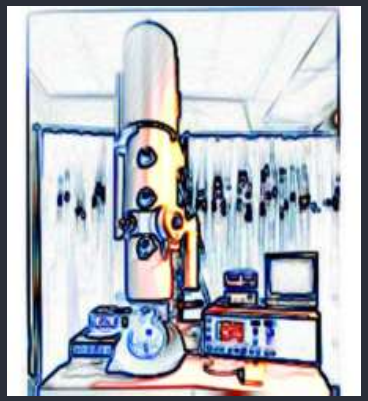
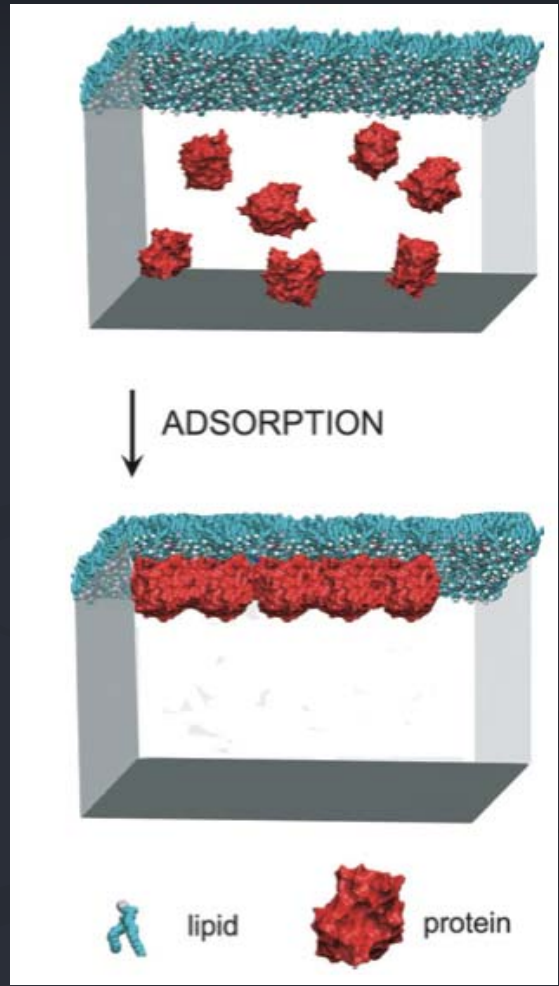
Monolayer Purification for Single Particle EM



Debbie Kelly
Walz Laboratory
Department of Cell Biology
Harvard Medical School
kelly@crystal.harvard.edu

Historical Background

- **Uzgiris and Kornberg (1983)**
  - 2D xtals of Ab on lipid Antigen
- **Darst *et al.*, 1988**
  - RNA polymerase
- **Avila-Sakar *et al.*, 1994**
  - 50S ribosome subunit
- **Kubalek *et al.*, (1994)**
  - His-tagged HIV1 RT



Dietrich and Venien-Bryan (20

Monolayer Structures

~ 90 projection maps

15 Cryo-EM

Only 7 Cryo-EM

3D Reconstructions

Current Specimen Limitations

- Screening conditions
- Fragile transfer step
- Specimen Flatness
- Alternative approach = Single particle EM

A combinatorial approach for protein purification / EM structural studies

- Develop the Ni-NTA monolayer technique as
  a novel purification method
- Single Particle Cryo-EM for 3D reconstruction
- Apply the methodology to a real system

Monolayer Purification Setup

1) Add protein sample well

Cell lysate w/ target (25 μl)

50mM Hepes+150 mM NaCl+imidazole

2) Cast monolayer

Filler / Ni-NTA lipid (1mg/ml in $CHCl_3$)

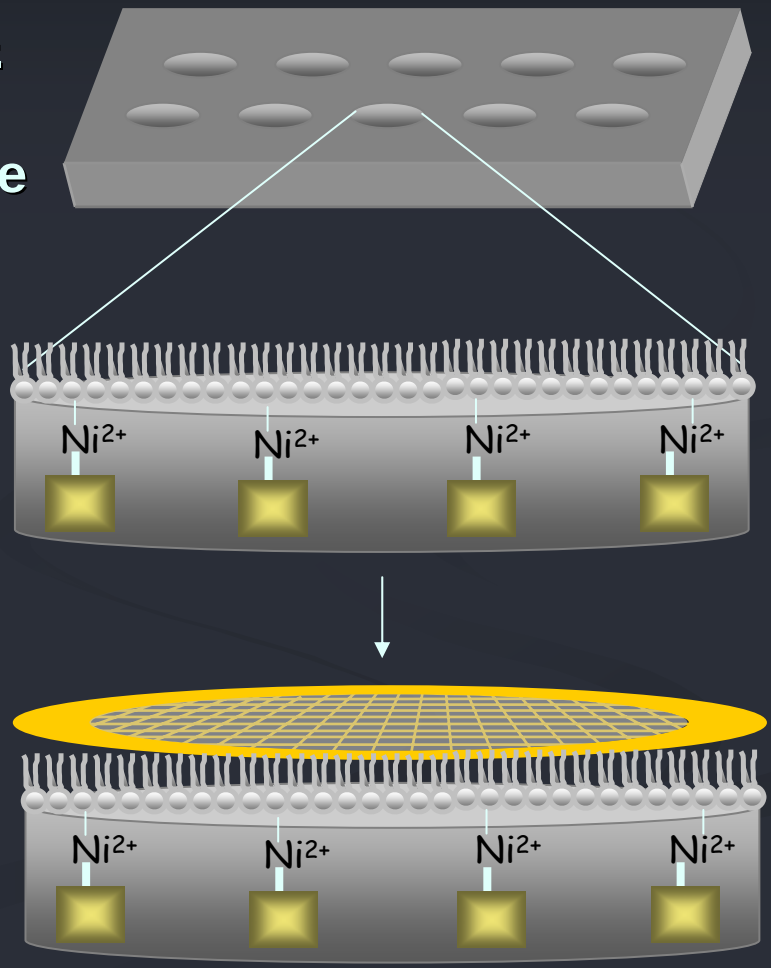
Apply 1μl Mix w/ Hamilton syringe

Incubate 15 - 30 min., 4°C

3) Sample with EM grid

Apply clean EM grid

Grid bar side on monolayer



Basic Considerations

- Biological sample preparation
- Grid preparation
- Transfer Step
- Neg. stain screening
- Vitrification
- Low-dose Imaging

Preparation of Cell Extracts

Insect cells
(Sf9)

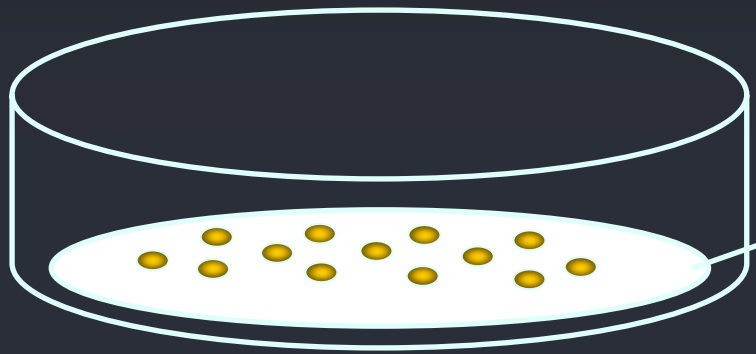
Bacteria
(E.coli, BL21)

QuickTime™ and a
TIFF (Uncompressed) decompressor
are needed to see this picture.

Mammalian cells
(293T)

- Grow His-tagged construct
- Lyse cells with lysozyme, sonication
- Obtained cleared lysate; ML input

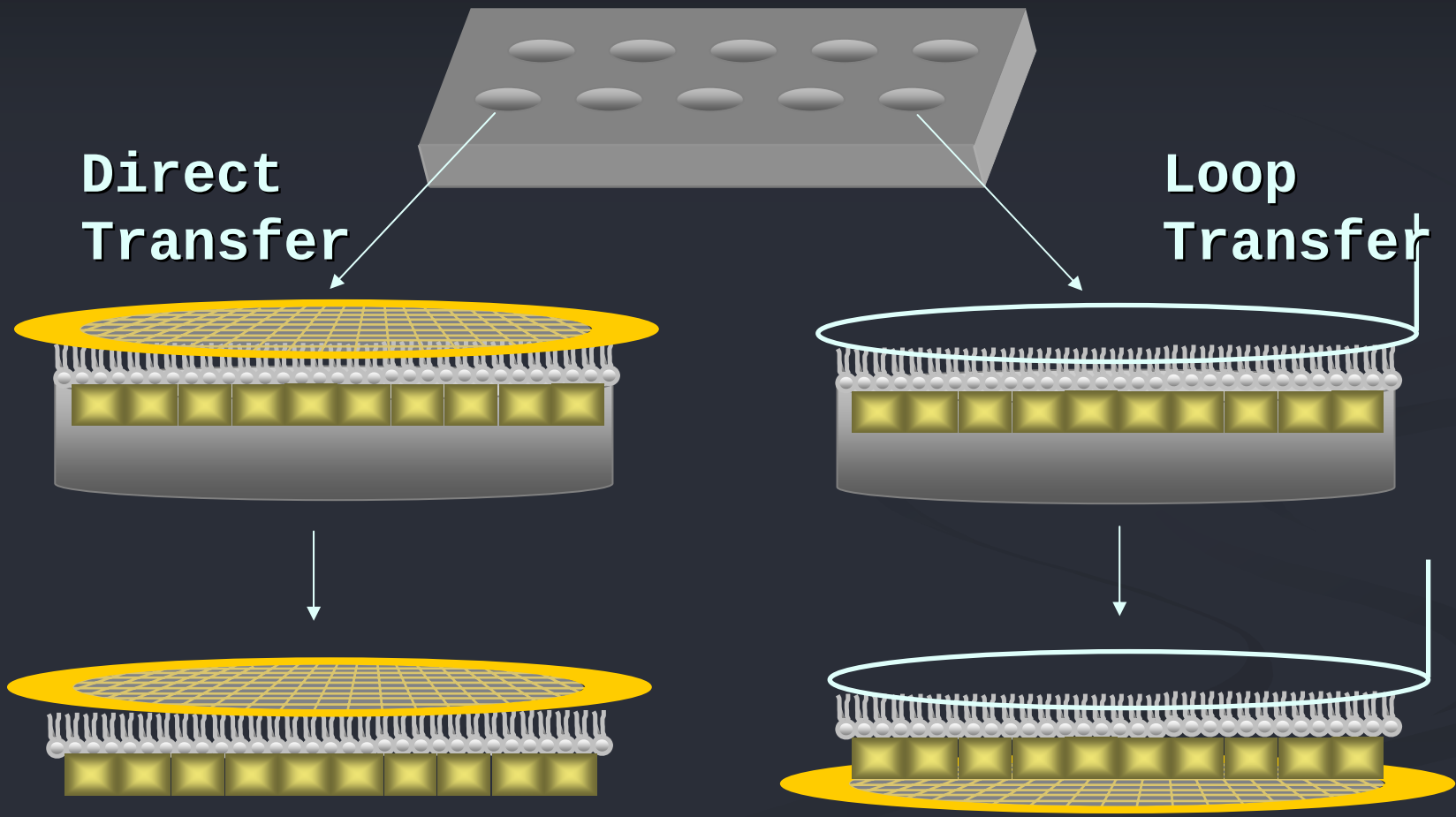
Quantifoil Grid Preparation



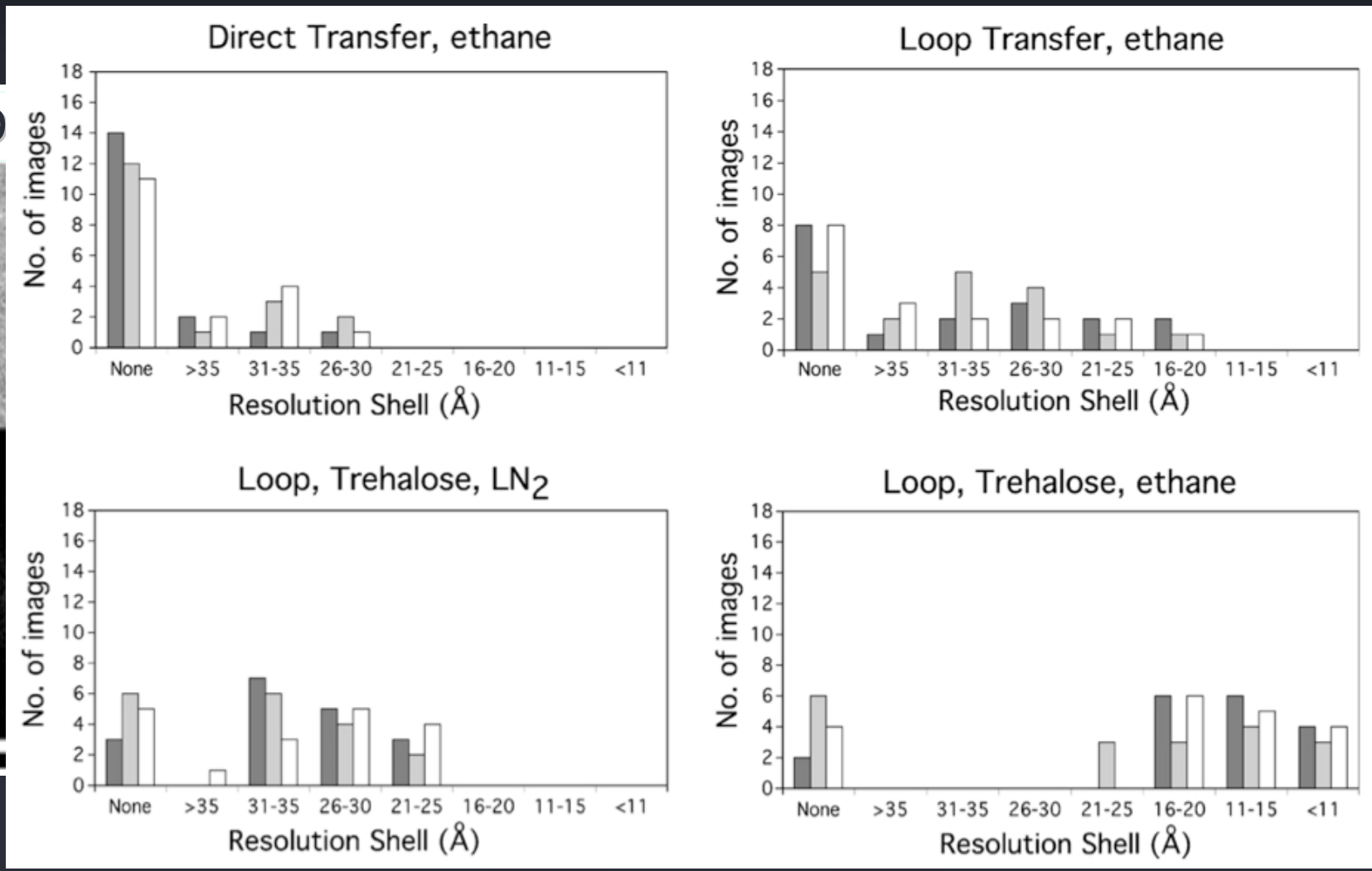
Whatman #1 paper
Saturated w/
Ethyl Acetate o/n
in hood

Bake for 1 hr
At least 100°C

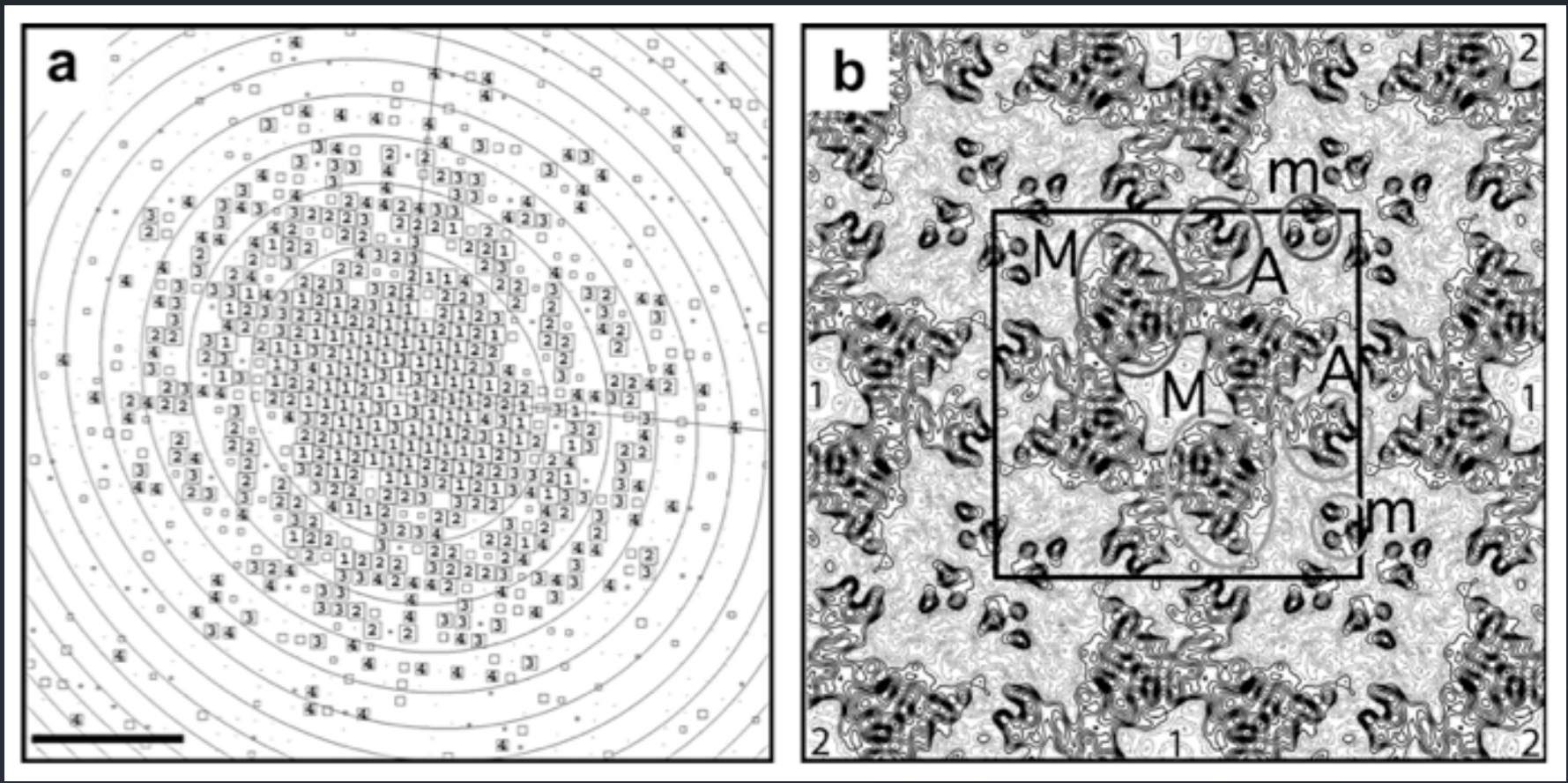
Transfer Methods



Direct Transfer vs. Loop transfer



7Å Projection Map of SbpA



Norville et al., JSB (in

Transfer of 2D crystal vs Single particles

- Crystals = Loop transfer / 5% Trehalose embedding /ethane
- Particles = Direct transfer /ethane

Basic Considerations

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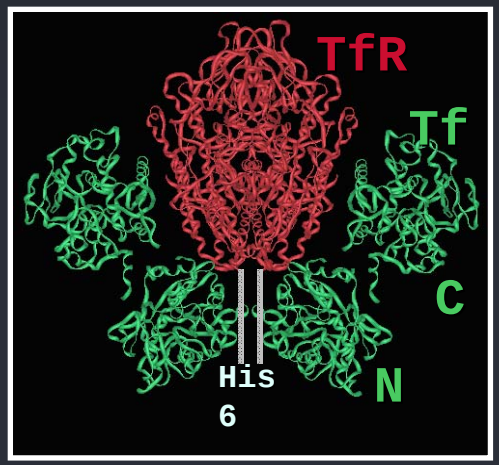
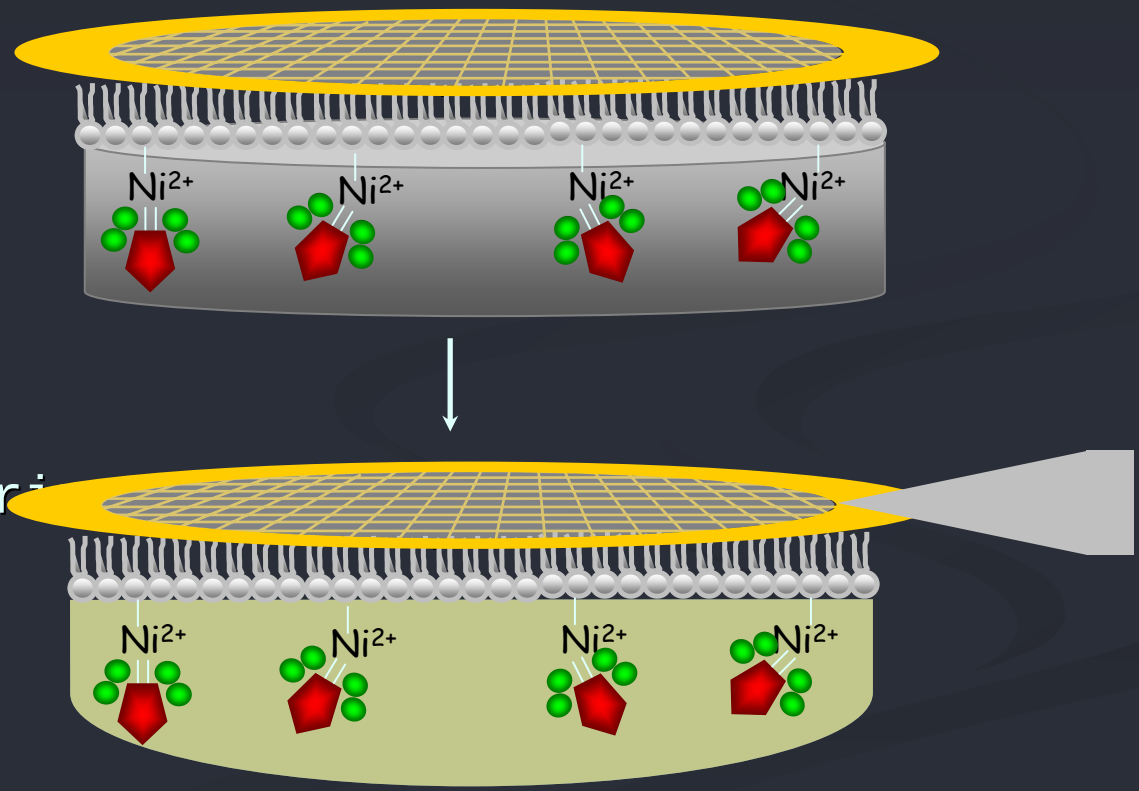
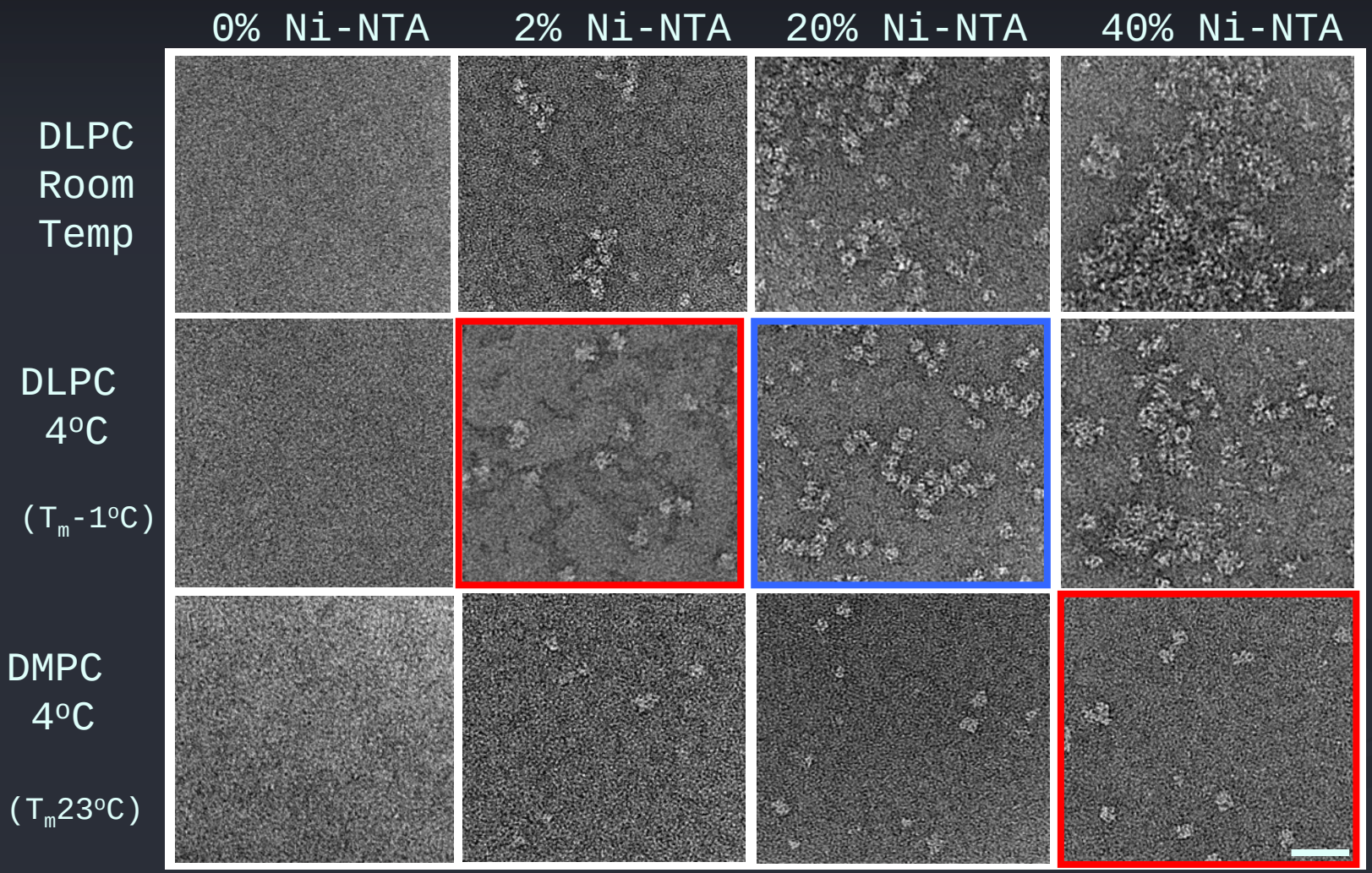
Negative Stain Screening
Test specimen: Tf-TfR complex



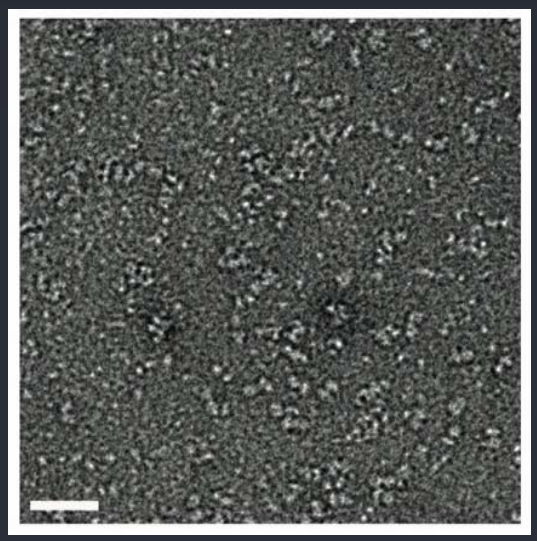
Transferrin-Transferri
Receptor Complex



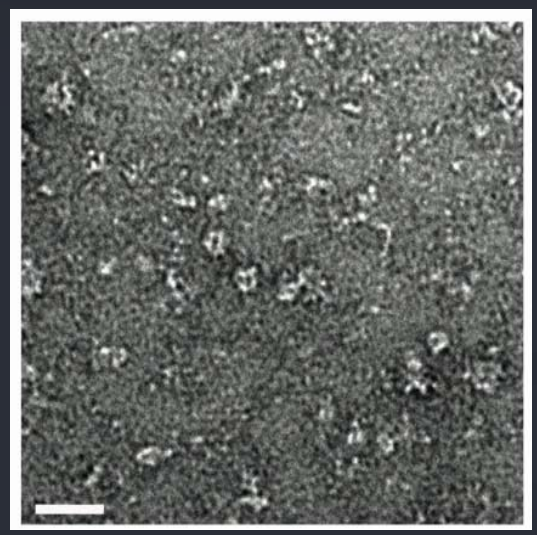
Negative Stain Screening



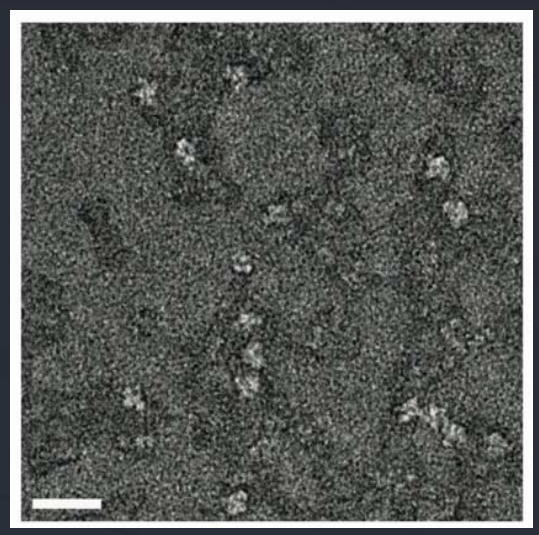
Screening Monolayer Purified Tf-TfR complex from Sf9 extracts



In Soln.

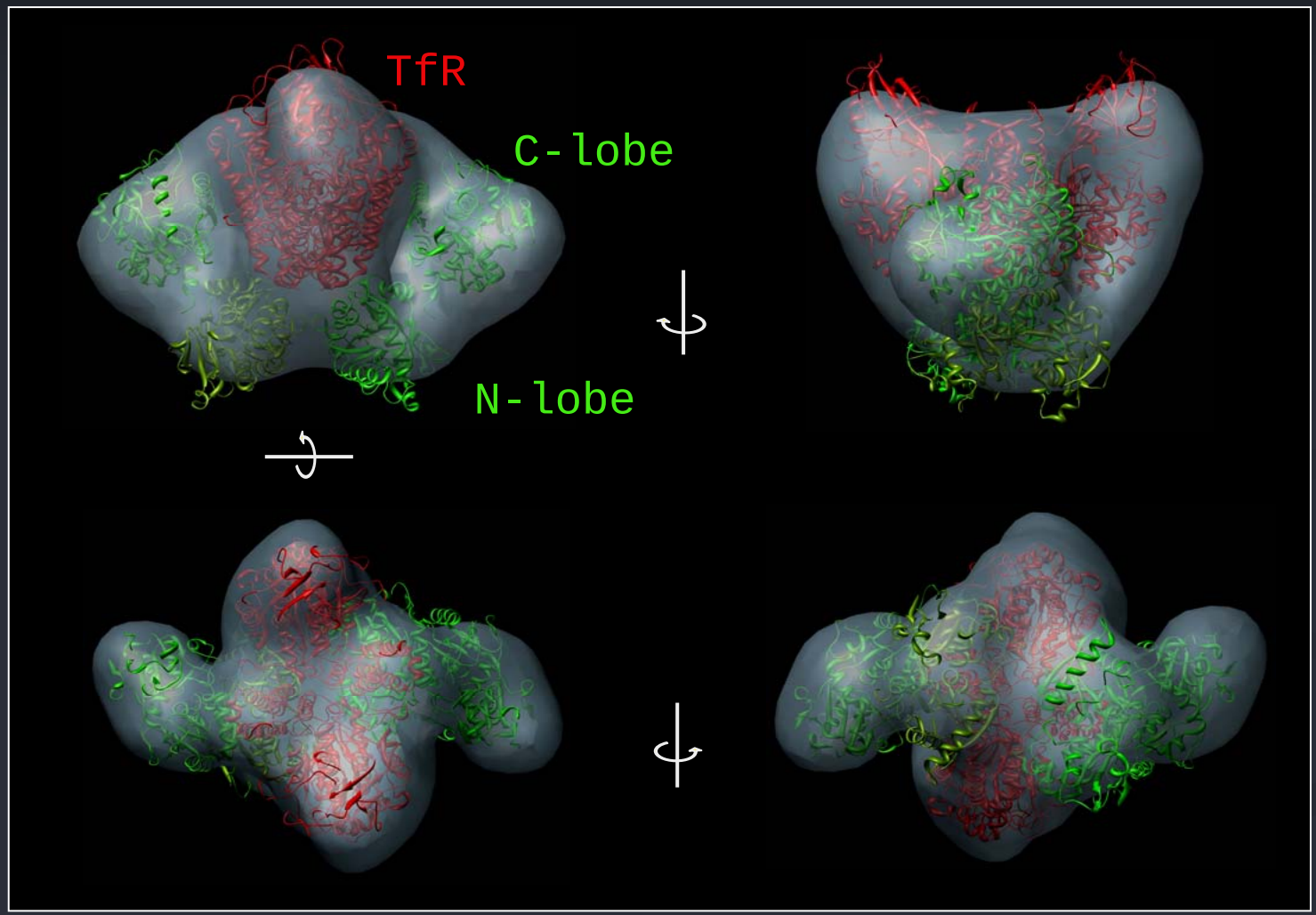


2% ML



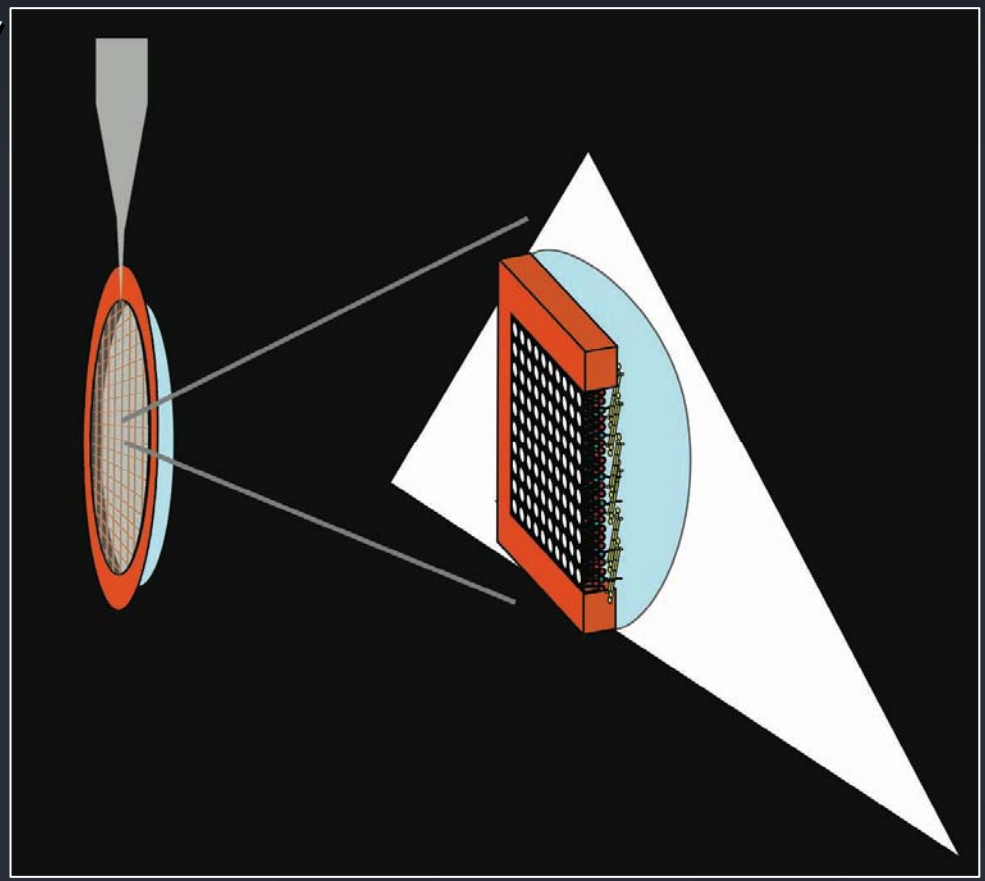
2% ML + 50 mM Imid.

Neg. stain reconstruction using RCT



Vitrification of ML specimens

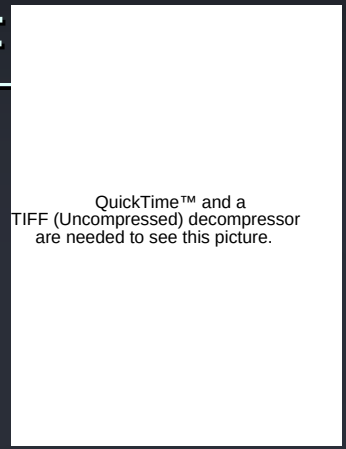
- Place “Grid bar” on top of ML
- Remove grid w/ forceps
- Blot 3μl sub-phase
- Plunge into ethane



Manual vs. Automated Freezing

- Manual
- Uncontrolled environment
- Calibrate blotting
- Consistent ice over entire grid

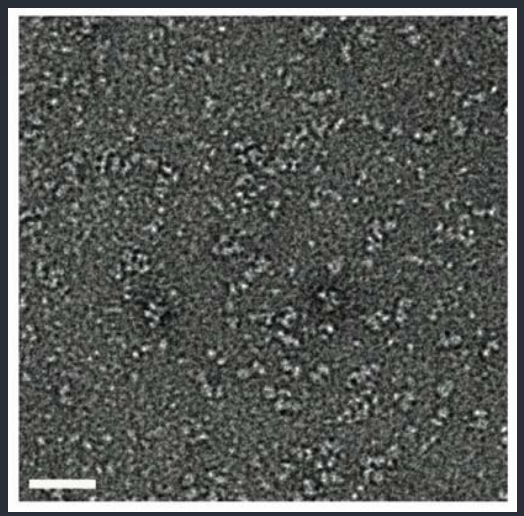
- Vitrobot (F
- Environment (22°C, 65% rh)
- Blotting time ∝ volume (μl)
- Gradient of vitreous ice



Low-dose imaging

- Tecnai F-20 operating at 200 kV
- Quantifoil 2/1 (2μm holes, 1μm spacing)
- Magnification = 50,000x
- Defocus = - 2 to - 5 μm
- 10 e^- / Å^2, 1 sec exposure
- Images on Film, scanned w/ 7μm step (1.4 Å / pixel, 3 x 3 sub-sampling)

Imaging Monolayer Purified Tf-TfR from Sf9 extracts



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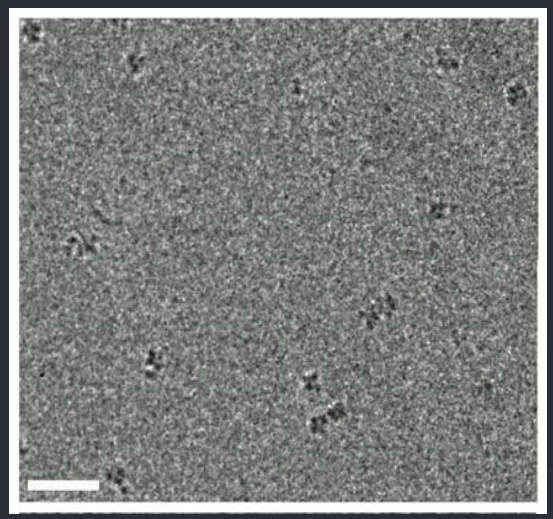
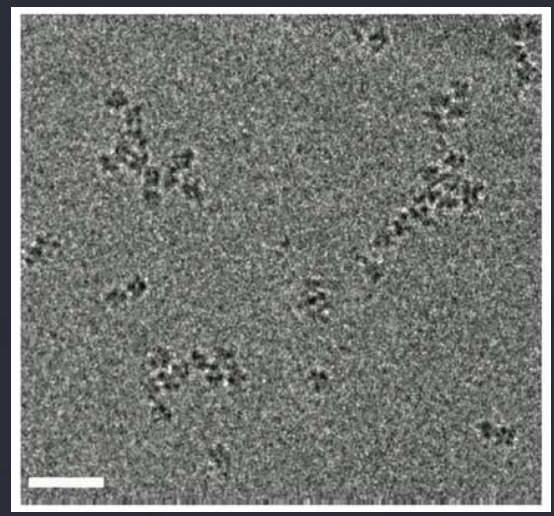
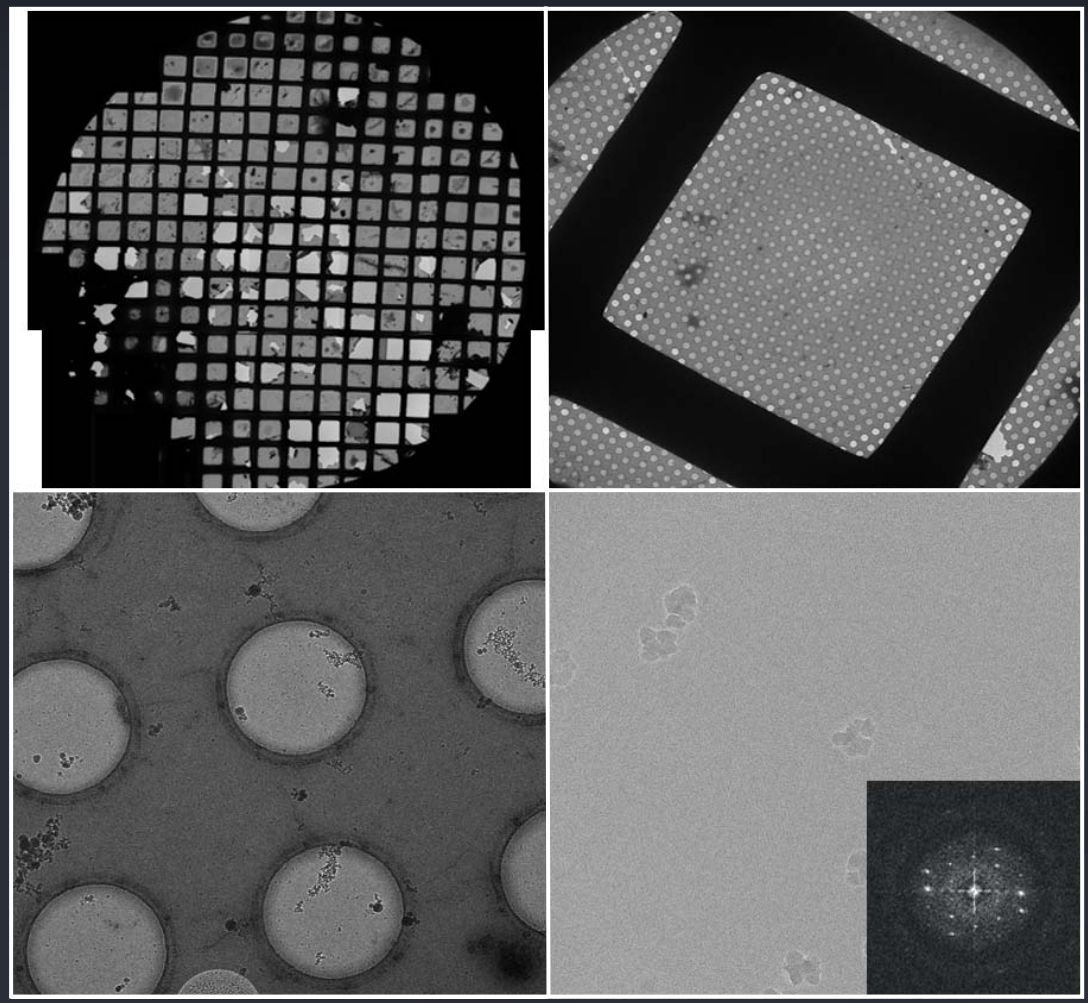


2% ML



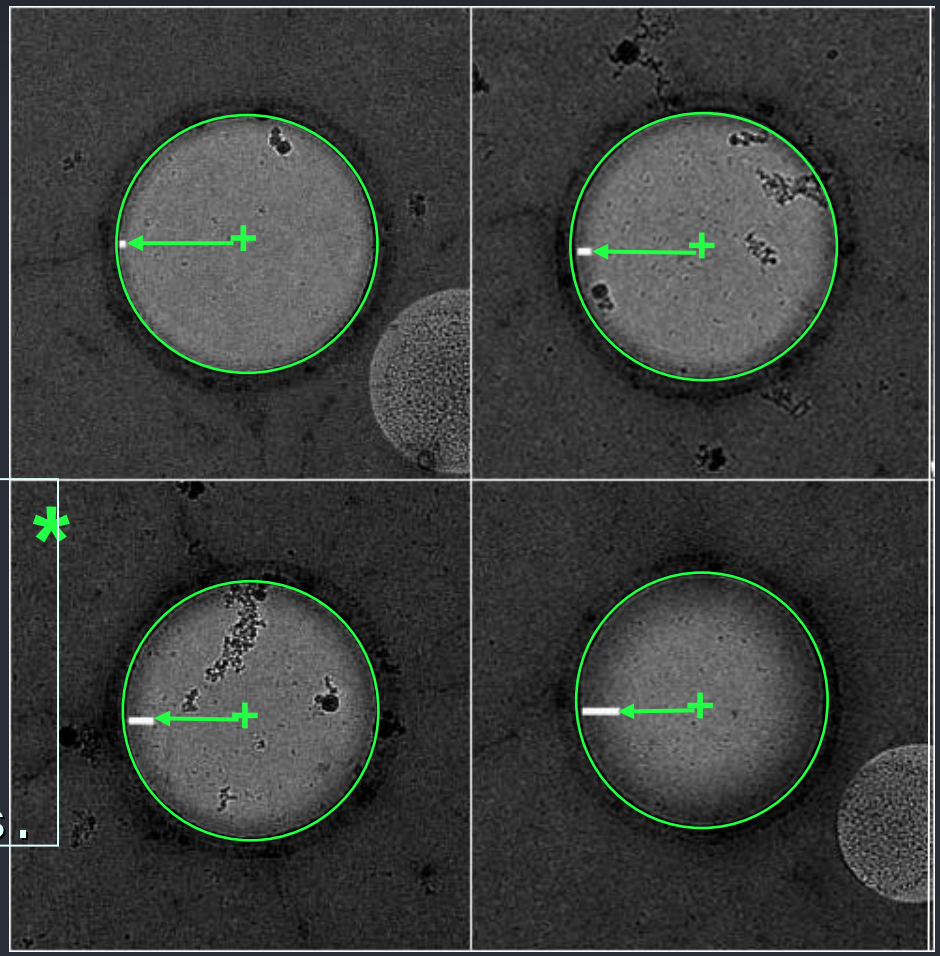
20% ML

Leginon for Screening ML Cryo-EM specimen



Taylor et al., JSB (in press)

Hole selection based on radial density function



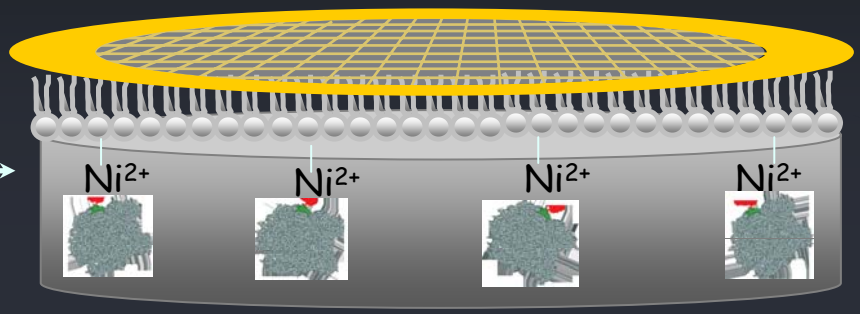
* 28,000 holes w/ crystals; 200 parts./hole

5 - 8 x 10^6 parts.

High-throughput Potential

1) Grow Construct

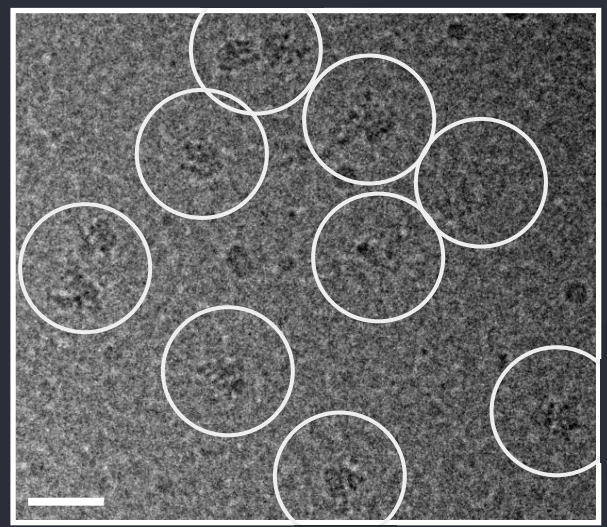
2) Prep. grid



3) Direct

4) Neg. Stain

Transfer screen



5) Vitrification

6) Cryo-EM

Current interests

- Adapt ML method for use with
  membrane proteins and other tags
- 3D reconstructions of native complexes
  large data sets and automated routines

Acknowledgements

- Danijela Dukovski
- Tom Walz

  Dept. of Cell Biology

  Harvard Medical School