XXI International Summer School "Nicola Cabrera"
New Frontiers in Scanning Force Microscopy: From Ultrahigh Vacuum to Biological Material (Residencia La Cristalera, Madrid, July 14-18th, 2014)

Molecular-scale Investigations of Solid-Liquid Interfaces by both FM-AFM and 3-Dimensional Force Mapping Method
– part 1 –

Hirofumi Yamada and Kei Kobayashi

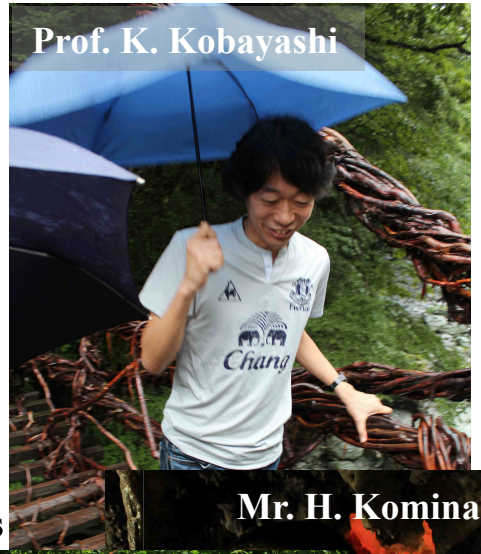
Department of Electronic Science & Engineering, Kyoto University

Liquid people

bR, Avidin

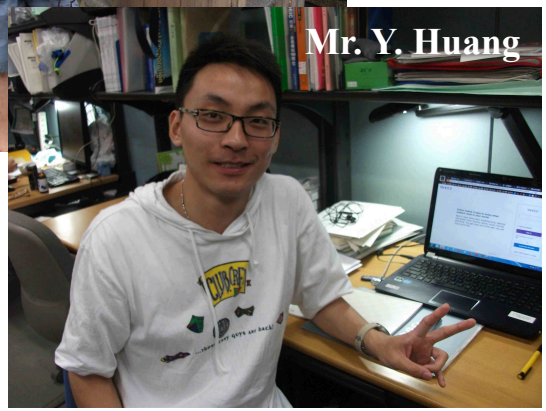
3D Charge mapping

Everything



DNA, IgG

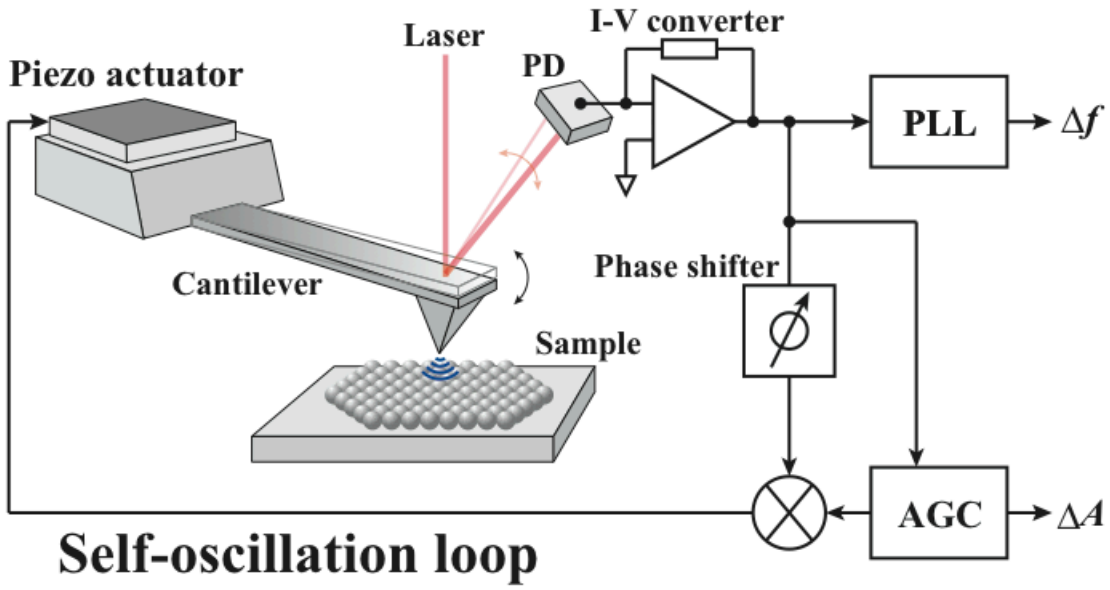
DNA, IgM

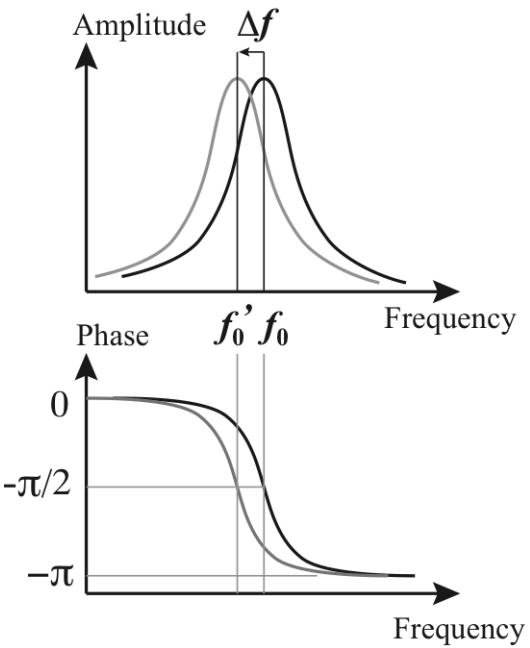


3D Hydration structures

Development of FM-AFM in liquids

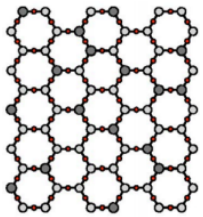
Schematic of FM-AFM



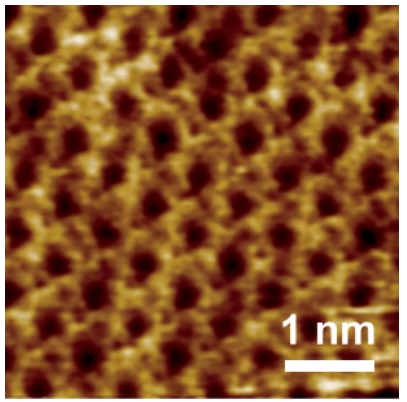


Two characteristic capabilities in FM-AFM

- **Atomic/molecular-scale imaging in liquids (part 1)**
  Highly sensitive force detection (n_{ds} < 10 fm/√Hz)
- **Quantitative force analysis (part 2)**
  $\Delta f \rightarrow F_{ts}$ *deconvolution*



in pure water



FM-AFM image of mica

Outline

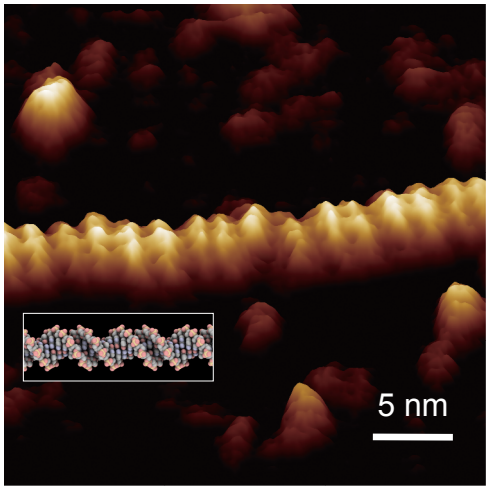
[part 1] Molecular-scale investigations of biomolecules by FM-AFM

- ★ **DNA**
- ★ **IgG antibody-antigen interactions**

[part 2] 2D/3D Force Mapping Method

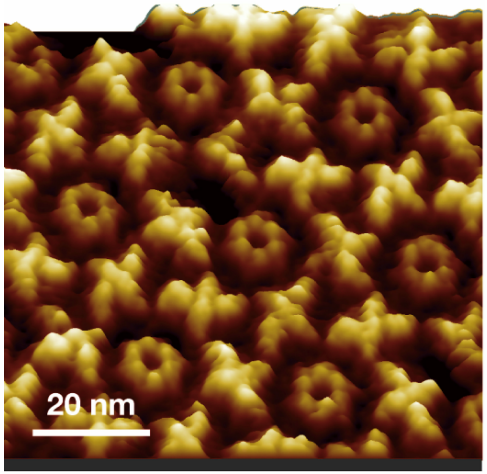
- Quantitative force analysis (required condition)
- Molecular-scale charge density mapping
- Visualization of molecular-scale 3D hydration structures (clay minerals)

DNA double helix



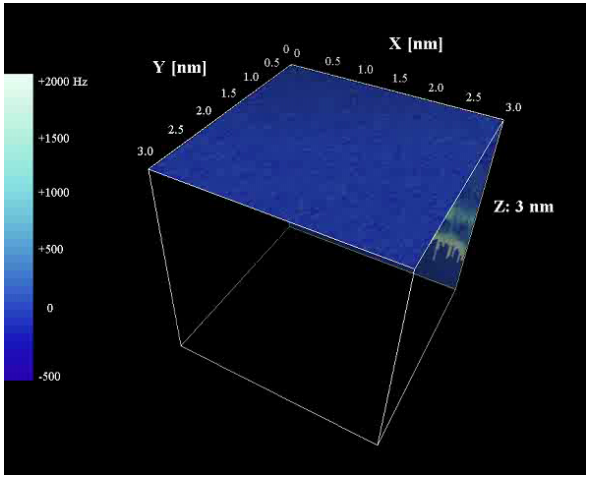
S. Ido, K. Kimura *et al.*,
ACS Nano 7, 1817 (2013).

IgG 2D crystal



S. Ido, H. Kimiya *et al.*,
Nature Materials 13 264 (2014).

3D force mapping of hydration layers *Movie in original file*



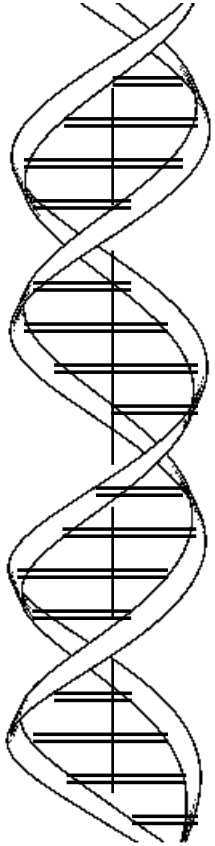
K. Kobayashi, N. Oyabu *et al*,
J. Chem. Phys. 138, 184704 (2013).

FM-AFM imaging of biomolecules in liquids

High-resolution imaging of DNA

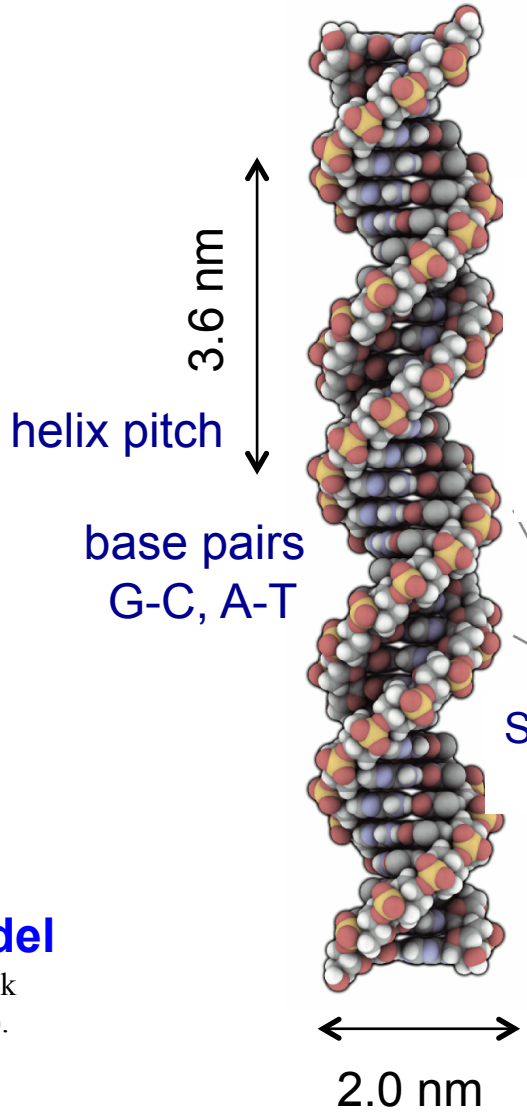
S. Ido, K. Kimura, N. Oyabu, K. Kobayashi, M. Tsukada, K. Matsushige, H. Yamada, *ACS Nano* 7, 1817 (2013).

DNA structure (B-form)



Watson-Crick Model

J. D. Watson & F. H. C. Crick
Nature **171,** 737-738 (1953).



No real-space imaging

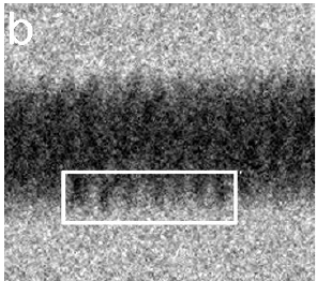
→ **DNA-protein complex**

Imaging in physiological environments

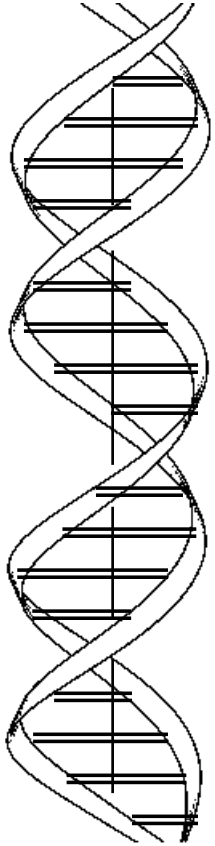
← **Present structural analysis using DNA crystal**

TEM imaging of a bundle of 7 DNA chains

Francesco Gentile *et al.*
Nano Lett. **12**, 6453 (2012).

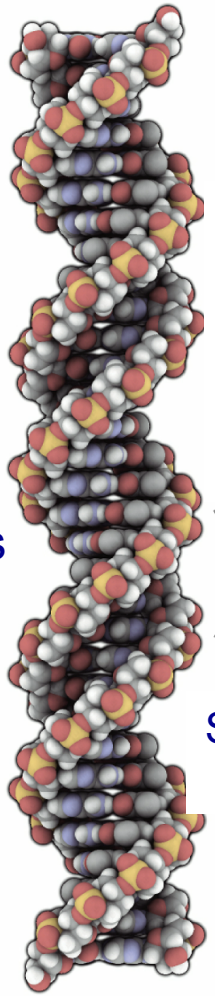


DNA structure (B-form)



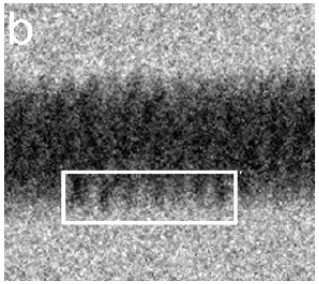
Watson-Crick Model

J. D. Watson & F. H. C. Crick
Nature **171,** 737-738 (1953).



TEM imaging of a bundle of 7 DNA chains

Francesco Gentile *et al.*
Nano Lett. **12**, 6453 (2012).



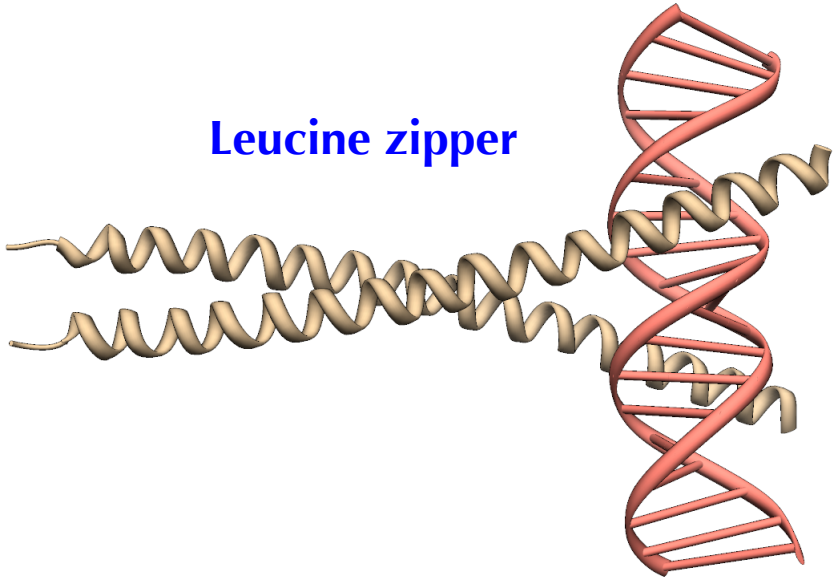
Protein-DNA Complexes

DNA-binding domain (DNA-binding protein motif)

- Leucine zipper
- Loop-sheet-helix
- ββα zinc finger
- Cro and Repressor
- .....

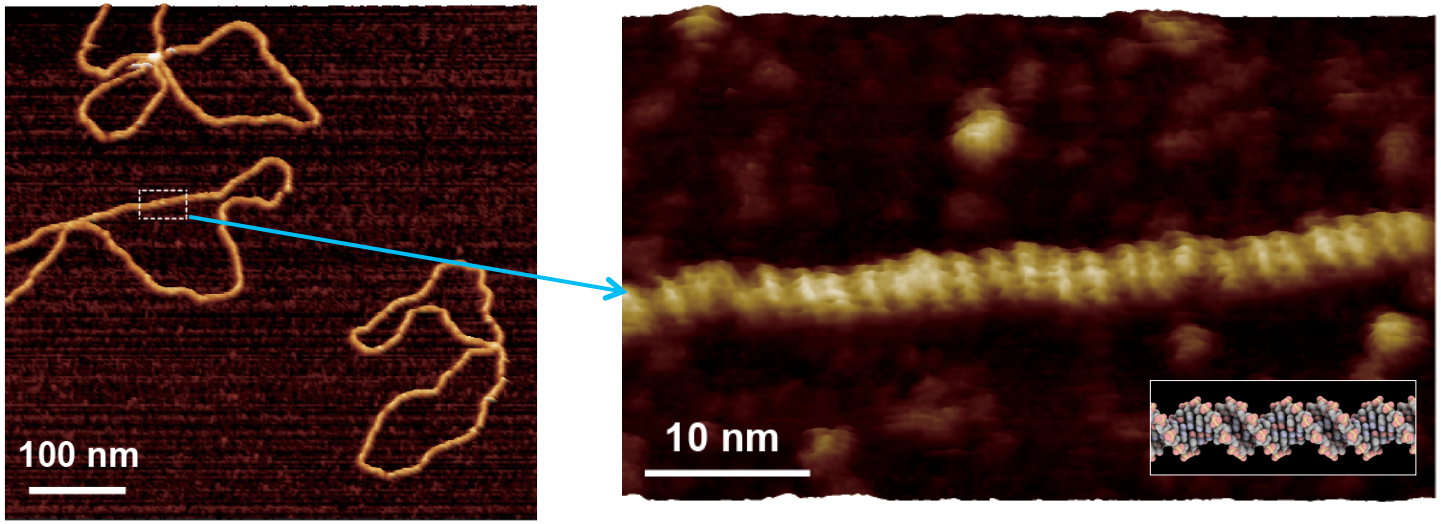
→ Real-space imaging is required for the analysis of DNA-binding domains.

Leucine zipper

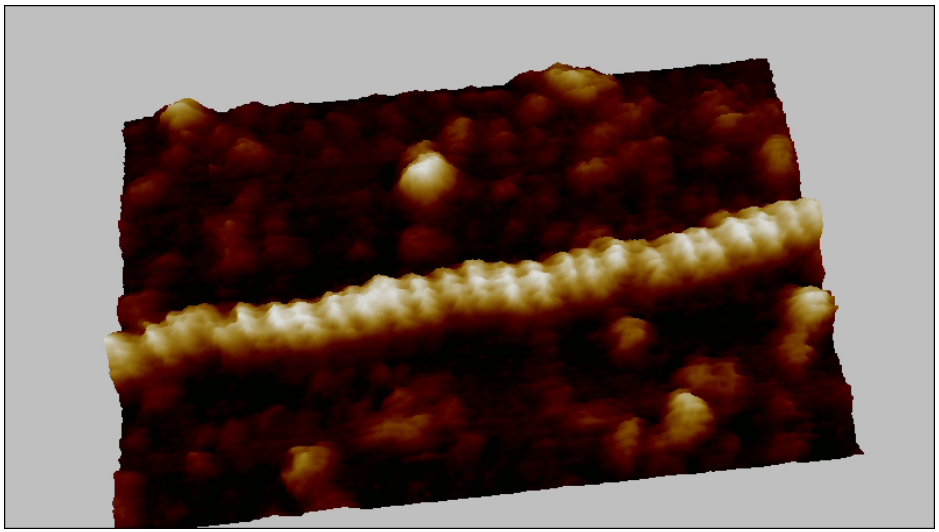


FM-AFM imaging of DNA molecules in liquid

➔ **High-resolution imaging of plasmid DNA (pUC18) in solution**



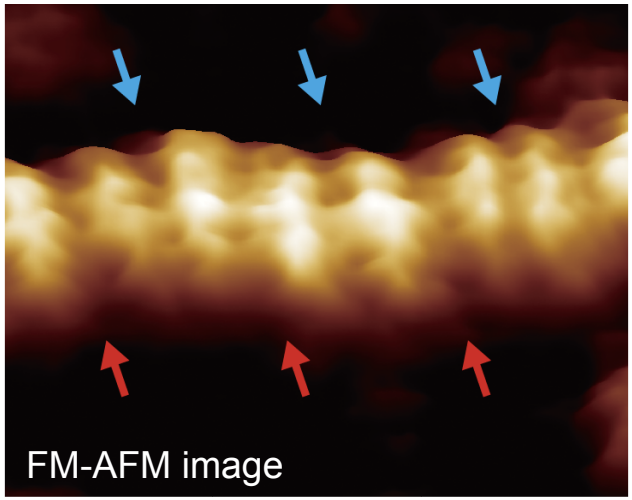
S. Ido et al, *ACS Nano* 7, 1817 (2013)

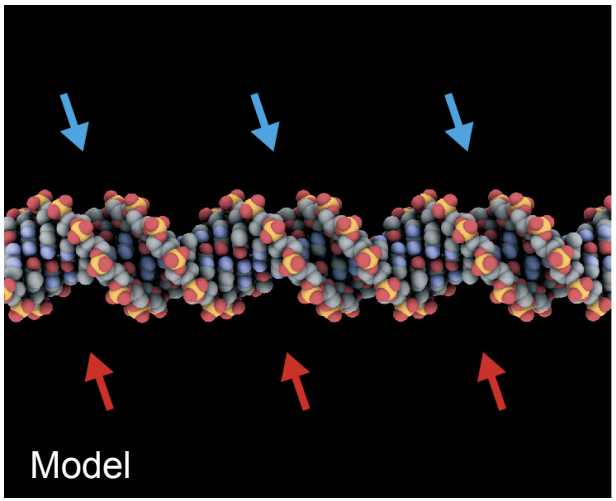


Movie in original file

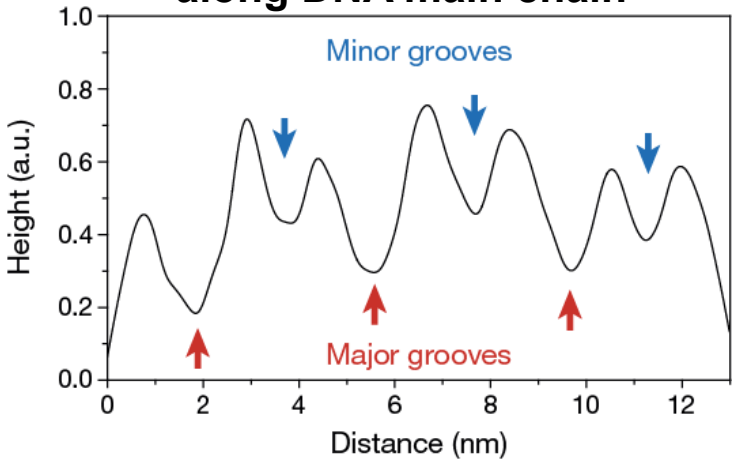
FM-AFM imaging of DNA molecules in liquid

➔: Major grooves, ➔: Minor grooves



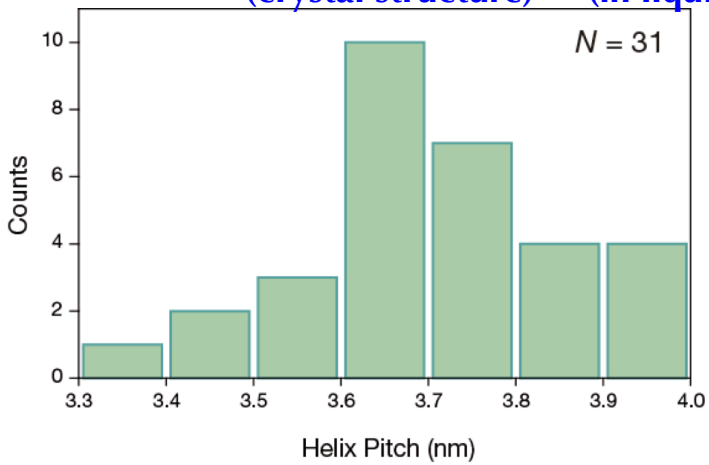


Cross sectional profile along DNA main chain



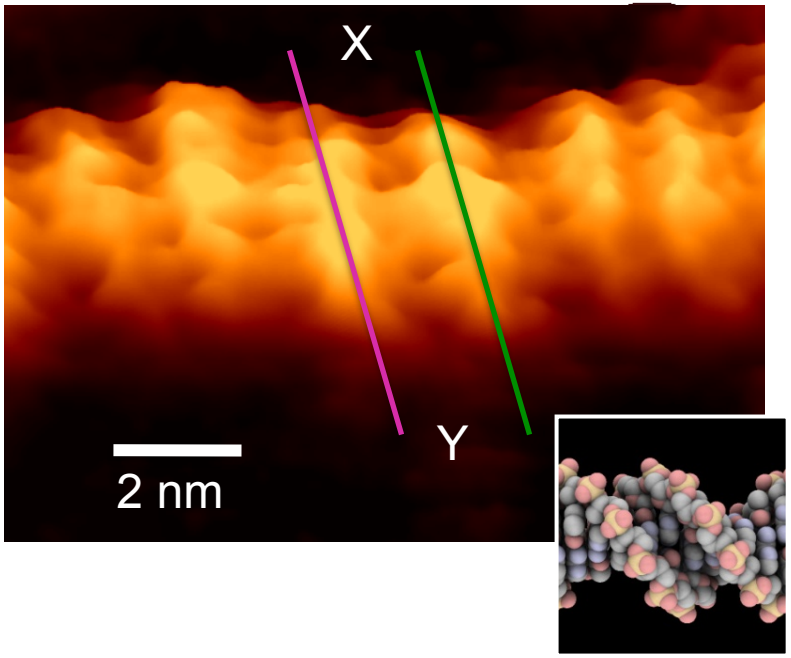
Helix pitch: 3.4 nm → 3.6 nm

(crystal structure) (in liquid)

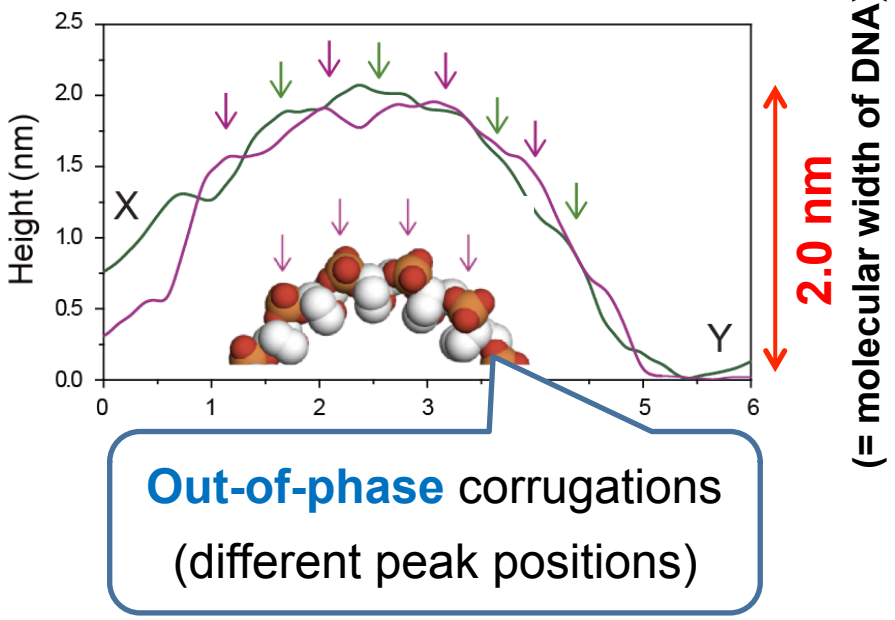


FM-AFM imaging of DNA molecules in liquid

➔ High-resolution imaging of plasmid DNA (pUC18)



Cross-section profiles on **neighboring DNA backbones** (along the X-Y lines)



Protrusions on backbones (➔, ➔) were identified with individual **phosphate groups** forming DNA backbones.

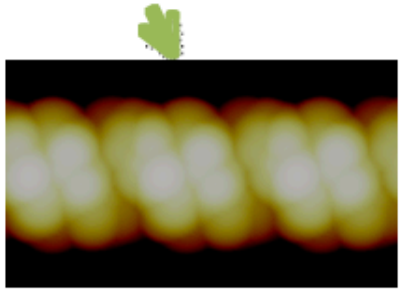
Comparison of simulation and experimental profiles

Simulatior : Geometric AFM Simulator (Prof. Tsukada et. al.)

Tip radius: 0.1 nm

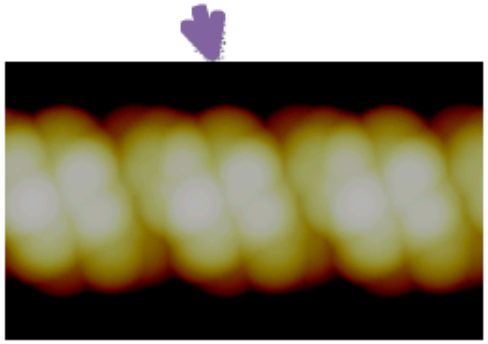
10 nm x 3.1 nm

Tip radius: 1.0 nm



10 nm x 5.8 nm

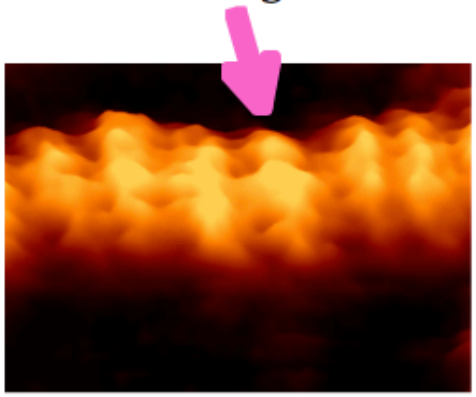
Tip radius: 2.0 nm

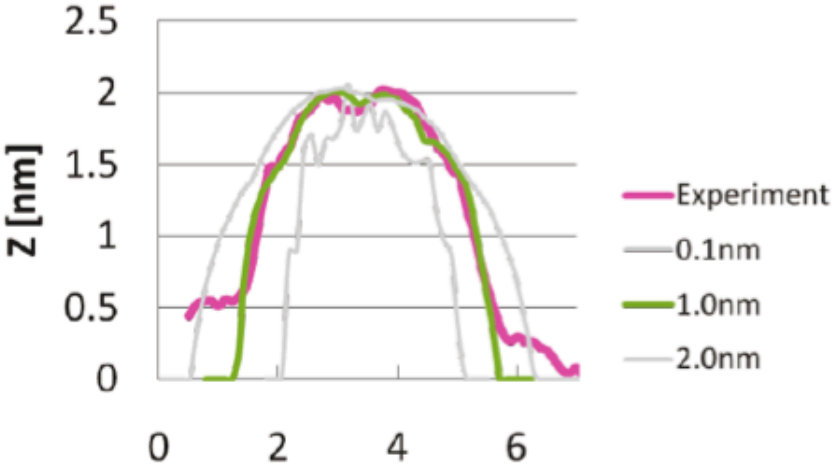


10 nm x 7.8 nm

Comparison of simulation & experimental profiles

FM-AFM image

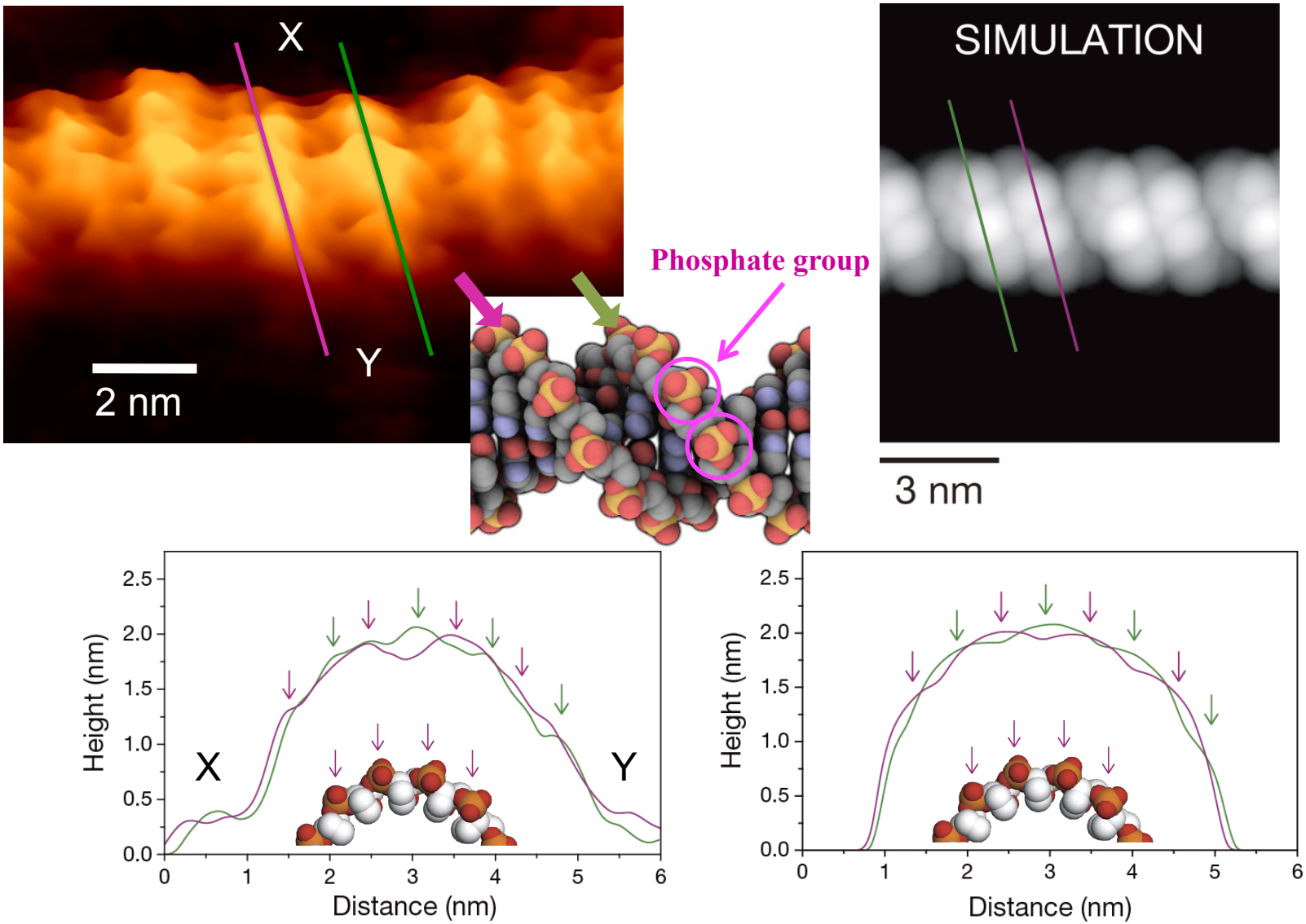




PDB ID: 1LAI (B-DNA)
Tip model: sphere + con
(cone angle: 10 deg)

Mini-tip radius: 1.0nm

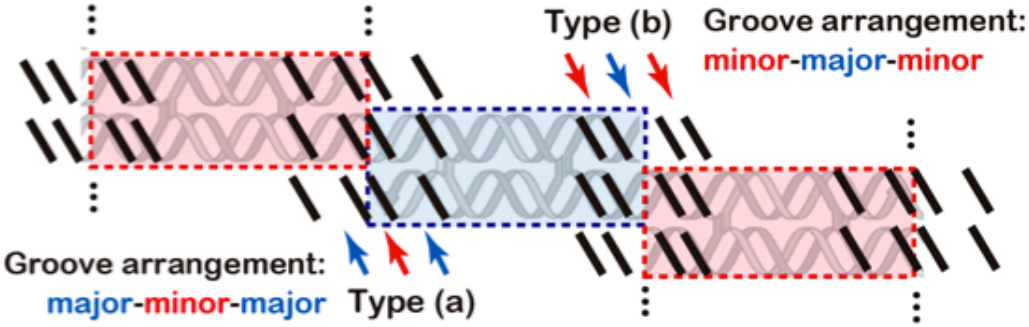
Imaging of individual phosphate groups

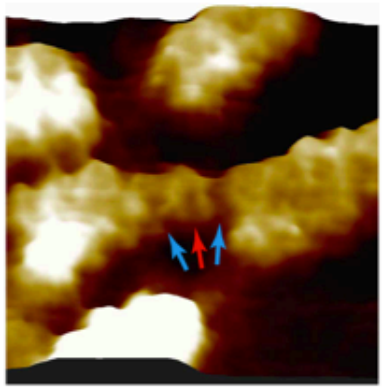


High-resolution FM-AFM imaging of DNA tiles

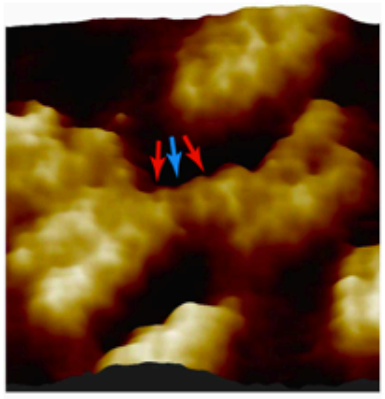
- Unit connections of the DNA tile

Two types of the groove arrangements appeared alternately





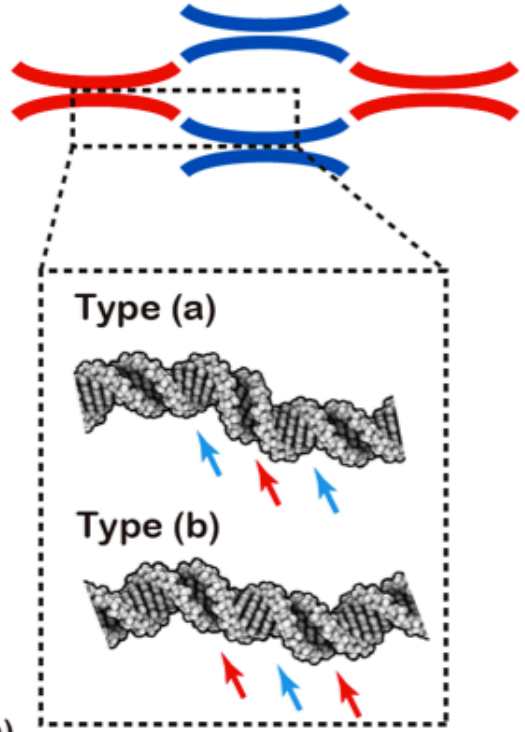
→ Groove arrangement: type (a)



→ Groove arrangement: type (b)

- DNA bending in the tile

DNA bending is induced by the charge repulsion between backbones



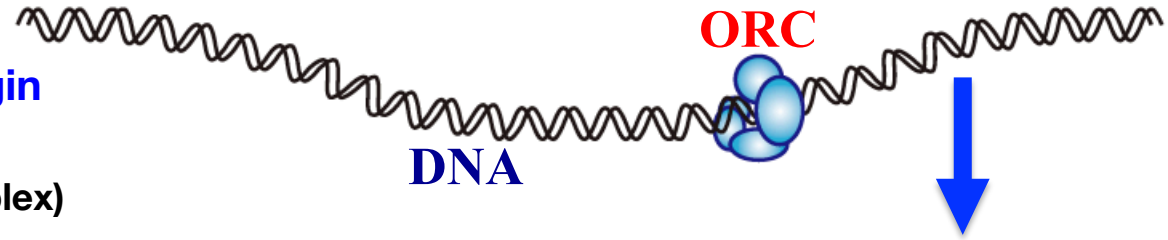
Formation of double-stranded structures between units were precisely imaged

Toward direct imaging of DNA replication process

Initial stage of DNA replication

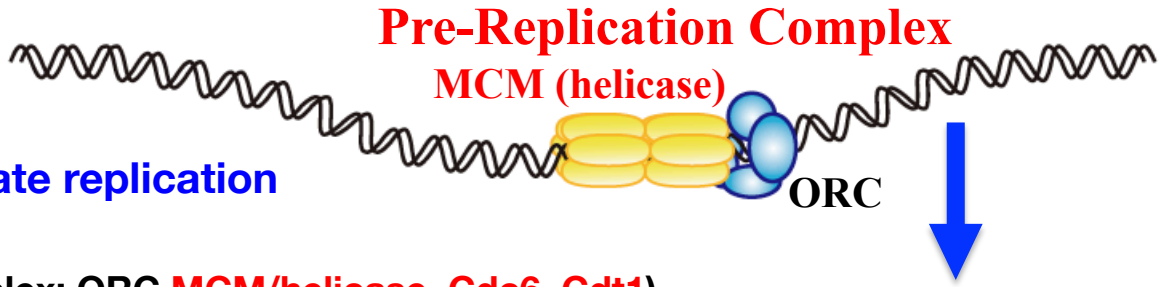
1. Determination replication origin

ORC binding to DNA
(ORC: Origin Recognition Complex)



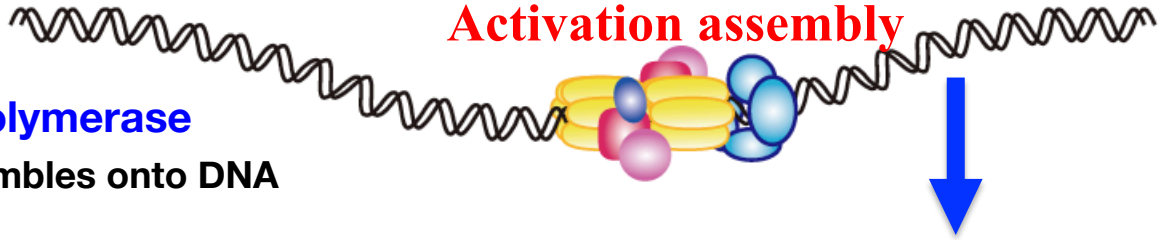
2. Licensing the complex to initiate replication

pre-RC formation
(pre-RC: pre-Replication Complex; ORC MCM/helicase, Cdc6, Cdt1)

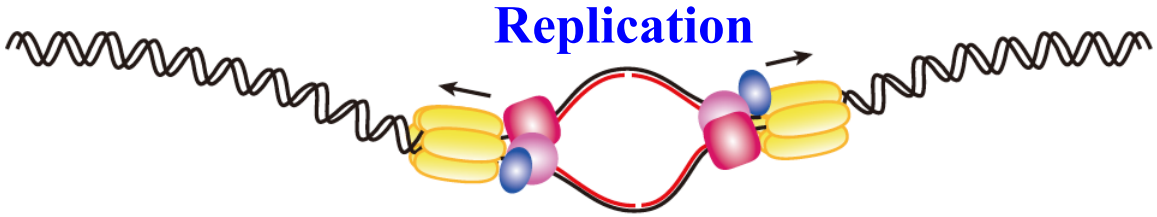


3. Activation of helicase, DNA polymerase

Several activation proteins assembles onto DNA
(Cdc45, GINS, ...)



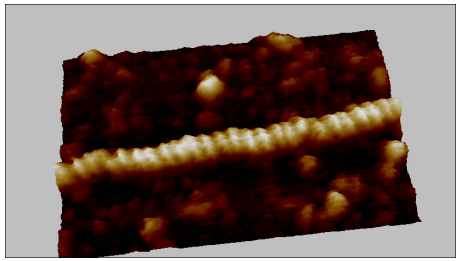
4. Start of replication



Summary of DNA study

Double helix structure

- Diameter: 2.0 nm
  (agree with the textbook value obtained by X-ray analysis)
- Helix pitch: 3.6 nm
  (slightly larger than the textbook value 3.4 nm)
- Individual phosphate groups with a spacing of 0.5 nm along the backbone chain were resolved.



Movie in original file

DNA-protein complex

DNA-ORC complex in the initial stage of DNA replication process was imaged.

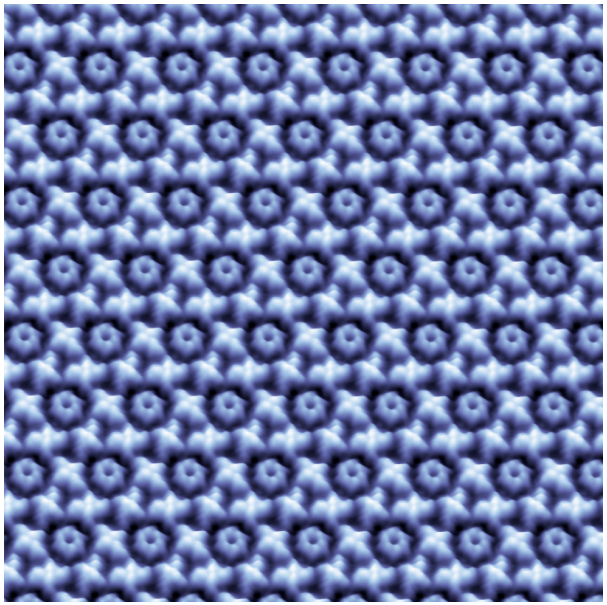
Charge distribution

Charge distribution around the DNA chain was visualized.

FM-AFM imaging of biomolecules in liquids

High-resolution imaging of IgG molecules

in collaboration with Dr. H. Kimiya at Panasonic Corp.

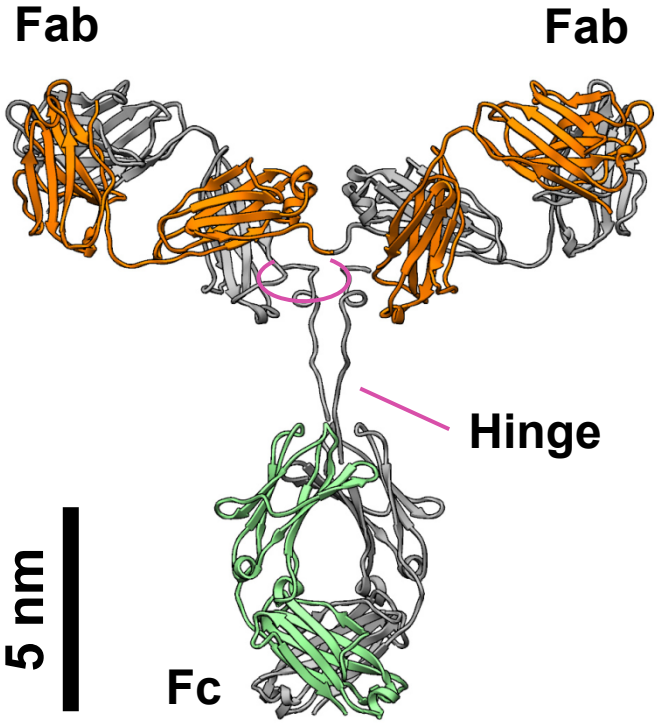


S. Ido, H. Kimiya, K. Kobayashi, H. Kominami, K. Matsushige, H. Yamada, *Nature Materials* 13 264 (2014).

Molecular structure of antibody molecule

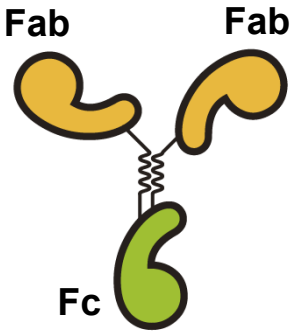
IgG molecule

Ig: Immunoglobulin
5 classes: G, M, A, D, E

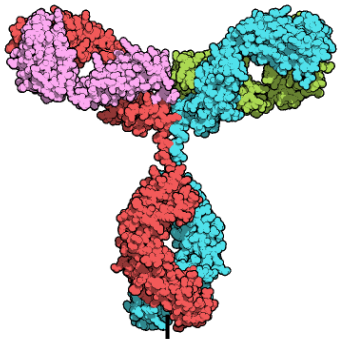


Y-shaped protein molecules:

- **Fab** (fragments, **antigen-binding**) regions
- **Fc** (fragment, **crystallizable**) region
- Flexible **hinge** region



Schematic representation
used in this study

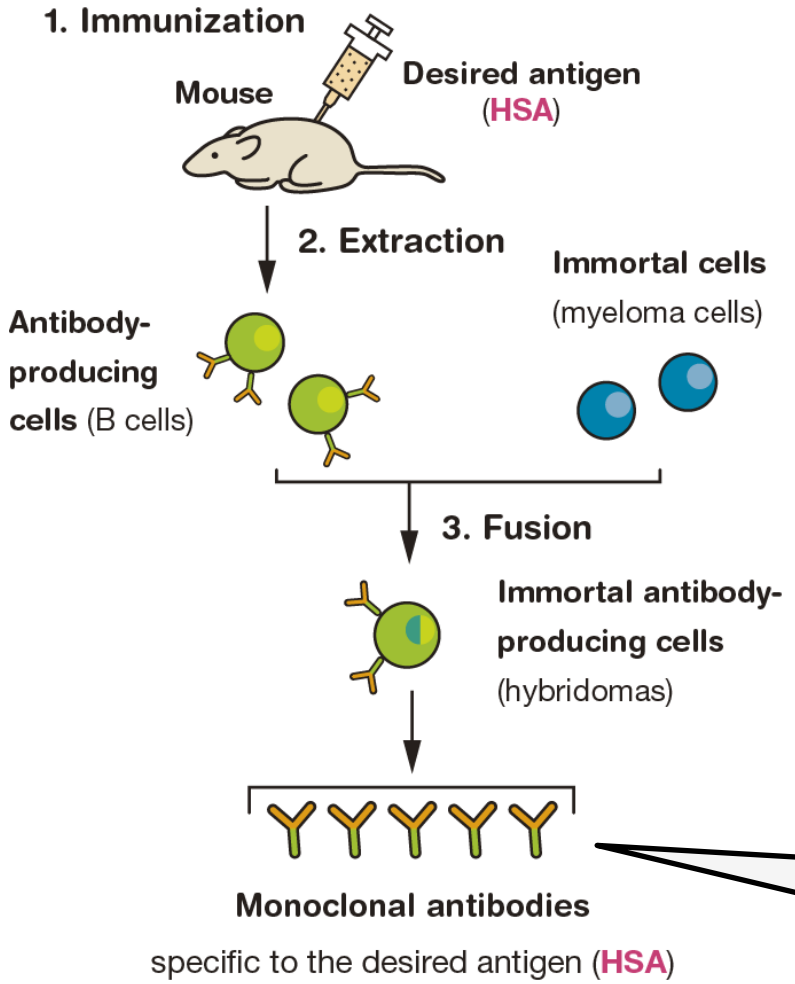


3D representation
(2-fold symmetry)

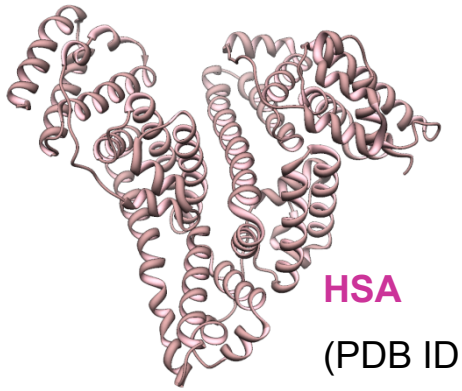
Flexibility of antibodies plays essential roles in **the immune system.**

Monoclonal antibodies

Production of monoclonal antibodies



- Sample: mouse monoclonal antibodies
- Class: immunoglobulin G (IgG)
- **Antigen:** human serum albumin (HSA)



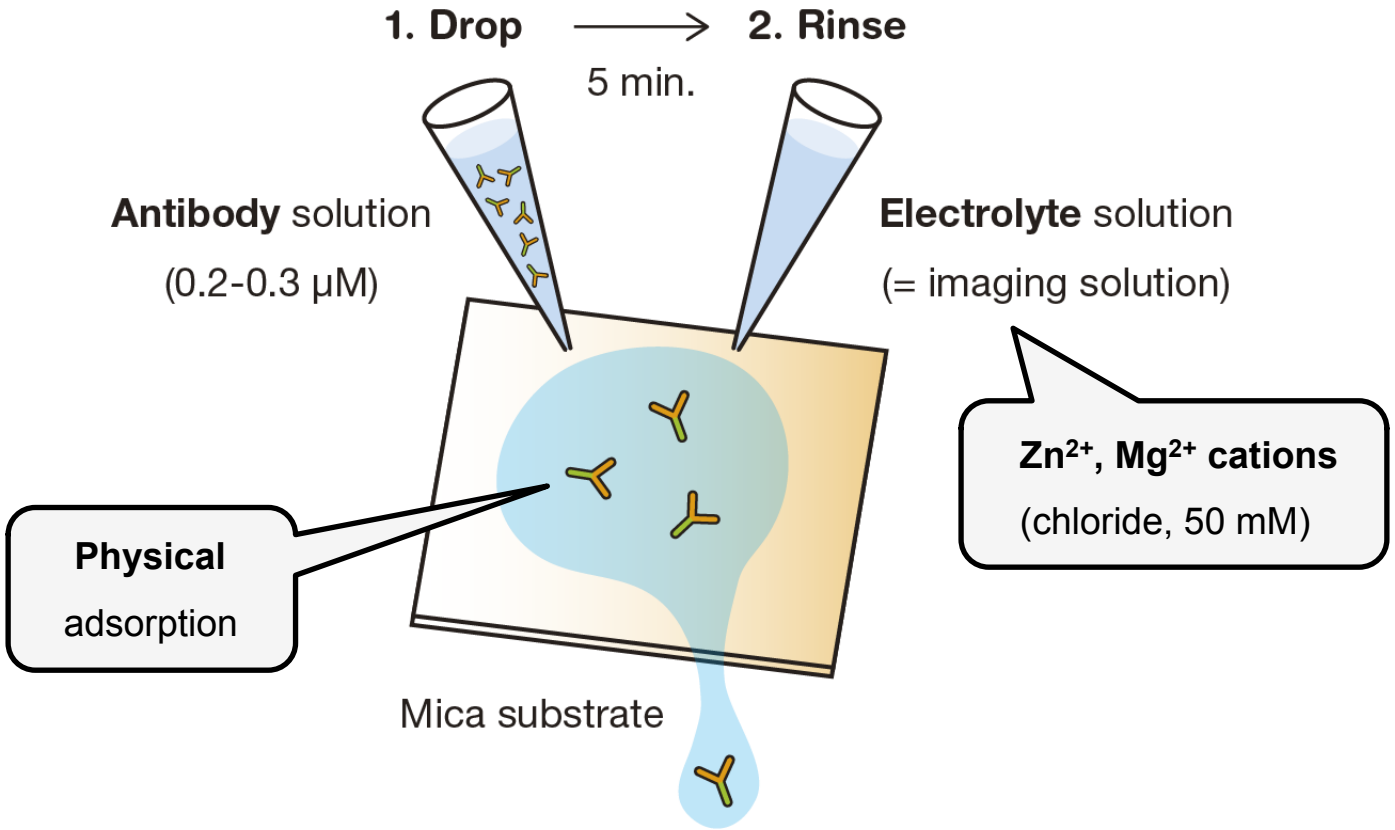
HSA

(PDB ID: 1E7I)

Homogeneous structures

from identical immune cells

Sample preparation for FM-AFM

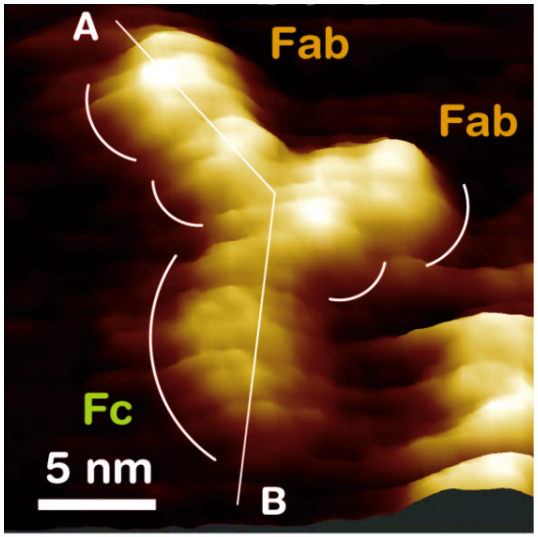


Both mica and antibodies are **negatively charged**.

➔ **Cations** binds antibodies onto mica by **electrostatic interactions**.

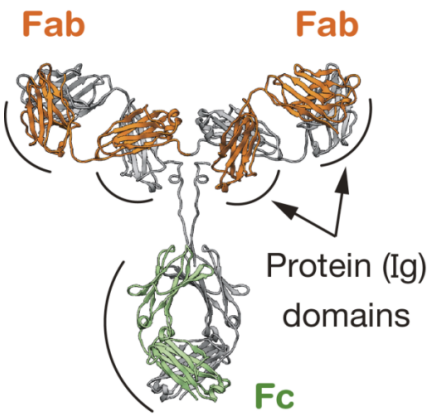
FM-AFM imaging of antibodies in $ZnCl_2$ solution

Y-shaped antibody structure

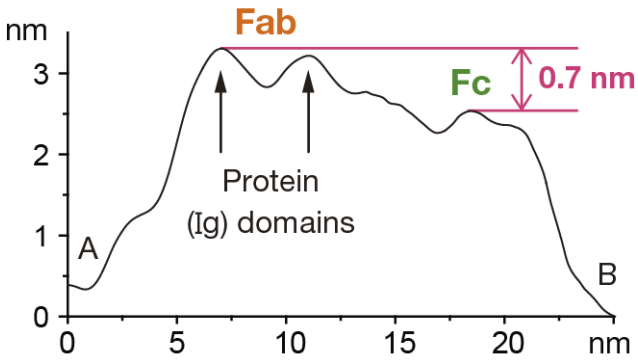


Mica substrate

→ monomer structure



Cross-section profile along A-B



Two arms were higher than **the other arm**.

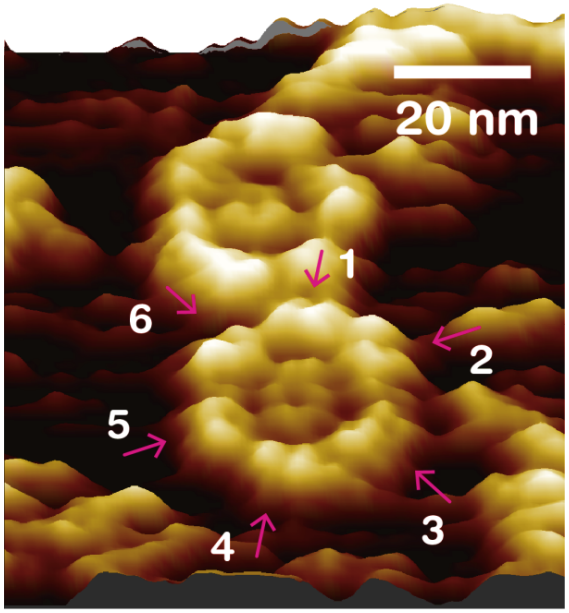
→ 2-**Fab** & 1-**Fc** regions were identified.

Antibodies were **individually** adsorbed onto a mica substrate.

➔ **Protein domain structures** (Ig domains) were resolved.

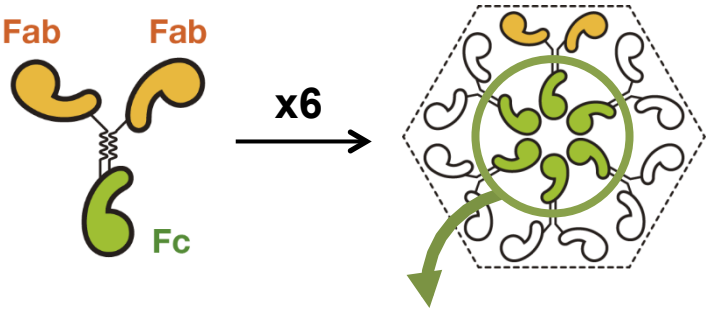
FM-AFM imaging of antibodies in $MgCl_2$ solution

Flower-like antibody structure



Mica substrate

→ Composition of six antibodies (hexamer)



Characteristic structure: center ring assembly of **6-Fc**

Side-on (hydrophobic†) interactions between **6-Fc** → hexamer formation

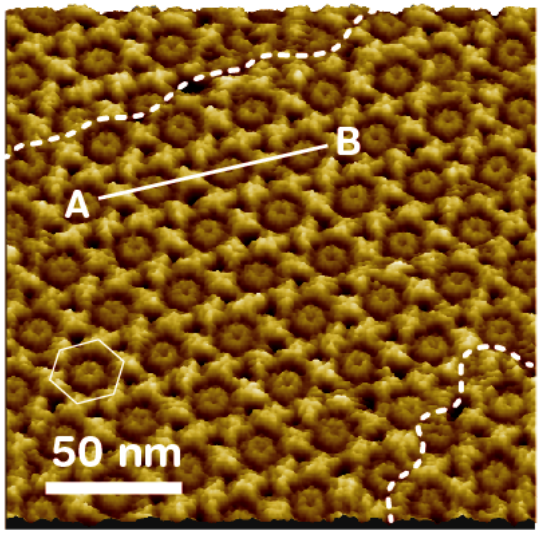
†Burton, D. R. *Trends Biochem. Sci* **15**, 64-69 (1990).

Antibodies were self-assembled into flower-like **hexamers**.

FM-AFM imaging of antibodies in $MgCl_2$ solution

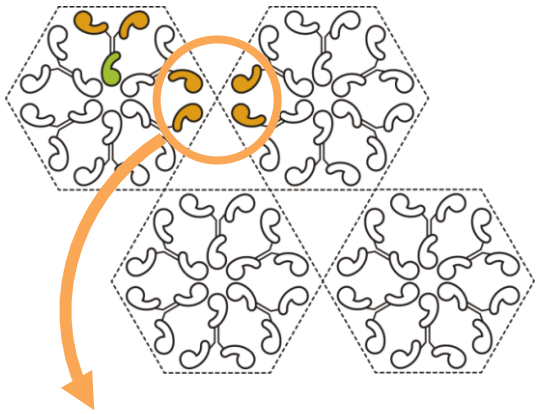
*condition of the **high surface concentration** of antibodies

2D crystal of antibodies



Mica substrate

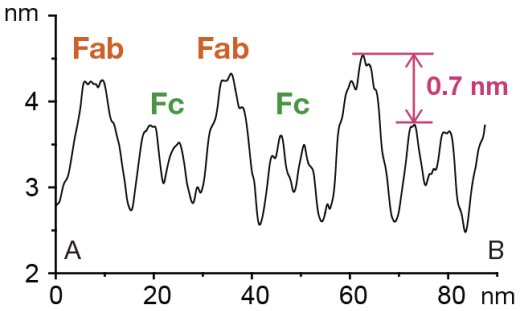
→ Composition of hexamers



Characteristic structure:

X-shaped assembly of **4-Fab**

Cross-section profile along A-B



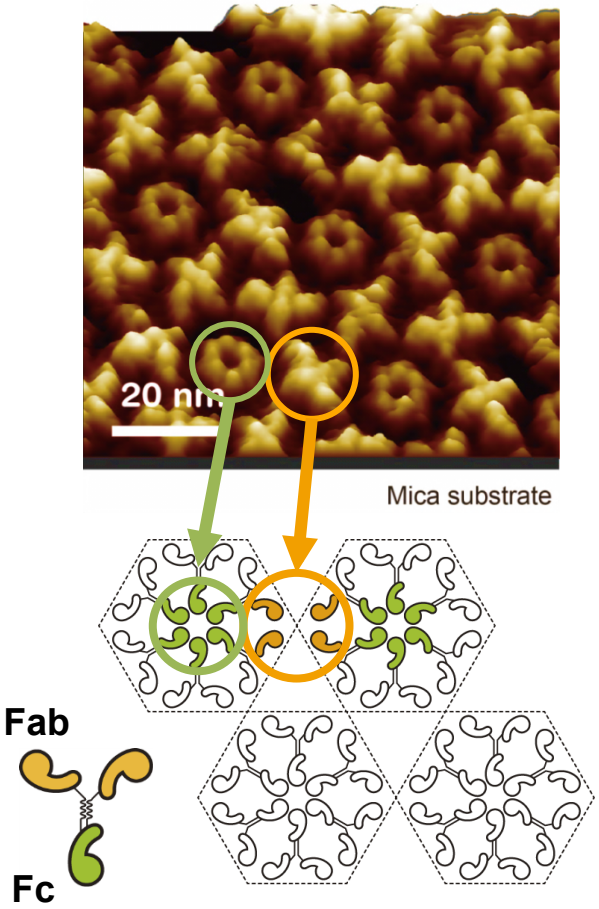
Fab regions were higher than **Fc** regions.

→ **Consistency** with the imaging of antibody monomers

Side-on interactions between **4-Fab** → 2D crystal formation

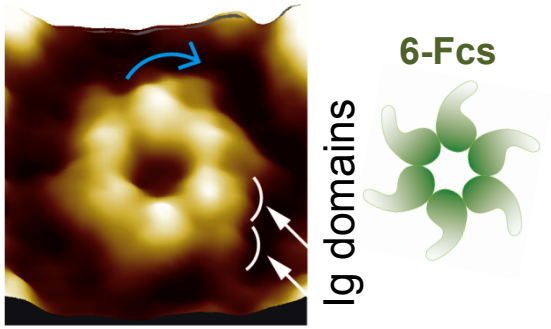
Antibody hexamers were self-assembled into **2D crystals**.

High-resolution images of the 2D antibody crystals



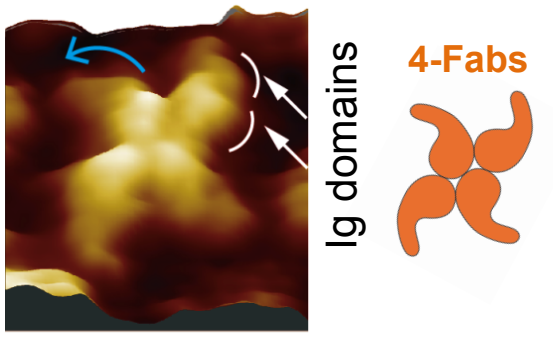
Characteristic structures in the antibody crystal

Ring assembly of **6-Fcs**



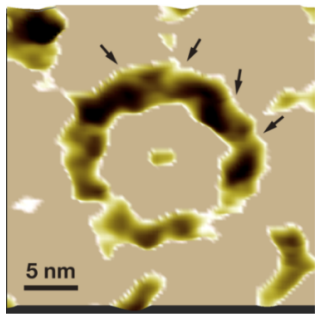
Right-handed bending

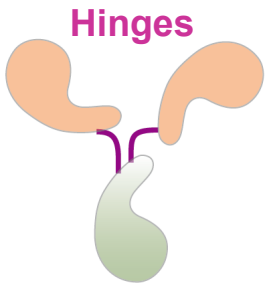
X-shaped assembly of **4-Fabs**



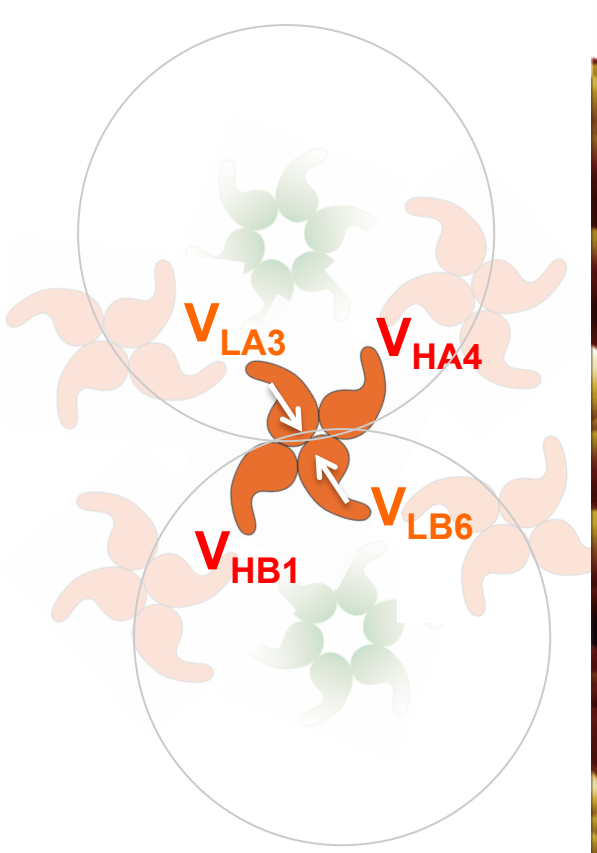
left-handed bending

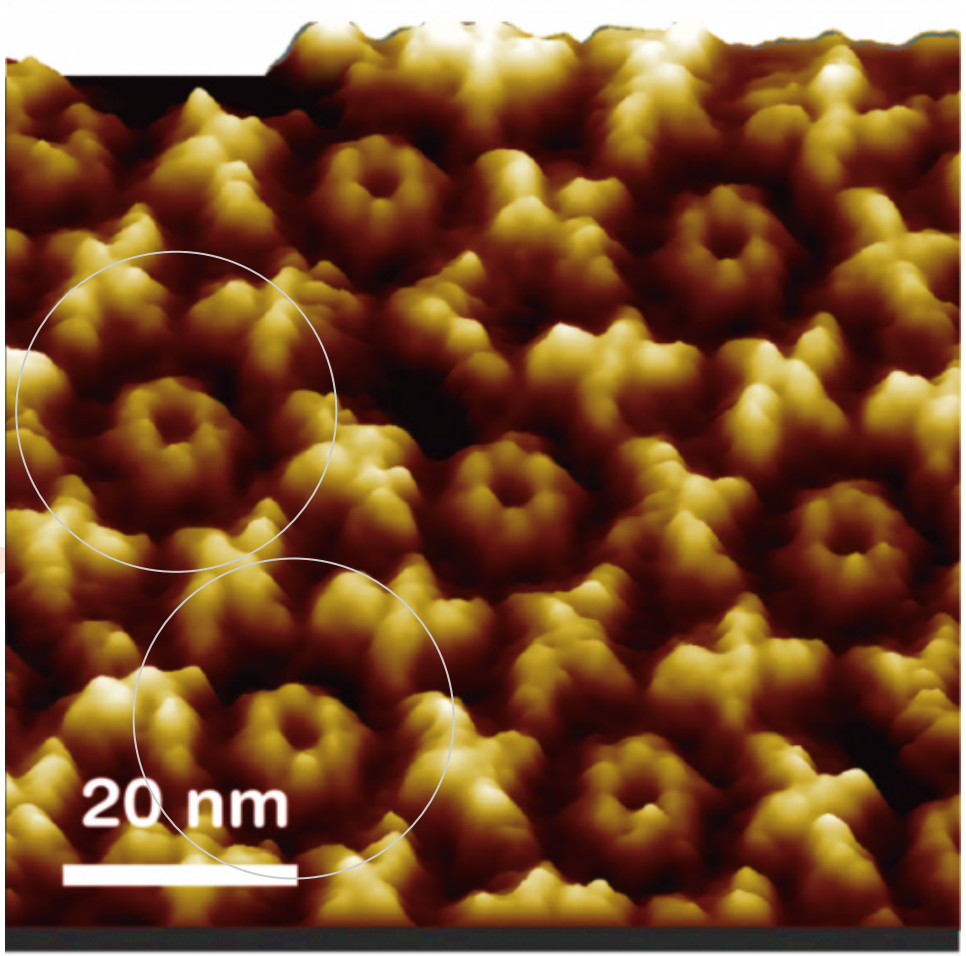
Hinge regions





IgG hexamer-hexamer interactions





Adjacent hexamers are tied together through V_L-V_L *interactions*.

Summary of the solution conditions

		Cationic species		
		Monovalent	**Divalent**	
		K^+, Na^+	**Mg^{2+}, Ca^{2+}**	**Zn^{2+}, Ni^{2+}** (transition metals)
Solution pH	**Neutral** (~pH7)	Weak binding -	**Moderate binding** **Crystallization** (T = 20 – 37°C†) 	Strong binding (Aggregation)
	Acidic (~pH5)	Weak binding -	Moderate binding (Disaggregation)	**Strong binding** **Disaggregation** 

- Imaging the 2D antibody crystals → **neutral** solution containing **Mg^{2+}** (Ca^{2+}) cations
- Imaging antibody monomers → **acidic** solution containing **Zn^{2+}** (Ni^{2+}) cations

†We also confirmed the antibody crystallization at **the body temperature of a mouse (37°C)**.

➔ We succeeded in **<u>reproducible</u> imaging of the antibody crystals**.

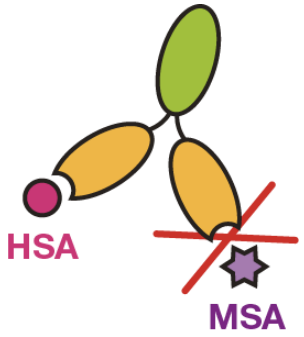
Is 2D IgG crystal biochemically active?

Check the biological activity of the antibody crystal !

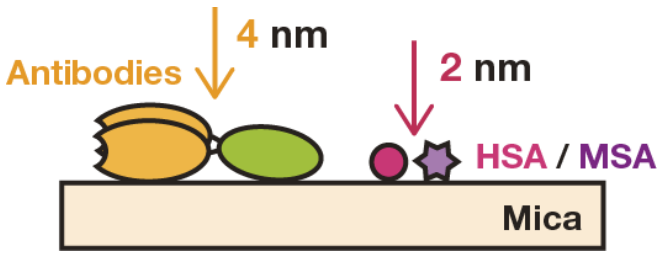
Imaging of the **2D antibody crystals** interacted with **antigenic molecules**

Sample

- **Anti-HSA** monoclonal antibodies
- **Serum albumin** (2 types):
  - **Antigenic:** human serum albumin (**HSA**)
  - **Non-antigenic**: mouse serum albumin (**MSA**)

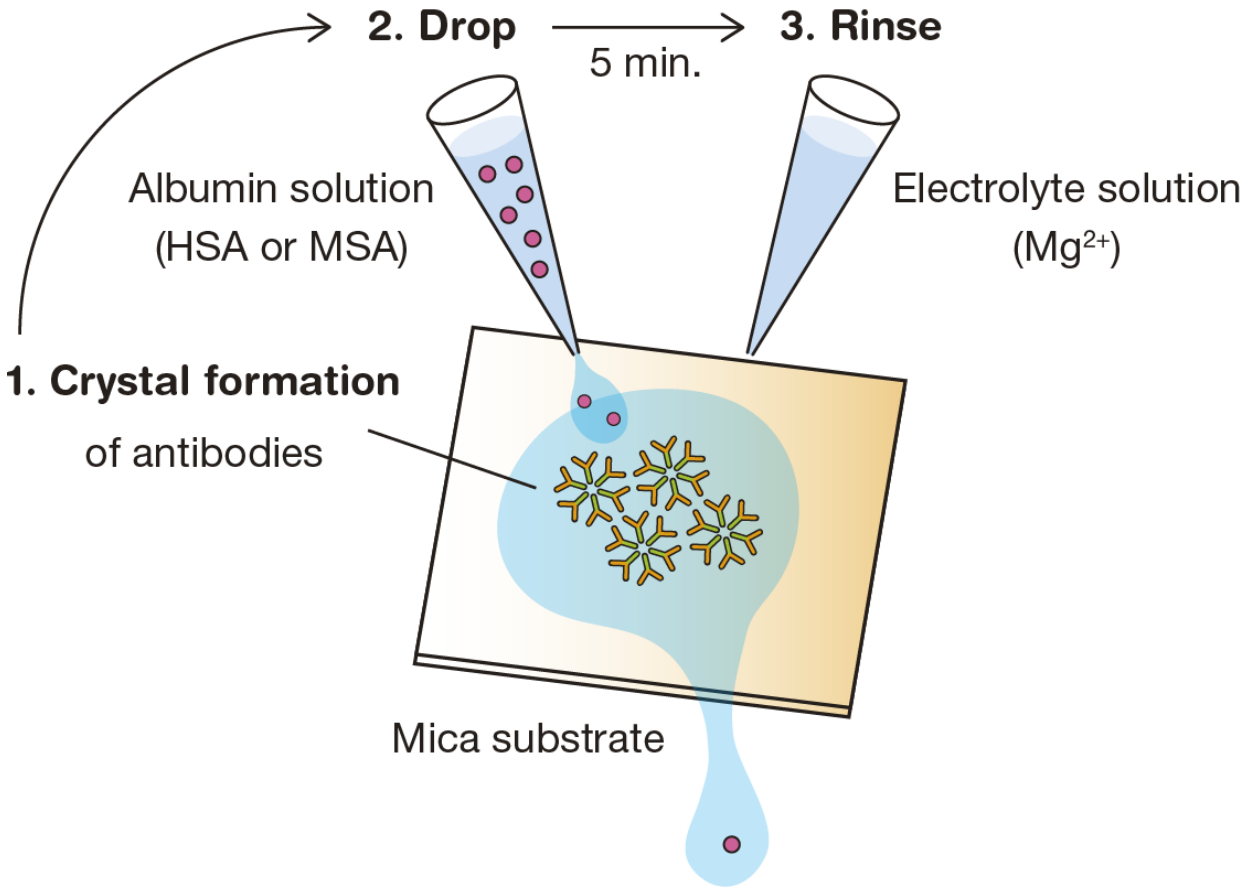


*Height difference from a substrate



➔ Antibodies were higher than the albumin (HSA / MSA) molecules.

Sample preparation for FM-AFM

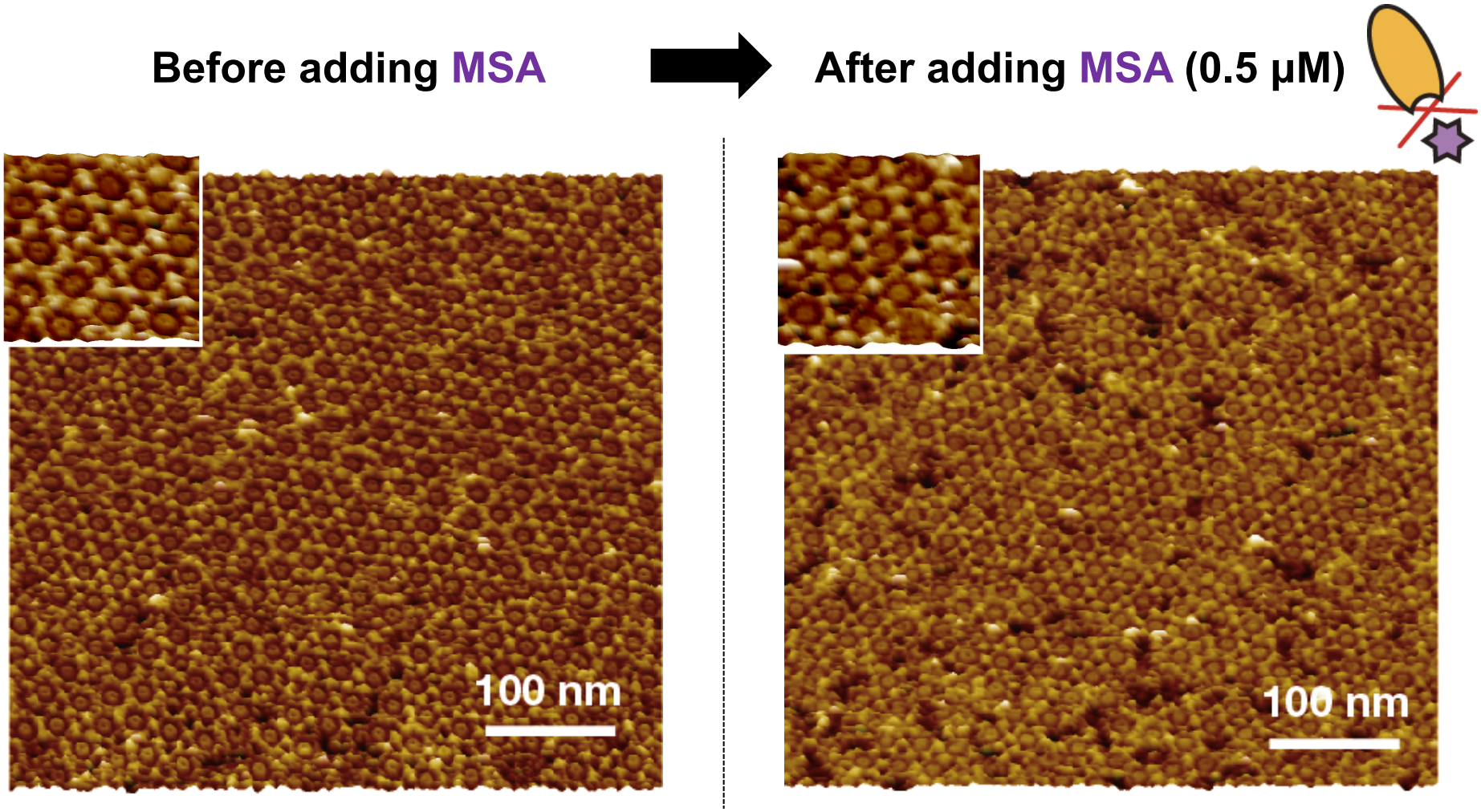


After the 2D crystallization of antibodies was confirmed,

antigenic / **non-antigenic** albumin (**HSA** / **MSA**) was interacted with the crystals.

2D antibody crystals with non-antigenic albumin (MSA)

Before adding MSA → **After adding MSA (0.5 μM)**



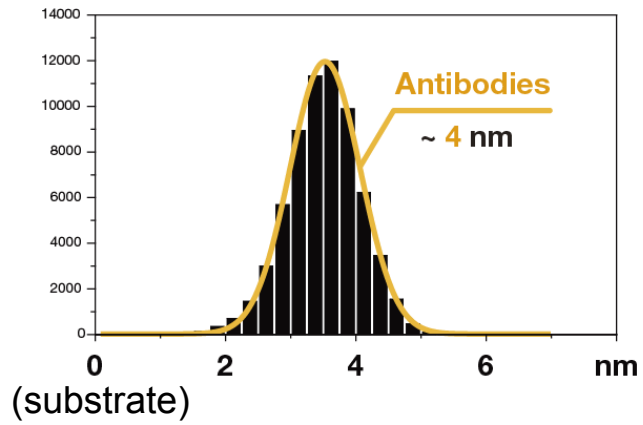
There was **NO significant difference** between two images.

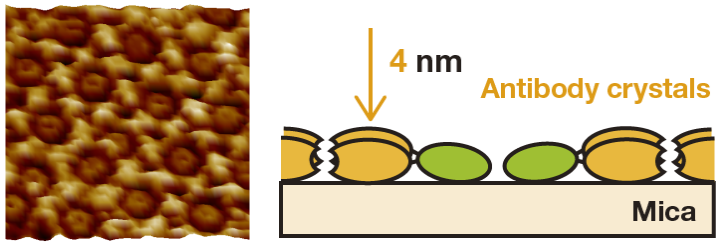
*Slight increase in defects → effect of rinse in the sample preparation

2D antibody crystals with non-antigenic albumin (MSA)

Before adding MSA

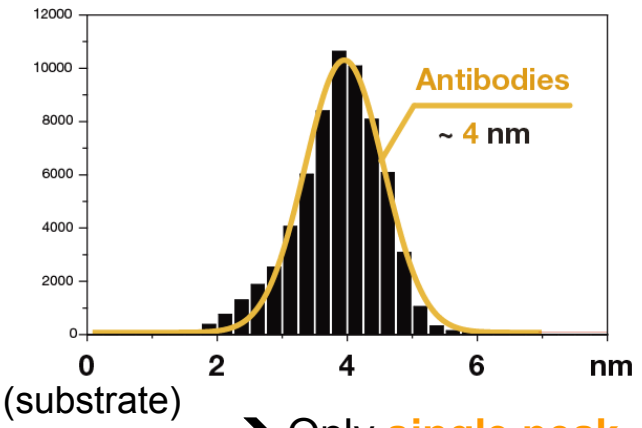
Height histogram from the substrate



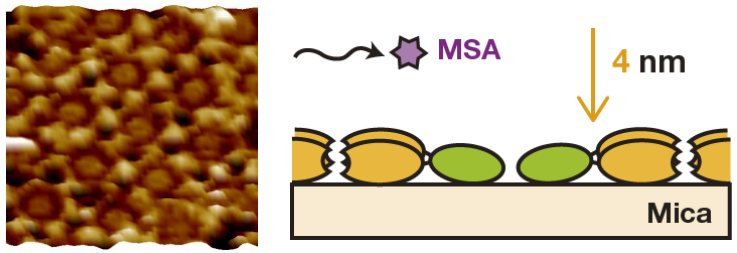


After adding MSA (0.5 μM)

Height histogram from the substrate



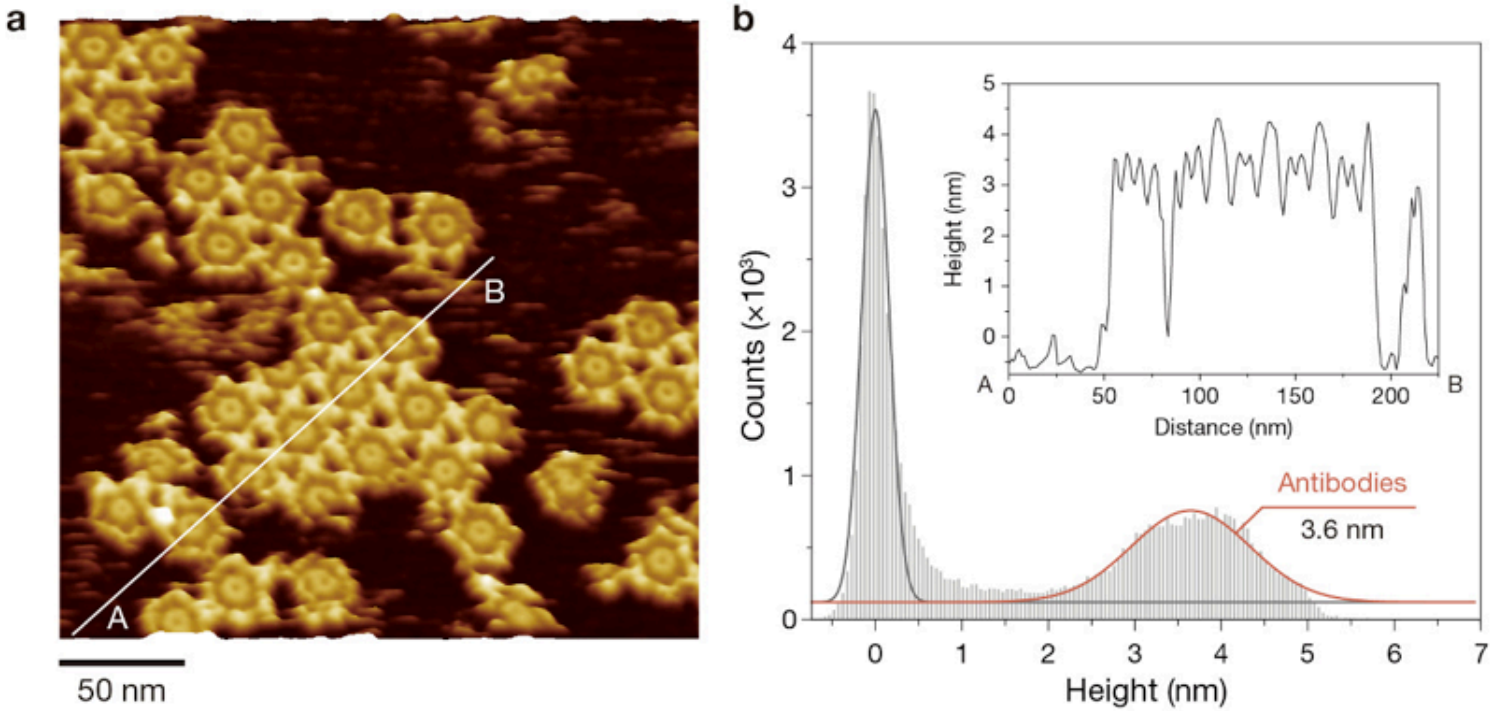
➔ Only **single peak**



Single peaks corresponded to the height of the 2D antibody crystal (**~4 nm**).

➔**Non**-antigenic albumin (MSA) **never interacted with the antibody crystals**.

Define substrate level in height histogram

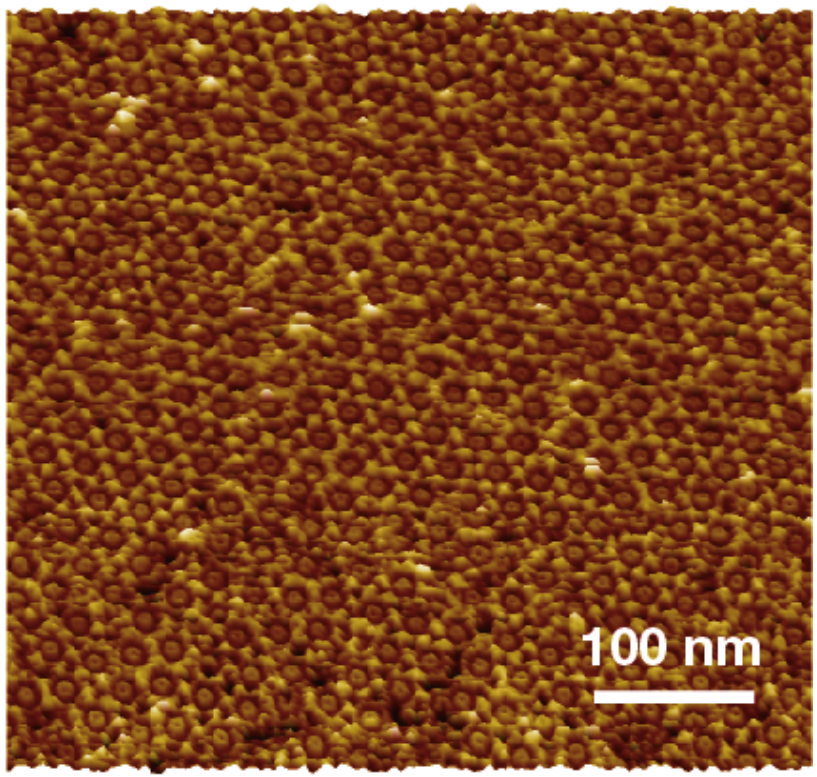


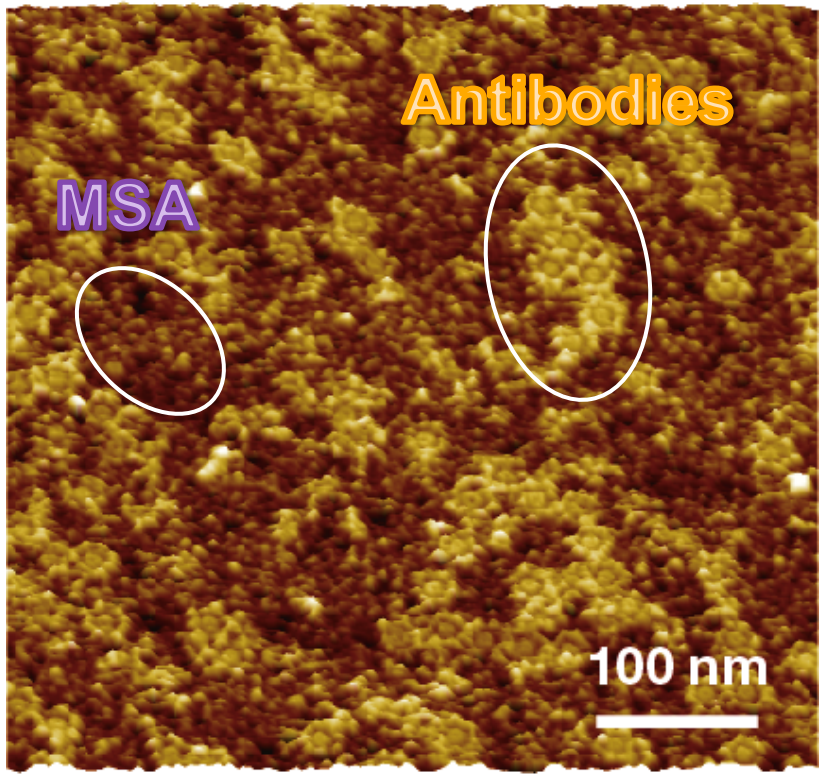
FM-AFM images of 2D antibody crystals in aqueous solution.
a, FM-AFM image of 2D IgG crystals in 50 mM $MgCl_2$ solution. The mica substrate is partially covered with the 2D IgG crystals.
b, Height histogram measured in **a**.

2D antibody crystals with non-antigenic albumin (MSA)

Before adding MSA

After adding high concentration of MSA (1.5 μM)





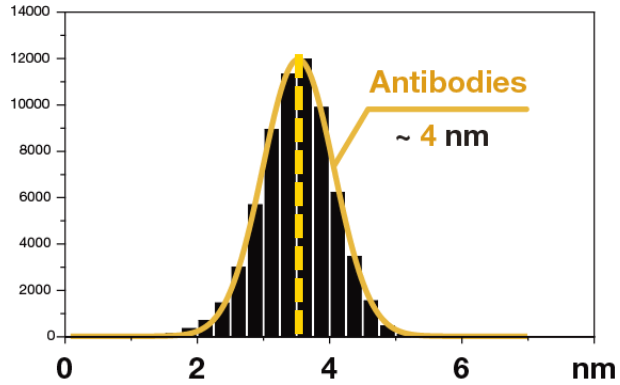
Lower domains were composed of highly-concentrated **MSA** molecules.

➔ **MSA** strongly adsorbed onto mica, but **never** interacted with antibodies.

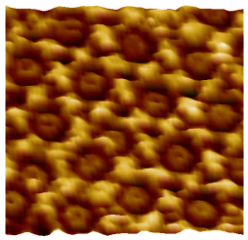
2D antibody crystals with non-antigenic albumin (MSA)

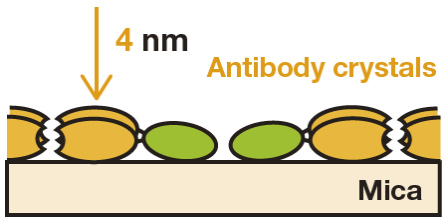
Before adding MSA

Height histogram from the substrate



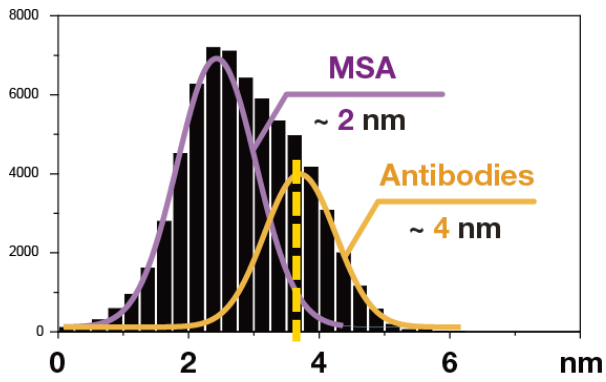
(substrate)





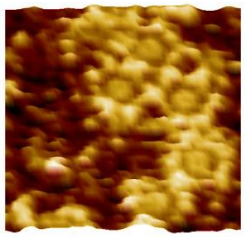
After adding high concentration of MSA (1.5 μM)

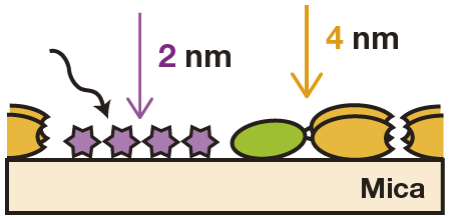
Height histogram from the substrate



(substrate)

➔ **Lower peak** appeared



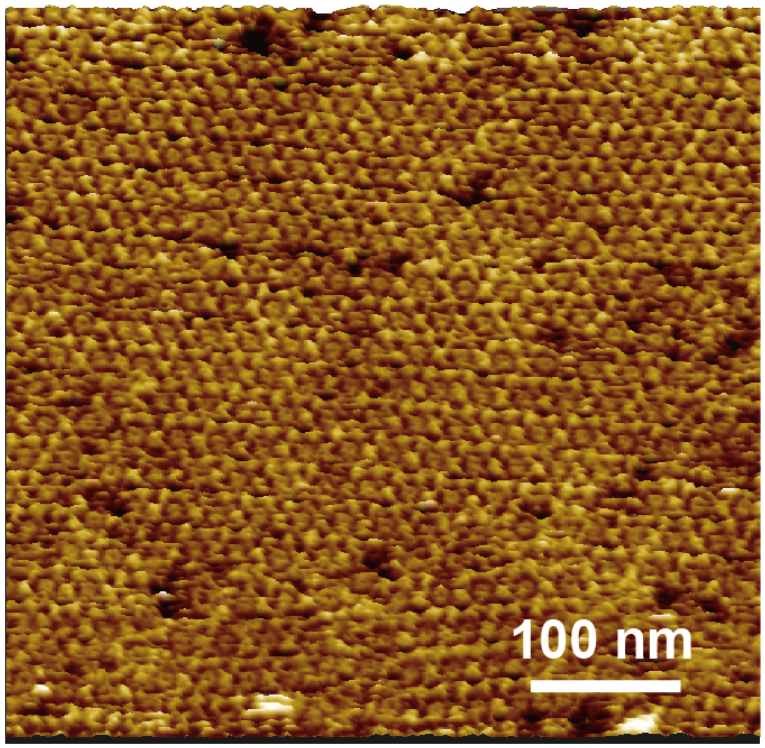


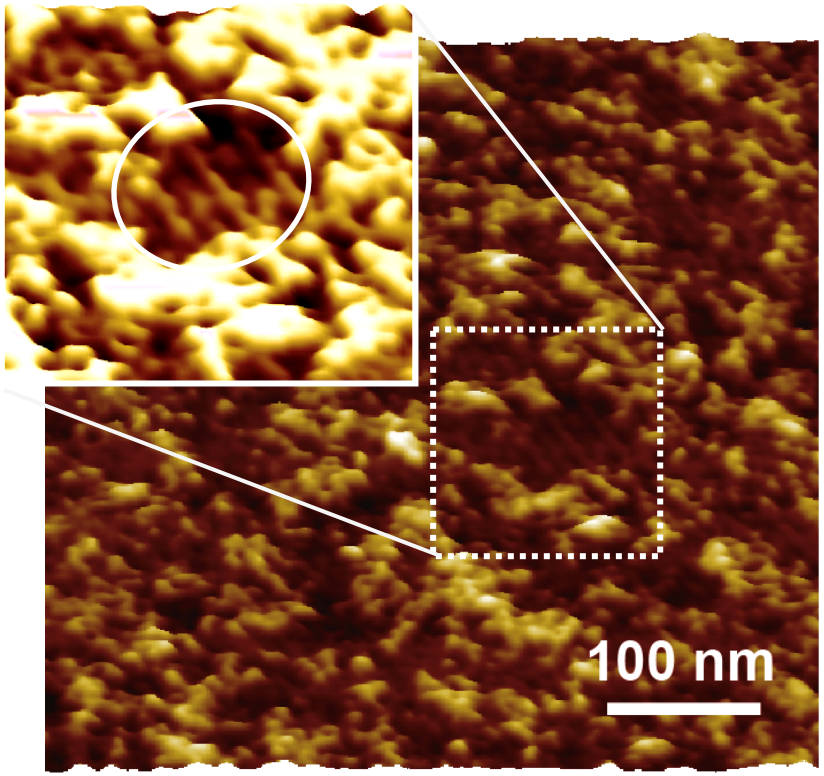
Lower peak corresponded to the height of **MSA** that replaced antibodies.

➔ We confirmed **non-antigenic** albumin **never** interacted with antibodies.

2D antibody crystals with antigenic albumin (HSA)

Before adding HSA → **After adding HSA (0.5 μM)**





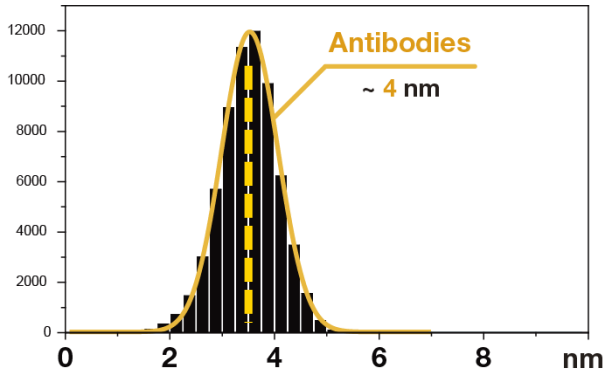
Surface topography was drastically changed.

➔ Antibody crystals were observed **beneath adsorbates (HSA)**.

2D antibody crystals with antigenic albumin (HSA)

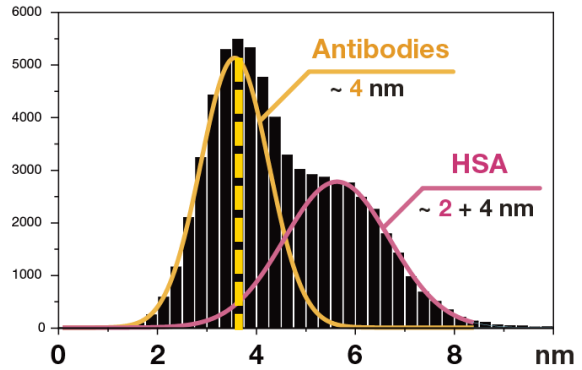
Before adding HSA → **After adding HSA (0.5 μM)**

Height histogram from the substrate



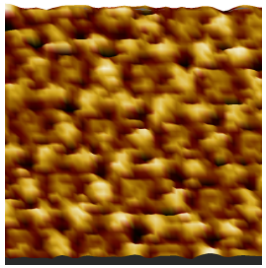
(substrate)

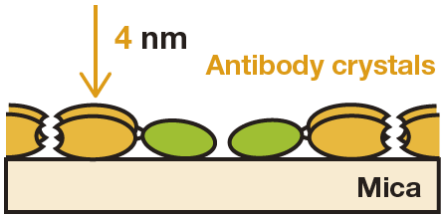
Height histogram from the substrate

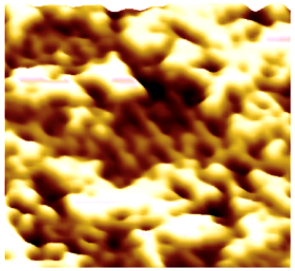


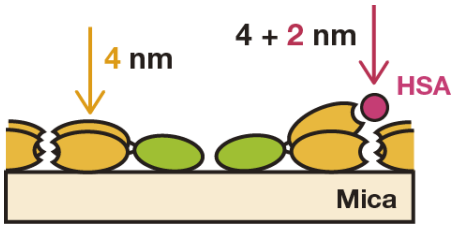
(substrate)

→ **Higher peak** appeared









Higher peak corresponded to the **HSA** **specifically bound to the 2D antibody crystals**

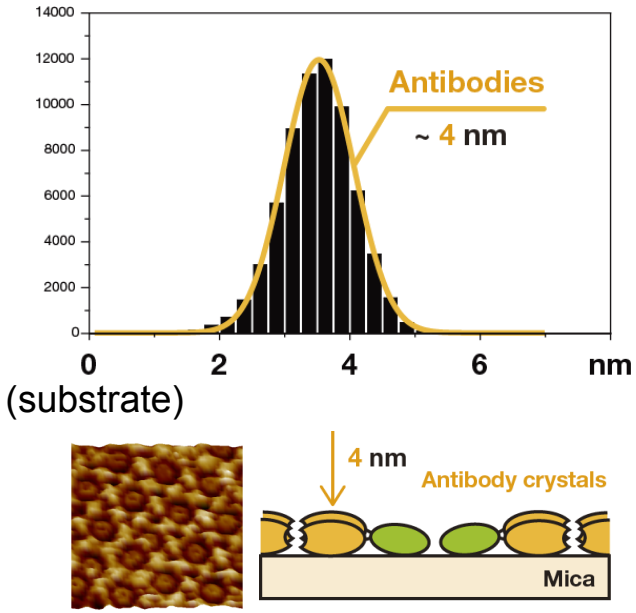
→ We confirmed the **biological activity** of the 2D crystals

Biochemical activity of 2D IgG crystal

Addition of MSA or HSA

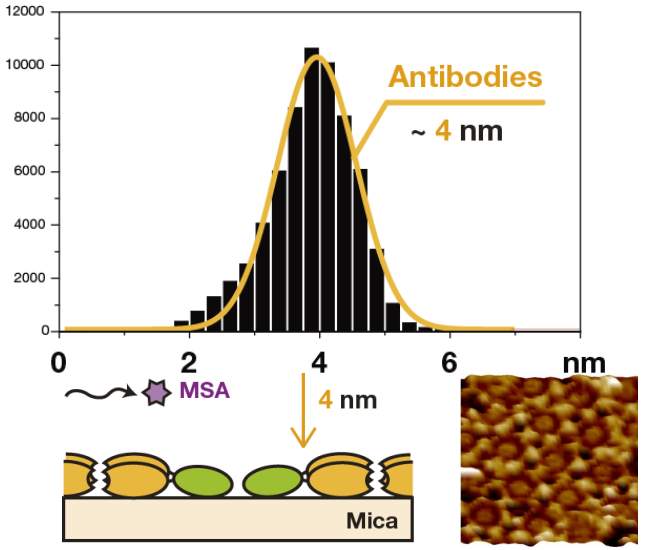
Initial 2D IgG crystal

Height histogram from the substrate

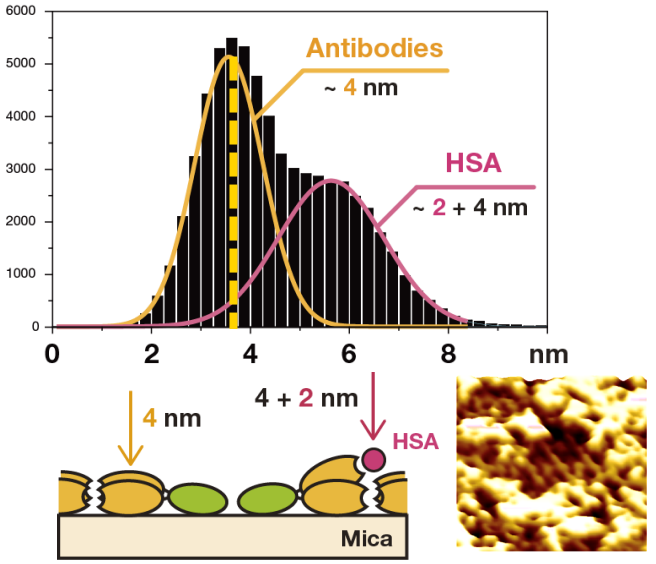


MSA
Non-antigenic molecule

Height histogram from the substrate



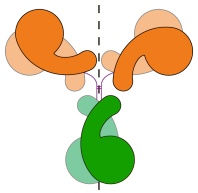
HSA
Antigenic molecule



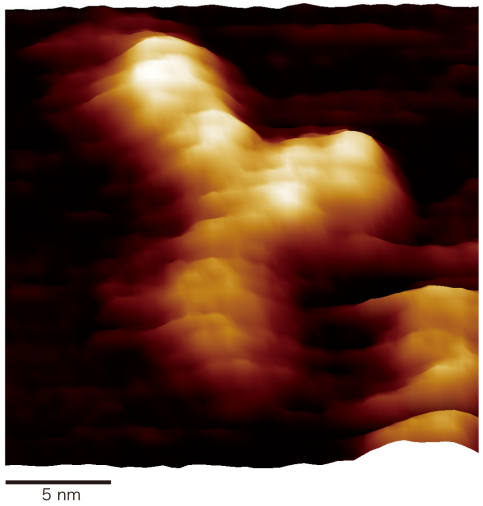
S. Ido et al. *Nature Mater.* **13**, 264 (2014)

Hexmer formation and 2D crystallization of different IgGs?

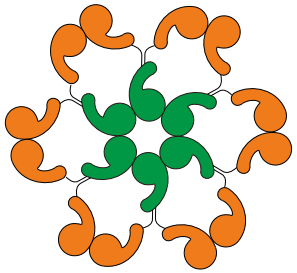
Isolated monomer

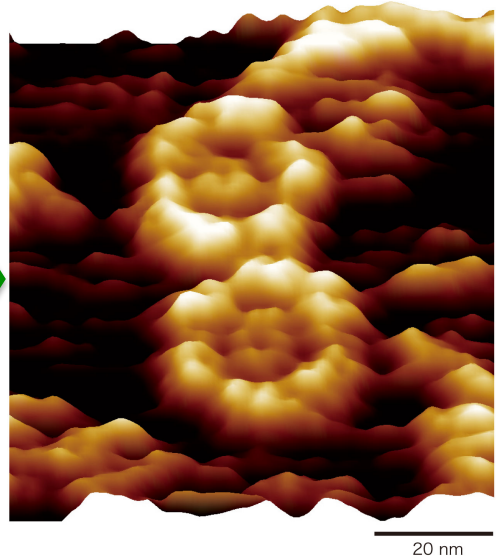


mouse IgG1

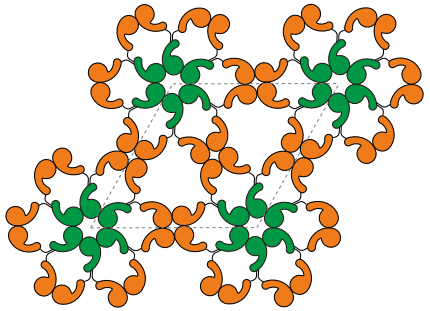


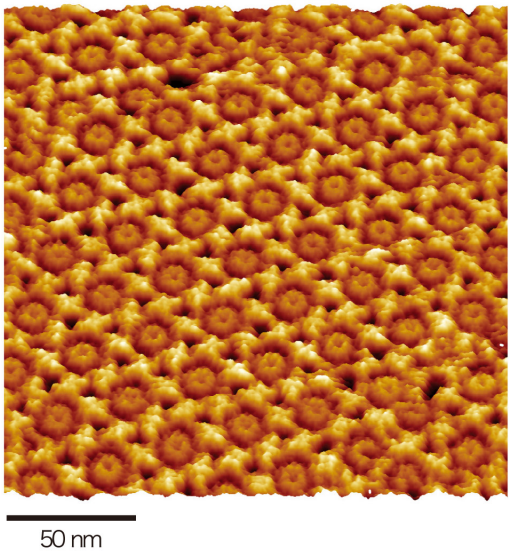
Hexamer formation





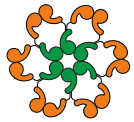
2D crystallization



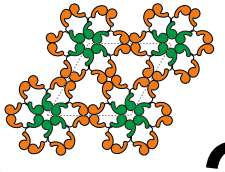


Hexmer formation and 2D crystallization of different IgGs?

FM-AFM images of IgGs
in 50 mM $MgCl_2$, 10 mM PBS.



Hexamer formation



2D crystallization

***mouse* IgG1**
with different paratope
(different Fabs, identical Fc)

?

?

***mouse* IgG2a**
(different Fabs and Fc)

?

?

***rat* IgG1**
(different Fabs and Fc)

?

?

Summary of IgG antibody study

Summary

Investigation on the solution condition for the 2D antibody crystallization

→ Improving reproducibility of the antibody crystallization

Imaging the antibody-antigen interactions on the 2D antibody crystals

→ Confirmation of biological activities of the antibody crystals

Future work

Difficulty in identifying antigen-binding site of the antibody crystals

→ Imaging of molecular structure of the immunocomplex