# CryoET as a method to understand the air-water interface

Alex J. Noble

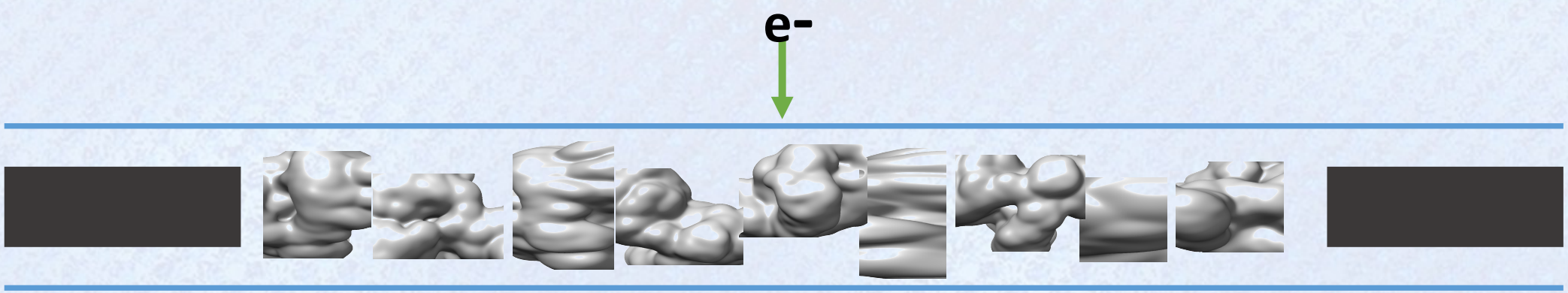
SIMONS ELECTRON MICROSCOPY

**NRAMM**

Manuscript in preparation

# Why Should I Care About the Air-water Interface?

Well, what does an **ideal sample** in holes **look like**?




- **Thin ice**: particle size + ~10-20 nm of space between air-water interfaces,
- **Non-overlapping particles** in beam direction,
- **Maximally concentrated particles** with **no particle-particle interactions**,
- **Randomly oriented particles**,
- Particle layer **perpendicular to the beam**,
- **No air-water interface interactions!**

# Why Should I Care About the Air-water Interface?

- No problem, my grids are perfect!

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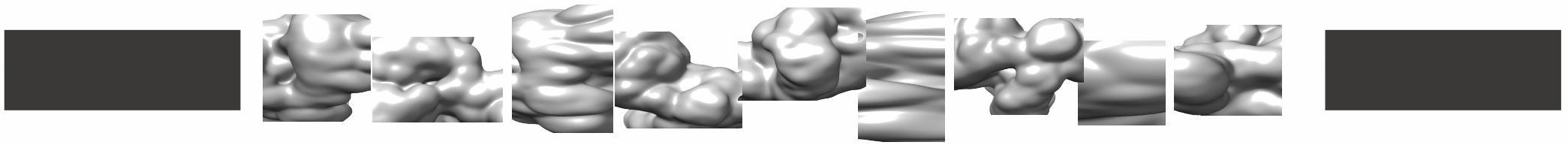


**<u>What do you think</u>: What percentage of samples are ideal?**

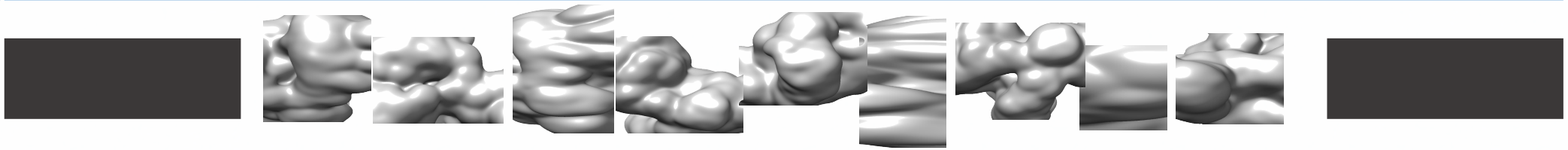
A) **<10%**

B) **25%**

C) **50%**

D) **75%**

# Why Should I Care About the Air-water Interface?



**What do you think: What percentage of samples are ideal?**

A) ~~<10%~~ <5%

B) 25%

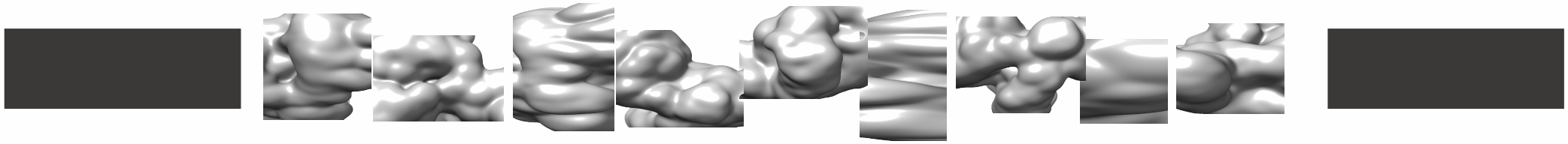
C) 50%

D) 75%

E) >90%

Why?

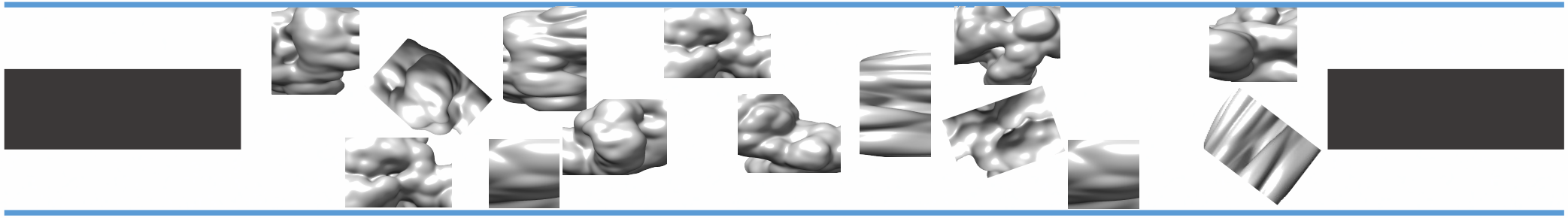
# Why Should I Care About the Air-water Interface?



**Of the >50 samples I've studied, <5% of samples are ideal**

- **Thin ice: ~2/3** of samples are have areas of **≤50 nm ice**,
- **Non-overlapping particles: ~2/3** of samples are have **single layer** areas,
- **Maximally concentrated particles** with **no particle-particle interactions:** **>1/2** of samples have areas where **particle saturation** is **between 60-90%**,
- **Randomly oriented particles: ~40%** of samples have **no apparent preferred orientations**,
- Particle layer **perpendicular to the beam: 80%** of samples have areas oriented **≤5°** with respect to the electron beam normal,

# Why Should I Care About the Air-water Interface?



**<u>What do you think</u>: What percentage of all particles I've studied by cryoET are at the air-water interface?**

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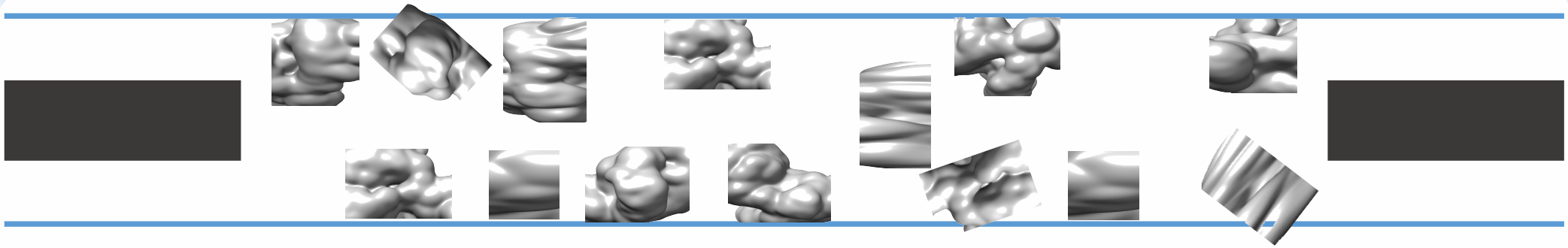
D) **75%**

E) **>90%**

# Why Should I Care About the Air-water Interface?



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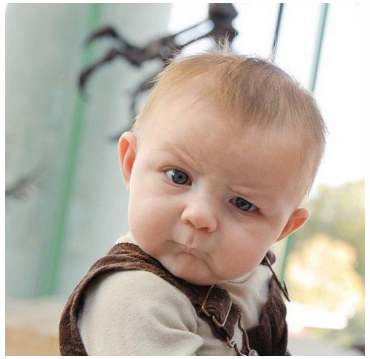

# Why Should I Care About the Air-water Interface?

# You Must Care About the Air-water Interface

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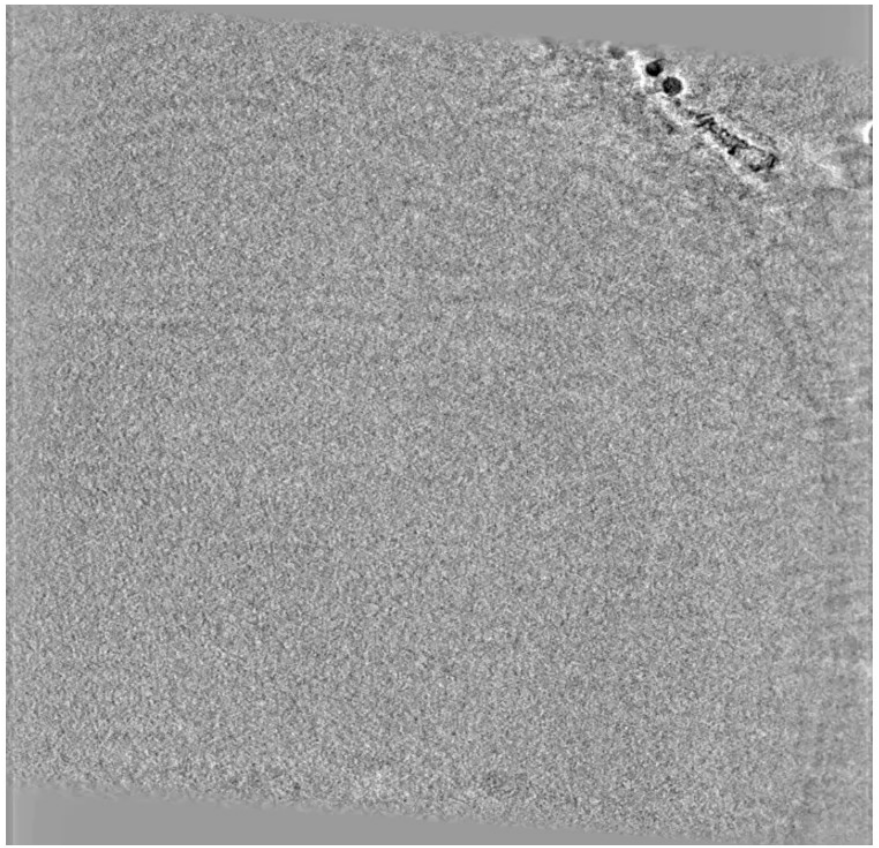
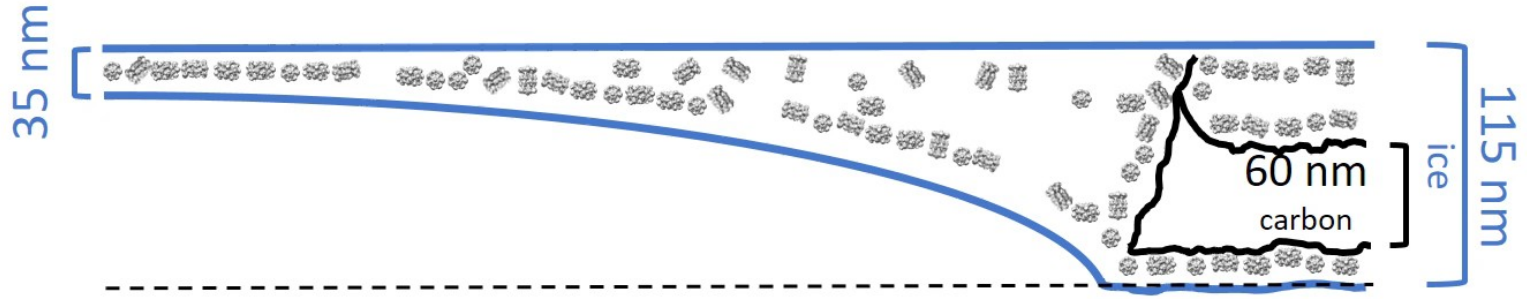
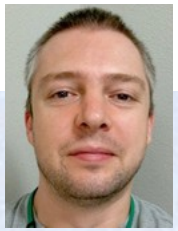


250 nm




**T20S Proteasome**



**Anchi Cheng**, **Radostin Danev**, Alex Noble

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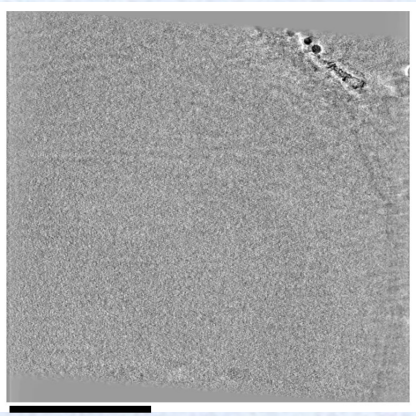
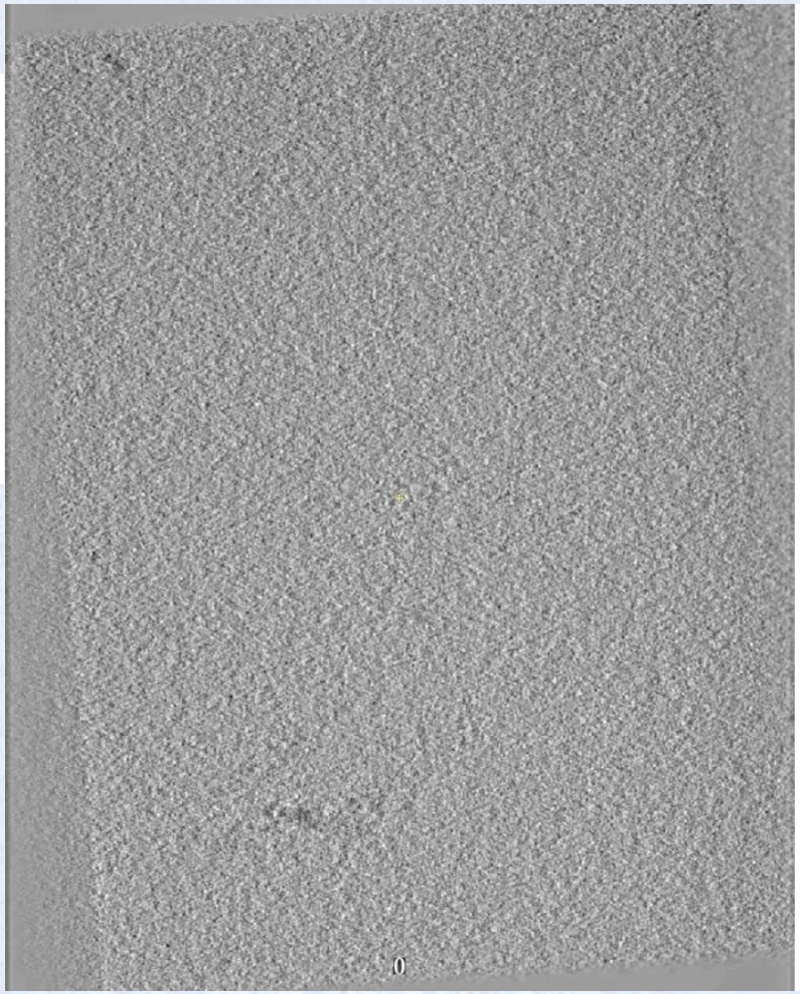
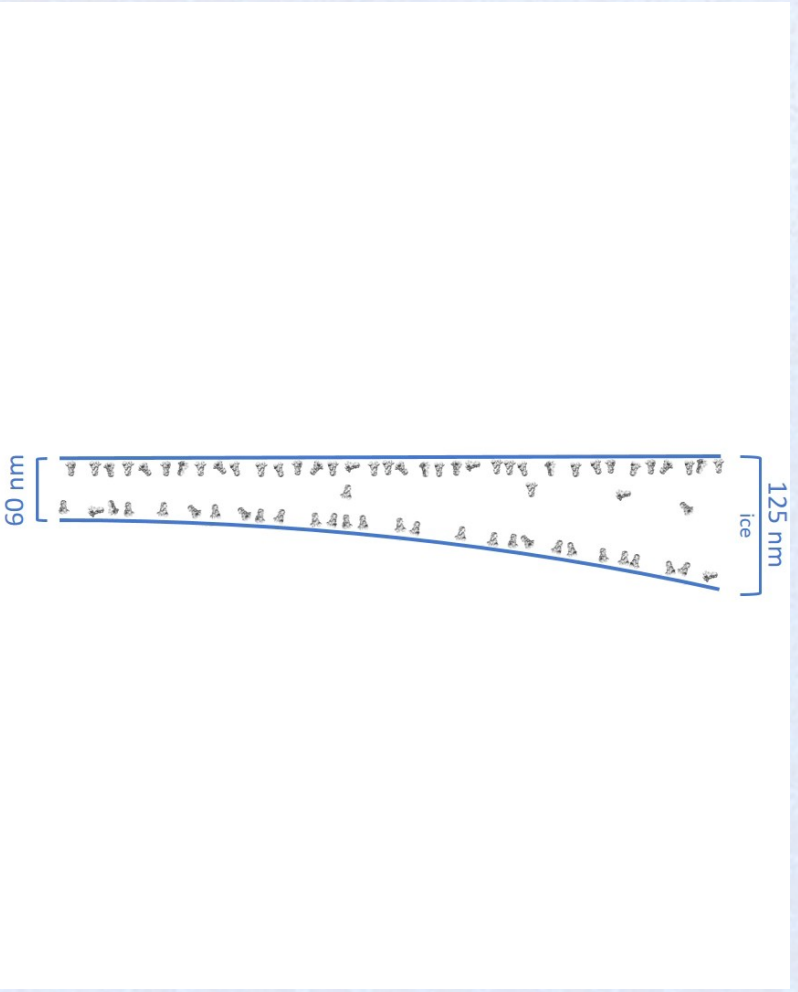
# You Must Care About the Air-water Interface





250 nm




**Hemagglutinin**

**Hui Wei**, Alex Noble

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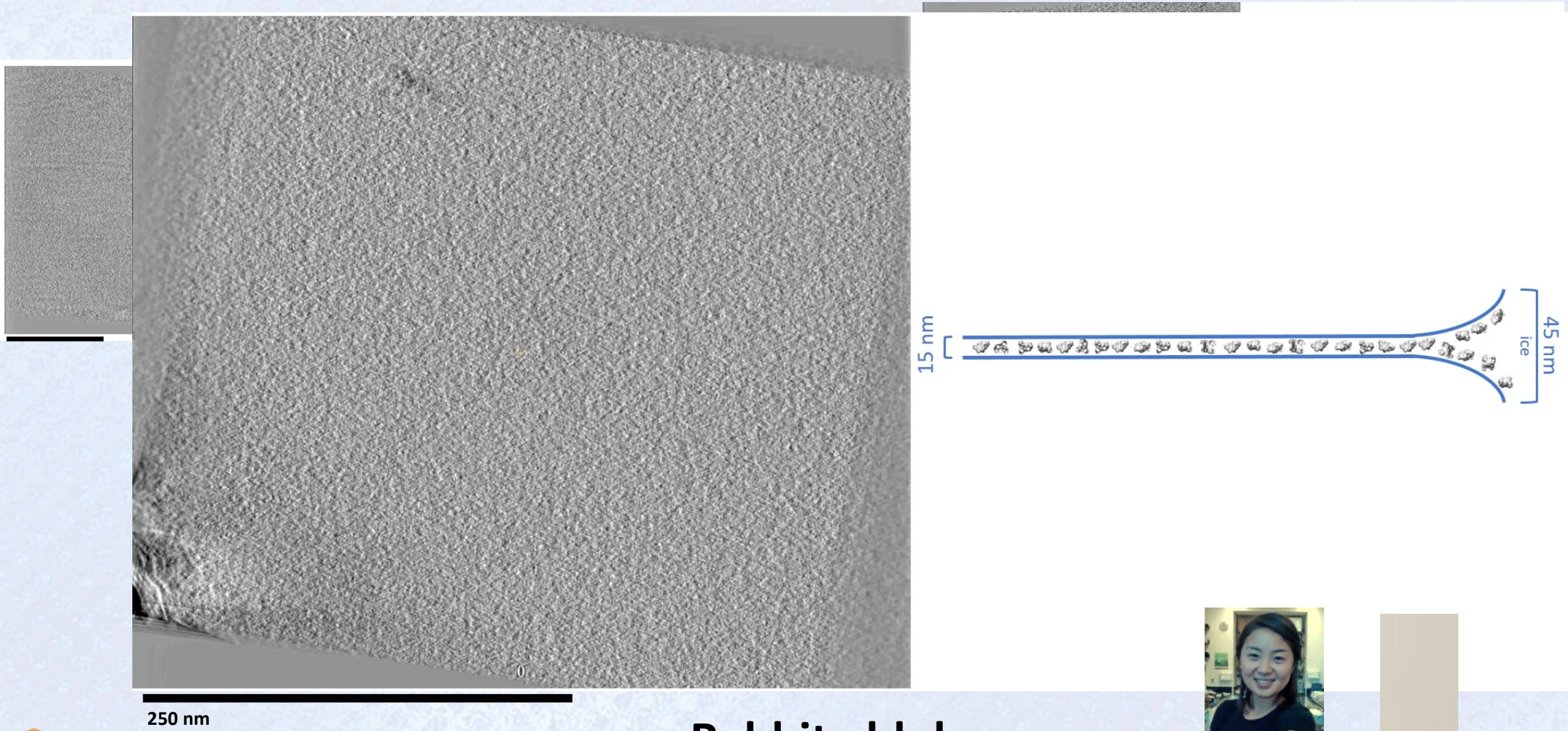
# You Must Care About the Air-water Interface




**Rabbit aldolase**



**Laura Kim, Venkata Dandey**, Alex Noble

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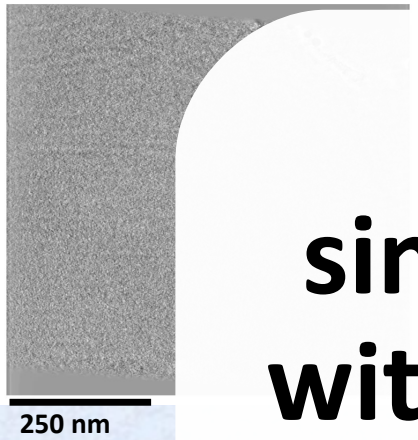
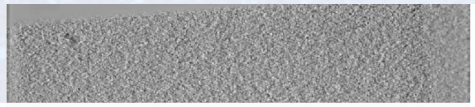
# You Must Care About the Air-water Interface

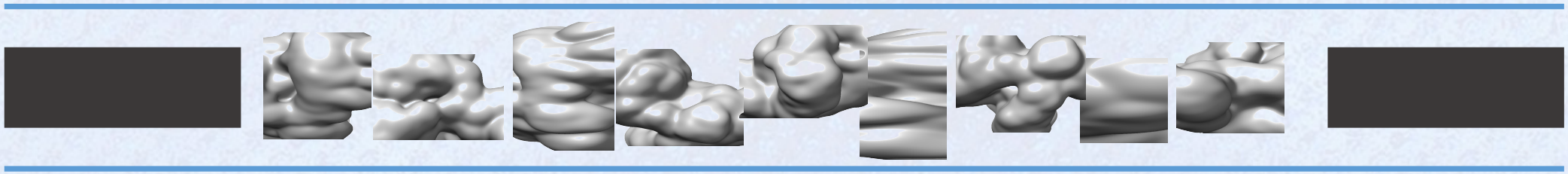


**Out of over 1,000 tomograms of single particle grids from over 50 preps with incubation times on the grid on the order of 1 seconds,**

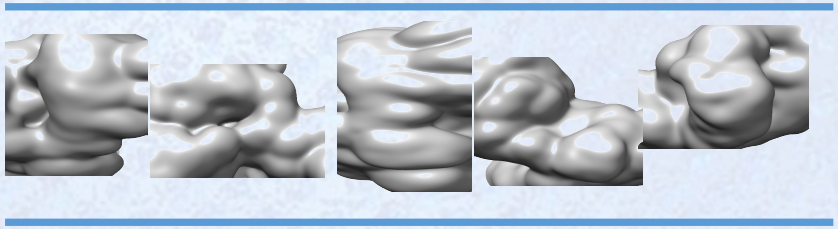
**about 90% of all particles are on an air-water interface**

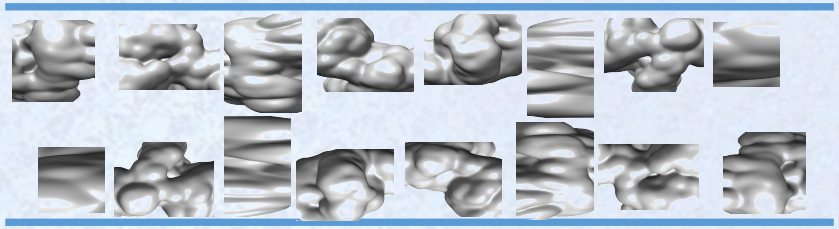
# You Must Care About the Air-water Interface



Center of holes

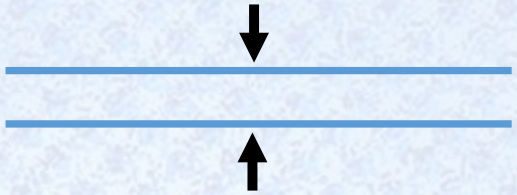
60%
one layer





20%
two layers

Ice thickness
(avg ± 1 stdev)

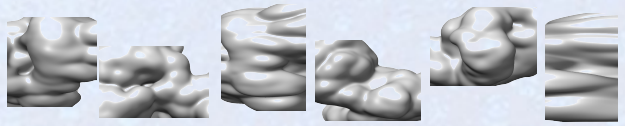


**Gold** Spotiton
31 ± 14 nm

**Carbon** Spotiton
47 ± 25 nm

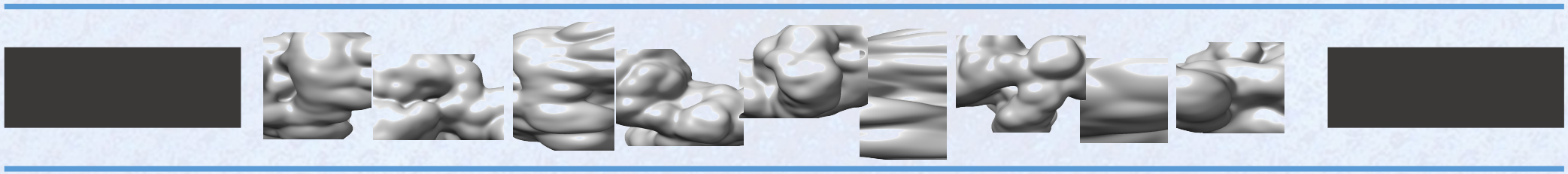
Holey **Carbon**
61 ± 37 nm

Particle layer tilt WRT e- beam
(avg ± 1 stdev)



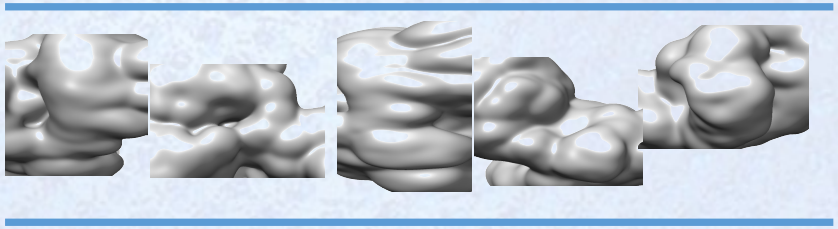
4.7 ± 3.1°

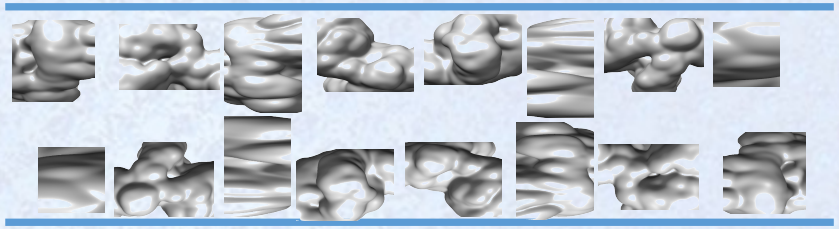
# You Must Care About the Air-water Interface



~100 nm from the edge of holes

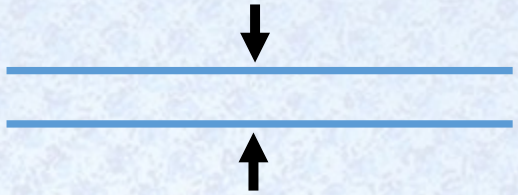
20%
one layer





60%
two layers

**Ice thickness**
**(avg ± 1 stdev)**

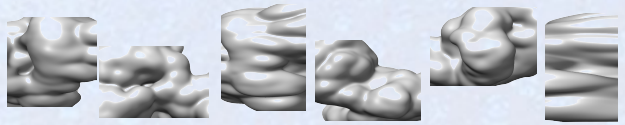


**Gold** Spotiton
**61 ± 11 nm**

**Carbon** Spotiton
**95 ± 32 nm**

Holey **Carbon**
**99 ± 24 nm**

**Particle layer tilt WRT e- beam**
**(avg ± 1 stdev)**



**6.9 ± 3.5°**

Ok, so my particles are likely adsorbed to an air-water interface…

Should I be worried?

# To Help Understand Protein Behavior at Interfaces We Turn to…

oil-water

air-water

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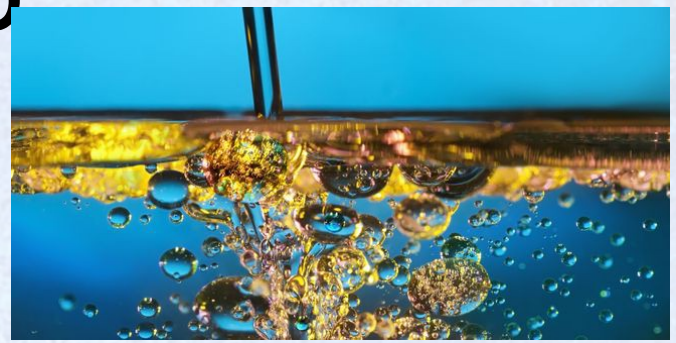
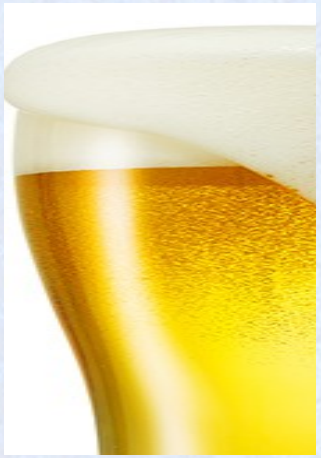
# To Help Understand Protein Behavior at Interfaces We Turn to

oil-water



air-water

# Food Colloids – Proteins in Emulsions and Foams



taste

texture

digestive

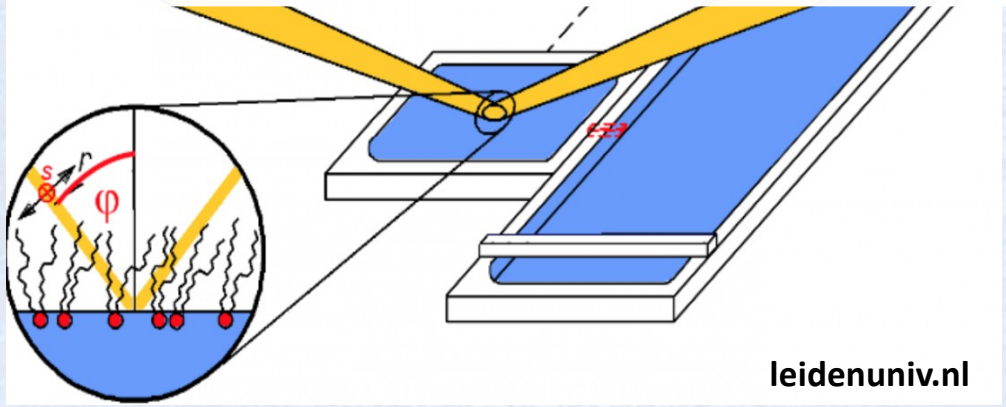
# Food Colloids – Proteins in Emulsions and Foams


LB trough experiments are conducted with very clean equipment

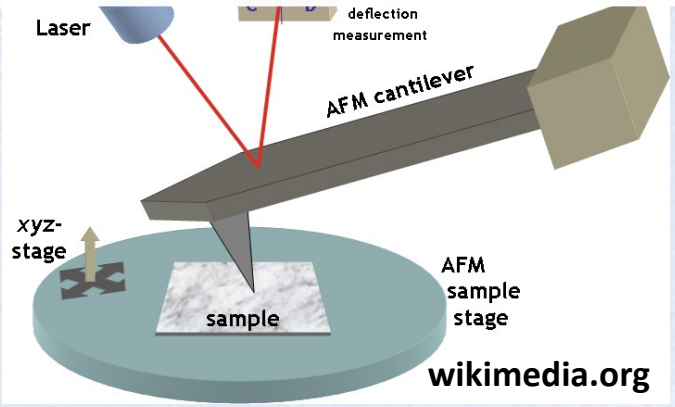
…

unlike cryoEM grid prep

Glaeser and Han, 2016

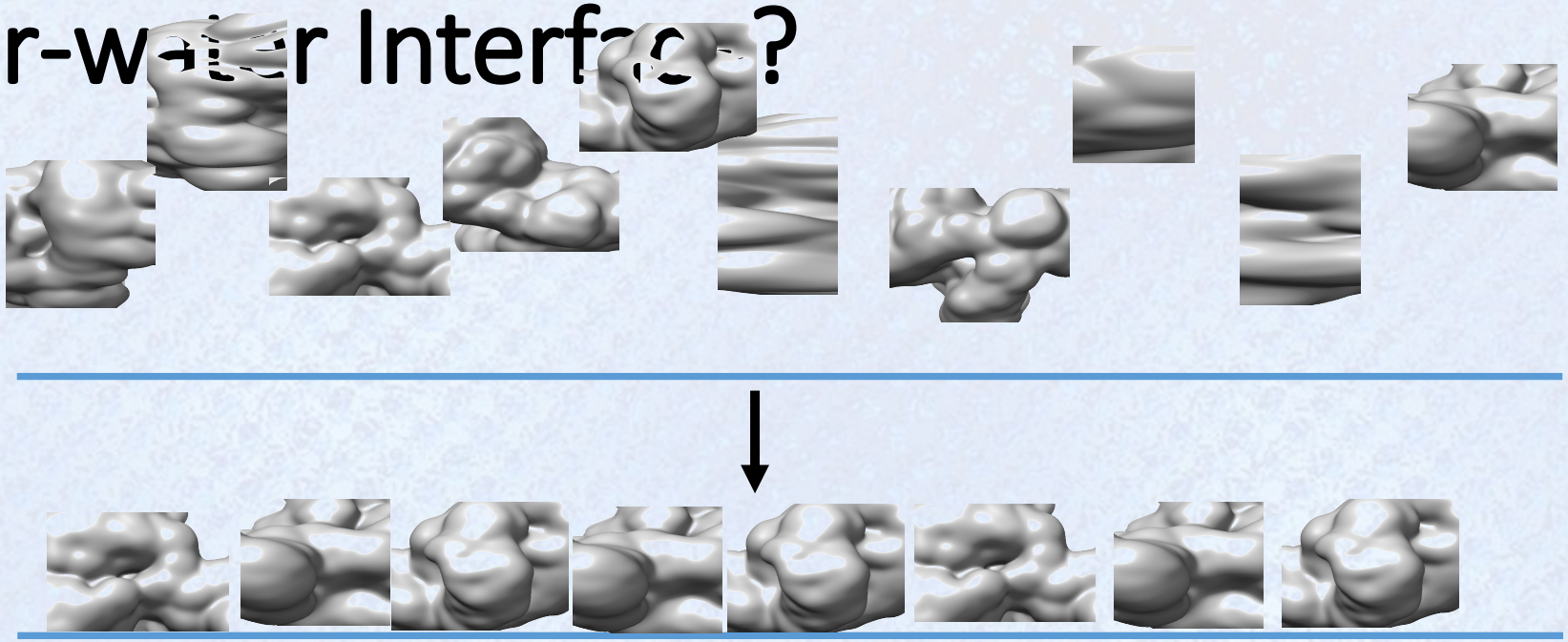



**IRRAS**




**AFM of LB films**

# How Quickly Might Bulk Particles Adsorb to the Air-water Interface?



**Bulk diffusion**

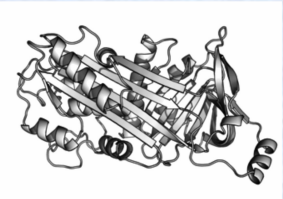
↓

**adsorption (tbd)**

**Theory: tbd ≃ 1 ms to 0.1 s** (Naydenova and Russo, 2017; Taylor and Glaeser, 2008)

**Food science: tbd ≃ 0.3 ms** (Kudryashova et al., 2005)

↳ Ovalbumin (45 kDa egg white protein)



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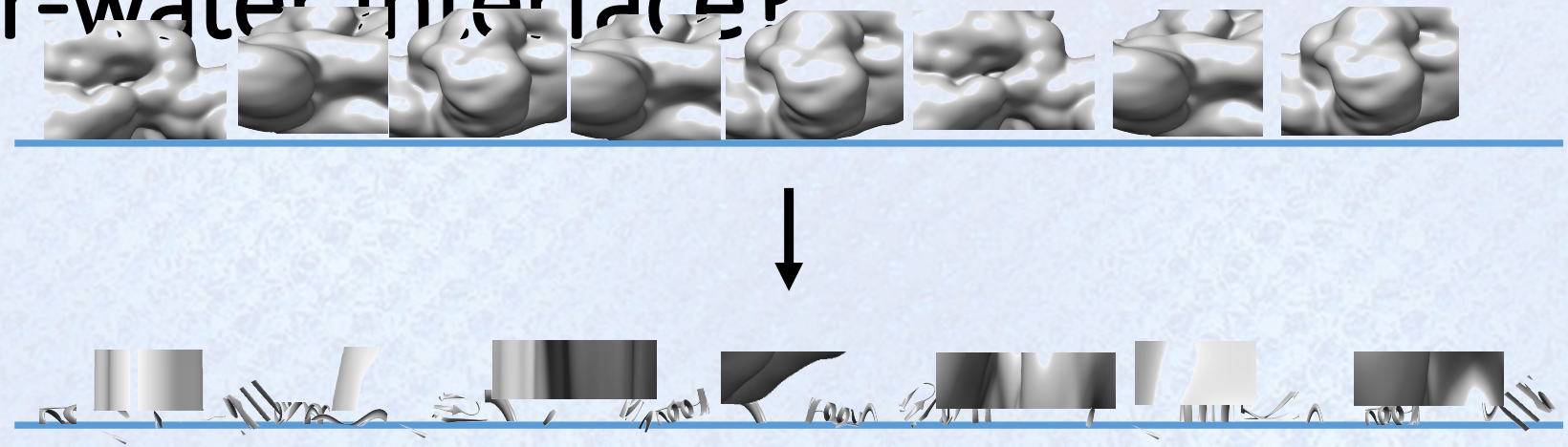
# How Quickly Might Adsorbed Particles Denature at the Air-water Interface?

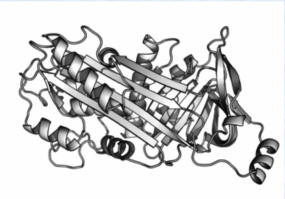


**Adsorbed particles**

**denaturation (tsd)**

**Food science: tsd ≃ 10+ ms** (Kudryashova et al., 2005)

Ovalbumin (45 kDa egg white protein)



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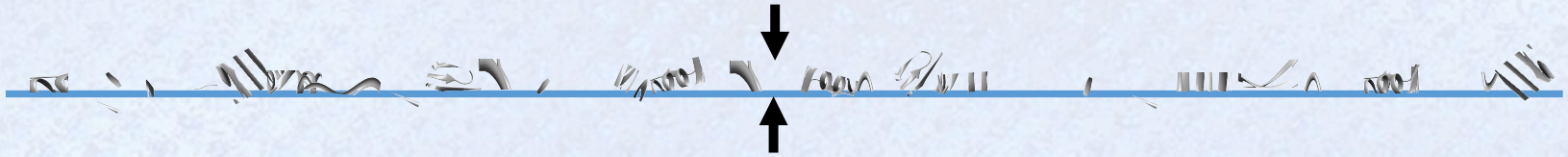
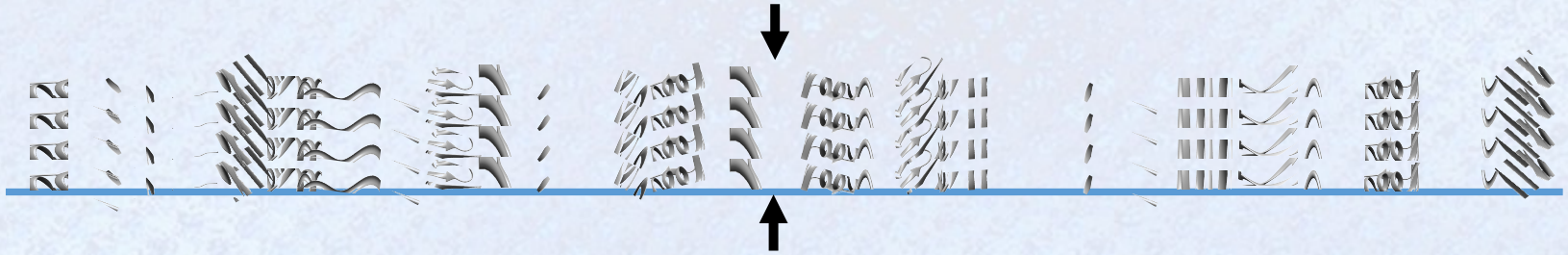
# How Thick are these Denatured Layers?



**Monolayers are ~1 – 10 nm thick**
**using ~0.1 mg/mL bulk protein**

(by IRRAS: van Vliet et al., 2002)
(by AFM: Gunning et al., 1996)

# How Thick are these Denatured Layers?



**Multilayers might be as thick as 50 nm!**

An IRRAS study of β-casein showed that a bulk protein concentration increase **from 0.1 to 100 mg/mL increased the denatured layer thickness from 5 to 50 nm**.

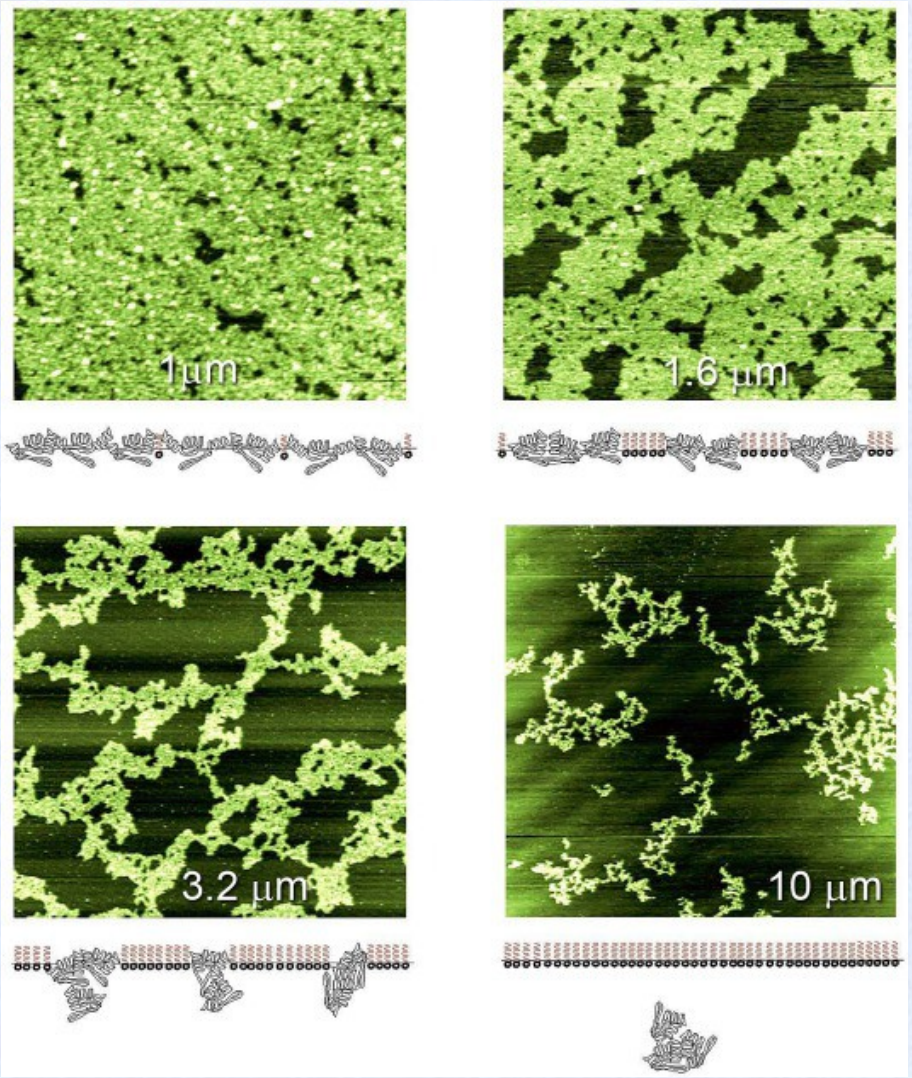
**(Meinders et al., 2001)**

# Are Denatured Protein Layers at the Air-water Interface Uniform?

Protein layer **displacement by surfactants** show non-uniform displacement

- Some proteins partially desorb
- Occurs nearly identically with different surfactants
- Denatured protein layers might **not be uniform**.

β-lactoglobulin + Tween 20

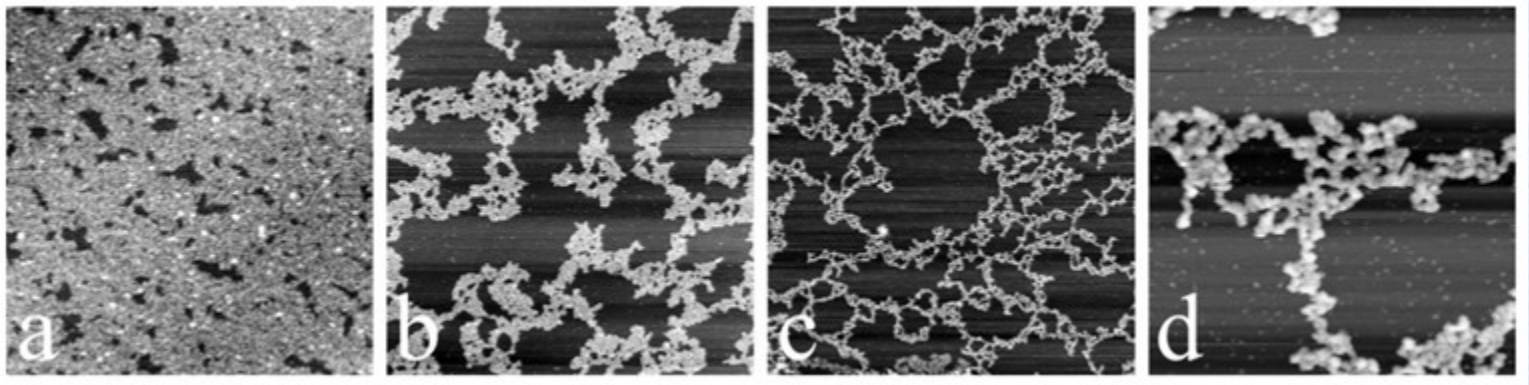



**Color indicates thickness**

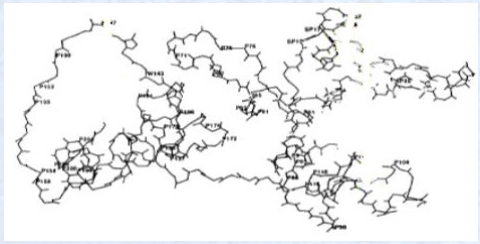
**Gunning and Morris, 2008 & 2017**

# Are Denatured Protein Layers at the Air-water Interface Uniform?

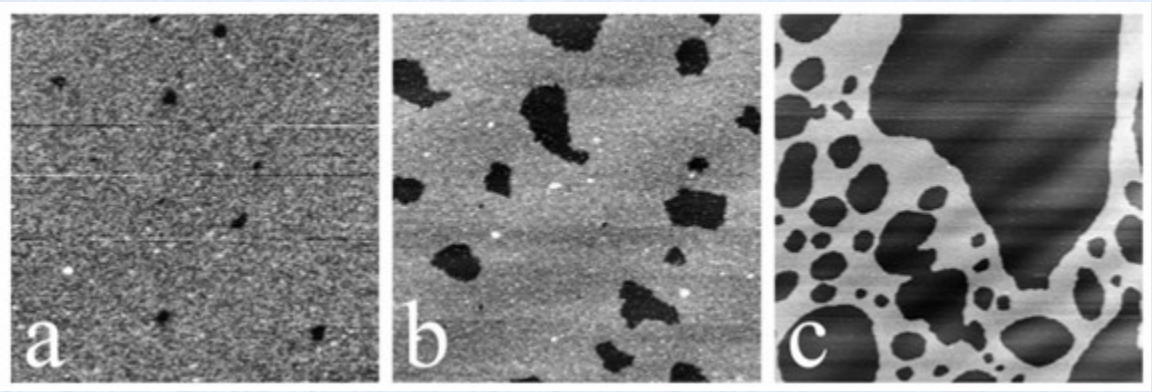
β-lactoglobulin + Tween 20




**Greyscale indicates thickness**



β-casein + Tween 20



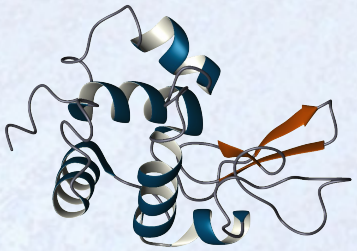

time

Disordered proteins form more uniform layers than globular proteins.

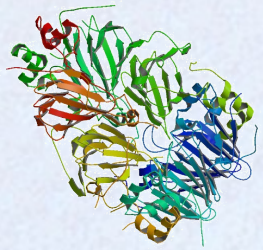


**Gunning and Morris, 2008**

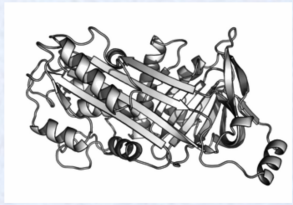
# Does the Protein Layer Strength Vary?



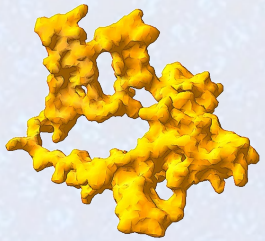
lys = lysozyme



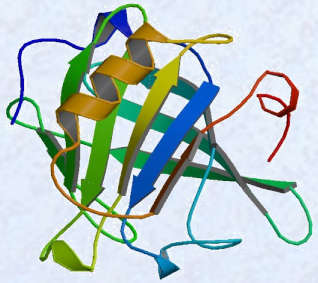
gly = glycinin



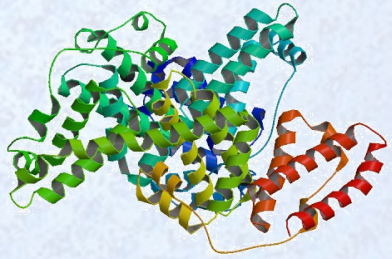
ova = ovalbumin



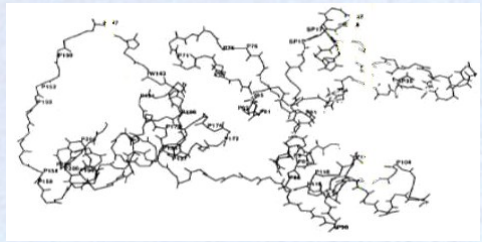
kcas = k-casein



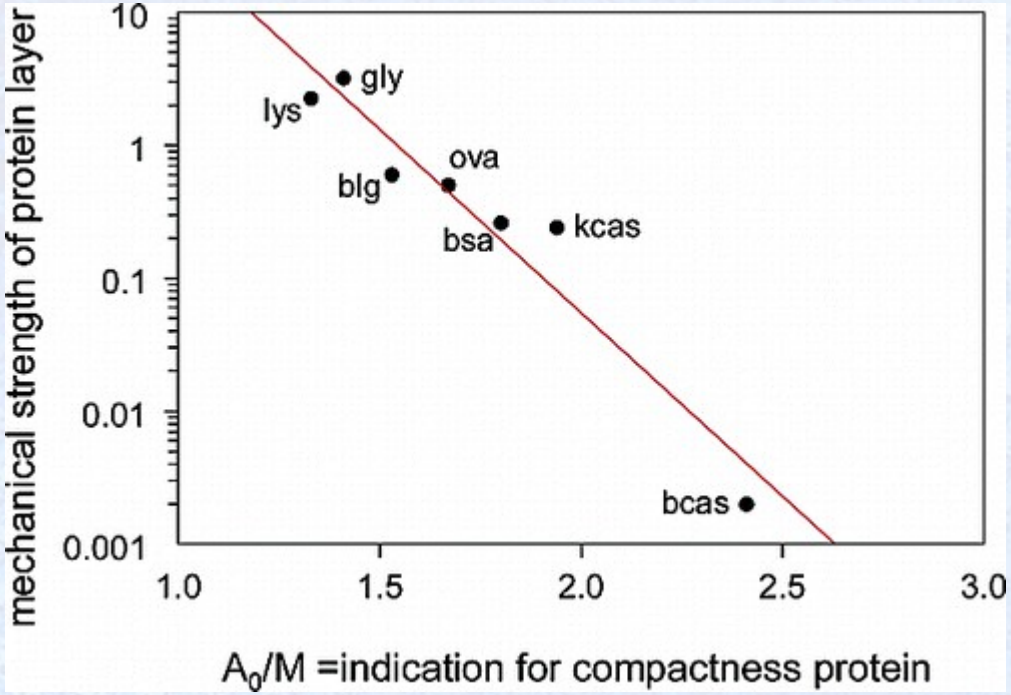
blg = β-lactoglobulin



bsa = bovine serum albumin



bcas = β-casein




A0 = Surface area, M = Mol. weight

Mechanical strength as measured by shear stress and compressibility before fracturing shows a correlation:

**The more globular a protein, the stronger the protein network.**

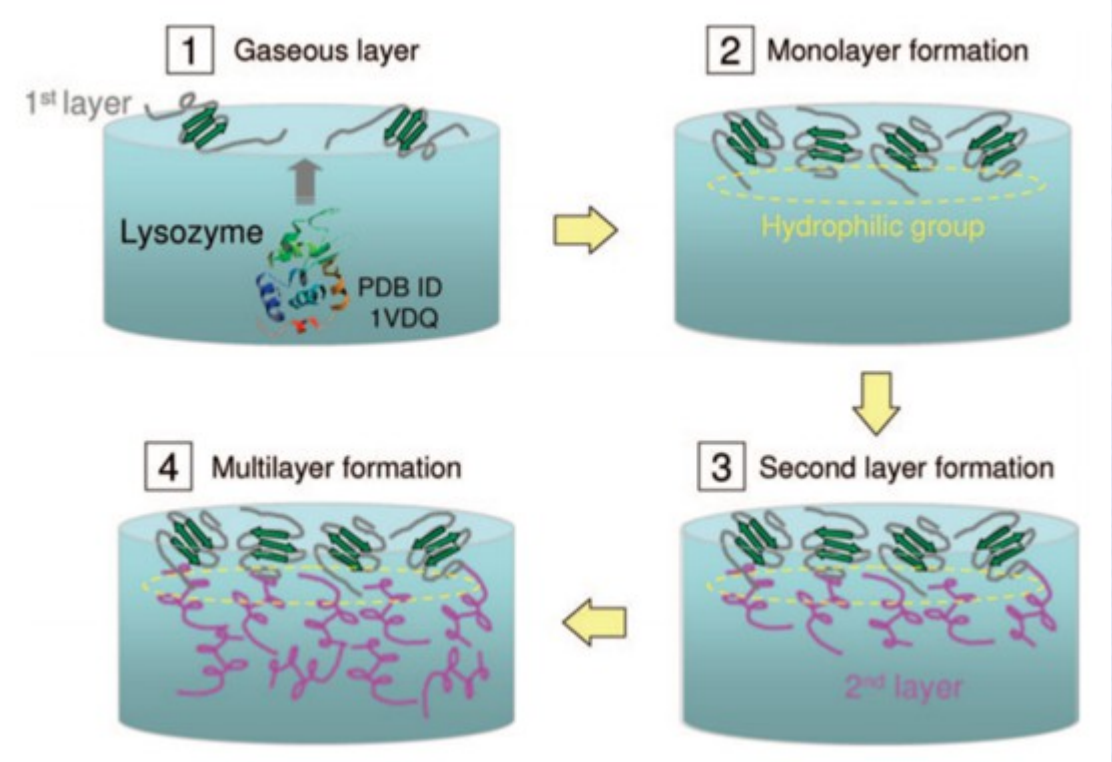
SIMONS ELECTRON MICROSCOPY

**Martin et al., 2005**

# Can Secondary Structure Survive at the Air-water Interface?

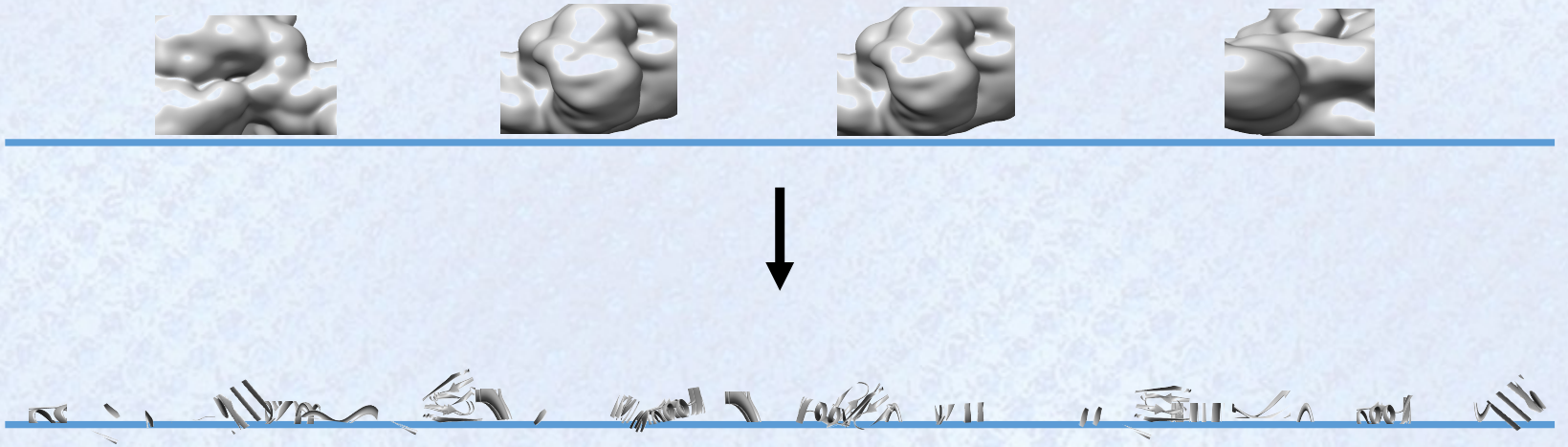
Lysozyme studies at the air-water interface show that **β-sheets survive**

- Most β-sheets are able to **re-structure their alternating hydrophobic-hydrophilic residues** and thus can survive air-water interfaces.




Yano et al., 2008

# Can Surviving β-sheets Interact at the Air-water Interface?



It has been shown that **intermolecular β-sheets can bind together**, strengthening the protein network.

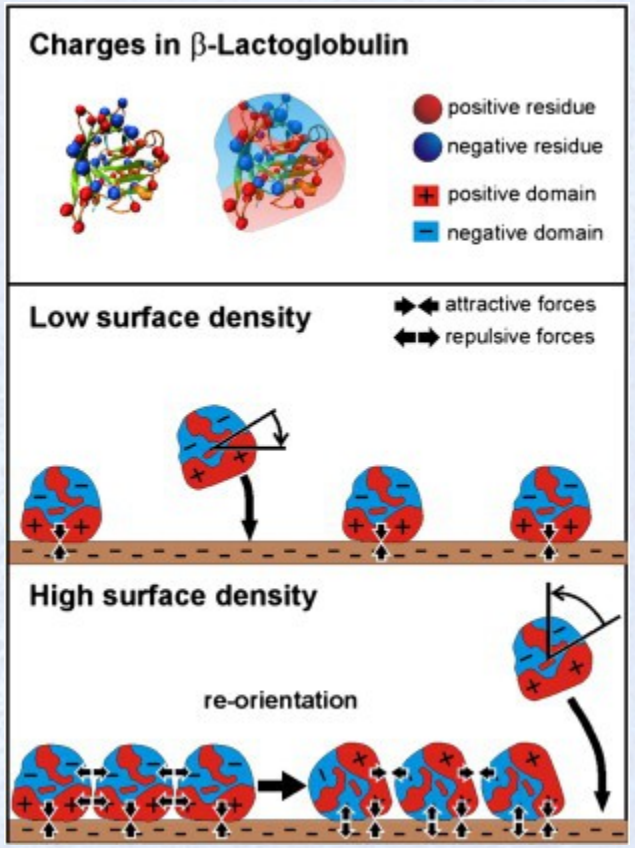


Renault et al., 2002
Martin et al., 2005

# Protein Concentration-dependant Preferred Orientation

**Surface-water protein** studies have shown that globular particles at **high interface concentrations might induce alternative preferred orientations**

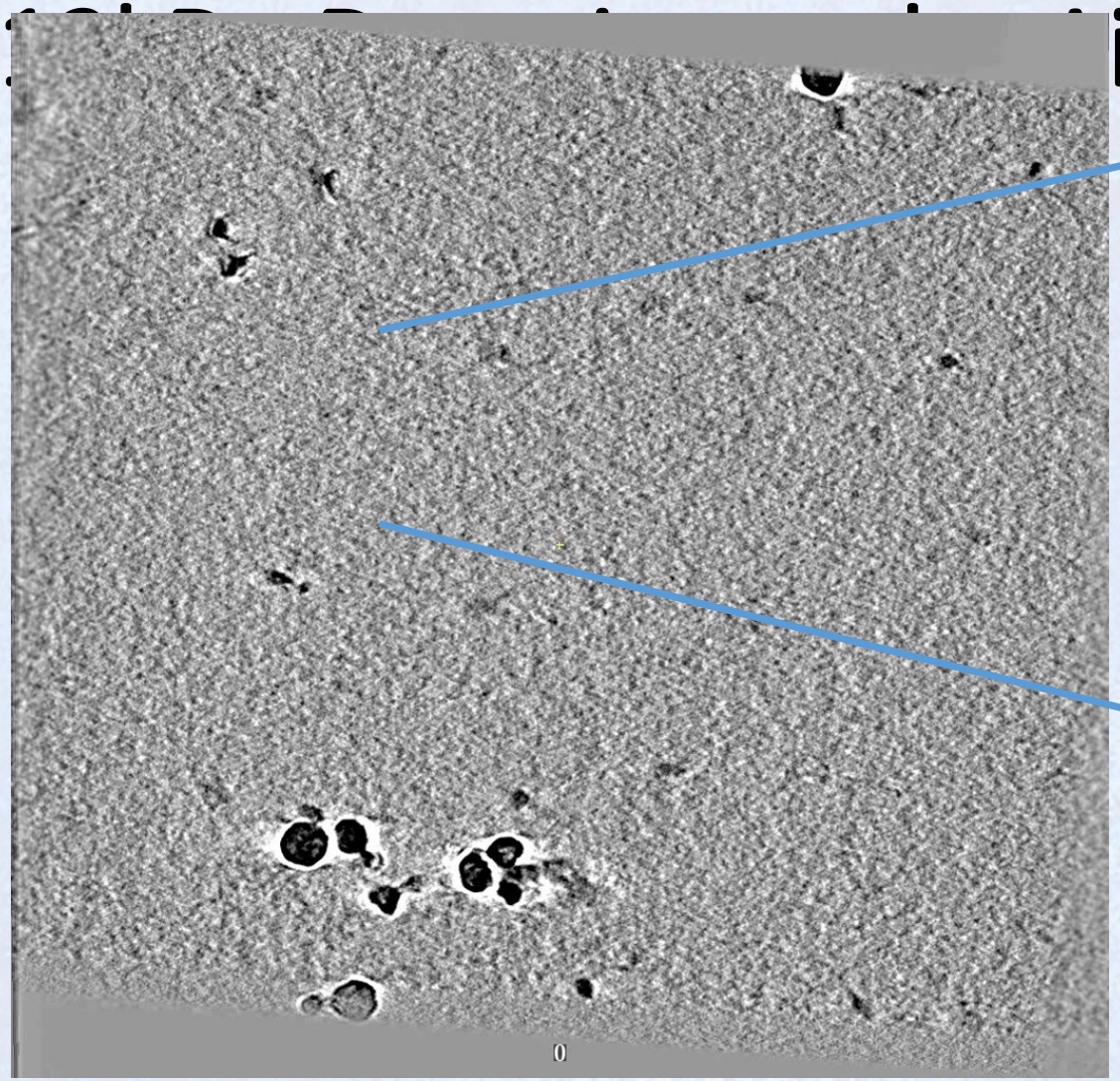
- Might also be applicable to air-water interfaces



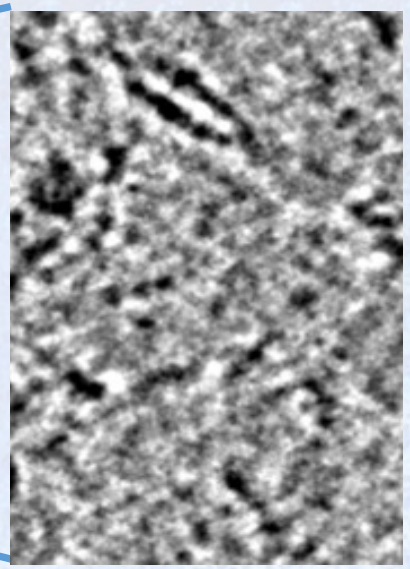

Rabe et al., 2011

# Ok, but do we see denatured proteins in cryoEM grids?

# Clustered Protocadherins CryoEM Grids Show [illegible]ir-water Interfaces

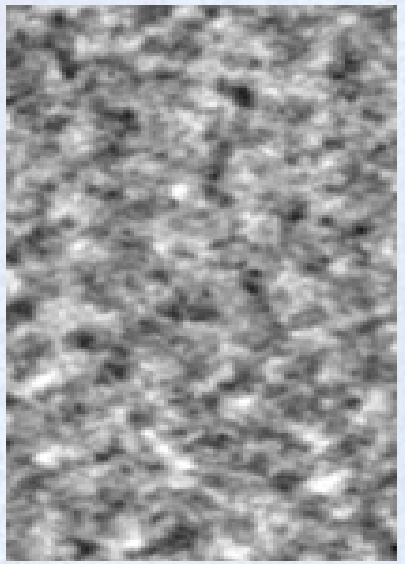



250 nm

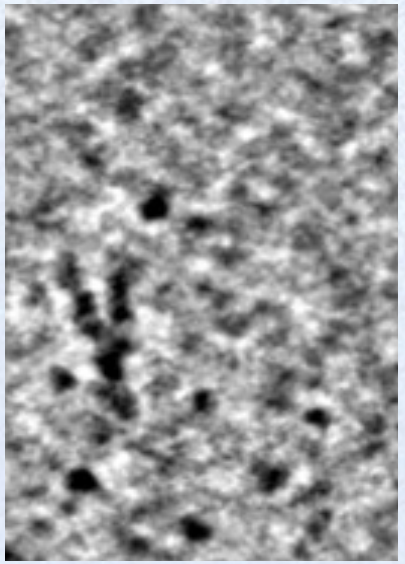


25 nm

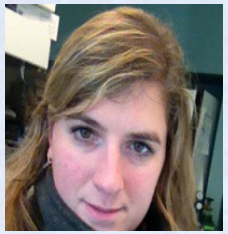
10 slices at air-water interface *with* sample layer



10 slices in ice

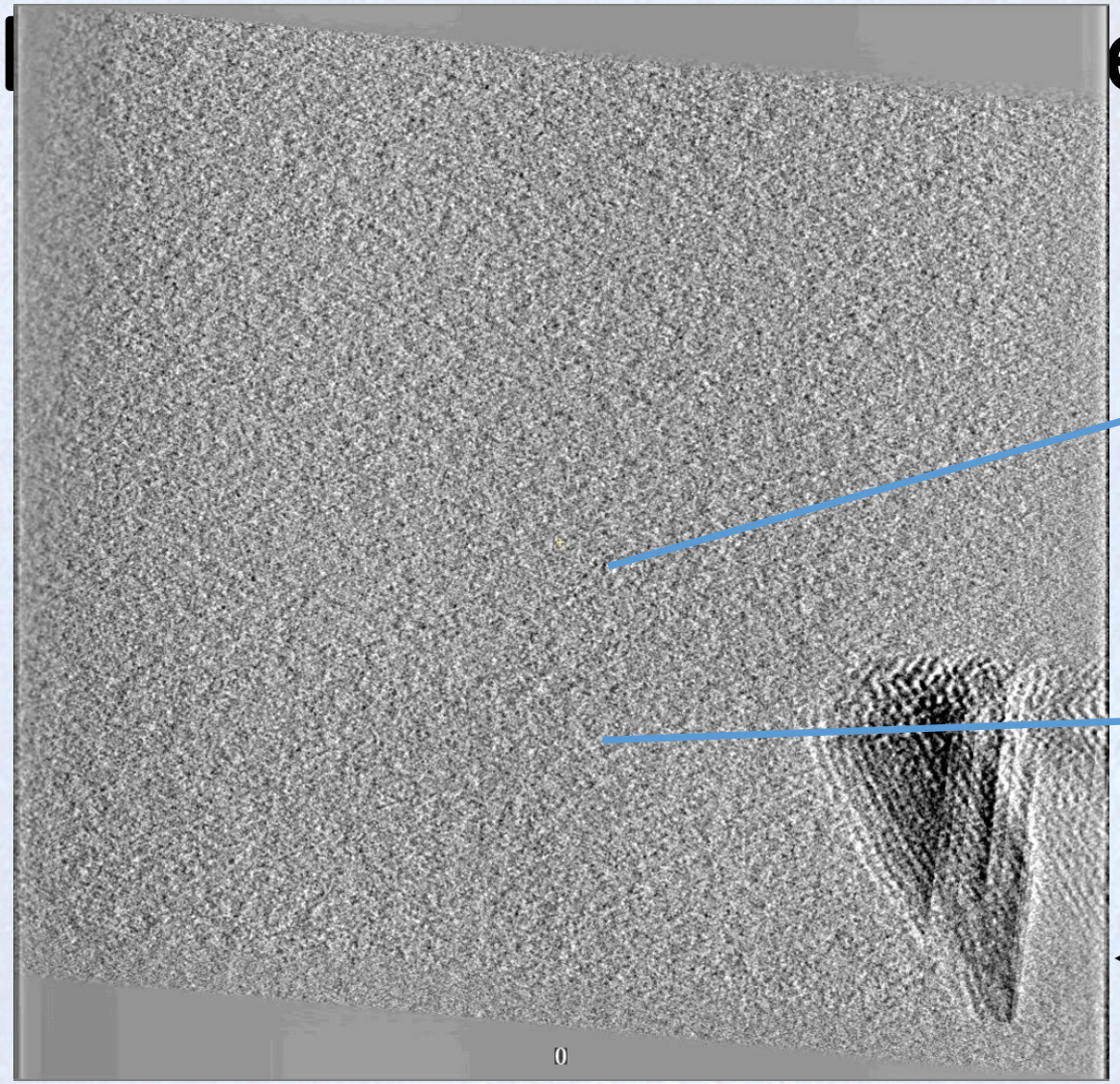


10 slices at air-water interface *without* sample layer

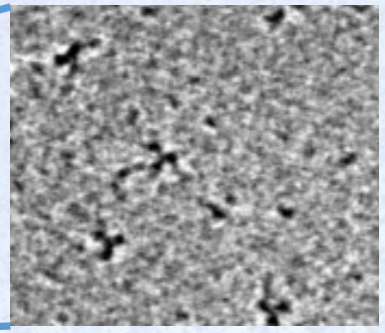


**Julia Brasch** & Alex Noble

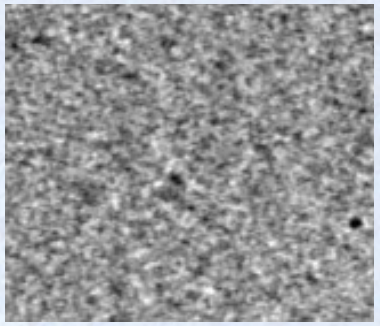
# HIV-1 Trimer CryoEM Grids Show
# e Air-water Interfaces



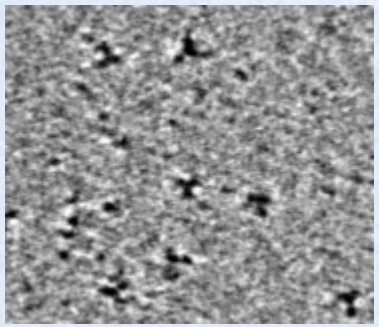

250 nm



Slice 45-55
(air-water/trimers)



Slice 85-95
(in ice)

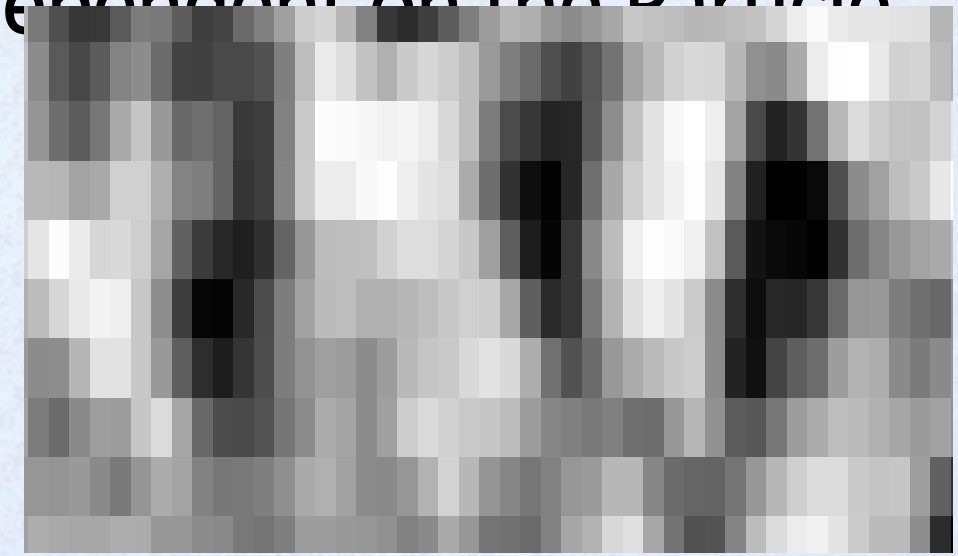


Slices 110-120
(air-water/trimers)

Spotiton nanowire

**Priyamvada Acharya** & Alex Noble

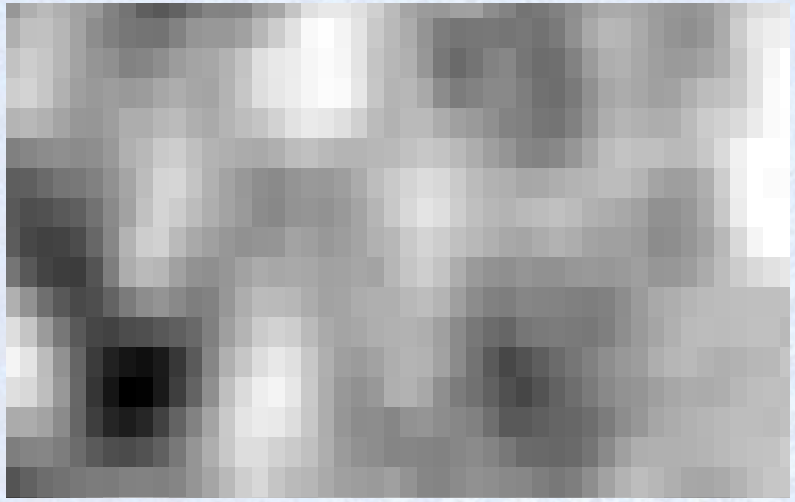
# CryoET Shows a Gradient of Visible Protein Denaturation Dependent on the Particle



**Hemagglutinin**

**Apoferritin**

10 nm slices
through tomograms



**GDH**



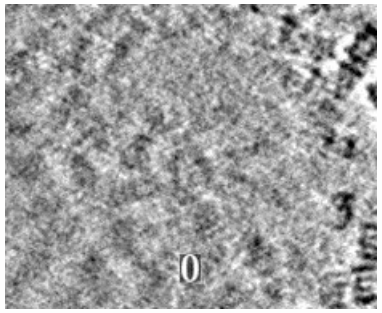
**T20S proteasome**

So some particles denature and some don’t?

Not so fast!

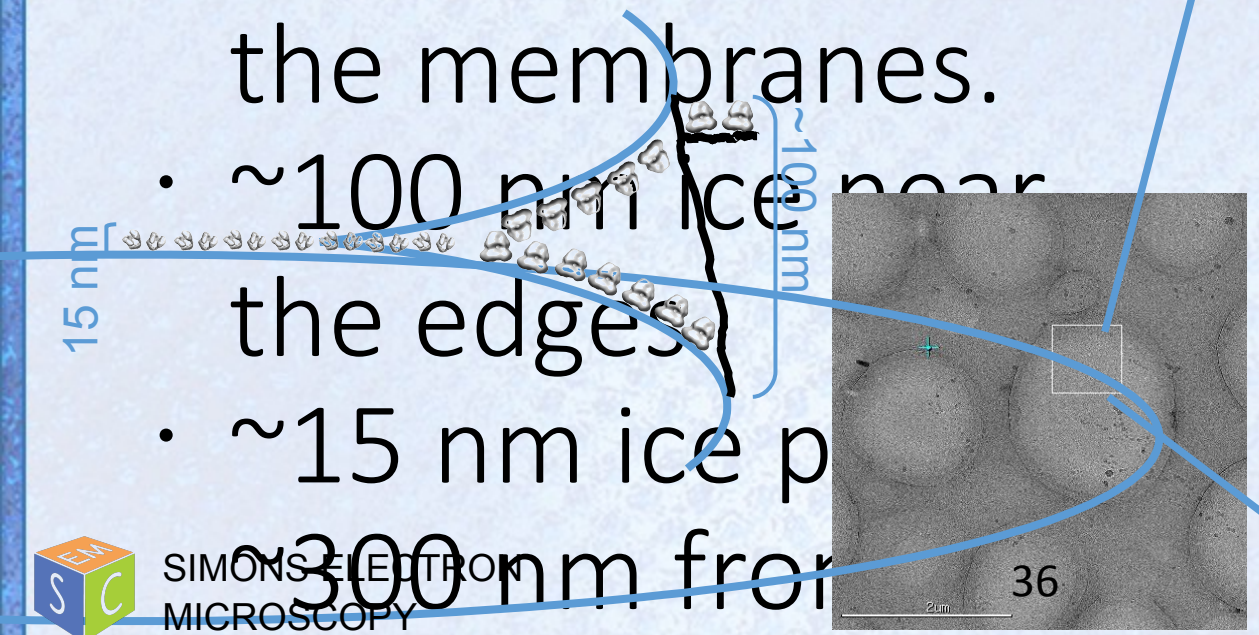
Proteasome

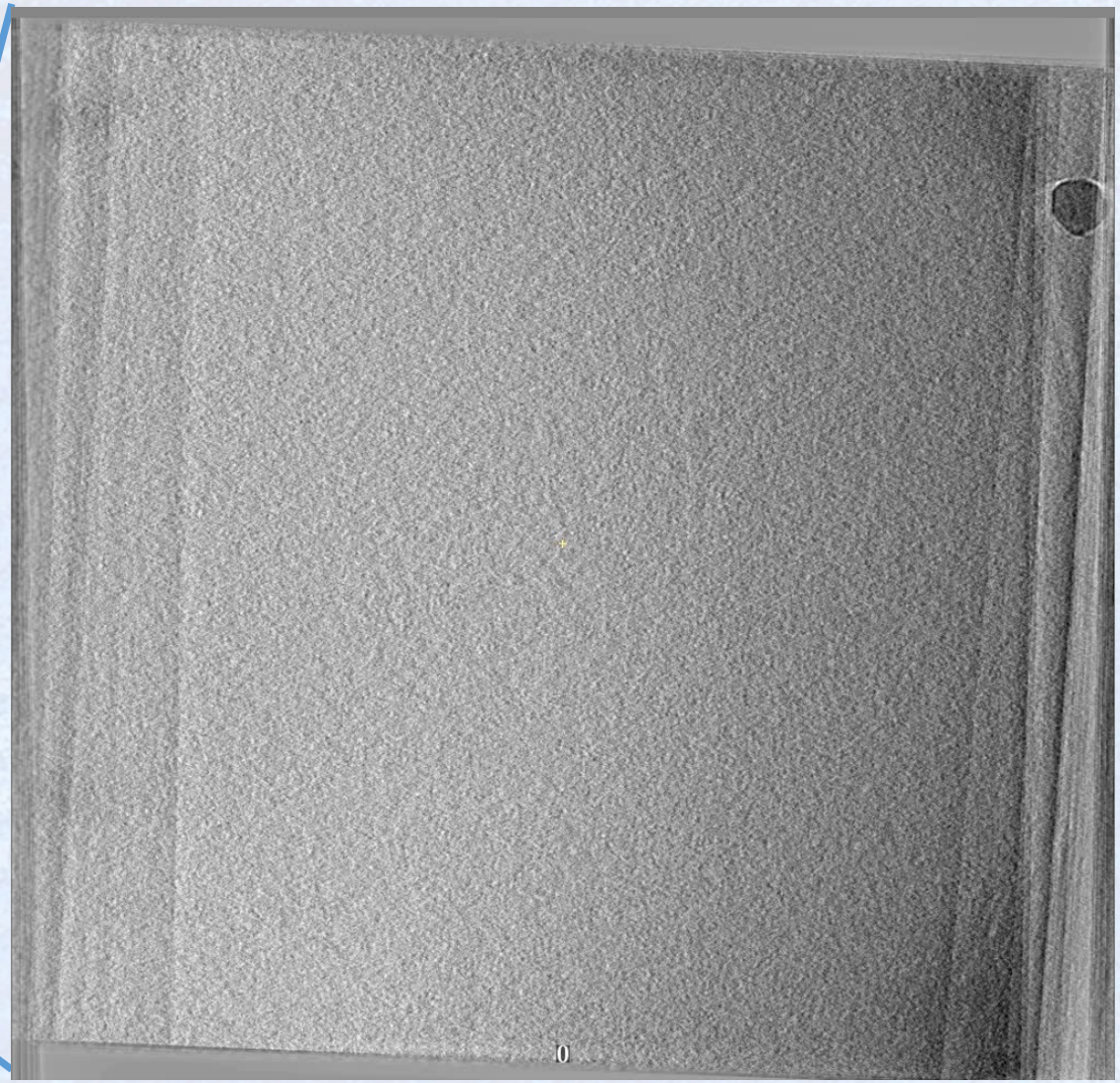
shows partial particles



# Apparent Membrane Denaturation May Occur in Thin Ice (Un-named Protein with Lipid Membrane)

- Thin ice with proteins with membranes may disassociate from the membranes.
- ~100 nm ice near the edges
- ~15 nm ice p
- ~300 nm fro



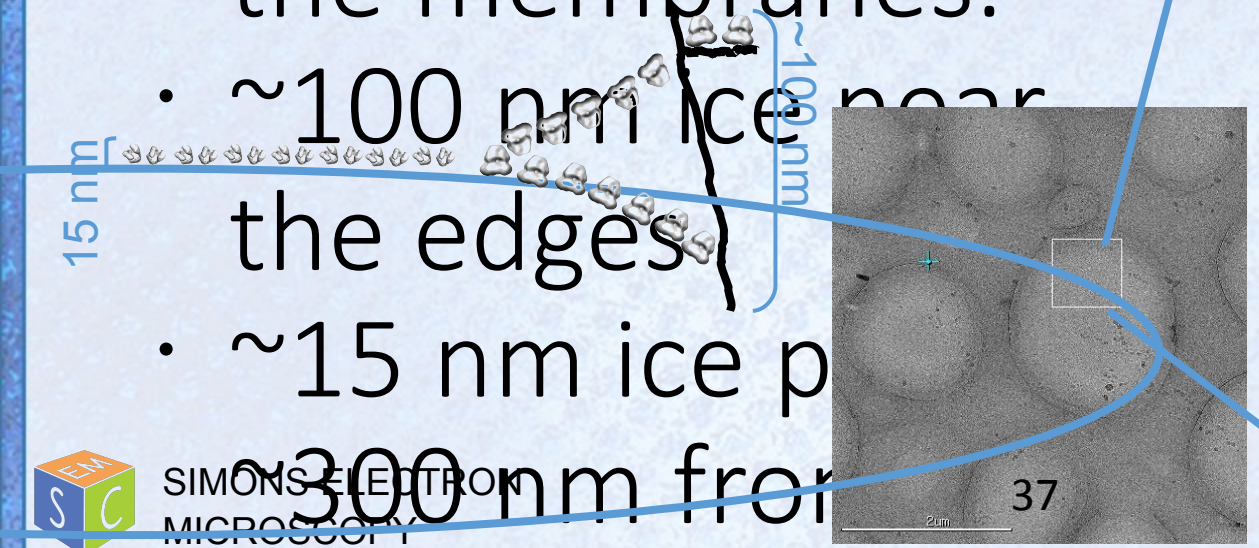



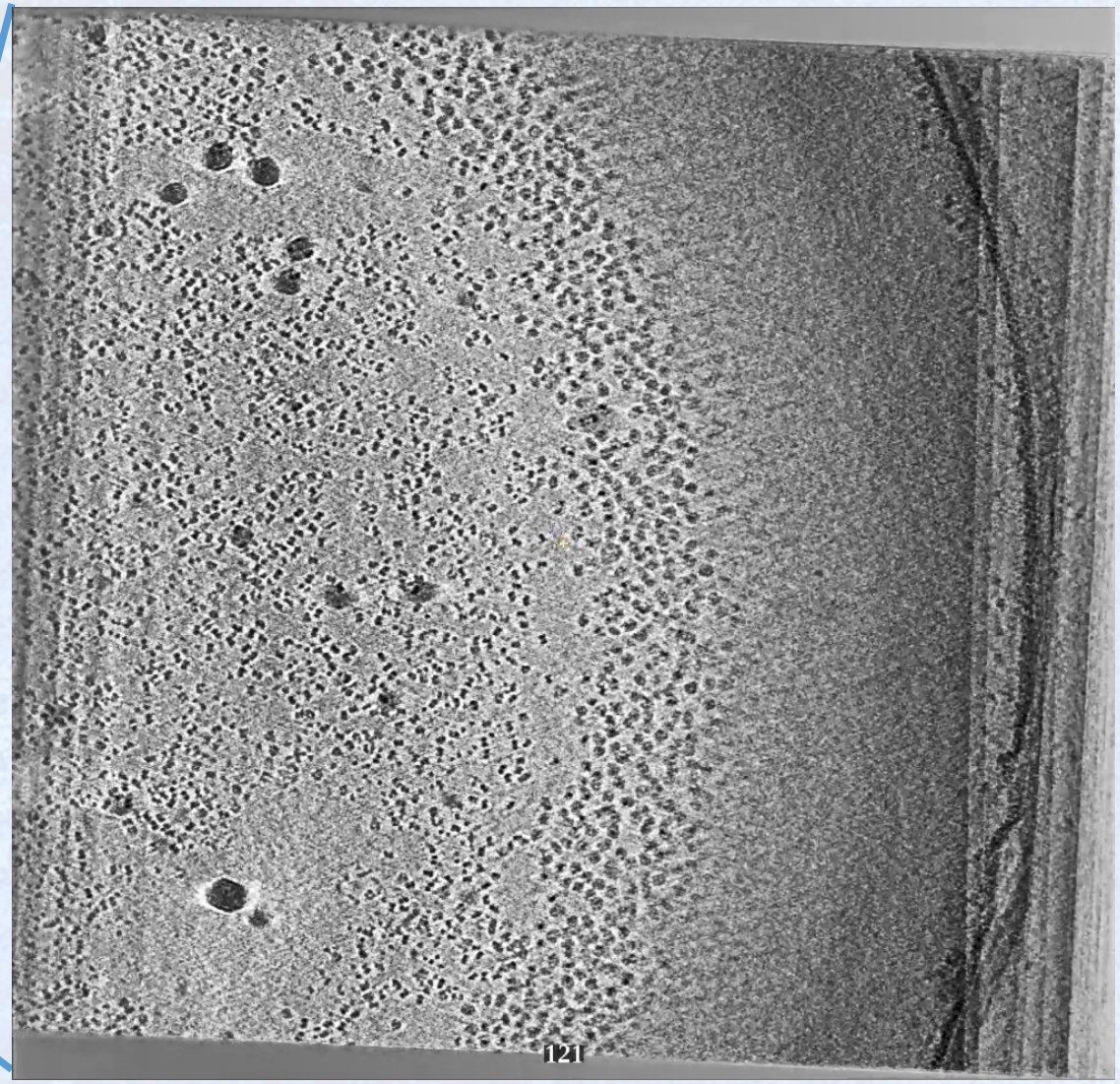

200 nm

Z-slices through tomogram/ice

# Apparent Membrane Denaturation May Occur in Thin Ice (Un-named Protein with Lipid Membrane)

- Thin ice with proteins with membranes may disassociate from the membranes.
- ~100 nm ice near the edges
- ~15 nm ice p
- ~300 nm fro




200 nm

Z-slices through tomogram/ice

Ok, now I'm scared of the air-water interface...

How do we avoid it?

# How Can We Avoid the Air-water Interface?

Standard ideas:

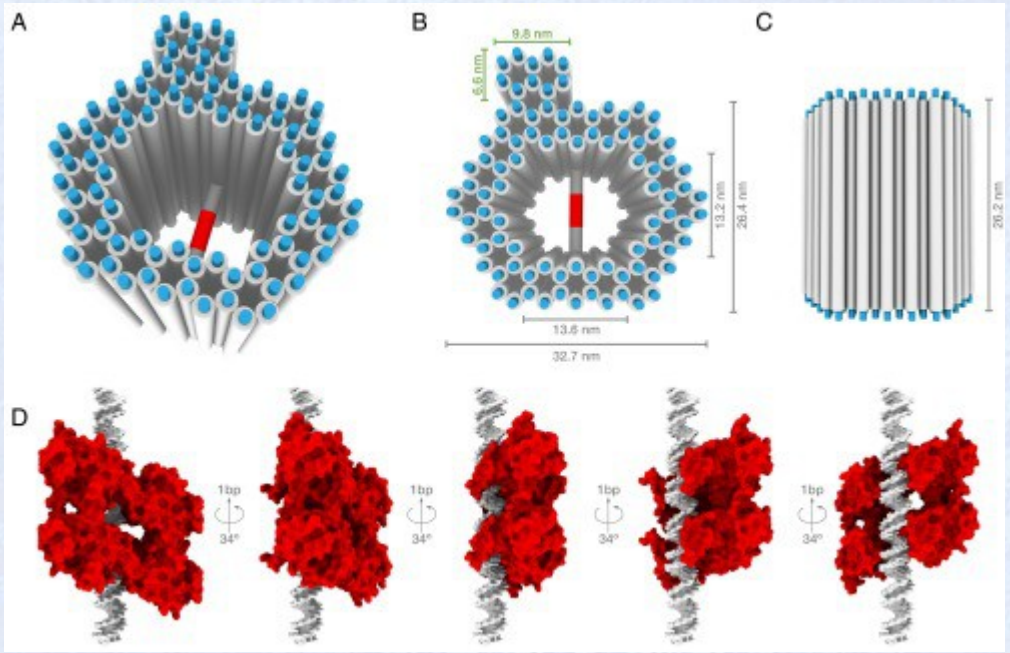
- Affinity grids
  - Carbon over holes
  - Streptavidin over holes
  - Ni-NTA grids
- Introduce surfactant to your protein solution

Some **non-standard ideas exist…**

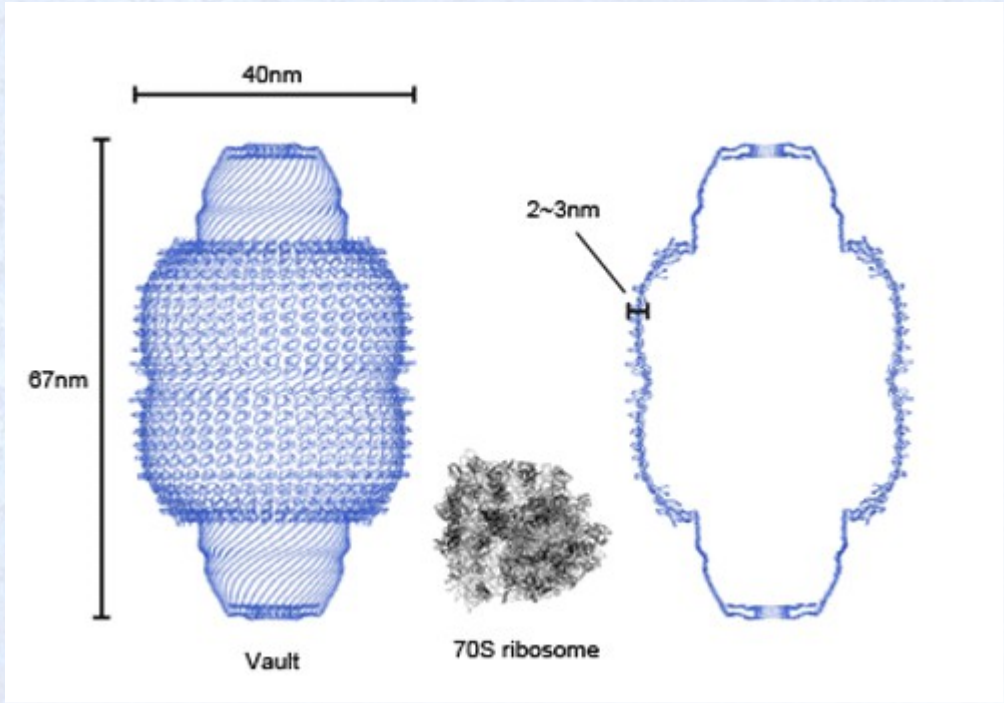
# How Can We Avoid the Air-water Interface?

A non-standard idea:

**Encapsulate particles individually** using a synthetic or protein capsule:



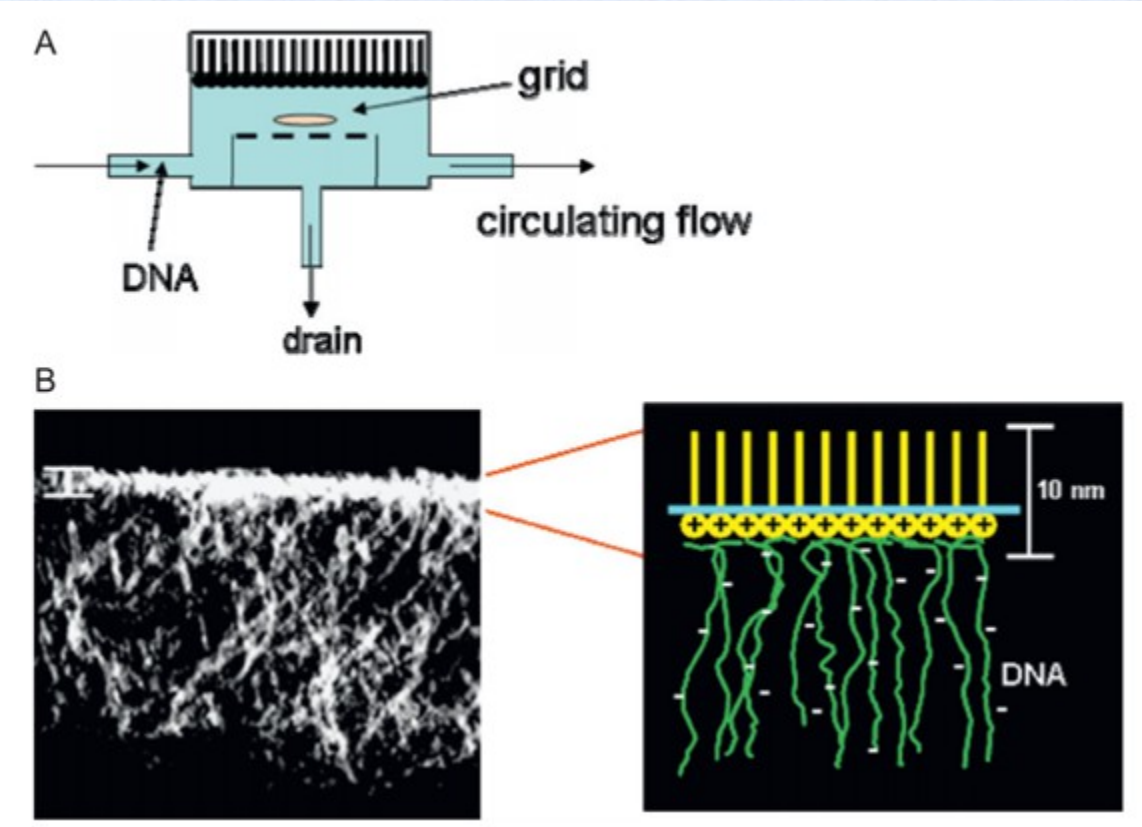

Martin et al., 2016



# How Can We Avoid the Air-water Interface?

A non-standard idea:

Apply a **lipid monolayer** to your grid and/or thin film of sample on the grid:




Vos et al., 2008

# How Can We Avoid the Air-water Interface?

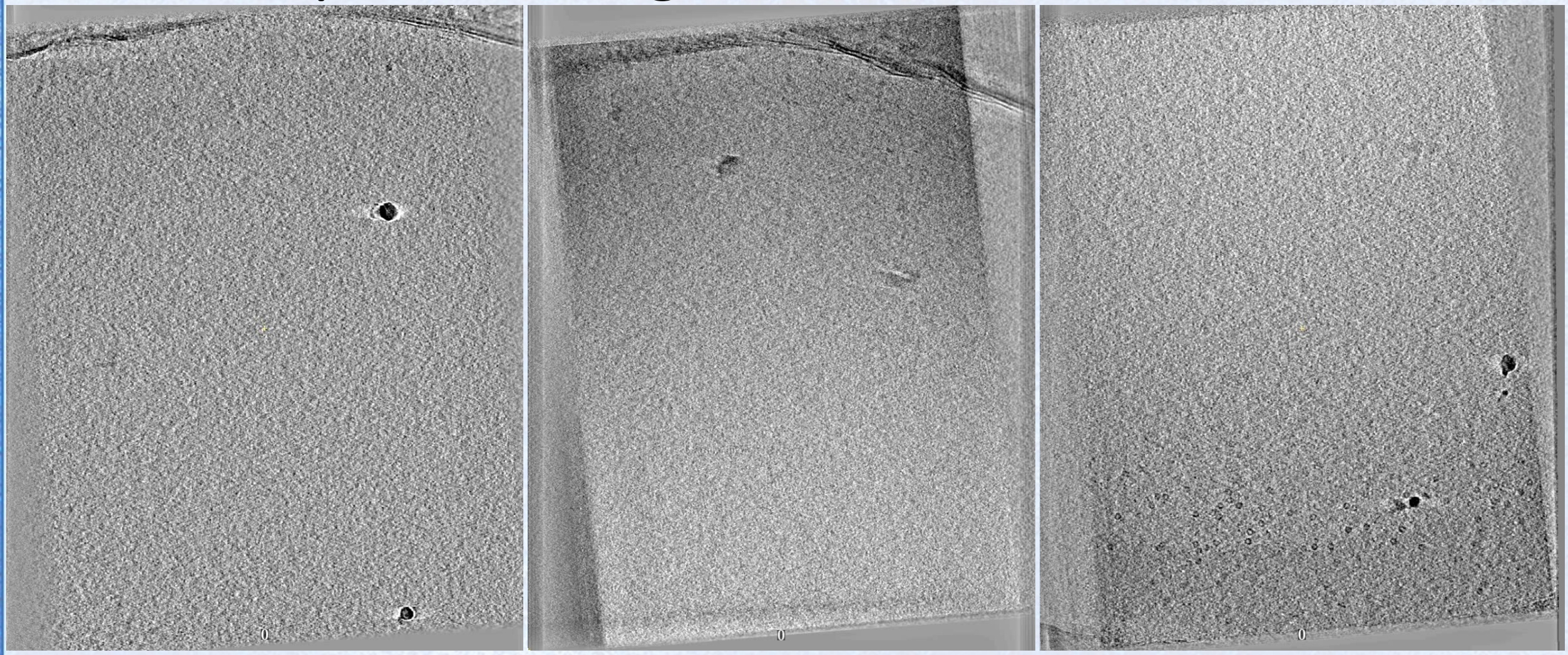
The best idea:

**Plunge faster** than the bulk and surface diffusion times (~10+ ms)

# Preliminary Spotiton Time Resolve Results

# Preliminary Spotiton Time Resolve Results

## 800 ms Spot-to-Plunge Time

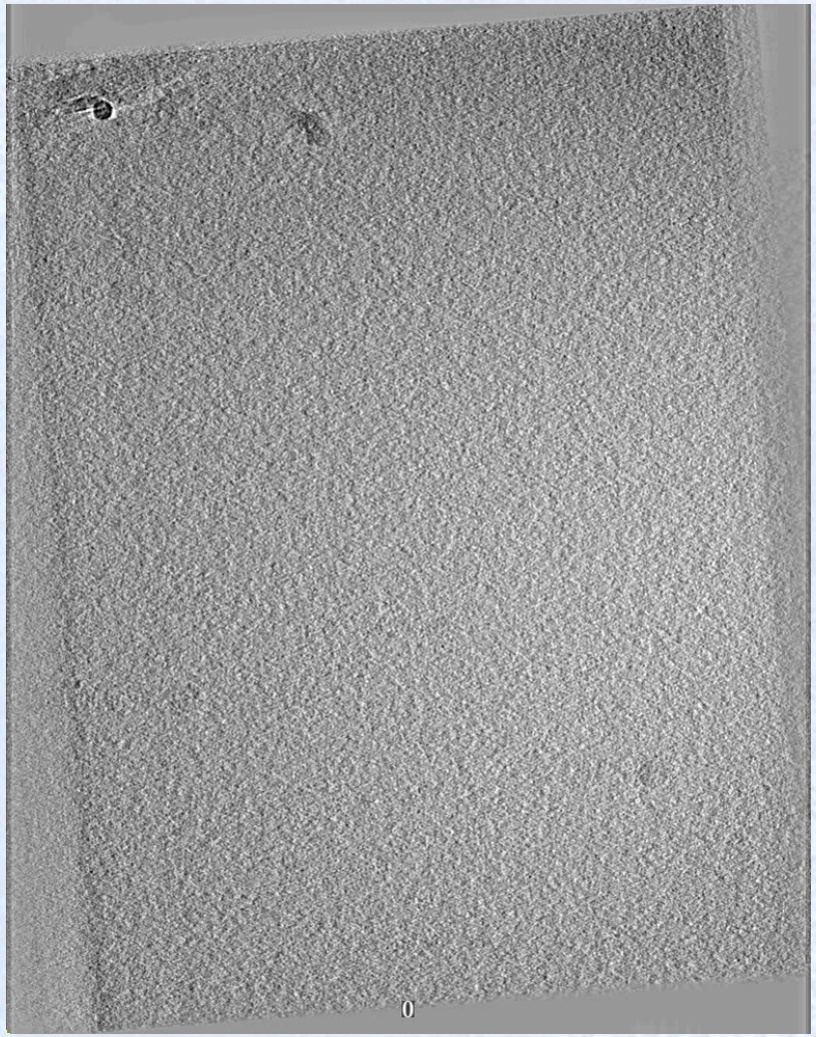


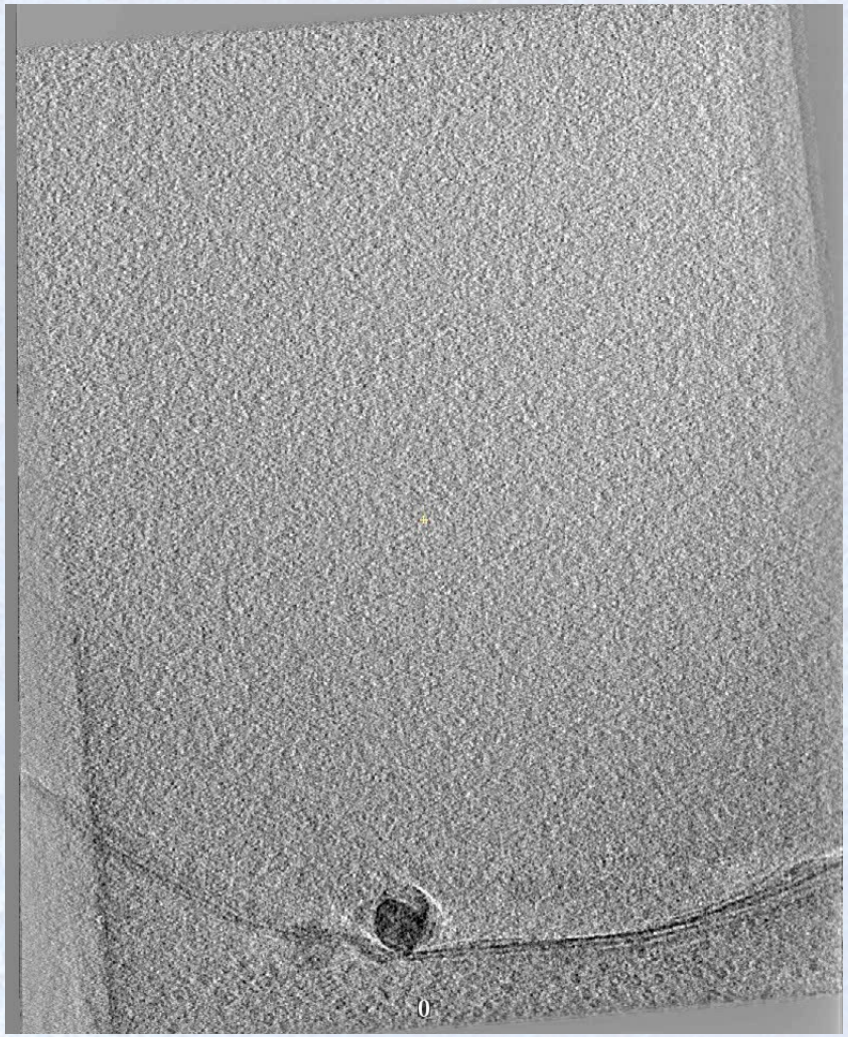
250 nm

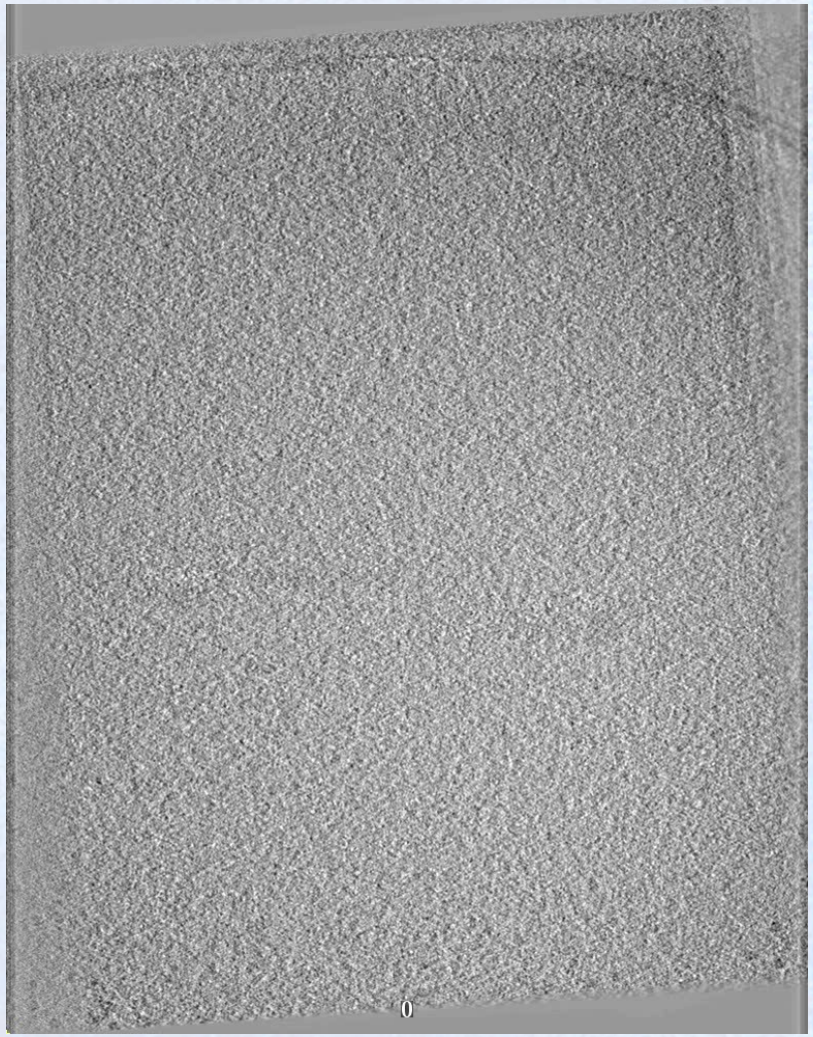
**Most particles are on the air-water interfaces**

# Preliminary Spotiton Time Resolve Results

## 170 ms Spot-to-Plunge Time







250 nm

**Particles are roughly evenly distributed in the ice in all directions!**

# Preliminary Spotiton Time Resolve Results

So it might be possible to **outrun the particle diffusion to the air-water interface!**

- These are preliminary results with a **low N value**.

# Summary

- The **ideal view** of single particles in one layer, thin ice, no preferred orientation, no air-water interface interaction is **rarely correct**.
- The **vast majority** of all particles are **adsorbed to the air-water interface**.
- Food science literature might lend some ideas as to **why**:
  - It might take **10+ ms** for proteins to diffuse to and at the air-water,
  - Denaturing might not be complete – **protein networks** can form.

# Acknowledgements


**Tomography**

Anchi Cheng

Hanspeter Winkler

**Spotiton Preparation**

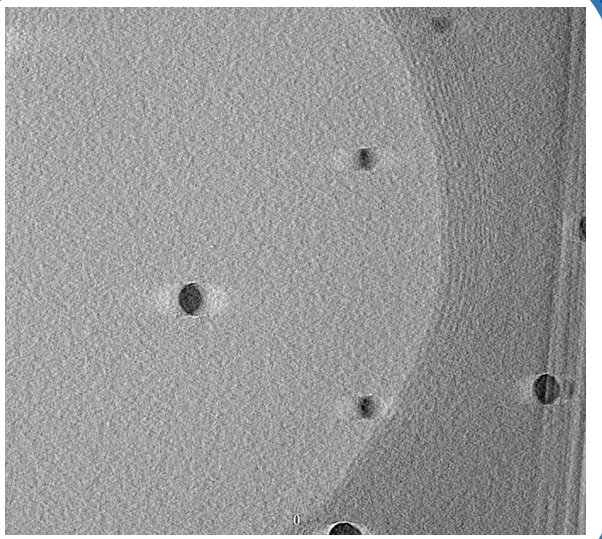
Venkat Dandey

Hui Wei

**Sample/Grid Preparation**




GDH



6mg/mL aldolase

Mtb Proteasome

32 kDa kinase

Thank you!
Questions?
Come see my poster for additional results!

SIMONS ELECTRON MICROSCOPY CENTER

Manuscript in preparation

| Sample # | Sample name | Grid Type | Ice thickness (center, edge, substrate) in nm ± a few nm | # of Layers (center, edge, substrate) | Apparent preferred orientation in layer? | Min. Particle/layer distance from air-water interface (nm ± a few nm) |
|---|---|---|---|---|---|---|
| 1*† | 32kDa kinase | Carbon Spotiton | (65, 45, --) | (0+, 0+, 0+) | Unknown | <5 |
| 2*† | 32kDa kinase | Gold Spotiton | (30, --, --) | (0+, --, --) | Unknown | <5 |
| 3*† | Insulin receptor (150 ms) | Carbon Spotiton | (--, 140, --) | (--, 1, --) | Some | 5 |
| 4*† | Insulin receptor | Gold Spotiton | (55, --, --) | (1-2, --, --) | No | 5 |
| 5*† | Hemagglutinin (800 ms) | Carbon Spotiton | (25-95, 100-210, --) | (0 or 2, 2, --) | Some | 5 |
| 6*† | Hemagglutinin (170 ms) | Carbon Spotiton | (60, 125, --) | (2, 2, --) | Some | 5 |
| 7*† | Hemagglutinin (150 ms) | Carbon Spotiton | (110-135, 95-110, --) | (2, 2, 2) | Some | 5 |
| 8* | HIV-1 Trimer Complex 1 | Carbon Spotiton | (75-210, --, --)** | (2+, --, --) | Yes | 5-10 |
| 9* | HIV-1 Trimer Complex 1 | Gold Spotiton | (20, --, --) | (1, --, --) | Some | 5 |
| 10* | HIV-1 Trimer Complex 2 | Carbon Spotiton | (50, 50, --) | (1, 1, 1) | Some | 5 |
| 11* | 147 kDa kinase | Gold Spotiton | (15, --, --) | (1, --, --) | Unknown | <5 |
| 12 | 150 kDa protein | Holey Carbon Spotiton | (35, 70, --) | (1, 2, --) | Some | <5 |
| 13 | Stick-like protein 1 | Carbon Spotiton | (80, --, --)** | (1, --, --) | No | <5 |
| 14 | Stick-like protein 2 (150 kDa) | Carbon CFlat | (100, 100, --)** | (1, 1, --) | Unknown | 5 |
| 15 | Stick-like protein 2 | Gold Spotiton | (135-180, --, --)** | (1, --, --) | Some | 5 |
| 16* | Clustered protocadherin | Carbon Spotiton | (60-90, --, --)** | (1, --, --) | Yes | 5 |
| 17* | Clustered protocadherin | Carbon Spotiton | (80-90, 100-140, 135)** | (1, 1, 1) | Yes | 5 |
| 18 | 200kDa protein | CFlat Carbon + Gold mesh | (40-60, 95, 110) | (1, 1, 2) | No | 5 |
| 19 | Small, popular protein | Carbon Spotiton | (30, 70, --) | (1, 2, 2) | No | 5 |
| 20* | Protein with bound lipids (deglycosylated) | Carbon Spotiton | (15, 90, 130) | (1, 2, 2) | Yes | <5 |
| 21 | Protein with bound lipids (glycosylated) | Gold Spotiton | (155, --, --)** | (2, --, --) | No | <5 |
| 22* | Lipo-protein | Holey Carbon | (0-95, 85-100, --) | Uniformly distributed in ice | Unknown | 5 |
| 23* | GPCR | Carbon Spotiton | (25, --, --) | (1, 2, --) | Some | 5 |
| 24* | Rabbit aldolase | Gold Spotiton | (15, 50, --) | (1, 2, --) | No | <5 |
| 25*† | Rabbit aldolase 6mg/ml | Carbon Spotiton | (60-110, 75-130, 85) | (2+, 2+, 2) | Some | 5 |
| 26 | Un-named protein | Holey Carbon | (35, --, 60) | (1, --, 2) | Yes | 5 |
| 27 | Un-named protein | Carbon Spotiton | (35 , 110, --) | (1, 2, --) | Yes | 5 |
| 28*† | DnaB Helicase | Gold Quantifoil | (50-55, 80-100, --) | (1+, 2, --) | No | 5 |
| 29* | Protein in nanodisc (0.58 mg/mL) | Gold Spotiton | (30, 65, --) | (1-2, 2, --) | No | 5-10 |
| 30*† | IDE | Carbon Spotiton | (25, 60, 95) | (1, 2+, 2) | Unknown | 5 |
| 31*† | IDE | Gold Spotiton | (50, --, --) | (1, --, --) | No | 5-10 |
| 32 | Small, helical protein | Gold Spotiton | (50, 75, --) | (1, 2, --) | Some | 5 |
| 33 | 300 kDa protein | Carbon Spotiton | (30, 100, --) | (1, 2, 2) | No | 5 |
| 34*† | GDH | Holey Carbon | (30, 85, 100) | (1, 1+, 3+) | Some | 5 |
| 35*† | GDH | Holey Carbon | (60, 120, 140) | (1, 2+, 3+) | Some | 5 |
| 36*† | GDH + 0.001% DDM (2.5 mg/mL) | Carbon Spotiton | (50, 125, 190) | (1-1+, 2+, --) | Some | <5 |
| 37*† | Apoferritin | Gold Spotiton | (25, --, --) | (1, --, --) | No | 5 |
| 38*† | Apoferritin | Gold Spotiton | (25, --, --) | (1, --, --) | No | 5 |
| 39*† | Apoferritin (1.25mg/mL) | Holey Carbon Spotiton | (30-50, 100, 105) | (1, 2+, 2) | No | 5 |
| 40*† | Apoferritin (0.5mg/ml) | Holey Gold Spotiton | (25-30, 55, --) | (1, 2, --) | No | <5 |
| 41*† | Apoferritin with 0.5 mM TCEP (800 ms) | Carbon Spotiton | (40-90, 145-175, --) | (1-2, 2+, 1) | No | 5 |
| 42*† | Apoferritin with 0.5 mM TCEP (170 ms) | Carbon Spotiton | (95, 120-135, --) | (2+, 2+, 1) | No | 5 |
| 43*† | Apoferritin | Holey Carbon Spotiton | (30, 125, 135) | (1, 2, 2+) | No | 5 |
| 44 | Protein with carbon over holes | Carbon Quantifoil | (110, 70-100, --) | (1+, 1+, --) | Some | 5-10 |
| 45 | Protein and DNA strands with carbon over holes | Carbon Quantifoil | (60, --, --) | (1+, --, --) | Some | 5-10 |
| 46 | Protein on streptavidin | Holey Carbon | (20-100, 80-120, --) | (0-2, 1-2, --) | No | 10 |
| 47*† | T20S Proteasome | Holey Carbon | (35, 115, 120) | (1, 2+, 3+) | Some | <5 |
| 48*† | T20S Proteasome | Holey Carbon | (125, 140-160, 150) | (2+, 2+, 2+) | Some | 5 |
| 49*† | T20S Proteasome | Gold Quantifoil | (50-75, --, --) | (1+, --, --) | Some | 5 |
| 50*† | Mtb Proteasome | Carbon Spotiton | (35, 80, 115) | (0, 1+, 1) | No | 5-10 |

| Sample # | Sample name | Air-water interface, particle behavior, and layer/ice angle (bottom, center) | Air-water interface, particle behavior, and layer/ice angle (bottom, edge) | Ice behavior (bottom) | Air-water interface, particle behavior, and layer/ice angle (top, center) | Air-water interface, particle behavior, and layer/ice angle (top, edge) | Ice behavior (top) | Notes |
|---|---|---|---|---|---|---|---|---|
| 1 | 32kDa kinase | A, B1 or B2 or B3 (50%), 8° | A, B1 or B2 or B3 (50%), 10° | C2 | A, B1 or B2 or B3‡ (50%), 8° | A, B1 or B2 or B3‡ (50%), 10° | C2 | Particles aggregate into clouds. |
| 2 | 32kDa kinase | A, A, B2 or B3 (50%), 4-8° | -- | C1 or C2 | A, A, B2 or B3‡ (50%), 4-8° | -- | C1 or C2 | Gold beads are glow discharge contamination. |
| 3 | Insulin receptor (150 ms) | -- | A, B2 or B3 (50%), 15° | C2 or C3 | -- | A, No particles, 9° | C2 or C3 | |
| 4 | Insulin receptor | A, B1 or B2 or B3 (100%), 3-5° | -- | C2 or C3 | A, B1 or B2 or B3‡ (100%), 3-5° | -- | C2 or C3 | Gold beads are glow discharge contamination. |
| 5 | Hemagglutinin (800 ms) | A2, No particles, 3-7° | A, B3 (40%), 5° or A, B3 (40%), 3° | C3 | A2‡, No particles, 3-7° or A, B3 (50%), 7° | A, B3 (50%), 5-7° | C3 | Where very thin ice in the center of holes excludes particles, protein fragments remain. |
| 6 | Hemagglutinin (170 ms) | A, B3 (30%), 3° | A, B3 (30%), 6-8° | C3 | A, B3 (30%), 3° | A, B3 (50%), 3° | C3 | |
| 7 | Hemagglutinin (150 ms) | A, B2 or B3 (30%), 6-7° | A, B2 or B3 (30%), 6-7° | C1 or C2 | A, B2 or B3 (80%), 6-7° | A, B2 or B3 (30%), 6-7° | C1 or C2 | |
| 8 | HIV-1 Trimer Complex 1 | A2, B1, B3 (30%), 1-5° | -- | C1, C2, or C3 | A2, B1, B3 (30%), 1-5° | -- | C1, C2, or C3 | Trimer domains and/or unbound receptors are adsorbed to air-water interfaces. |
| 9 | HIV-1 Trimer Complex 1 | A2, B3 (80%), 6° | -- | C2 | A2, B3‡ (80%), 6° | -- | C2 | Trimer domains and/or unbound receptors are adsorbed to air-water interfaces. |
| 10 | HIV-1 Trimer Complex 2 | A, B2 or B3 (70%), 3° | A, B2 or B3 (70%), 3° | C2 | A, B2 or B3‡ (70%), 3° | A, B2 or B3‡ (70%), 3° | C2 | |
| 11 | 147 kDa kinase | A, B2 or B3 (50%), 0° | -- | C2 or C3 | A, B2 or B3‡ (50%), 0° | -- | C2 or C3 | Gold beads are glow discharge contamination. |
| 12 | 150 kDa protein | A, B2 or B3 (60%), 7-10° | A, B2 or B3 (60%), 8° | C2 or C3 | A, B2 or B3‡ (60%), 7° | A, B2 or B3 (40%), 9° | C2 or C3 | |
| 13 | Stick-like protein 1 | A and A2, B4 and B5 (1%), 10° | -- | C2 | A2, B4 and B5 (50%), 10° | -- | C2 | |
| 14 | Stick-like protein 2 (150 kDa) | A2, B3 and B4 and B5 (70%), 7° | A2, B3 and B4 and B5 (70%), 7° | -- | A2, B3 and B4 and B5‡ (70%), 7° | A2, B3 and B4 and B5‡ (70%), 7° | -- | Determinations are not accurate due to over focusing and minimal tilt angles. |
| 15 | Stick-like protein 2 | A2, B3 (80%), 0° | -- | C2 or C3 | A2, B3 (0%), 0° | -- | C2 or C3 | Note 1. Note 2. |
| 16 | Clustered protocadherin | A2, B3 (80%), 3-10° | -- | C2 or C3 | A2, No particles, 3-10° | -- | C2 or C3 | Note 1. Note 2. |
| 17 | Clustered protocadherin | -- | A2, No particles, 2-7°or A2, B3 (70%), 5° | C3 | -- | A2, B3 (70%), 7° or A2, No particles, 7° | C3 | Note 1. Note 2. Two tomograms have one orientation, one has the opposite. |
| 18 | 200kDa protein | A, B2 or B3 (60%), 2° | A, B2 or B3 (50%), 4° | C3 | No particles or A, B2 or B3‡ (60%), 2° | A, No particles, 11° | C3 | |
| 19 | Small, popular protein | A, B2 or B3 (90%), 6° | A, B2 or B3 (90%), 9° | C2 | A, B2 or B3‡ (90%), 6° | A, B2 or B3 (90%), 1° | C3 | |
| 20 | Protein with bound lipids (deglycosylated) | A, B3 (70%), 4° | A, B3 (80%), 10° | C3 | A, B3‡ (70%), 4° | A, B3 (80%), 11° | C3 | Lipid membrane dissociates from protein in center. |
| 21 | Protein with bound lipids (glycosylated) | A, B3 (50%), 10° | -- | C2 or C3 | A, B3 (60%), 4° | -- | C2 or C3 | |
| 22 | Lipo-protein | No particles or A, B2, 3° | A, B3, 11° | C3, C4 | No particles or A, B2‡, 5° | A, B3, 11° | C3, C4 | Particles are uniformly distributed in the ice. |
| 23 | GPCR | A, B2 or B3 (70%), 3° | A, B2 or B3 (60%), -- | C3 | A, B2 or B3‡ (70%), 3° | A, B2 or B3 (60%), -- | C3 | |
| 24 | Rabbit aldolase | A, B2 or B3 (90%), 3-9° | A, B2 or B3 (80%), 6° | C3 | A, B2 or B3‡ (90%), 3-9° | A, B2 or B3 (80%), 10° | C3 | |
| 25 | Rabbit aldolase 6mg/ml | A, B1, B2 or B3 (90%), 5° | A, B1, B2 or B3 (90%), 5° | C2 or C3 | A, B1, B2 or B3 (90%), 5° | A, B1, B2 or B3 (90%), 5° | C2 or C3 | |
| 26 | Un-named protein | A, B3 (40%), 0-3° | -- | C2 or C3 | A, B3‡ (40%), 0-3° | -- | C2 or C3 | |
| 27 | Un-named protein | A, B3 (80%), 2° | A, B3 (60%), 4-6° | C3 | A, B3‡ (80%), 2° | A, B3 (60%), 4-9° | C3 | |
| 28*† | DnaB Helicase | A, B2 or B3 (90%), 1° | A, B2 or B3 (90%), 4° | C3 | A, B2 or B3 (<5%), 1° | A, B2 or B3 (<5%), 1° | C2 | Gold flakes from Quantifoil are on the top. |
| 29* | Protein in nanodisc | A, B2 (80%), 8-10° | A, B2 (80%), 8-10° | C2 or C3 | A, B2‡ (80%), 8-10° | A, B2‡ (80%), 8-10° | C2 or C3 | |
| 30*† | IDE | A2, B2 or B3 and B4 and B5 (50%), 0° | A2, B1, B2 or B3 and B4 and B5 (50%), 5° | C3 | A2, B2 or B3 and B4 and B5‡ (50%), 0° | A2, B1, B2 or B3 and B4 and B5 (50%), 2° | C3 | Note 1. |
| 31*† | IDE | A, B2 or B3 (95%), 0-4° | -- | C2 | A, B2 or B3 (95%), 0-4° | -- | C2 | |
| 32 | Small, helical protein | A, B2 or B3 (80%), 5° | A, B2 or B3 (70%), 3° | C3 | A, B2 or B3‡ (80%), 5° | A, B2 or B3 (70%), 7° | C3 | |
| 33 | 300 kDa protein | A or A2, B2 or B3 (70%), 7° | A or A2, B2 or B3 (50%), 13° | C3 | A or A2, B2 or B3‡ (70%), 7° | A or A2, B2 or B3 (50%), 9° | C3 | |
| 34*† | GDH | A, B3 (70%), 10° | A, B1, B3 (50%), 1° | C2 | A, B3‡ (70%), 10° | A, B1, B3 (50%), 16° | C3 | Note 2. Some free-floating particles stack between layers. |
| 35*† | GDH | A, B3 (40%), -- | A, B1, B3 (40%), 10° | C3 | A, B3‡ (40%), -- | A, B1, B3 (40%), 2° | C2 | |
| 36*† | GDH + 0.001% DDM | A, B3 (40%), 4° | A, B1, B3 (40%), 7° | C2 | A, B3‡ (30%), 4° | A, B1, B3 (30%), 6° | C3 | Some free-floating particles stack between layers. |
| 37*† | Apoferritin | A2, B2 or B3 (50%), 4-6° | -- | C2 or C3 | A2, B2 or B3‡ (50%), 4-6° | -- | C2 or C3 | Note 1. Note 2. |
| 38*† | Apoferritin | A2, B2 or B3 (60%), 4-12° | -- | C2 or C3 | A2, B2 or B3‡ (60%), 4-12° | -- | C2 or C3 | Note 1. Note 2. |
| 39*† | Apoferritin 1.25mg/ml | A2, B2 or B3 (40%), 4° | A2, B1, B2 or B3 (40%), 6° | C3 | A2, B2 or B3‡ (40%), 4° | A2, B1, B2 or B3 (30%), 4° | C3 | Note 1. Note 2. |
| 40*† | Apoferritin 0.5mg/ml | A2, B2 or B3 (20%), 5° | -- | C2 or C3 | A2, B2 or B3‡ (20%), 1° | -- | C2 or C3 | Note 1. Note 2. |
| 41*† | Apoferritin (800 ms) | A2, B2 or B3 (40%), -- or A2, B2 or B3 (50%), 3° | A2, B1, B2 or B3 (40%), 5-9° | C3 | A2, B2 or B3 (40%), -- or A2, B2 or B3‡ (50%), 3° | A2, B1, B2 or B3 (40%), 2-8° | C3 | Note 1. Note 2. About 5-10% of all particles are free-floating. |
| 42*† | Apoferritin (170 ms) | A2, B1, B2 or B3 (40%), 5° | A2, B1, B2 or B3 (40%), 0-5° | C3 | A2, B1, B2 or B3 (40%), 0° | A2, B1, B2 or B3 (40%), 5° | C3 | Note 1. Note 2. About 1/3rd of all particles are free-floating. |
| 43*† | Apoferritin | A2, B3 (70%), 5° | A2, B1, B3 (50%), 10° | C3 | A2, B3‡ (70%), 5° | A2, B1, B3 (60%), 3° | C3 | Note 1. Note 2. |
| 44 | Protein with carbon over holes | Carbon, B1 (30%), B3 (60%), 5° | Carbon, B1 (30%), B3 (60%), 5-9° | C2 | A, B3 (5%), 5° | A, B3 (5%), 5° | C1 or C2 | Note 3. |
| 45 | Protein and DNA strands with carbon over holes | A, No particles, 2-3° | -- | C2 or C3 | Carbon, B1 (20%), B3 (60%), 2-3° | -- | C2 | Some free-floating particles make contact with particle layer. Most free-floating particles are attached to DNA strands. |
| 46 | Protein on streptavidin | Streptavidin, B2 (10-30%), 0° or Streptavidin, No particles, 12° | Streptavidin or A2, 2 (10-30%), 12° | C1, C2, or C3 | Streptavidin, B2 (10-30%), 0° or Streptavidin‡, No particles, 12° | Streptavidin, 2 (10-30%), 13-14° | C1, C2, or C3 | Note 1. Some holes have a layer of streptavidin only on top, some have a layer on top and bottom. Particles are attached to streptavidin and sometimes the apposed air-water interface. |
| 47*† | T20S Proteasome | A, B3 (80%), 3° | A, B1 (5%), B3 (80%), 14° | C3 | A, B3‡ (80%), 3° | A, B1 (5%), B3 (20%), 3° | C2 | Note 2. Note 3. |
| 48*† | T20S Proteasome | A, B3 (10%), 2-5° | A, B3 (10%), 2-5° | C2 | A, B1 (20%), B3 (90%), 5-7° | A, B1 (20%), B3 (95%), 5-7° | C3 | Note 3. |
| 49*† | T20S Proteasome | A, B1 (10%), B3 (80%), 11° | -- | C3 | A, B3 (2%), 11° | -- | C2 | Note 2. Note 3. |
| 50*† | Mtb Proteasome | -- | A, B1, B2 or B3 (30%), 6° | C3 | -- | A, B1, B2 or B3 (30%), 11° | C3 | |