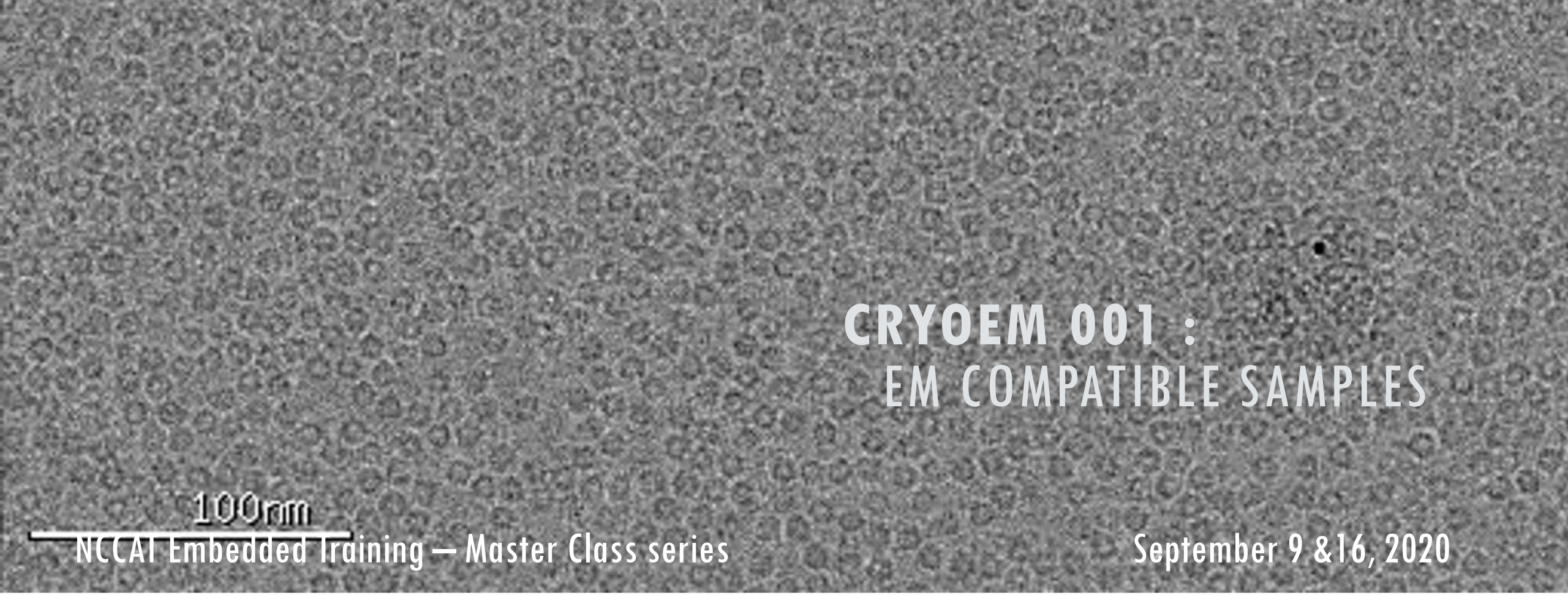


CRYOEM 001 :
EM COMPATIBLE SAMPLES

NCCAT Embedded Training – Master Class series

September 9 &16, 2020

NATIONAL CENTER FOR
CRYOEM ACCESS & TRAINING

New York Structural
Biology Center

SIMONS ELECTRON
MICROSCOPY CENTER

CRYOEM 001 : SINGLE PARTICLE MASTERCLASS

Introduction to cryoEM: SPA

Building a cryoEM toolkit

EM compatible samples

EM support films and grids

Sample preparation

Tools of the trade:

- microscopes and detectors

Microscope operations

Data collection strategies

Data assessment & QC

Data processing:

- cryoEM IT infrastructure
- On-the-fly feedback
- 3D Reconstruction

Visualization and validation

- Transmission Electron Microscopy: A Textbook for Materials Science

By David B. Williams, C. Barry Carter

THE QUALITY OF YOUR DATA IS AT LEAST DIRECTLY PROPORTIONAL TO THE QUALITY OF YOUR SPECIMEN.

TECHNIQUES TO FRAME THE START

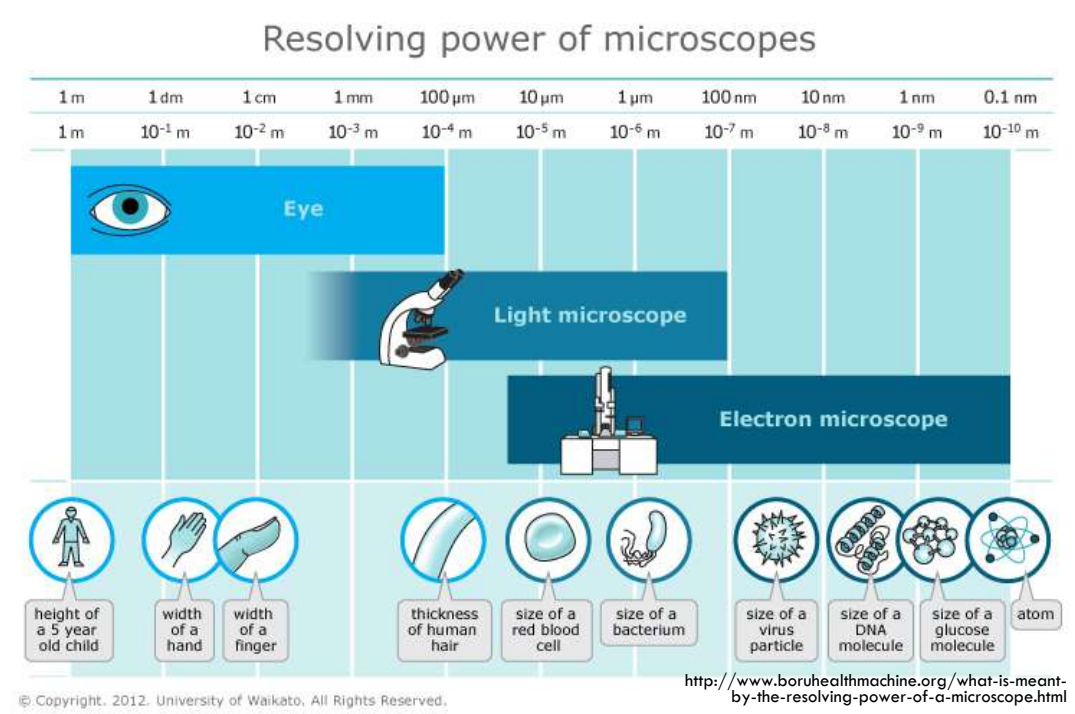
Cells

Organelles

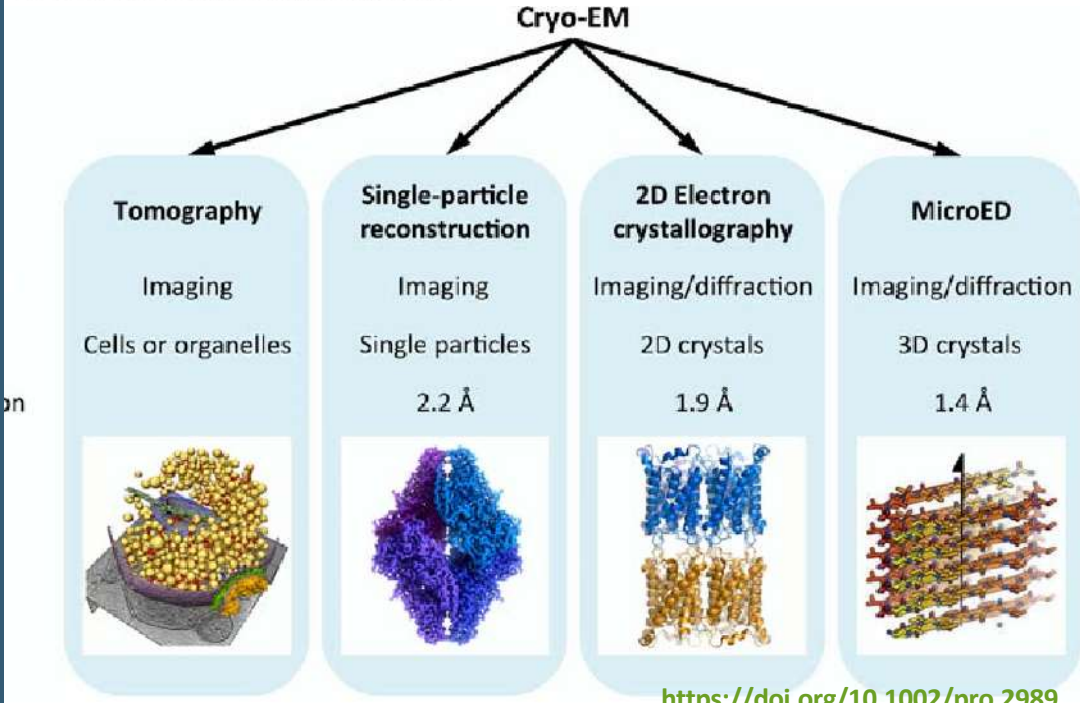
3D Crystals

2D Crystals

Individual Macromolecules

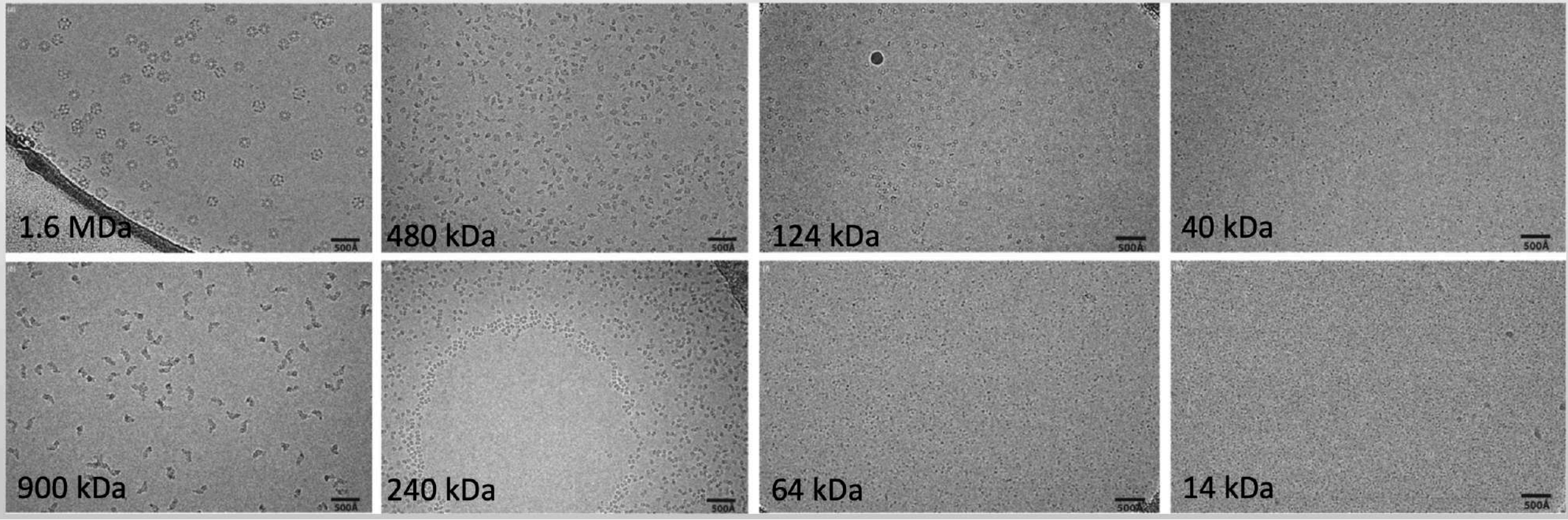


http://www.boruhealthmachine.org/what-is-meant-by-the-resolving-power-of-a-microscope.html



https://doi.org/10.1002/pro.2989

DOES SIZE MATTER?



Kutti R. Vinothkumar *et al*., 2016

CONSIDERATIONS FOR SAMPLES THAT WILL BE ANALYZED BY CRYOEM

- specimens must be thin
- vacuum in the microscope
- radiation damage
- low signal:noise
- charging

SAMPLES SUITABLE FOR EM

A major limiting factor for structure determination is specimen preparation.

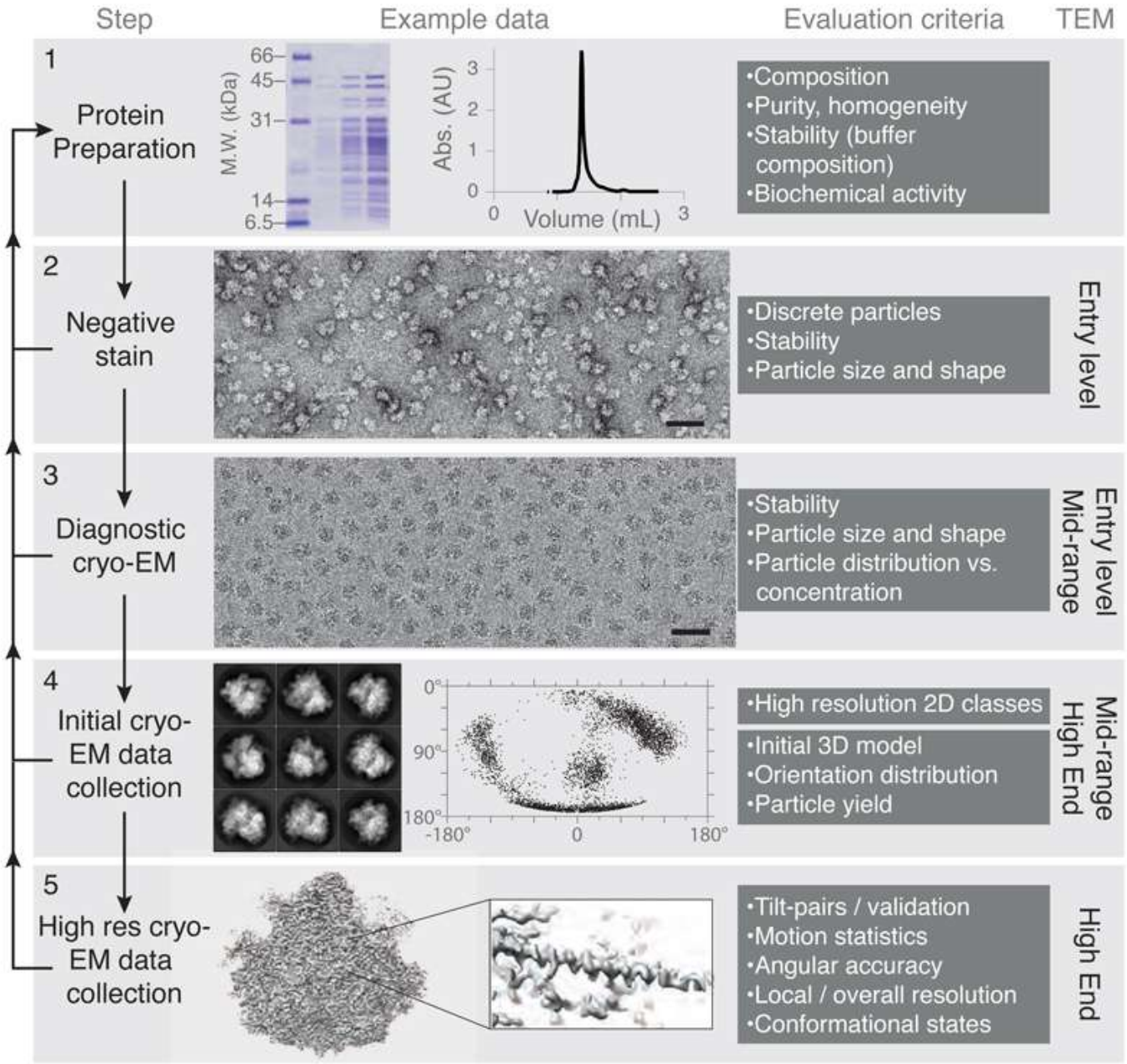
The origin of this limitation is two-fold in nature:

1. During the creation of a thin layer of water for vitrification and imaging, specimens are exposed to surfaces and conditions which are very different from the inside of a test tube or cell. The effects of these on the molecules and complexes are not known *a-priori*, and can be difficult to remedy if destructive to the specimen.
2. Specimen preparation for cryo-EM is a delicate process that still requires skilled handling and careful technique through a number of detailed preparation steps. This often confounds novice and experienced microscopists alike by making it difficult to distinguish problems with the specimen from problems in technique and methods.

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5140023/

THE OPTIMIZATION WORKFLOW

Structure determination by cryo-EM.
A systematic approach to 3D structure determination is shown. In the left column, the major steps are listed. Each step should be performed successively and only after one has been completed successfully should the scientist move onto the next step. In the second column, example data are shown for ribosomes (details in text). Scale bars on the micrographs are 500 Å. Each step should be evaluated with the criteria listed in the third column, returning to earlier steps for troubleshooting.

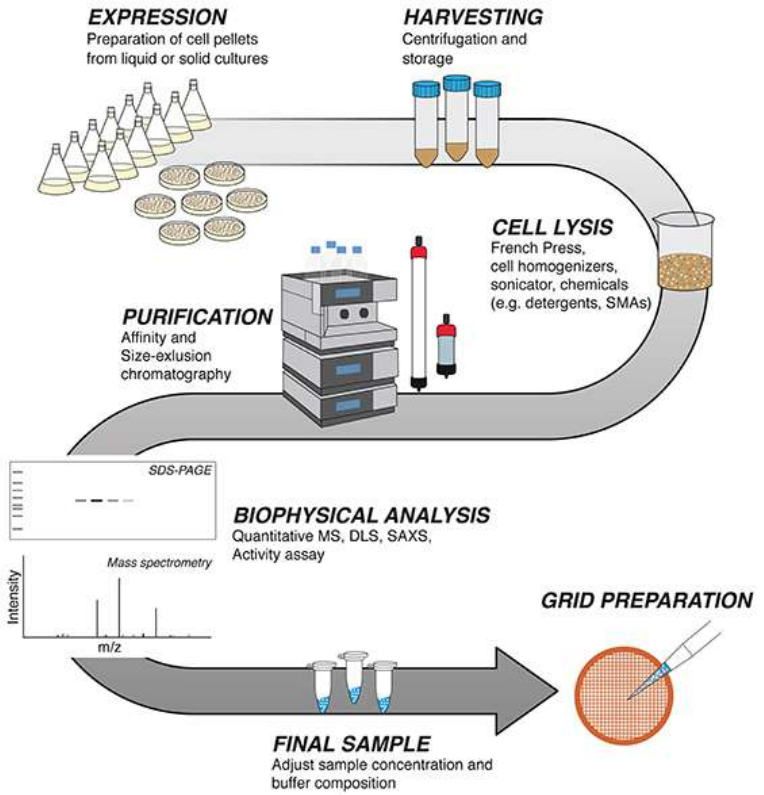


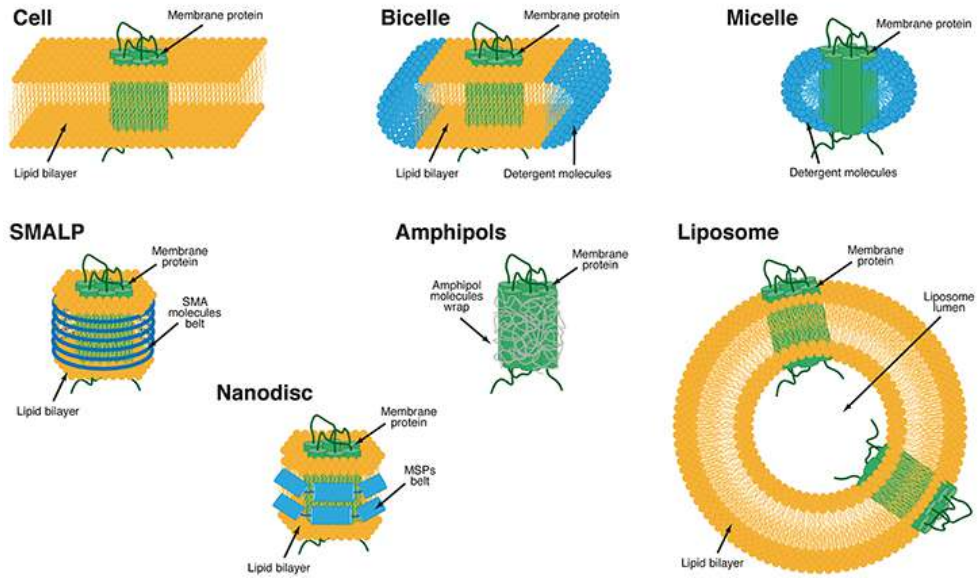
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5140023/

CRYOEM IS STILL STRUCTURAL BIOLOGY

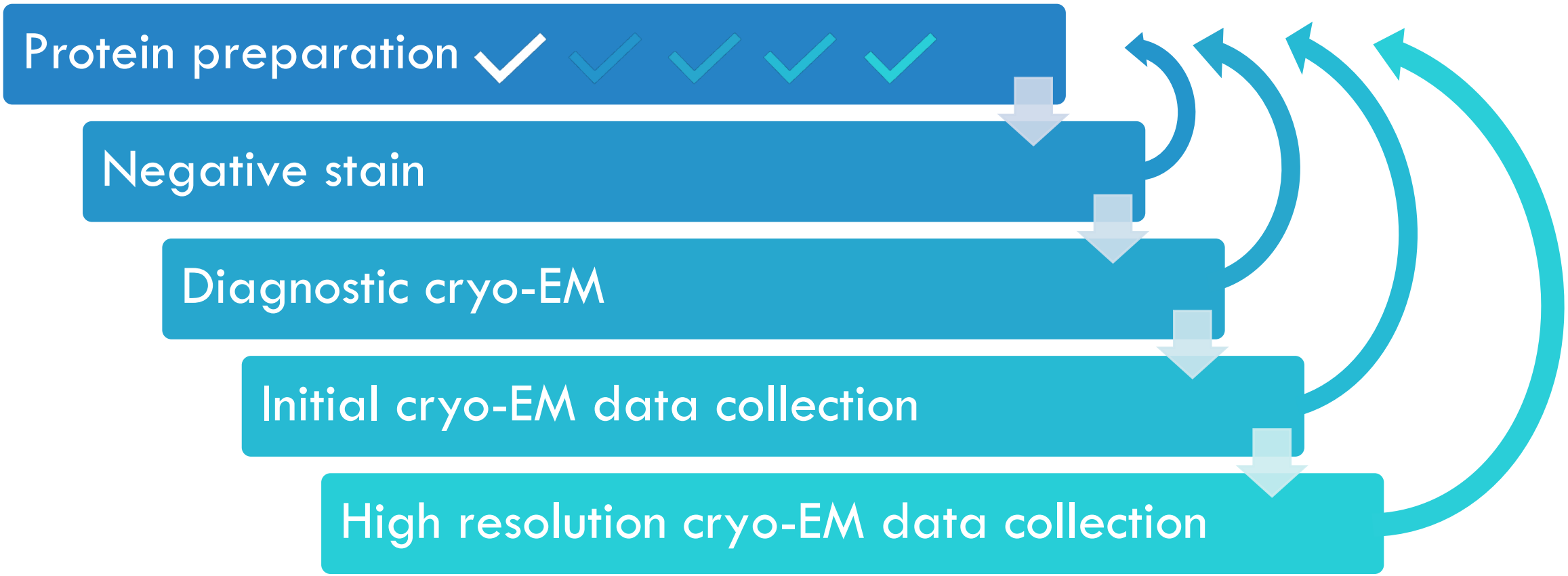
- The first step in a systematic approach is to evaluate several biochemical and biophysical aspects of the protein sample, such as composition, purity, homogeneity, stability, and biochemical activity.
- Contaminating proteins or degradation products may interfere with complex stability and subsequent computational analysis of the particle images, wasting resources on the more time-consuming and expensive cryo-EM data collection and image processing steps. Evaluation of sample homogeneity by negative staining electron microscopy before cryo-EM grid preparation will help not only to validate that the correct purification protocol has been followed, but will also ensure that no contaminants or degradation products are present in the protein sample.
- Prior knowledge of the protein molecular weight and oligomeric state(s), and buffer composition (salt concentration, pH, co-factors, cryo-protectants and other additives) in which the protein is stable can remarkably facilitate cryo-EM grid preparation.

https://www.frontiersin.org/articles/10.3389/fmolb.2018.00074/full

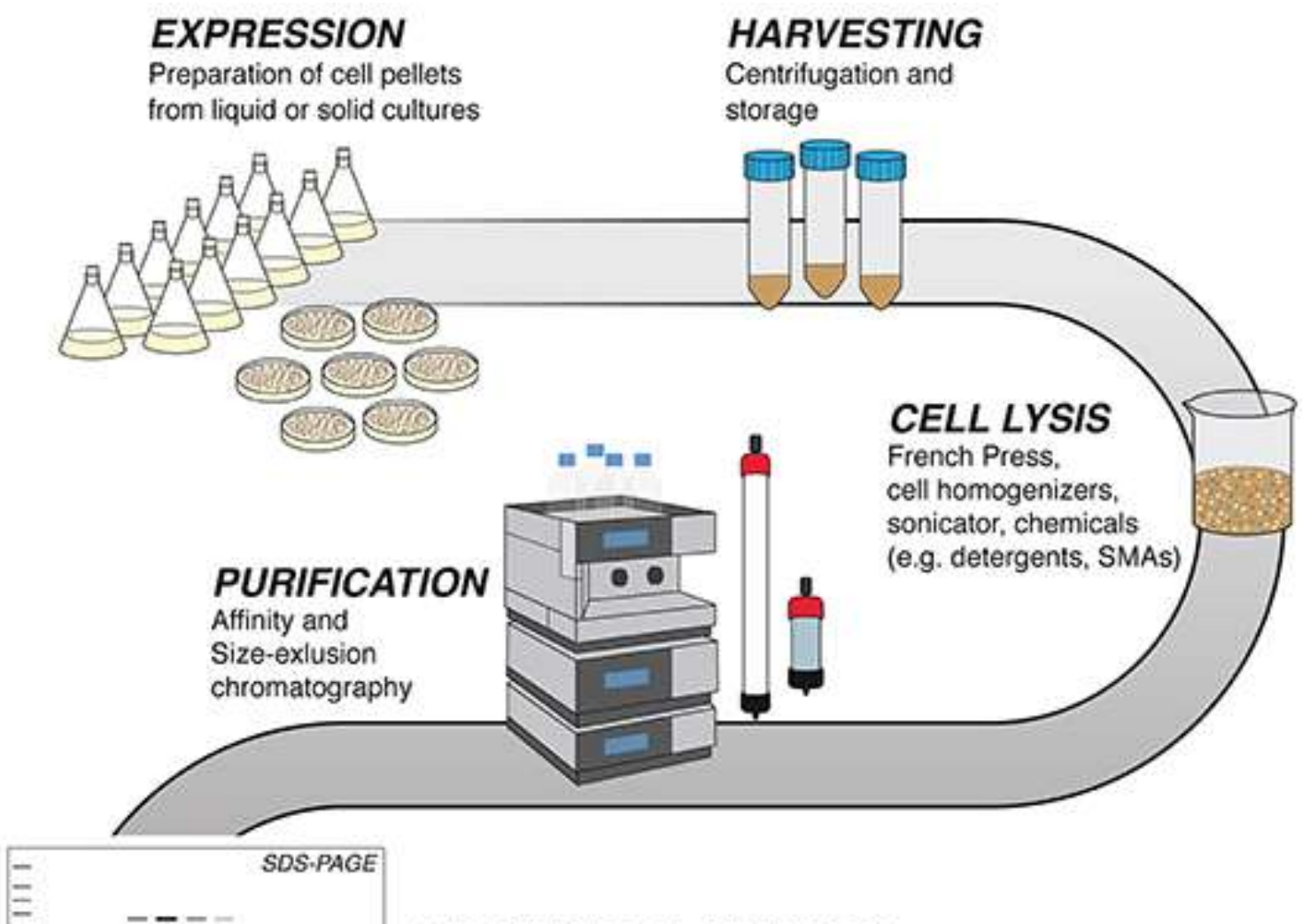




THE OPTIMIZATION WORKFLOW



PROTEIN PREPARATION



Molecular Biology

- Construct design
- Tags
- Genomic expansion
- Expression system

Biochemistry

- Composition
- Purity homogeneity
- Stability
- Biochemical activity

Markers

- Fabs/Nanobodies
- Fluorophores

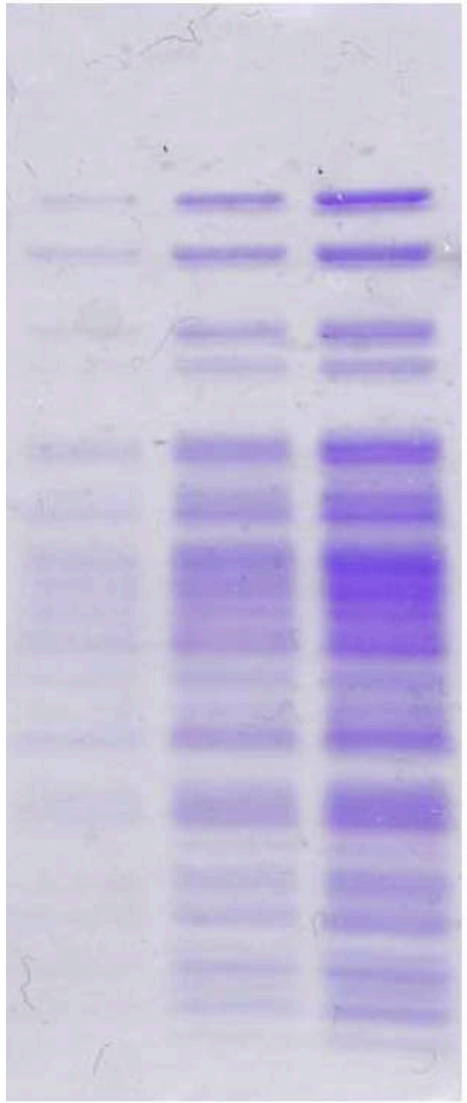
PROTEIN PREPARATION

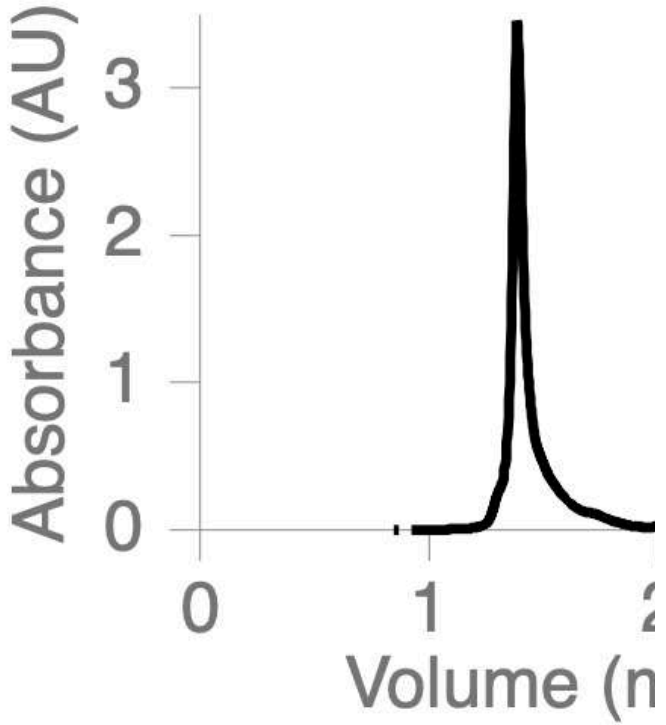
Specimen homogeneity

SDS-polyacrylamide gel electrophoresis (PAGE), native-PAGE, Silver stain

size exclusion chromatography

mass spectrometry

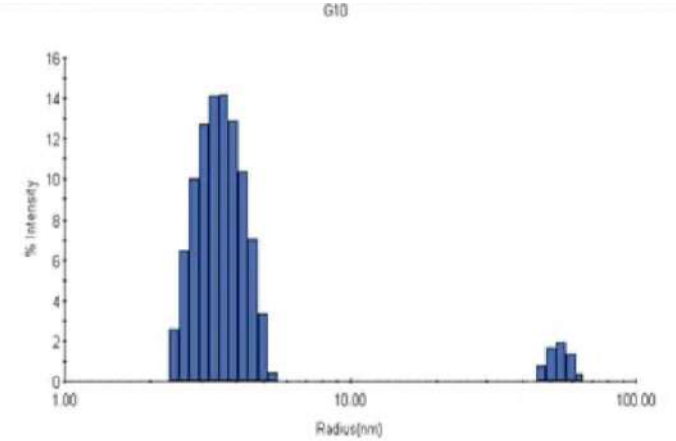




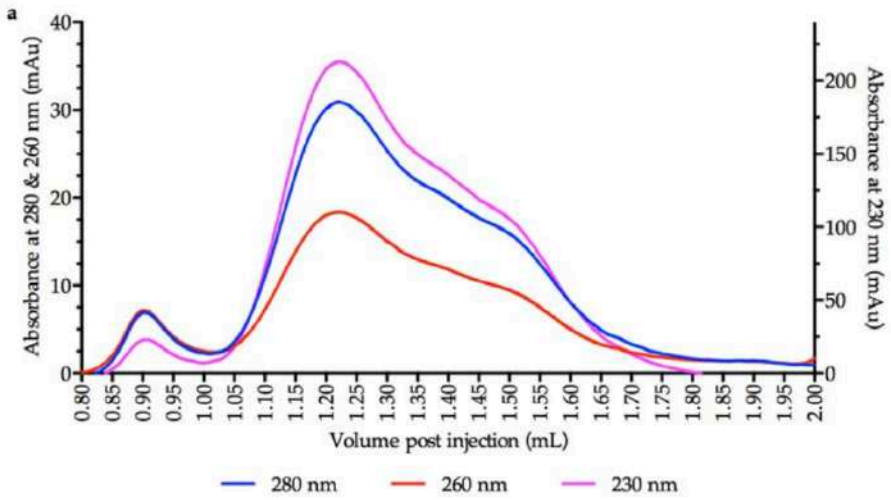
HOMOGENEITY OF SAMPLES

Optimize buffer conditions

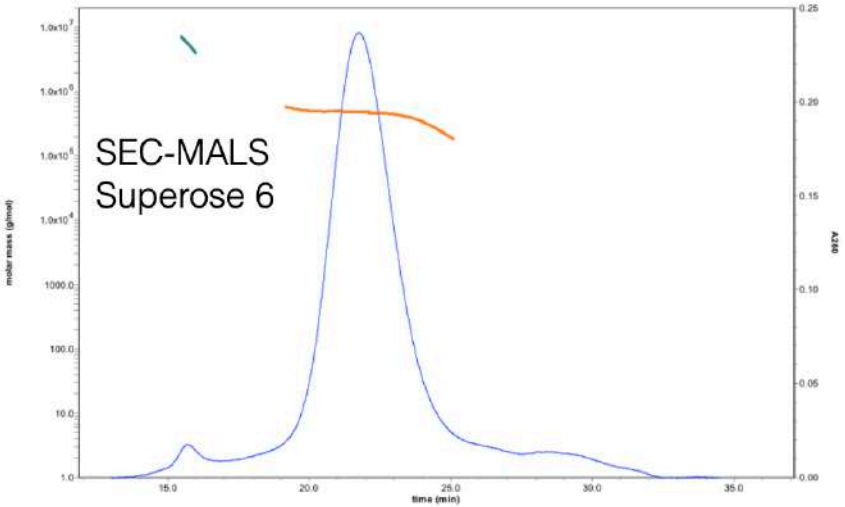
(salt, pH, detergent, etc…)



DLS
Dynamic Light Scattering



SEC
Size Exclusion Chromatography

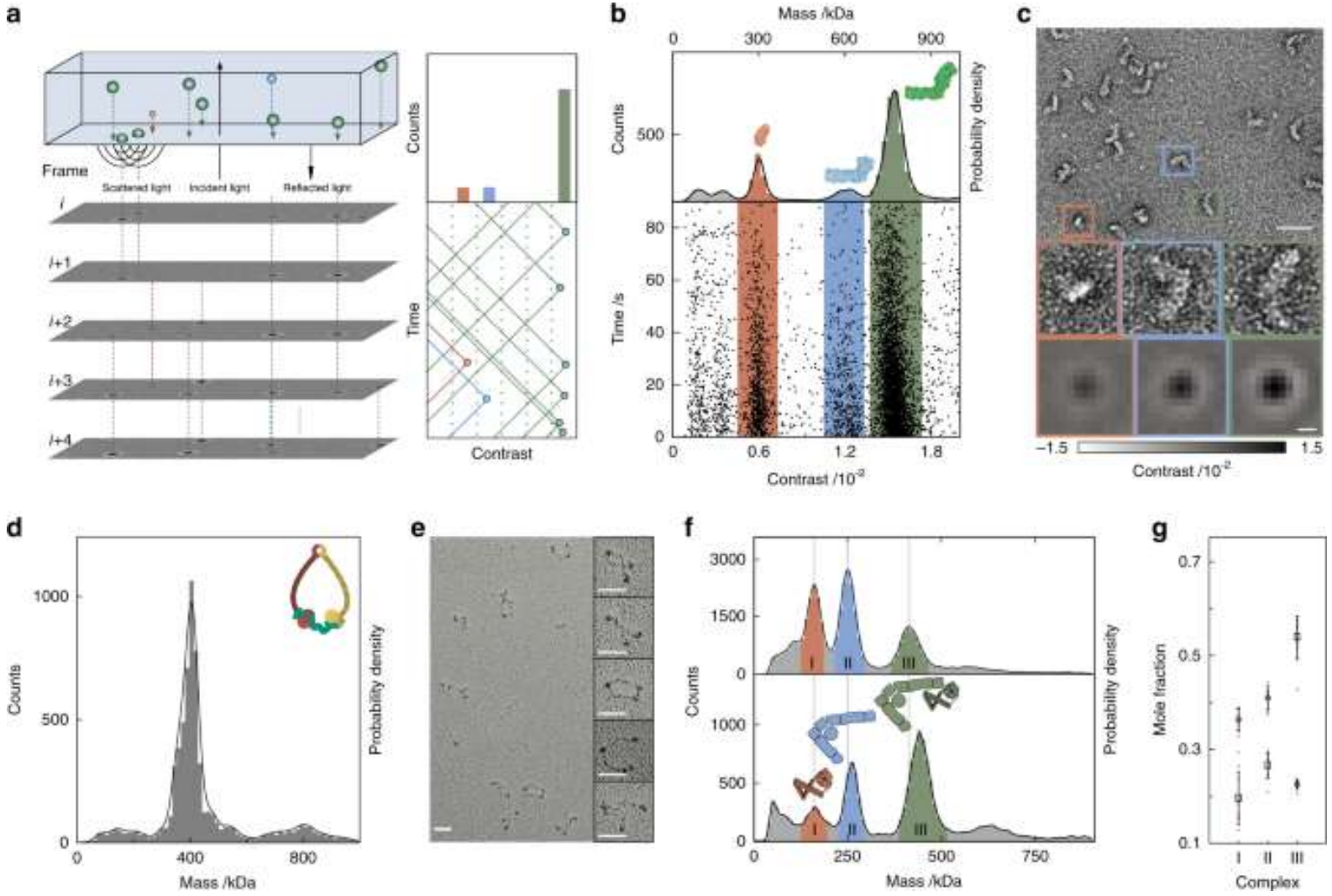


SEC-MALS
Size Exclusion Chromatography coupled to Multi Angle Light Scattering

HOMOGENEITY OF SAMPLES

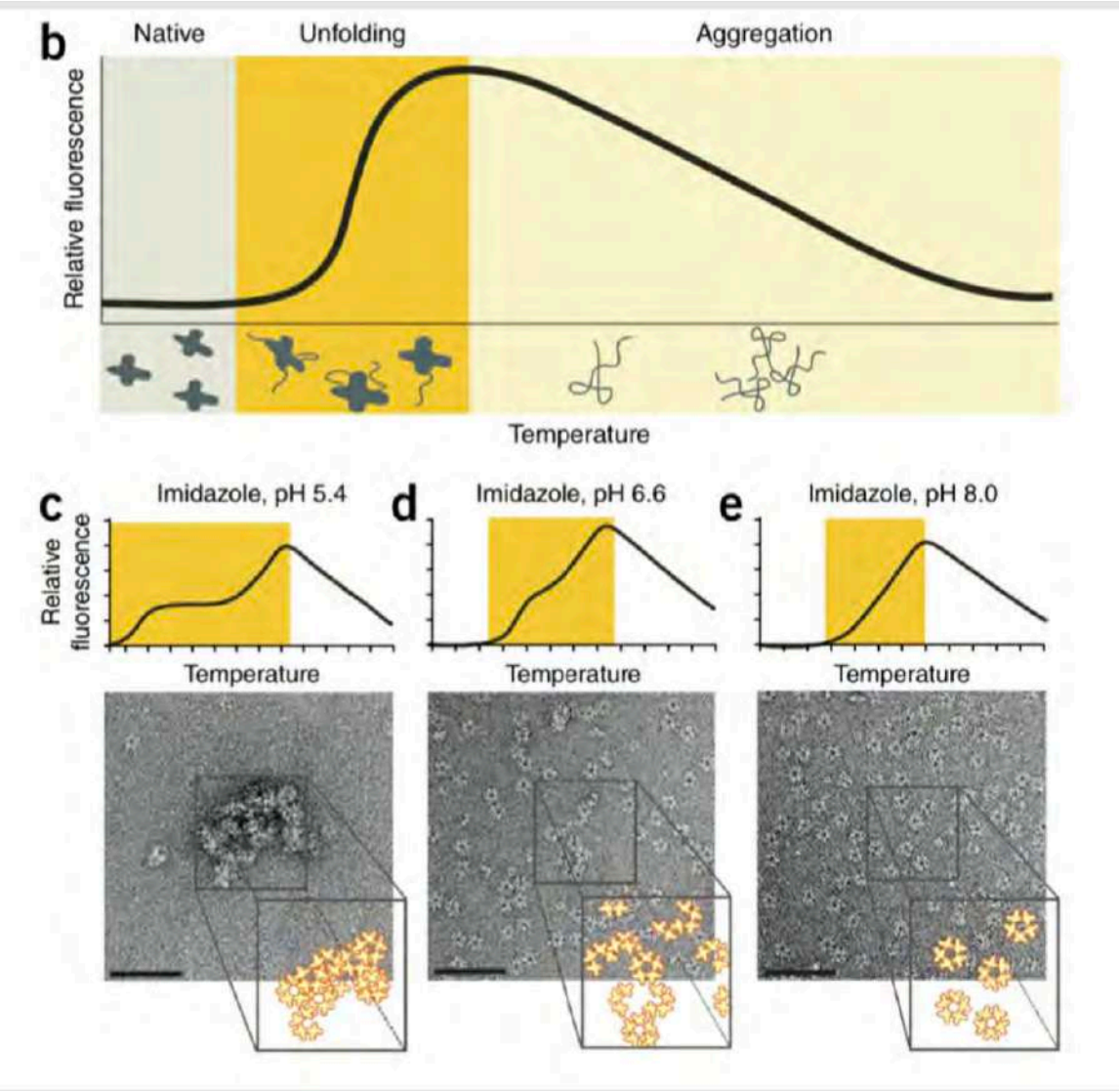
Mass photometry as a general method for characterizing biomolecular heterogeneity.

https://www.nature.com/articles/s41467-020-15642-w



SCREENING BUFFER CONDITIONS

sample stability can then be optimised by changing buffer conditions (e.g. salt, pH, detergent)



ProteoPlex thermal stability assay.

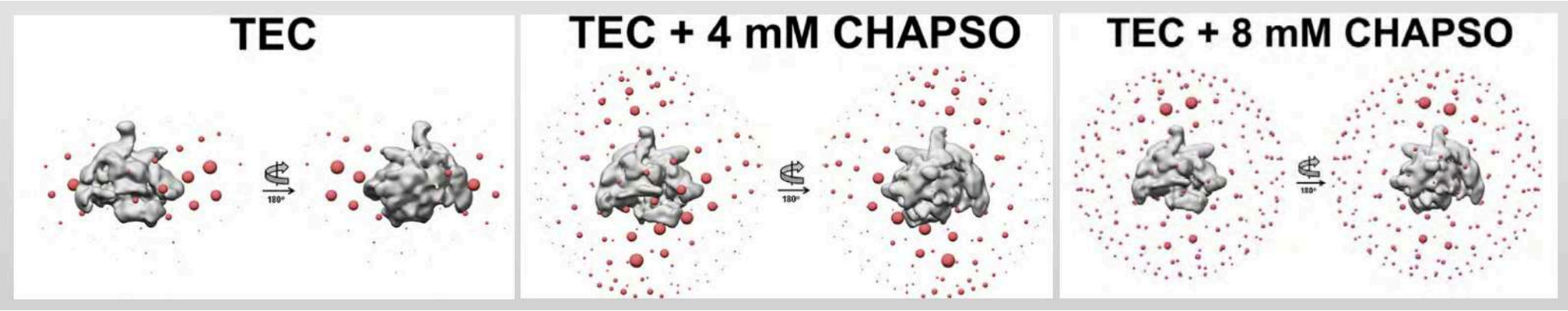
Holger Stark: Max Planck Institute for Biophysical Chemistry, Göttingen, Germany

Ashwin Chari et al., 2015

BUFFER COMPATIBILITY

Detergents can be used to relieve preferred orientation at air-water interface

- CHAPSO helps TEC (a bacteria RNA polymerase) distribute into diverse orientation.



James Chen et al., 2019

HOW MUCH SAMPLE IS NEEDED

Rules of thumb:

2 – 4 µl / grid

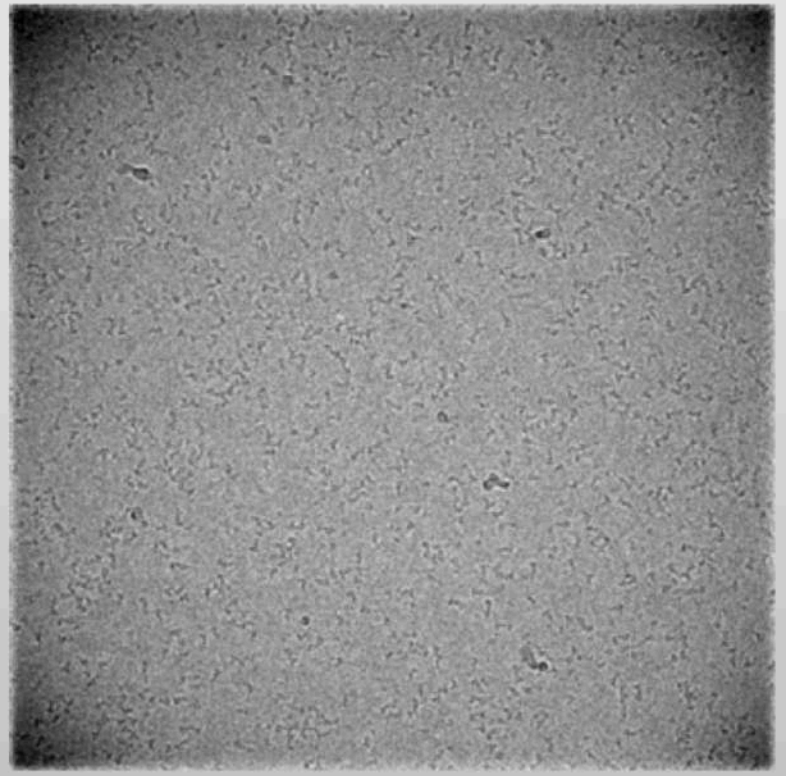
50 nM – 5 µM concentration

EM specimens are typically prepared using 3 µl protein solution at a concentration of 0.05 – 5 µM. Thus, it is essential for the protein complex to remain intact at these concentrations. If the dissociation constant (Kd) for the subunits is known, one can calculate whether it is expected to remain intact . Experimentally, one can run the protein complex on a size exclusion column repeatedly, at decreasing concentrations, to ensure it will not dissociate at the concentration required for cryo-EM.

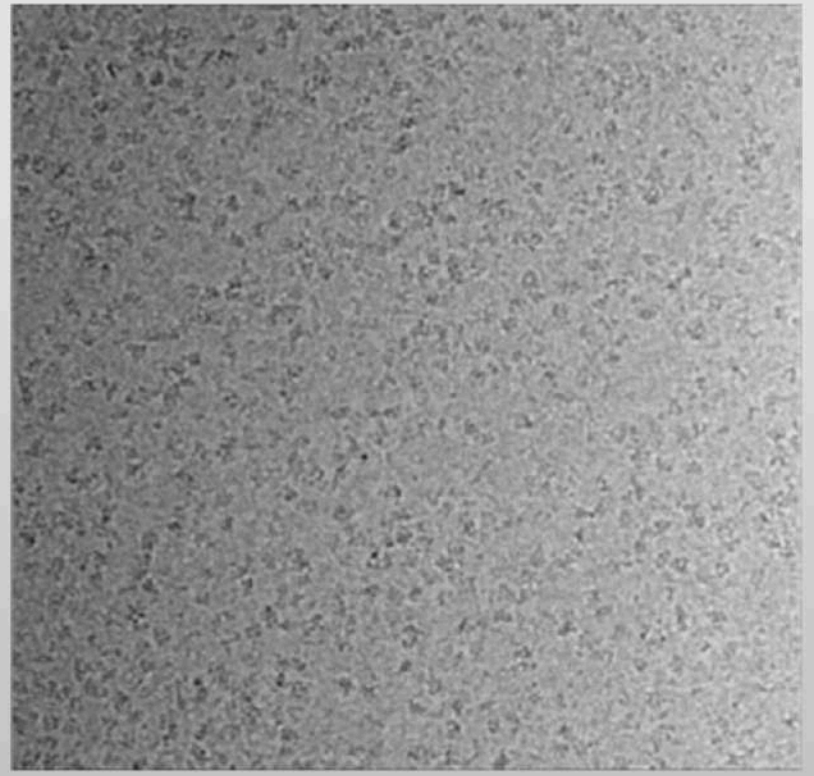
HOW MUCH SAMPLE IS NEEDED

For negative staining, 0.01-0.05 mg/ml.

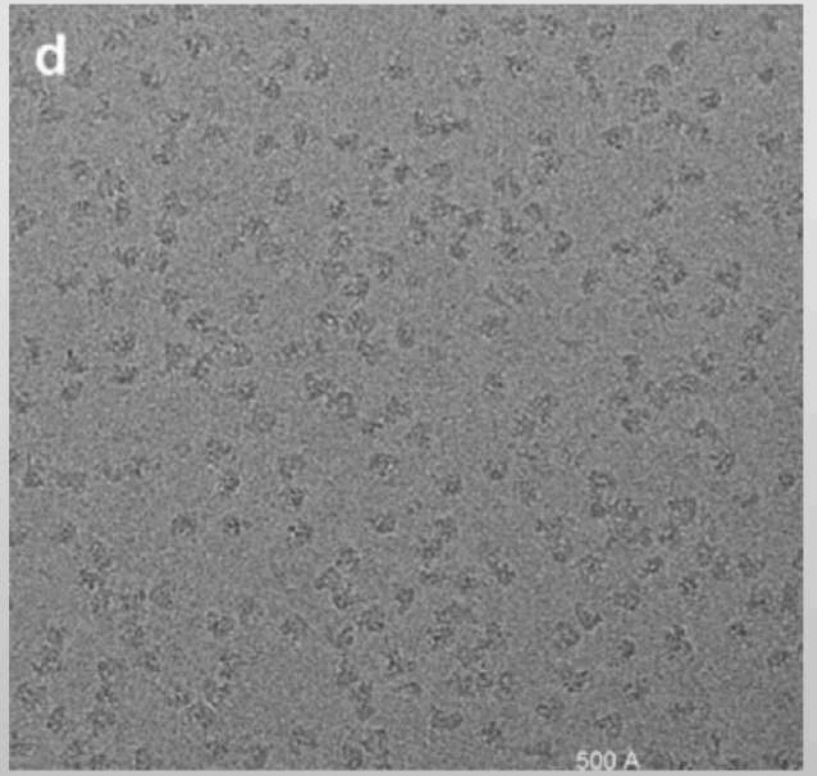
For cryo-EM, 0.1-5 mg/ml.



CTF3 complex, 130 kDa
0.2 mg/ml



Cas12a-AcrVA4/5 complex, 200 kDa
3 mg/ml



APC/C complex, 1.2 MDa
0.1 mg/ml on continuous carbon film

-slide from Leifu Chang

WHAT IF MY COMPLEX DISSOCIATES AT LOW CONCENTRATIONS?

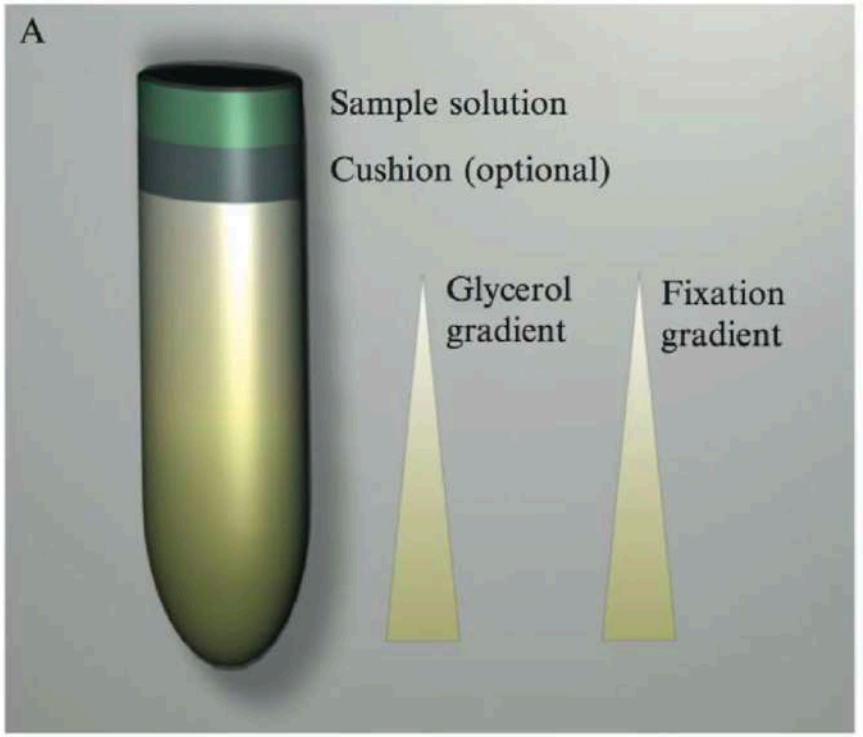
• Work at higher concentrations, adjust plasma and blotting

• Buffer conditions

• Crosslinking can stabilize protein- protein interactions (between subunits or domains)

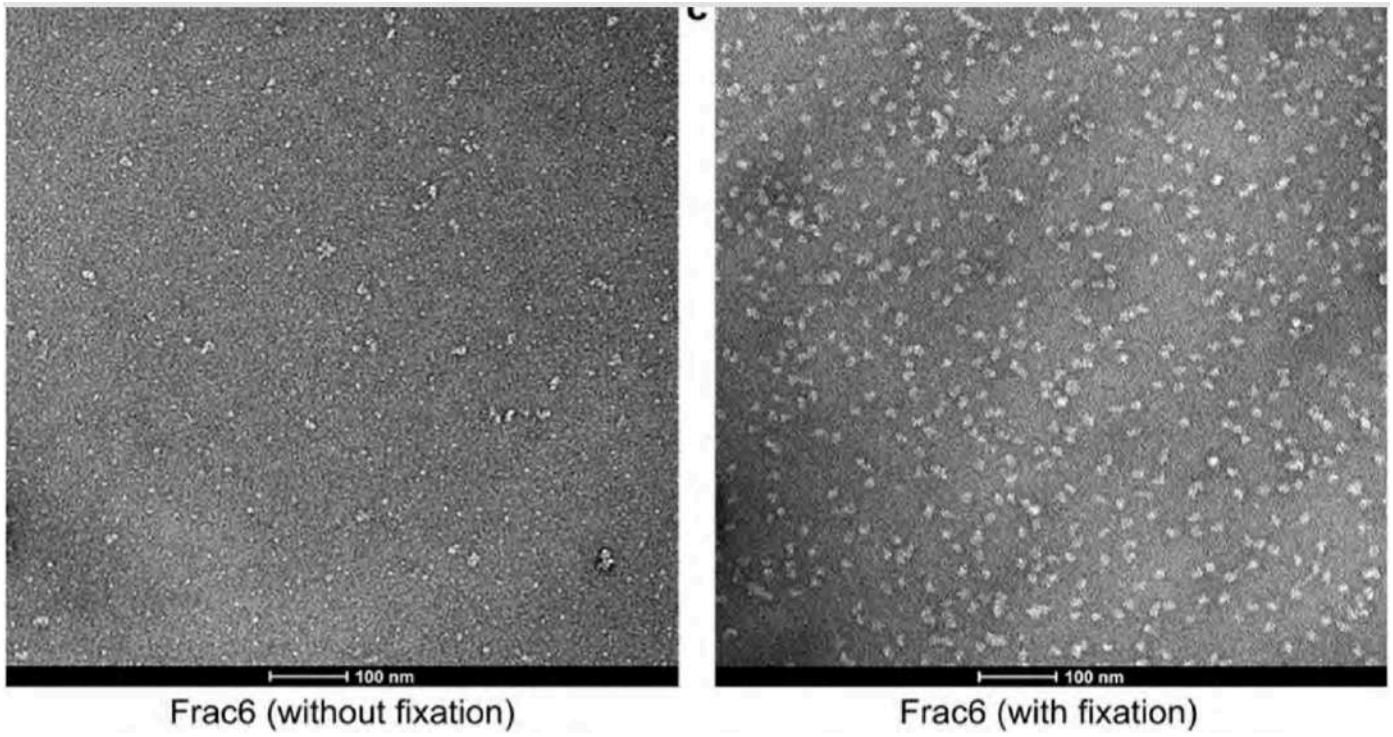
– Changes surface properties so can change particle orientations on grid

– Must minimize or remove aggregates due to intra-complex crosslinks



Kastner *et al*, 2008 | Stark, 2010

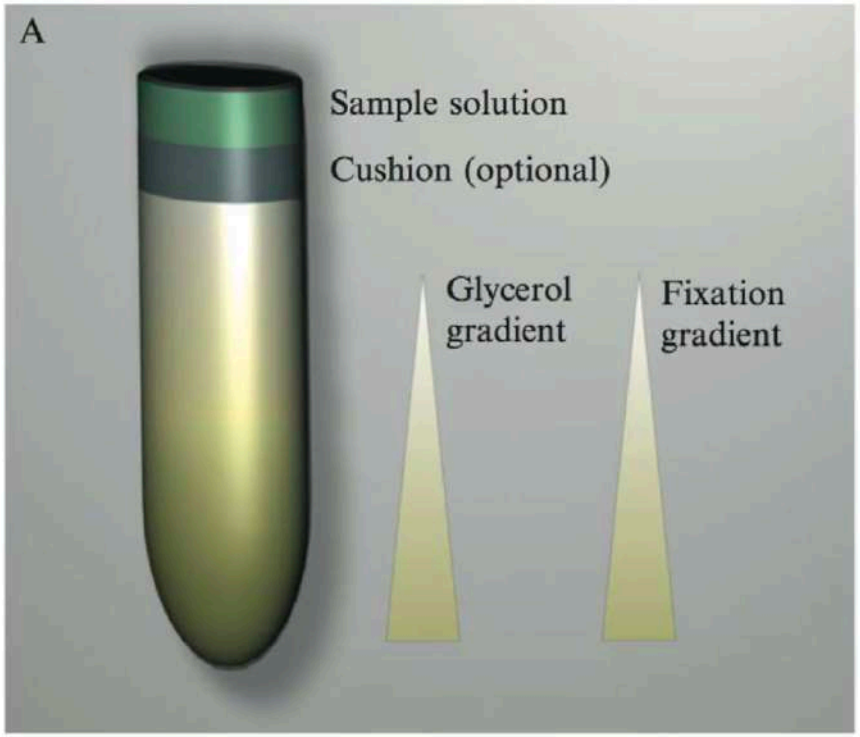
WHAT IF MY COMPLEX DISSOCIATES AT LOW CONCENTRATIONS?



Frac6 (without fixation)

Frac6 (with fixation)

Li *et al*, 2018



Kastner *et al*, 2008 | Stark, 2010

ADDITIONAL CONSIDERATIONS: MEMBRANE PROTEINS

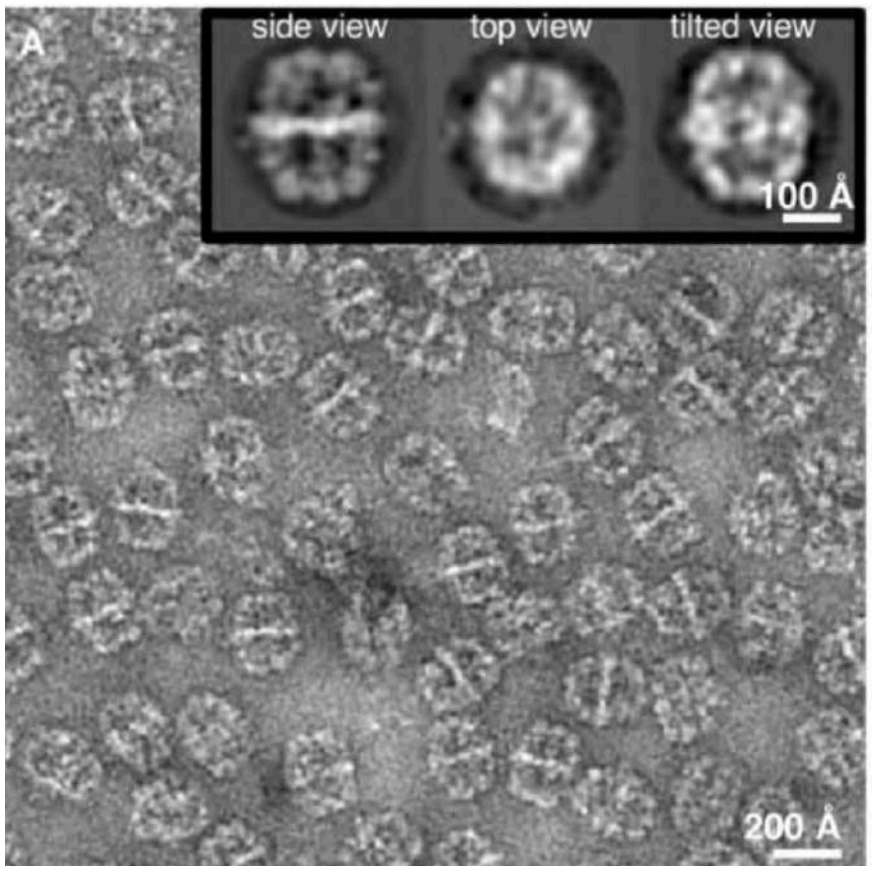
Choice of Detergents

- Amphipols: a hydrophilic backbone and hydrophobic side chains
- Amphipols have been successfully used in cryo-EM for many membrane proteins

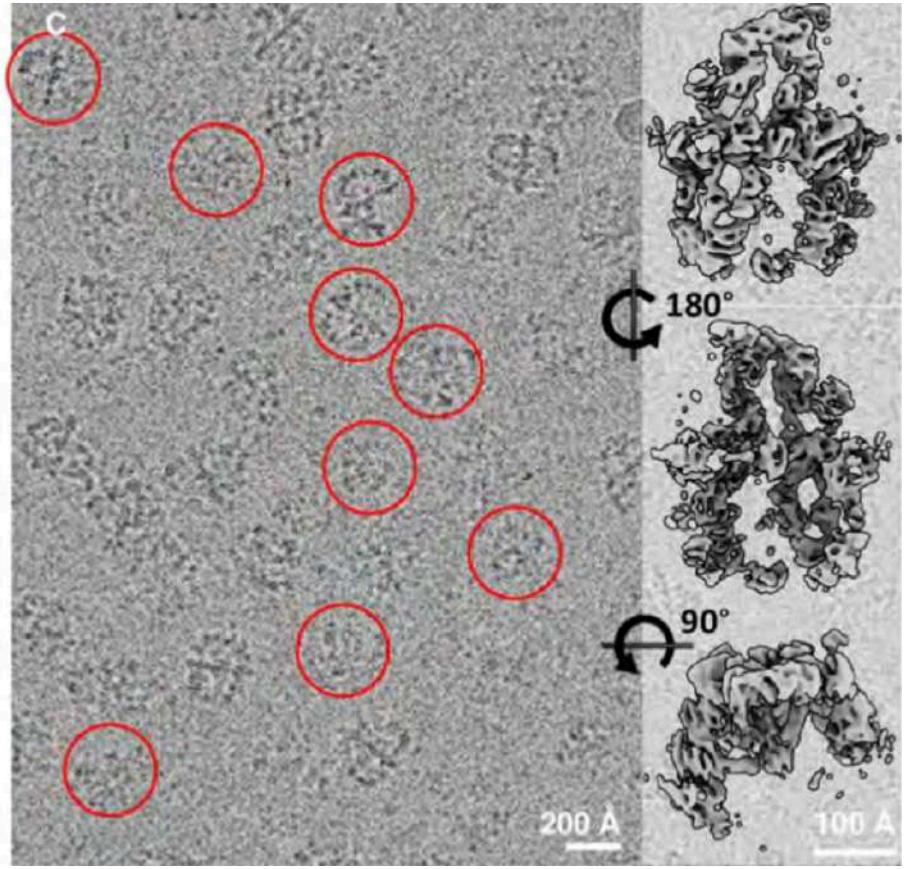
Nanodiscs

- MSP- Nanodiscs can stabilize proteins and offer membrane proteins a native bilayer.
- SMALPS- Endogenous bilayers may be used

ADDITIONAL CONSIDERATIONS: DENATURATION



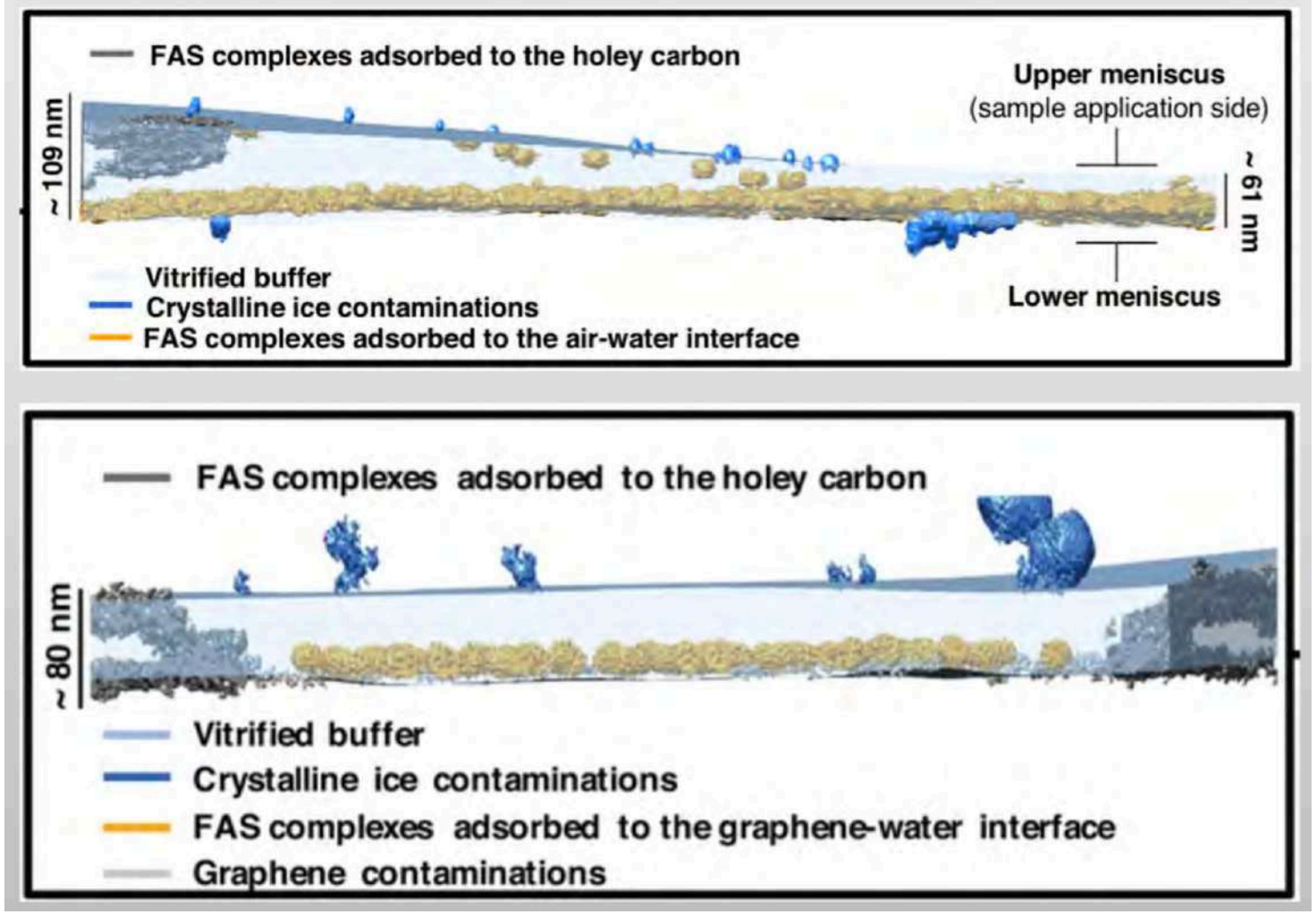
negative stain



cryoEM

D'Imprima *et al.*, 2019

ADDITIONAL CONSIDERATIONS: DENATURATION



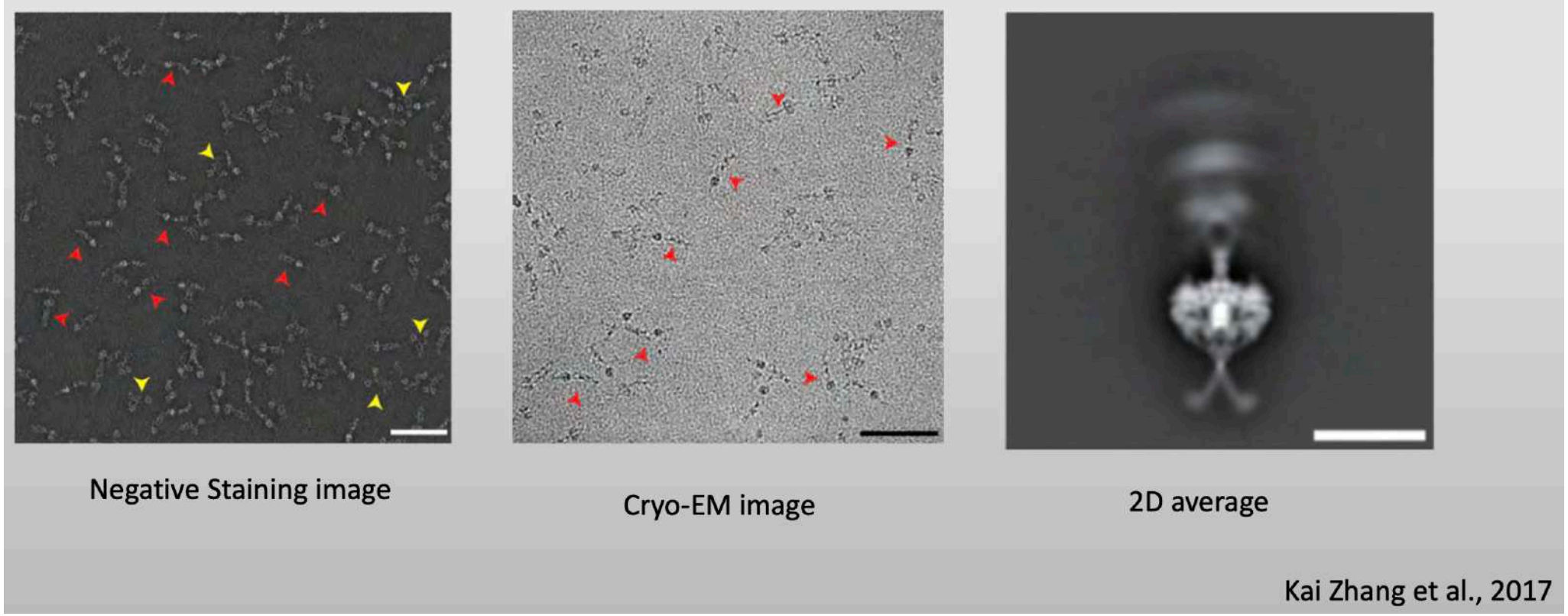
without graphene

with graphene

D'Imprima *et al.*, 2019

ADDITIONAL CONSIDERATIONS: FLEXIBILITY

Dynein has a globular motor domains and flexible tail.



EM COMPATIBLE SAMPLES

Moving from a **trial-and-error** process to a **controlled** and **reproducible** method

Questions?

BONUS SLIDES

Viewing Thermo Fisher S...

Today's Experts

Practical tips for GPCR cryo-EM

Patrick Sexton, PhD

Monash Institute of Pharmaceutical Sciences, Monash University

- PhD Pharmacology, Univ. of Melbourne (Australia)
- >30 years experience studying GPCRs
- With Denise Wootten, Rado Danev >50 GPCR structures determined by cryo-EM (~25 ≤ 2.5 Å)

Denise Wootten, PhD

Monash Institute of Pharmaceutical Sciences, Monash University

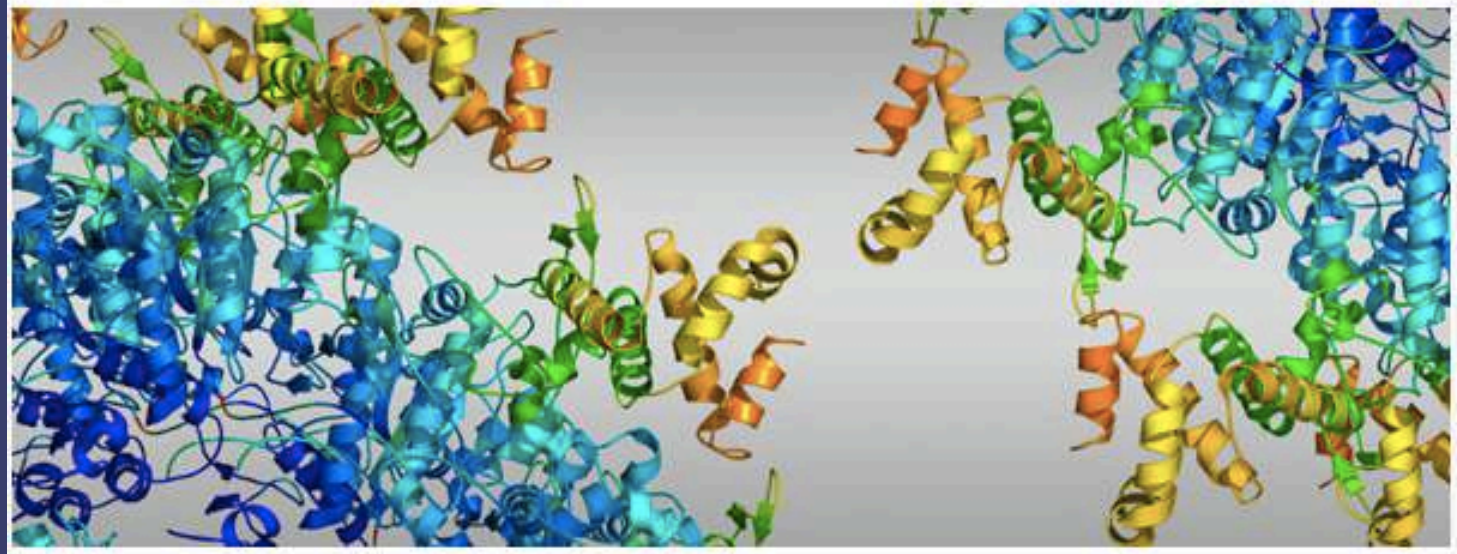
- PhD Biochemistry, Univ. of Birmingham (United Kingdom)
- Biochemistry and pharmacology of GPCRs
- With Patrick Sexton, Rado Danev >50 GPCR structures determined by cryo-EM (~25 ≤ 2.5 Å)

Radostin Danev, PhD

Graduate School of Medicine, The University of Tokyo

- PhD Biophysics, National Institute for Physiological Science, Japan
- Cryo-EM methods development
- With Patrick Sexton, Denise Wootten >50 GPCR structures determined by cryo-EM (~25 ≤ 2.5 Å)

ThermoFisher SCIENTIFIC



Join a discussion on sub-2.5 Å cryo-EM structure determination of GPCRs for drug design

Wed, Sep 9, 2020, 8 p.m. EDT | 5 p.m. PDT | 10 a.m. AEST | 9 a.m. JST

Attend our upcoming *Ask the Experts* Q&A session on routine sub-2.5 Å cryo-EM structure determination of GPCRs for drug design. This rapidly developing field is constantly producing new and exciting biological and pharmacological discoveries. Ask questions and get answers from leading academic investigators in the field.

You'll learn about:

- GPCR biochemistry and purification for cryo-EM
- GPCR sample preparation for cryo-EM
- High-resolution single-particle cryo-EM imaging and 3D reconstruction of GPCRs

BONUS SLIDES

Cryo-EM sample preparation

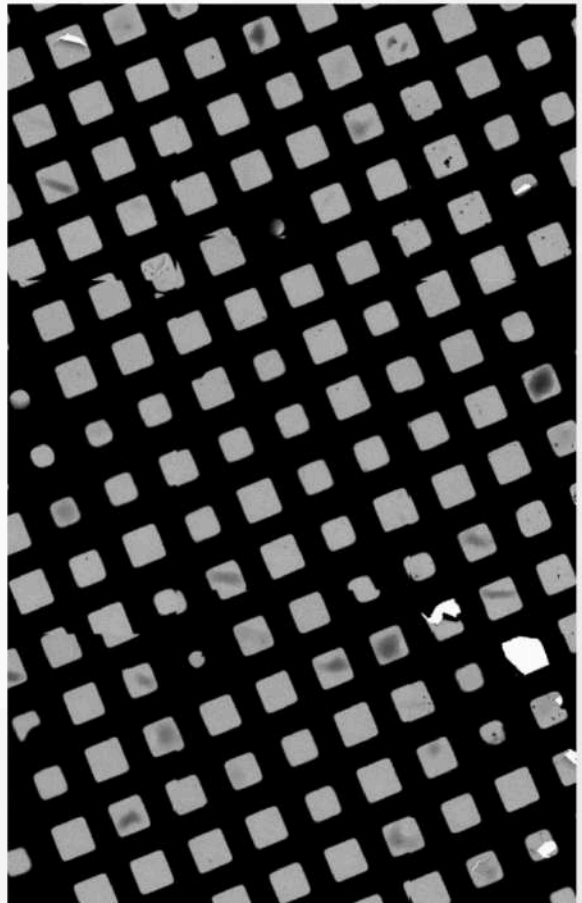
Viewing Thermo Fisher S...

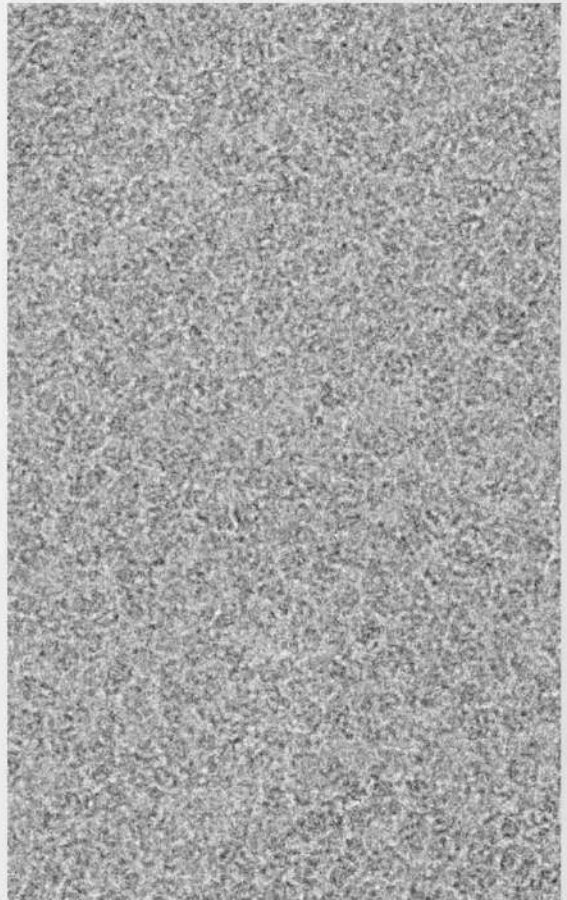
MONASH University

東京大学 THE UNIVERSITY OF TOKYO

- **The quality of the cryo-EM sample governs the outcome of the experiment!**

- We optimized the plunging parameters for ice thickness consistency and grid coverage
- Our blot time is relatively long: 10 s
- For every new sample, depending on the initial concentration, we prepare 2-3 grids with 2x dilution in-between
- GPCR sample concentrations in the range 3 – 7 mg/ml work best.
- Avoid as much as possible lower concentrations!
- Gold foil grids (UltrAuFoil) improved the consistency of getting uniformly thin ice and reduce beam-induced motion.





BONUS SLIDES

Viewing Thermo Fisher S...

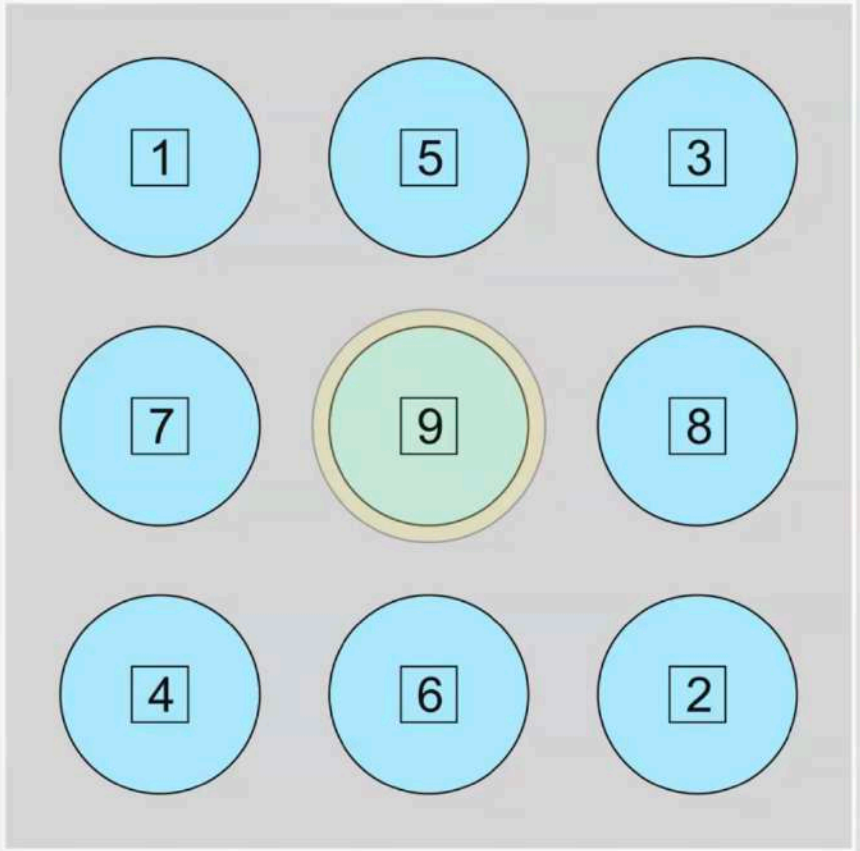
Cryo-EM data acquisition strategy

MONASH University

東京大学 THE UNIVERSITY OF TOKYO

- **Collect on the thinnest possible ice that still has good particle coverage !**

- We used 3 x 3 = 9-hole beam-image shift data acquisition scheme, 1 image/hole, realized with home-made scripts in SerialEM
- Defocus range: 0.5 – 1.5 µm. Start at the high end on the first hole and reduce the defocus step-wise for each hole in the pattern, e.g. 1.4 → 1.3 → 1.2 → … → 0.6
- Use an energy filter with <15 eV slit
- Do not use super-res (K3), select pixel size ≈ 1/3 the resolution you are hoping to get. Use EER with Falcon 4.
- Throughput: 1 sample/day ≈ 1 structure/day; ~5,500 movies
- Collect non-gain-normalized compressed TIFFs/EER. prepare your own gain reference with Relion



BONUS SLIDES

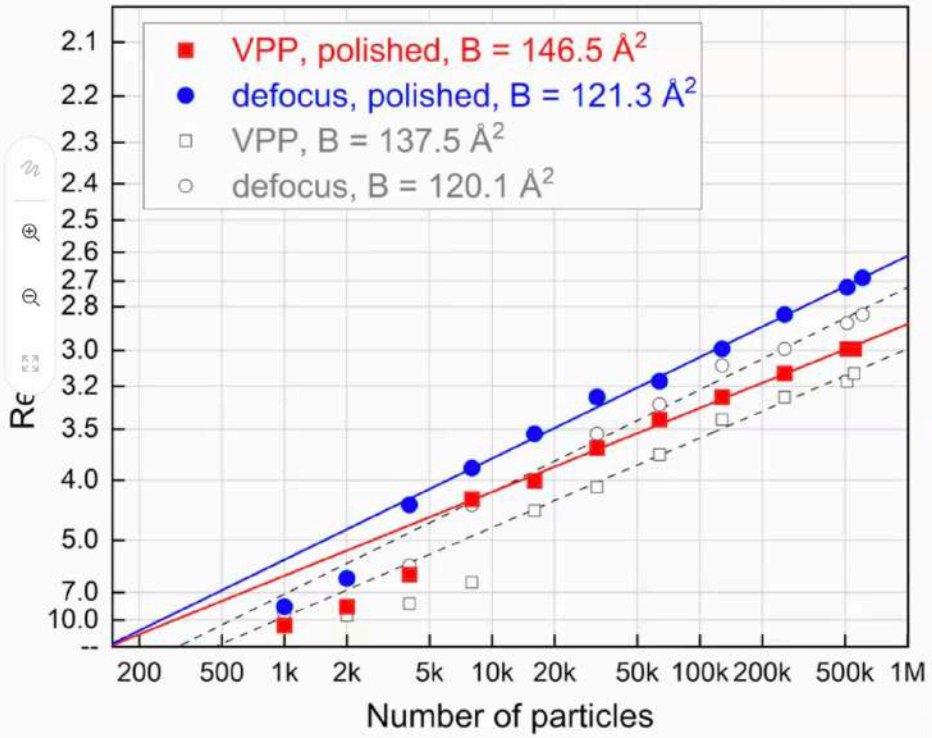
Performance factors

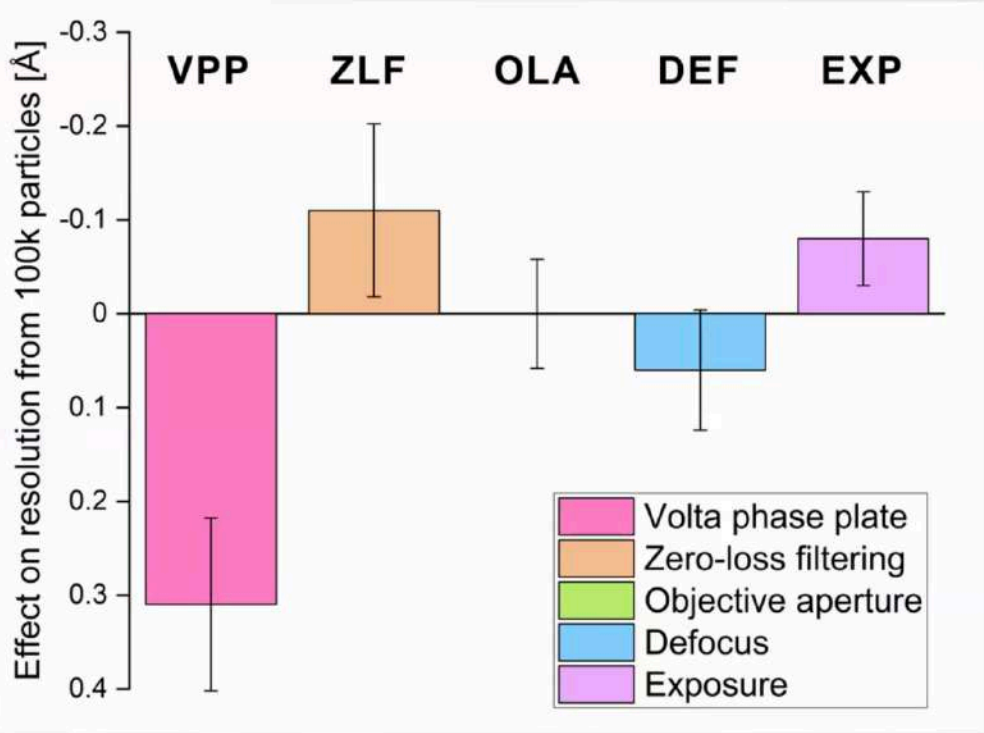
Viewing Thermo Fisher S...

MONASH University

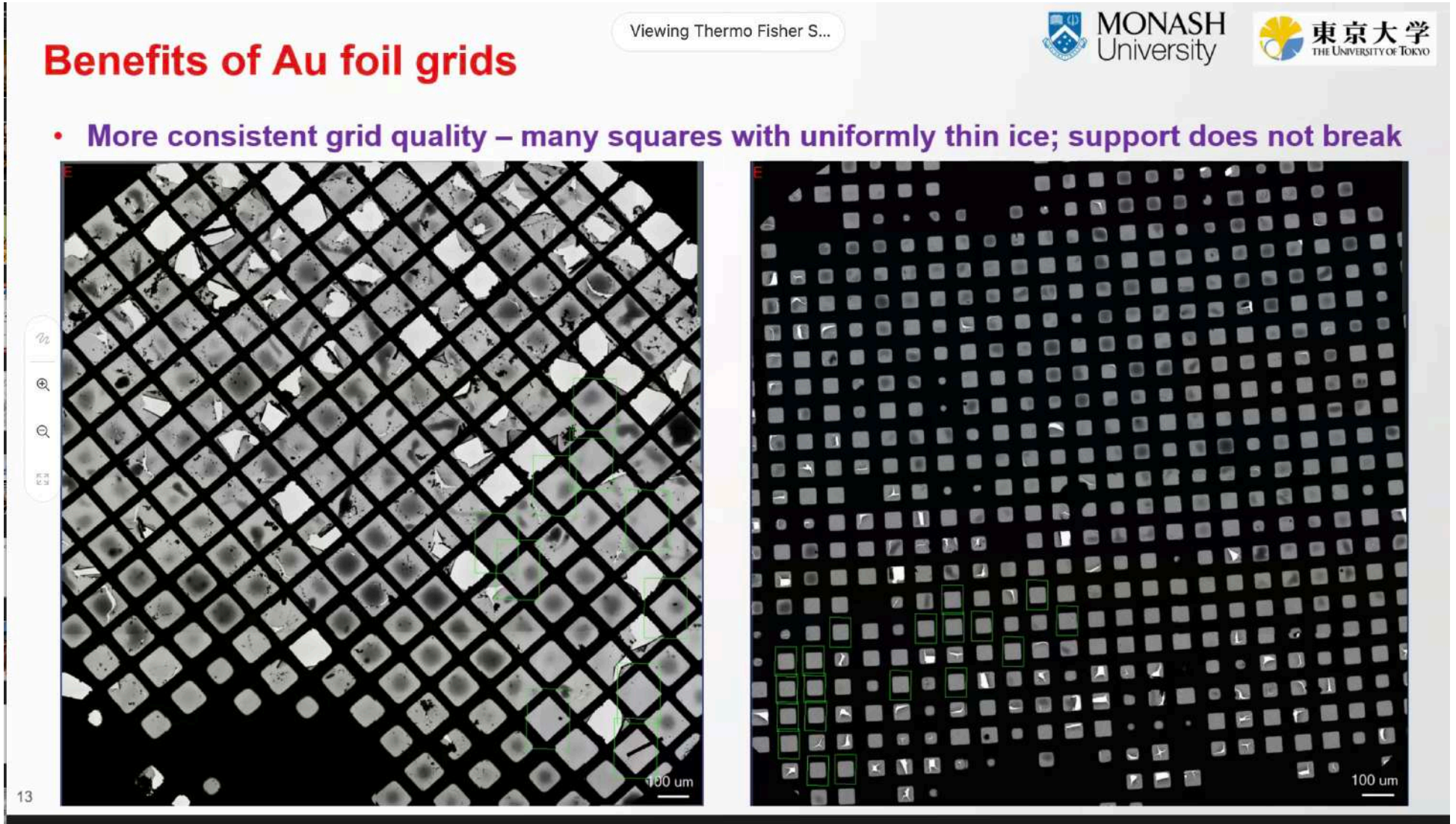
東京大学 THE UNIVERSITY OF TOKYO

- Do not use VPP; use zero-loss filtering; defocus <1.5 µm; total exposure ≥60 e/Å^2





BONUS SLIDES



WHAT NEXT?

cryoEM 001 : Single Particle Masterclass

1. Building a cryoEM toolkit
2. EM compatible samples
3. EM support films and grids
4. Sample preparation
5. Tools of the trade: microscopes and detectors
6. Microscope operations
7. Data collection strategies
8. Data assessment & QC
9. Data processing:
   - cryoEM IT infrastructure
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   - 3D Reconstruction
10. Visualization and validation