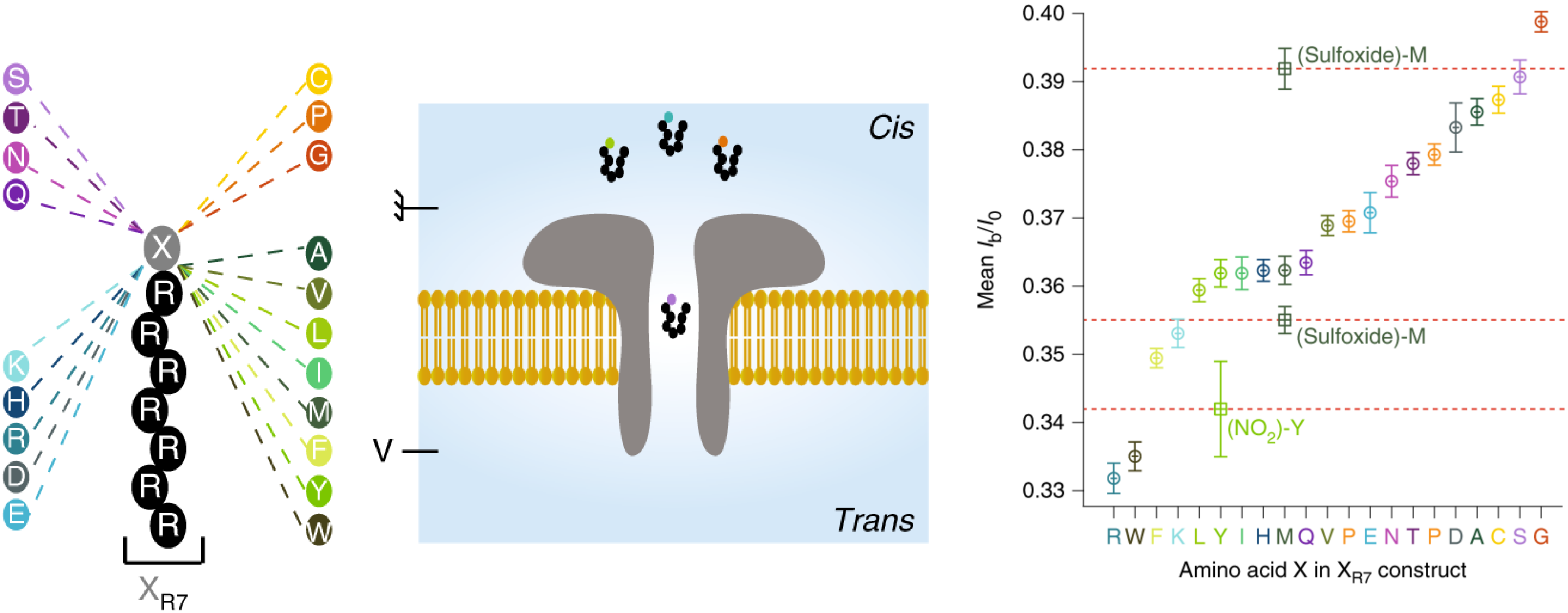


A nanopore-based method was provided to

- **identify and quantify a series of angiotensins with one unit difference in real time.**
- **reveal crosstalk effects between ACE and ACE2 in a mixed enzyme reaction system.**
- **evaluate the effects of SARS-CoV-2 SP in renin-angiotensin system.**

Attempts to Detect 20 Natural Amino Acids

A varied terminal "X" was chemically attached to an arginine heptapeptide, which acts as a polycationic carrier to ensure the unidirectional entry and travel of XR_7 peptides

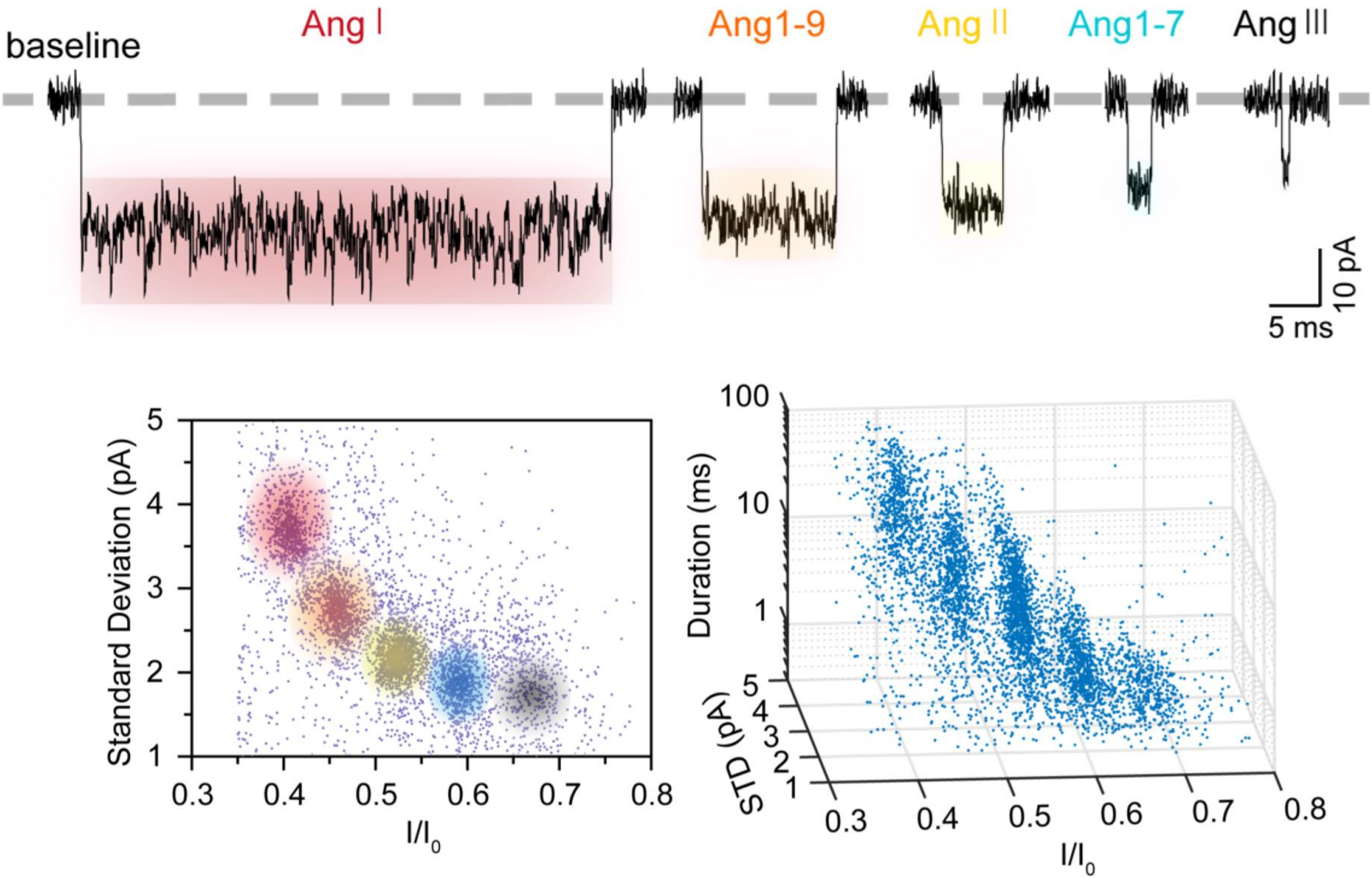


distinct current blockades in wild-type aerolysin can be used to identify 13 of the 20 natural amino acids.

H. Ouldali, K. Sarthak, T. Ensslen, F. Piguet, P. Manivet, J. Pelta, J. C. Behrends, A. Aksimentiev, A. Oukhaled, *Nat. Biotechnol.* **2020**, *38*, 176.

Distinguishing Ang Peptides in the Mixture

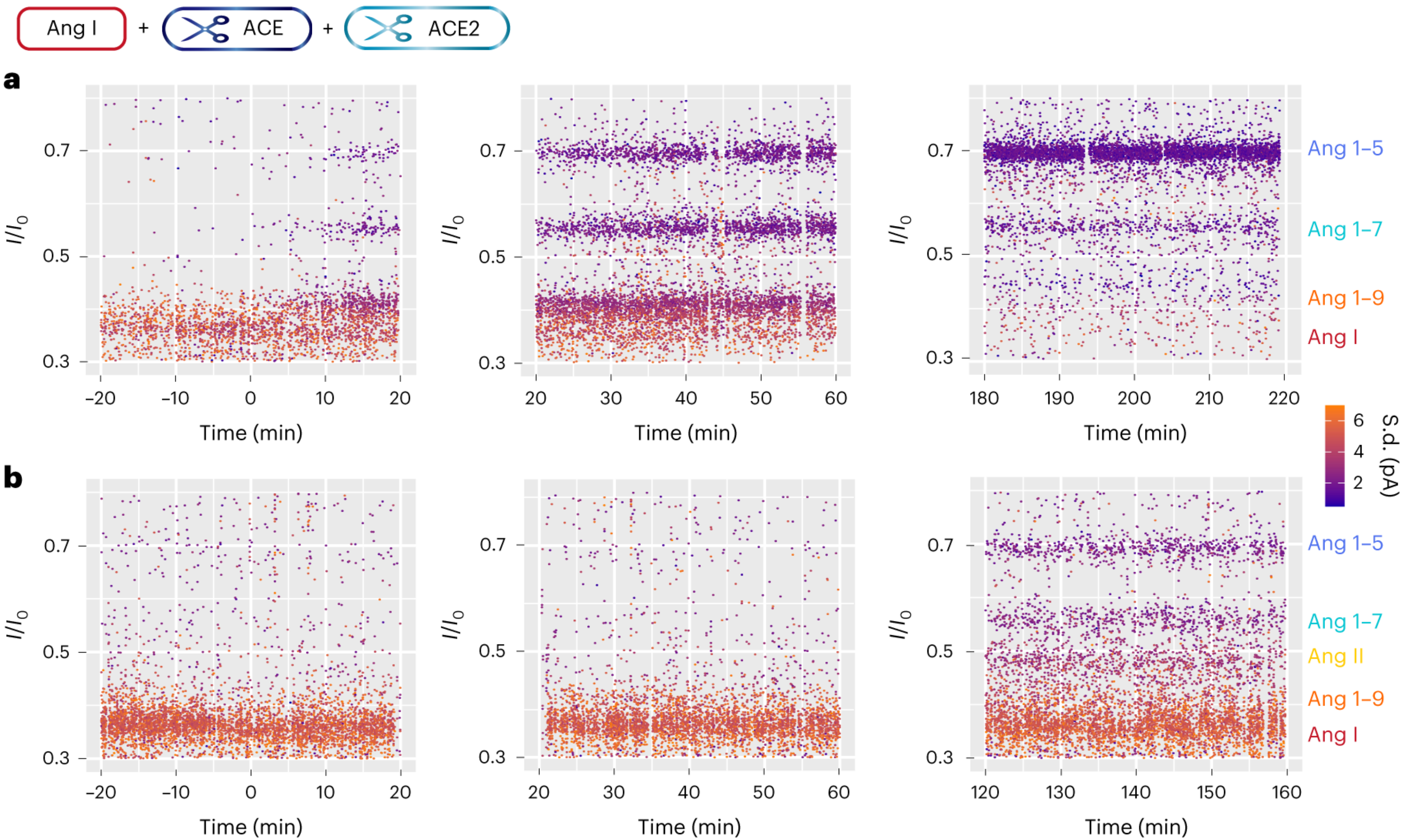
Current fluctuation corresponding to each Ang peptide



T232K aerolysin could identify all the five Ang peptides in the mixture.

Enzymatic degradation of Ang I by ACE2 and ACE

I/I_0 of the events versus reaction time while Ang I is cleaved by the enzyme mixture (a: 36 nM ACE and 23 nM ACE2, b: 36 nM ACE and 1.2 nM ACE2)

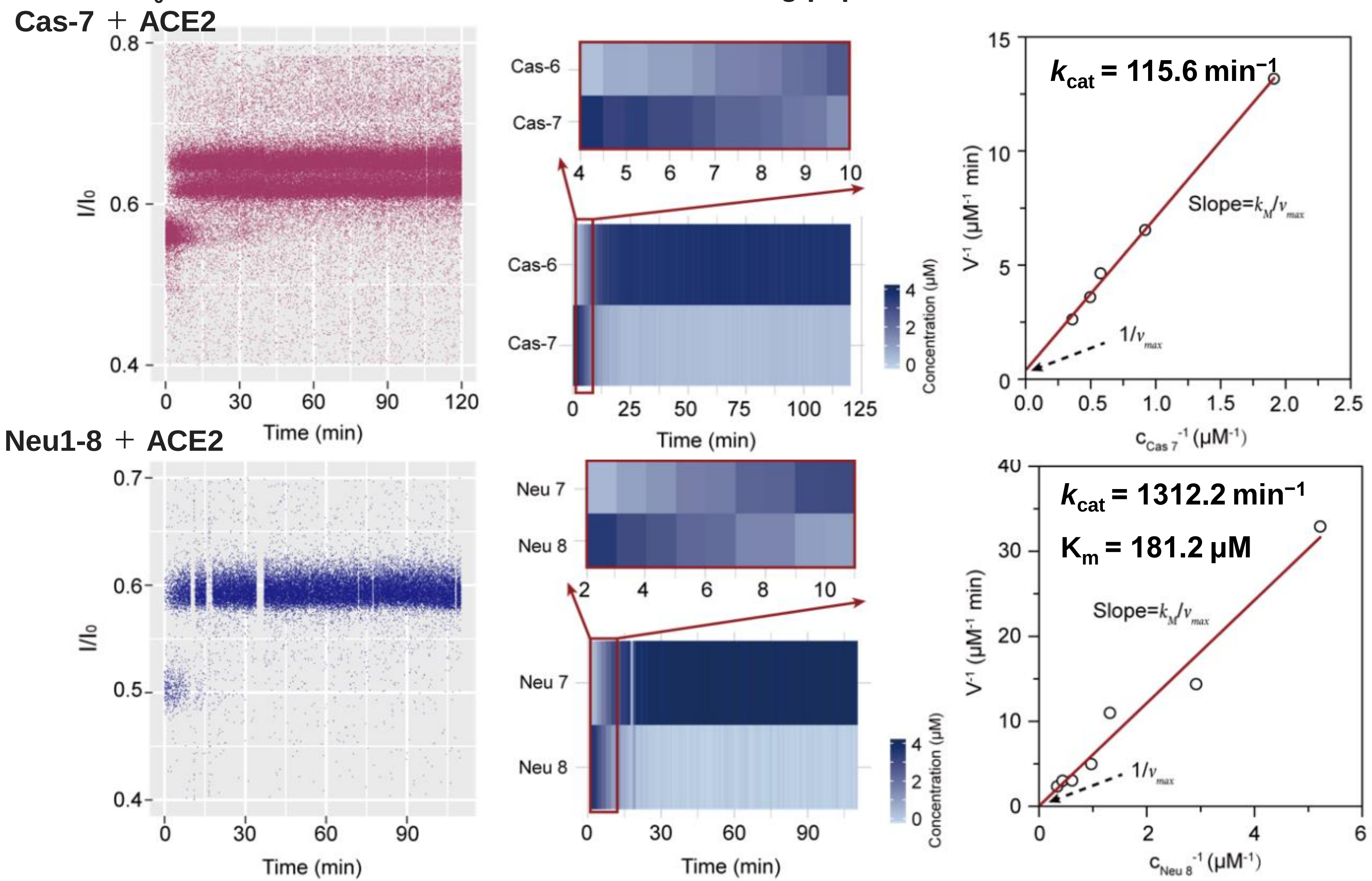


Hydrolysis of Different Substrates by ACE2

capture efficiency normalization

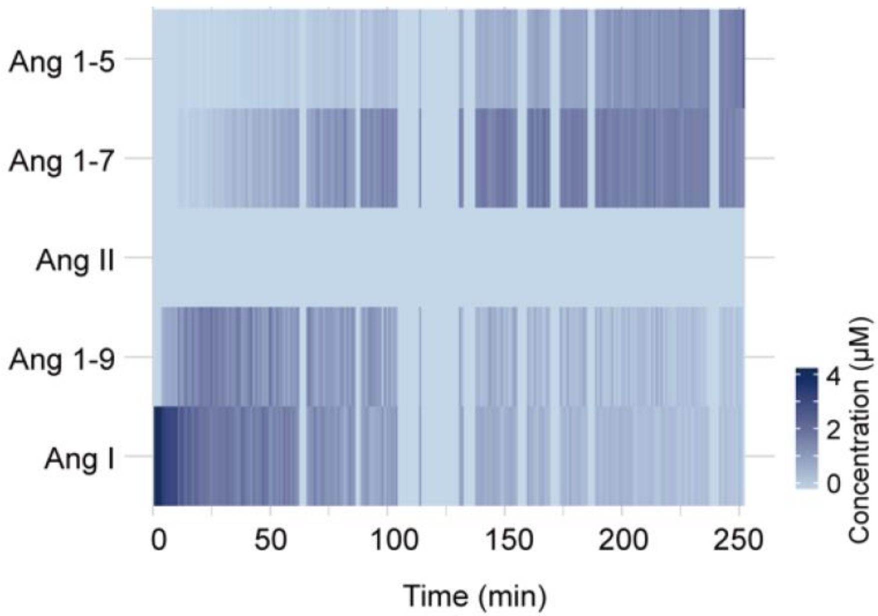
Michaelis-Menten equation
$v = k_{cat}[E]_0[S]/(K_m+[S])$

I/I_0 versus reaction time | Evolution of Ang peptides | Lineweaver-Burk plot

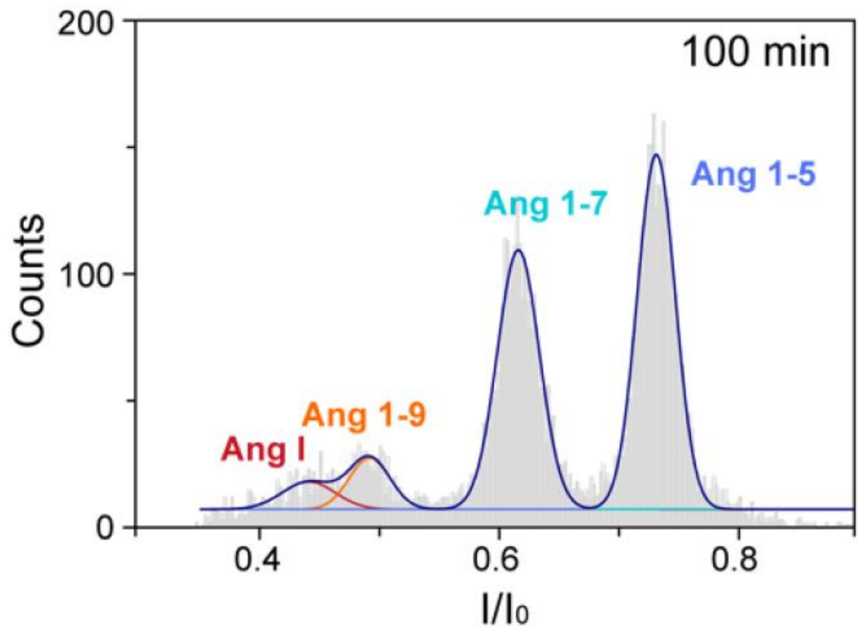


Influence of Chloride Anion

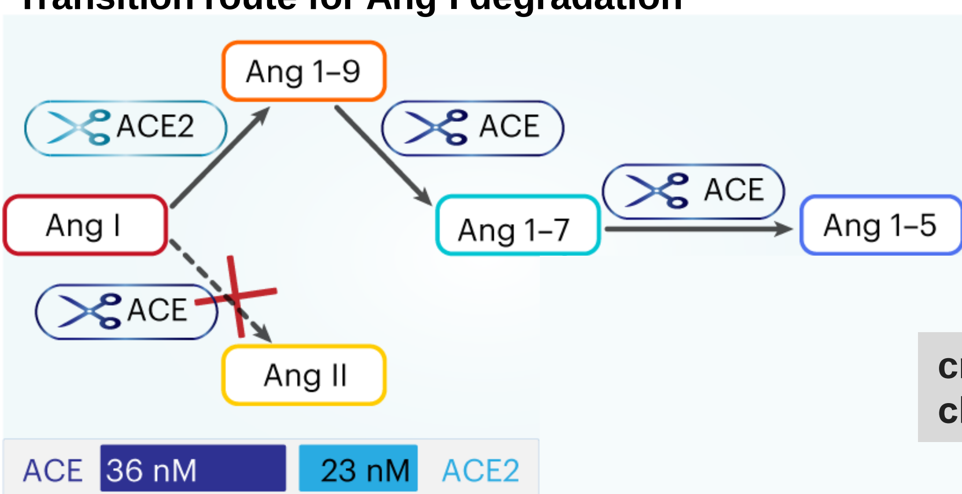
0.50 M KCl



0.15 M KCl



Transition route for Ang I degradation

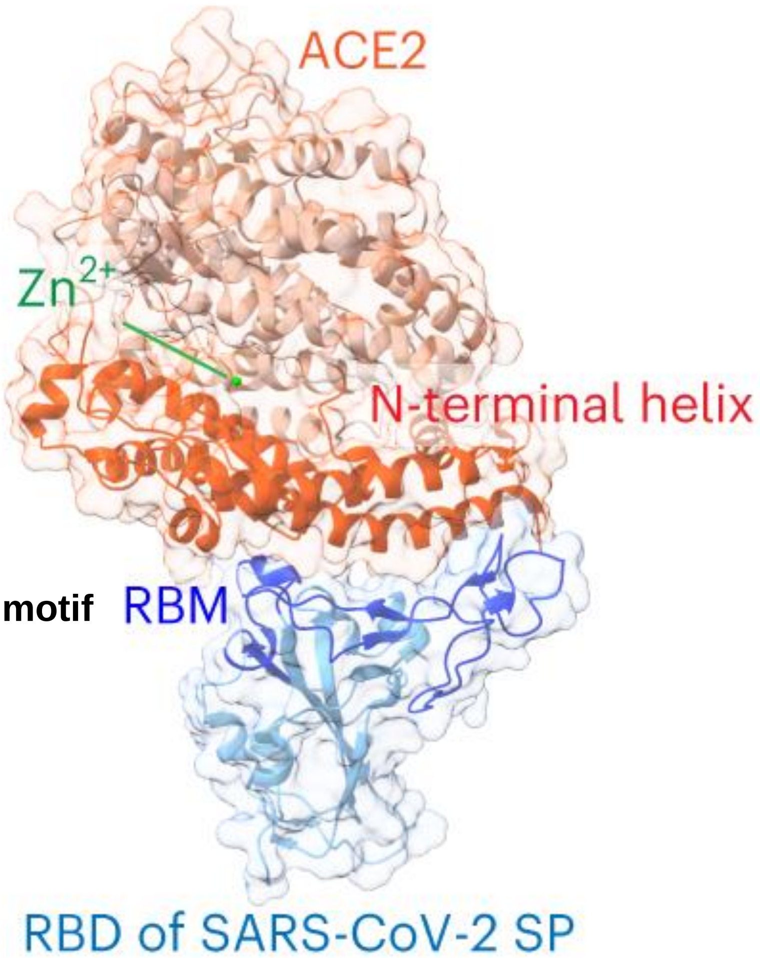


crosstalk between ACE and ACE2 was stable at chloride concentrations ranging from 0.15 M to 1.0 M.

SARS-CoV-2 Spike Receptor-Binding Domain

Illustration of the binding between the SARS-CoV-2 SP and ACE2 (PDB 6M0J)[1]

receptor-binding motif

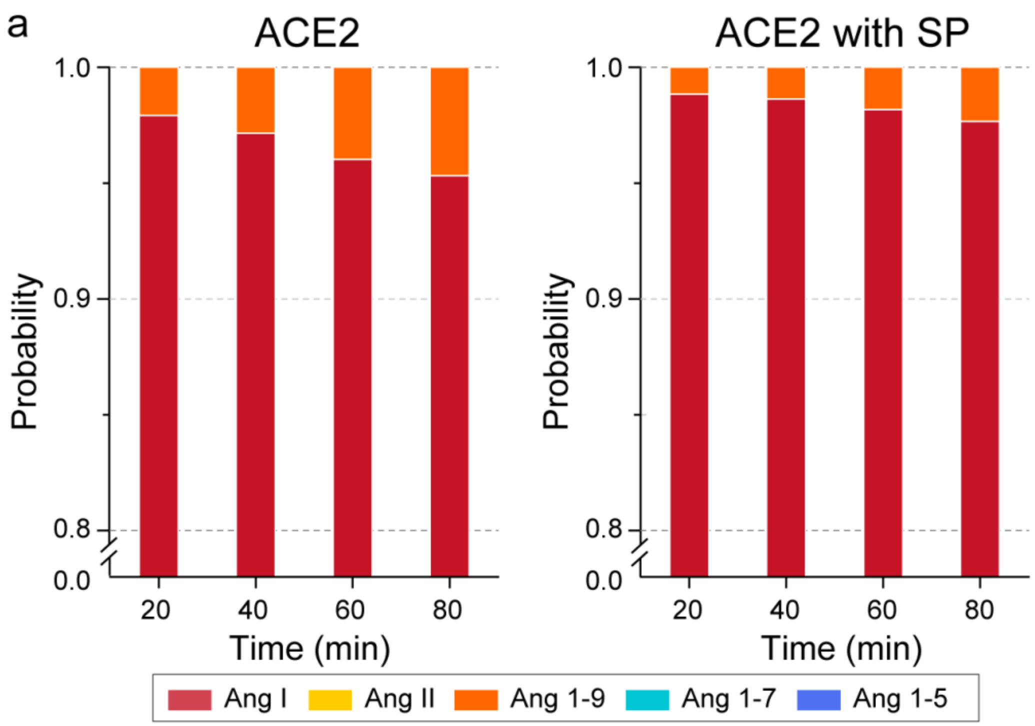


1. Lan, J.; Ge, J.; Yu, J.; Shan, S.; Zhou, H.; Fan, S.; Zhang, Q.; Shi, X.; Wang, Q.; Zhang, L.; Wang, X. *Nature* **2020**, *581*, 215.

Influence of the SARS-CoV-2 Spike Protein

Time-dependent probability of Ang peptides

by ACE2 (ACE2:SP = 1.2 nM:17 nM)



by ACE and ACE2 (ACE:ACE2:SP = 1:X:0.74)