CHM 165,265 / BIMM 162 / BGGN 262 Spring 2013

Lecture Slides

Jan 31, 2013

CHM 165,265 / BIMM 162 / BGGN 262 Spring 2013

Announcements for Jan 31, 2013

Reading assignment for next Thursday:

Keep an eye on TED class site for new assignments

Midterm Exam: Tuesday 8:00-9:20 am

Covers material presented through today's lecture and all lecture notes (pp. 1-208 for CHM 265/BGGN 262 students and pp.1-123,146-208 for CHM 165/BIMM 162 students)

'Virtual' homework: Answers to all sets will be posted outside NSB 4-105 *after* Friday's help session

Recitation session: Friday 5:00-6:00 pm in York 4080A

Last help session before midterm exam on Feb 5

CHM 165,265 / BIMM 162 / BGGN 262 Winter 2013

3D Electron Microscopy of Macromolecules

Midterm *next* Tuesday, Feb 5, 2013
8:00 - 9:20 AM

Peterson Hall, Room 103
Bring pencils, a ruler, and a calculator

Covers material presented through end of Jan 31st lecture. See ANNONUCEMENTS slide for further important details

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Help session THIS Friday, Feb 1st
York 4080A 5:00-6:00 PM

I.E OPERATION OF THE TEM

KEY CONCEPTS FROM LECTURE #7

- Recording Images Digitally (on CCD or DDD):

Nyquist Criterion

Pixel size of digital image must be **AT LEAST two times <u>FINER</u>** than the desired or expected resolution of the magnified electron image

- Recording Images Digitally (on DDD):

DDD Advantages:

Pixel resolution comparable to film (5-6 μm vs. ~ 5-10 μm)
Immediate image access (and much faster than CCD)
Detects electrons directly as opposed to indirectly in a CCD
Large dynamic range
Strict linear response with electron dose
Amenable (like CCDs) to numerous automated microscopy tasks

DDD Disadvantages:

Limited number of pixels (~4k by 4k vs. ~16k by 20k), hence **small field of view**
High upfront cost

DDD Designs:

HPDs (**H**ybrid **P**ixel **D**etectors) vs. MAPS (**M**onolithic **A**ctive **P**ixel **S**ensor)
MAPS better suited for high resolution TEM work

I.F OTHER MODES OF TEM OPERATION

KEY CONCEPTS FROM LECTURE #7

- Electron Diffraction

Crystalline specimens
Patterns are series of rings (random oriented samples) or discrete lattice of sharp spots (single crystals)

- Dark Field EM

Only uses scattered electrons
High contrast
More radiation damage

- High Resolution, High Voltage Microscopy TEM

Very short λ electron beam delivers highest potential resolution
Study thick specimens (up to several microns)

- Tilt and Stereo TEM

Reveal 'hidden' aspects of specimen
Stereo doesn't reveal full 3D structure of specimen

- Low Temperature TEM (to be discussed)

- Electron Energy Loss Spectroscopy (EELS)

Use different wavelengths of inelastically scattered electrons to locate specific atoms in the specimen

- X-ray Microanalysis

Scan small electron probe across specimen and measure wavelengths of emitted X-rays to characterize atoms in specimen

Slide not shown in class lecture

II.A SPECIMEN PREPARATION TECHNIQUES

KEY CONCEPTS FROM LECTURE #7

The Goal: Obtain TEM images that represent the specimen in its native state as **faithfully** as possible

Obstacles:
- **Contrast**
- **Thickness**
- **Dehydration**
- **Radiation Damage**

Grids/Support Films:

3mm copper grids + surface on which to deposit samples

Most common support films: C and C-stabilized plastic

Ideal qualities:
- Amorphous
- Good conductor
- Adequate physical strength
- Thin (low electron scattering)

II.A SPECIMEN PREPARATION TECHNIQUES

MORE CONCEPTS FROM LECTURE #7

- Thin Sectioning

Mostly used with **tissue** samples
Sectioning needed to get specimen thin enough for TEM
Four major steps: fixation, dehydration and embedding, sectioning, and staining
Resolution generally limited to ~40-50 Å

- Negative Staining

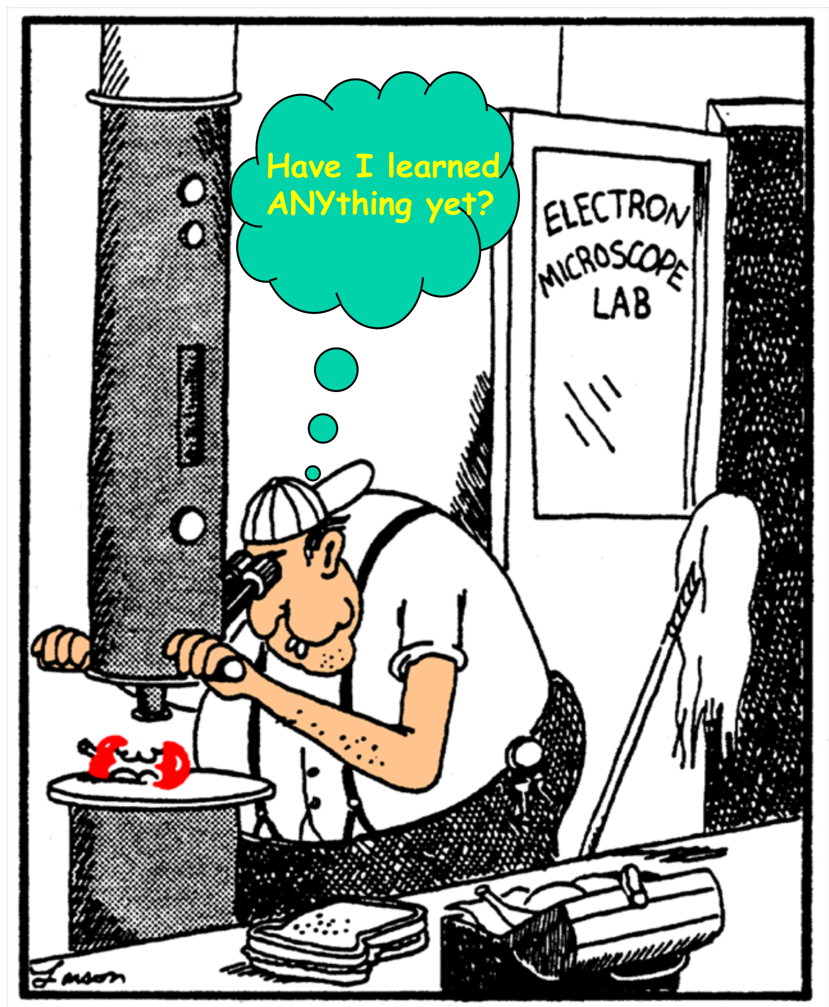
Mostly used with **macromolecules and macromolecular complexes**
Quick and easy
Increases mass thickness (gives excellent aperture contrast)
Yields good resolution (15-25 Å) and reasonable preservation.

- Metal Shadowing

Used with **particulate samples**, replicas, and freeze-fractured/etched cells to view **surface features**

- Freeze Drying/Etching/Fracture

Mostly used with **cells** to view membranes and particle distributions in membranes
Specimen preservation much better than air-drying



TOPICS

- Principles of TEM
  - Electrons, lenses and optics
- Design of TEM
  - Components top to bottom
- Contrast and image formation
  - Electron scattering from object
- Optimizing TEM performance
  - Alignment assures ‘best’ images
- Operation of TEM
  - “What do all these buttons do?”
- Other modes of TEM
  - Many ways to ‘observe’ specimens
- Specimen preparation for TEM
  - Getting specimen ready
- Radiation damage
  - Less is better
- 3D reconstruction
  - Specimen 3D structure from 2D images

§ II: The Specimen

II.A. Biological Specimen Preparation Techniques

- II.A.1 Specimen Support Films
- II.A.2 Thin Sectioning
- II.A.3 Negative Staining
- II.A.4 Metal Shadowing
- II.A.5 Freeze Drying/Etching/Fracture (183-187)
- II.A.6 Unstained and Frozen-Hydrated

§ II: The Specimen

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II.A.6 Unstained and Frozen-Hydrated

Goals

- Preserve native 3D structure of specimen
- Enhance specimen contrast **WITHOUT** stains or metal shadow
- Eliminate all preparation-induced artifacts (*e.g.* fixation, dehydration)
- Record images good to atomic resolution
- Preserve native structure
- Preserve native structure
- Preserve native structure
- Preserve native structure

Is that perfectly clear?

II.A.6 Unstained and Frozen-Hydrated

Vitrification

- Process of converting materials to glass

- In cryoTEM, aqueous samples are rapidly cooled (vitrified) to ~ -180° C to prevent bulk water from forming ice crystals

- **Primary advantage:** preserve 'native' structure of specimen (no chemical fixatives or stains)

- Devitrification occurs if water warms above -135° C

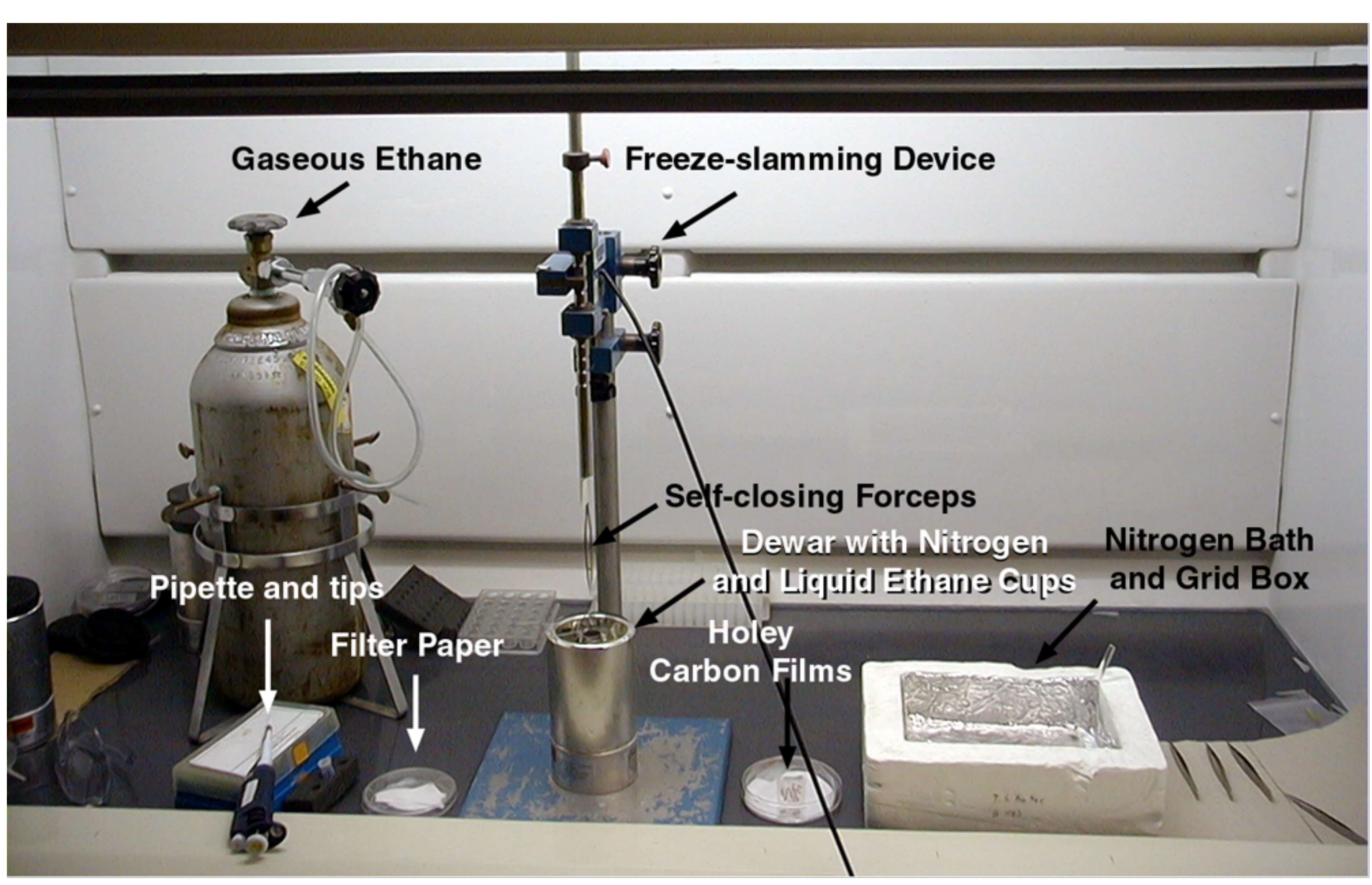
II.A.6 Unstained and Frozen-Hydrated

Cryo-EM Procedure

- Prepare carbon or **holey-carbon** grids
- Glow discharge grid to make surface hydrophilic
- Apply **2-5 μl** specimen to grid
- Blot grid nearly (not totally) dry
- Plunge freeze (**vitrify**) sample in ethane slush
- Transfer grid to cold holder
- Insert cold holder into microscope
- → Search grid for ‘good’ specimen
- → Adjust defocus and stigmate **off** the ‘good’ specimen
- → Record **minimal exposure** image
- Take a deep breath and repeat last 3 steps…

Cryo-EM Procedure

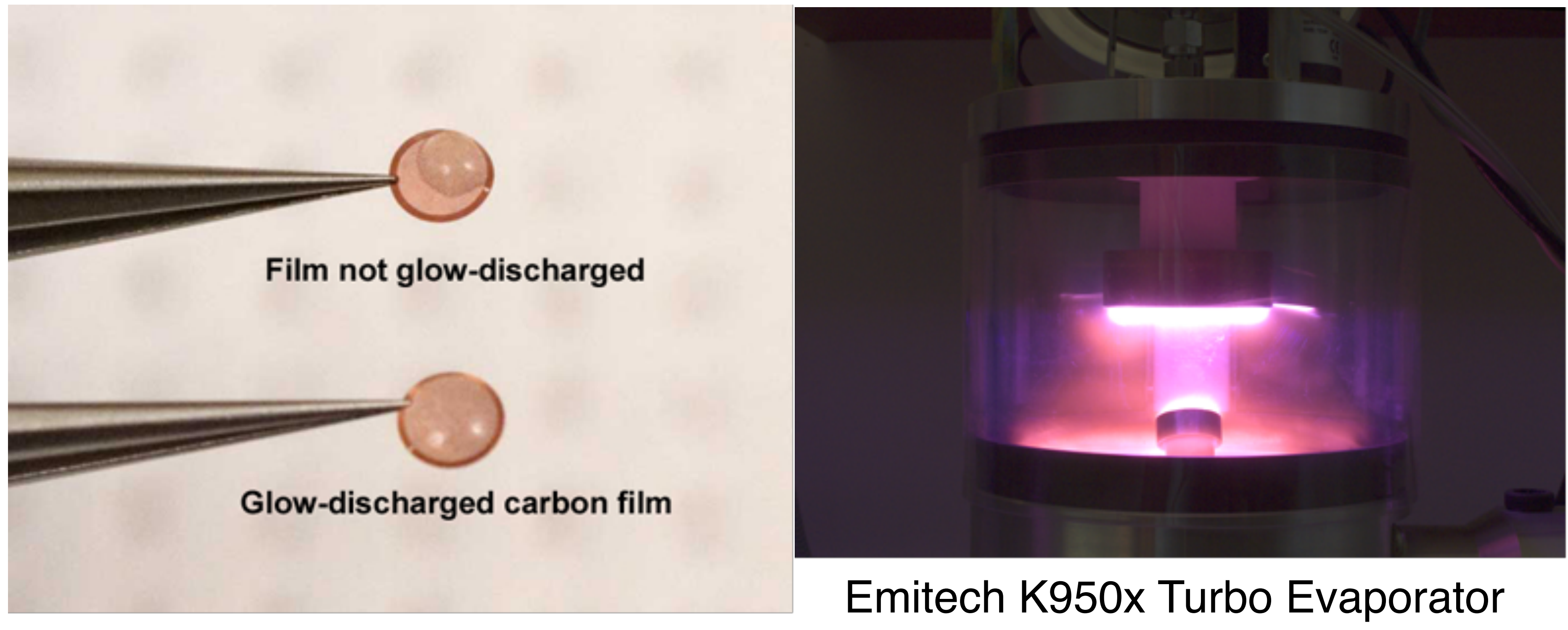
Sample Preparation Equipment



Cryo-EM Procedure

Glow Discharging Grids

Hydrophilic support film helps the sample to spread evenly across grid

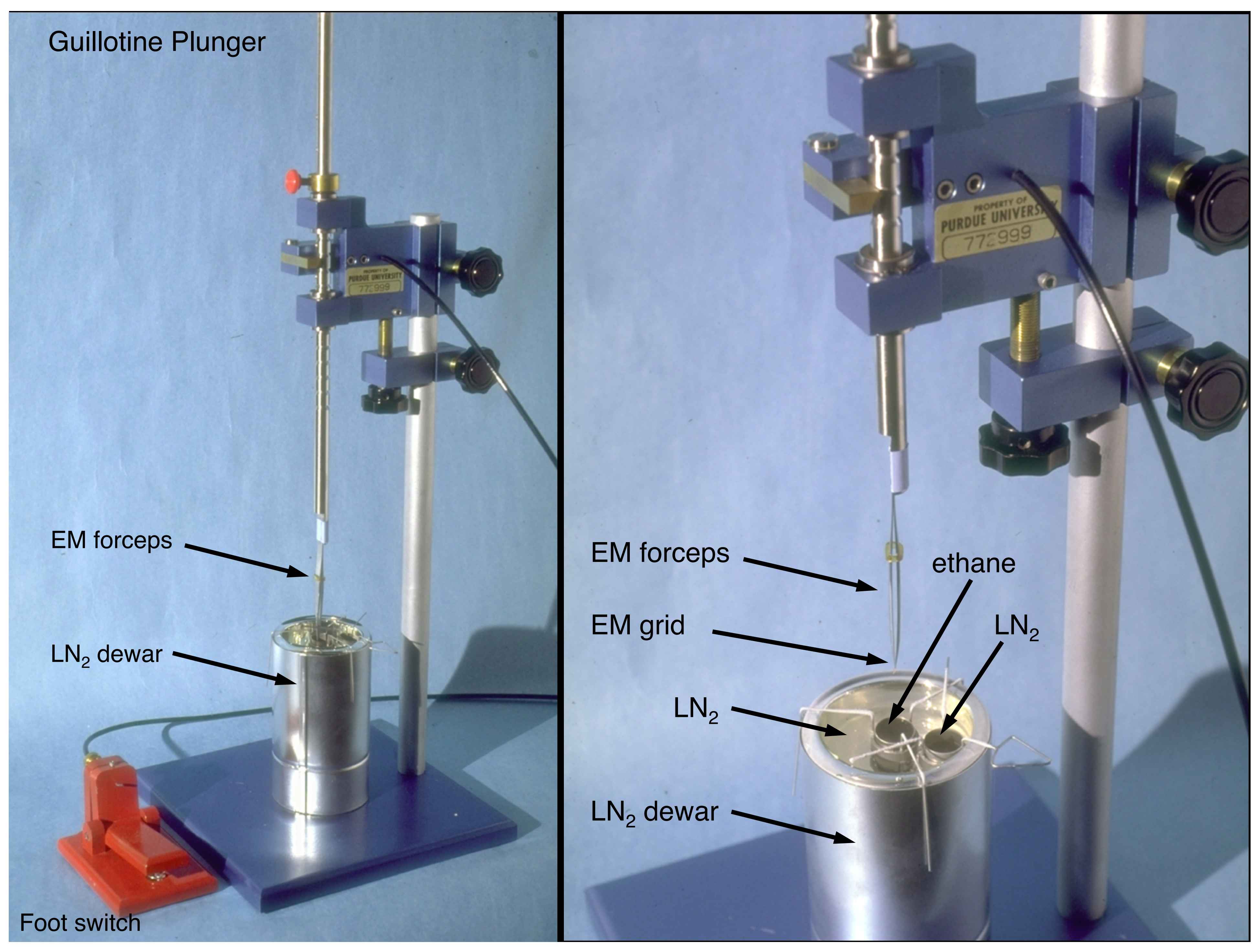


Emitech K950x Turbo Evaporator

Cryo-EM Procedure

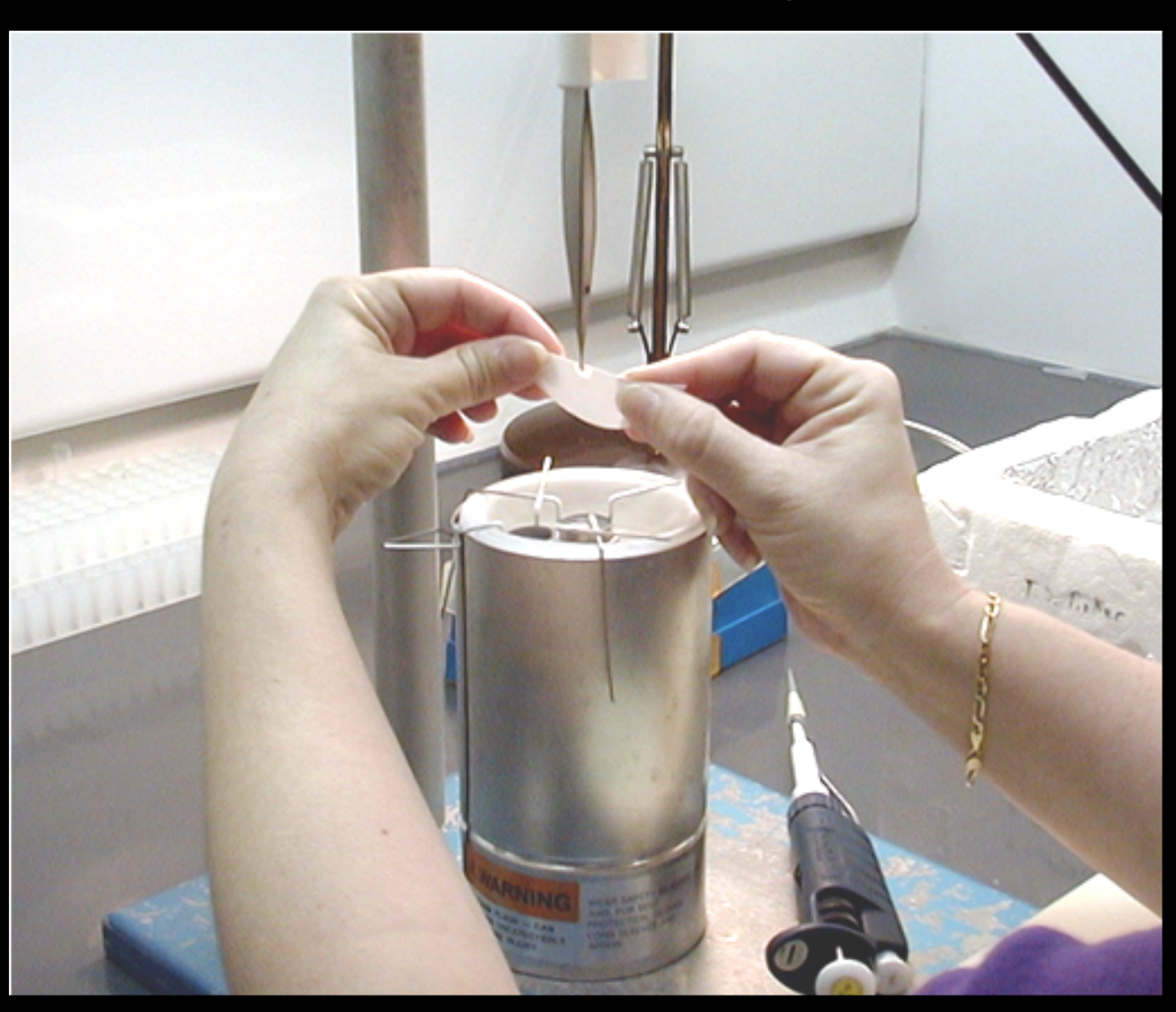
Quick freezing:

Time to get cool real fast


Guillotine Plunger
PROPERTY OF
PURDUE UNIVERSITY
EM forceps
LN_2 dewar
Foot switch
EM forceps
ethane
EM grid
LN_2
LN_2
LN_2 dewar

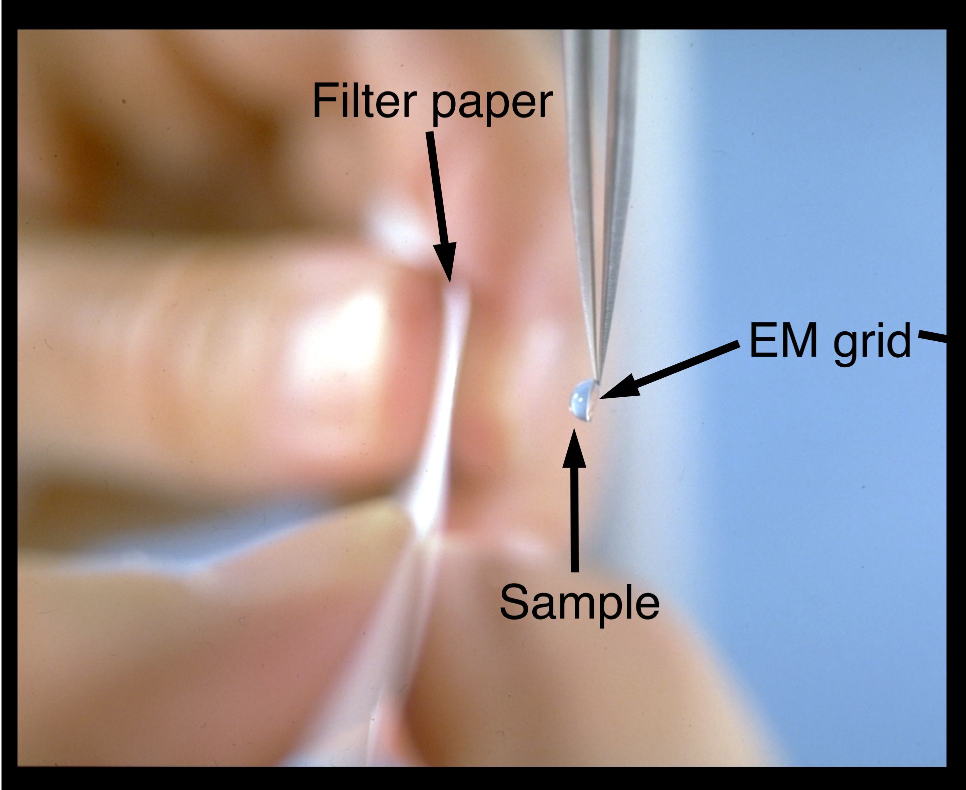
Cryo-EM Procedure

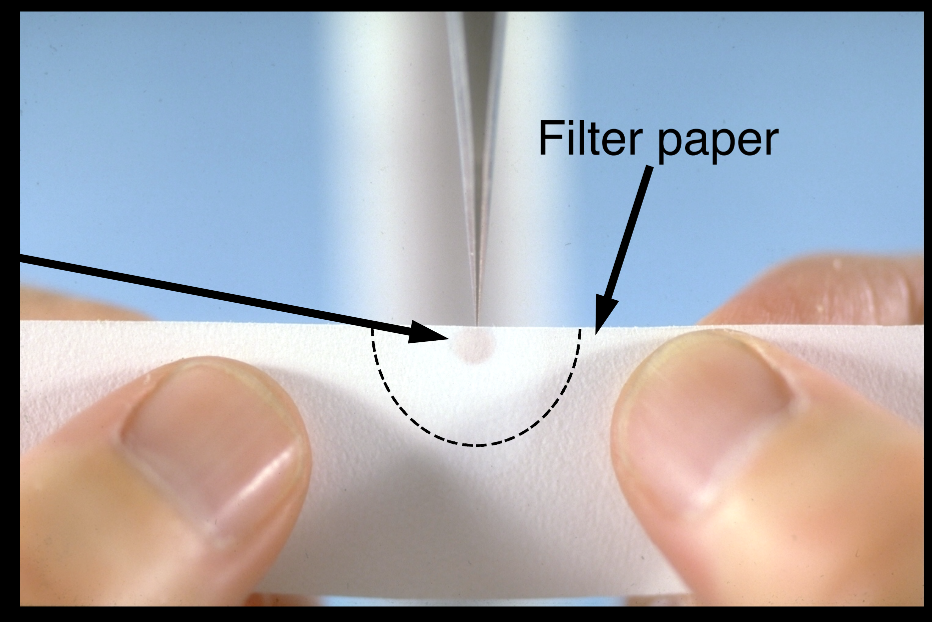
Sample Blotting



Cryo-EM Procedure

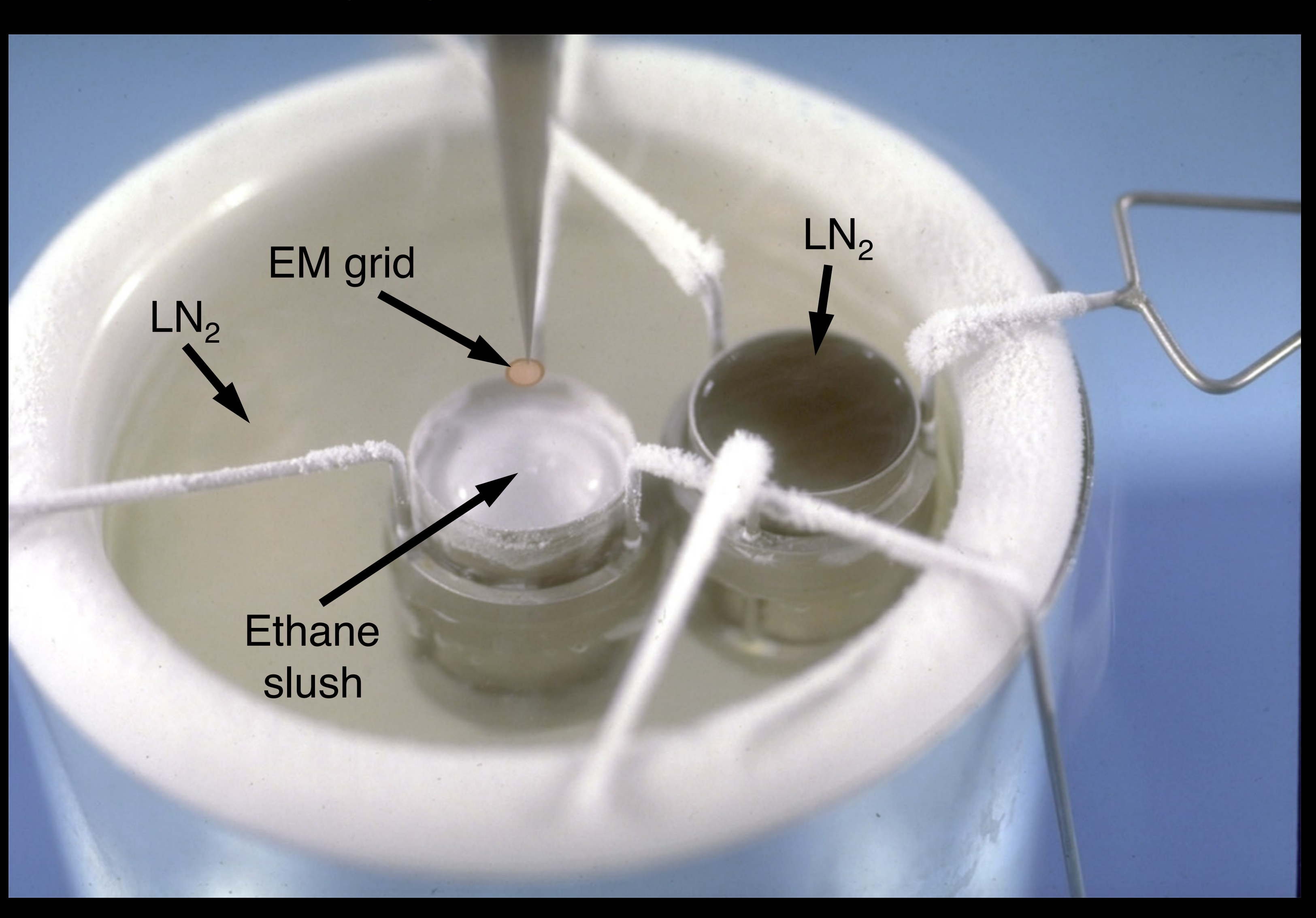
Sample Blotting





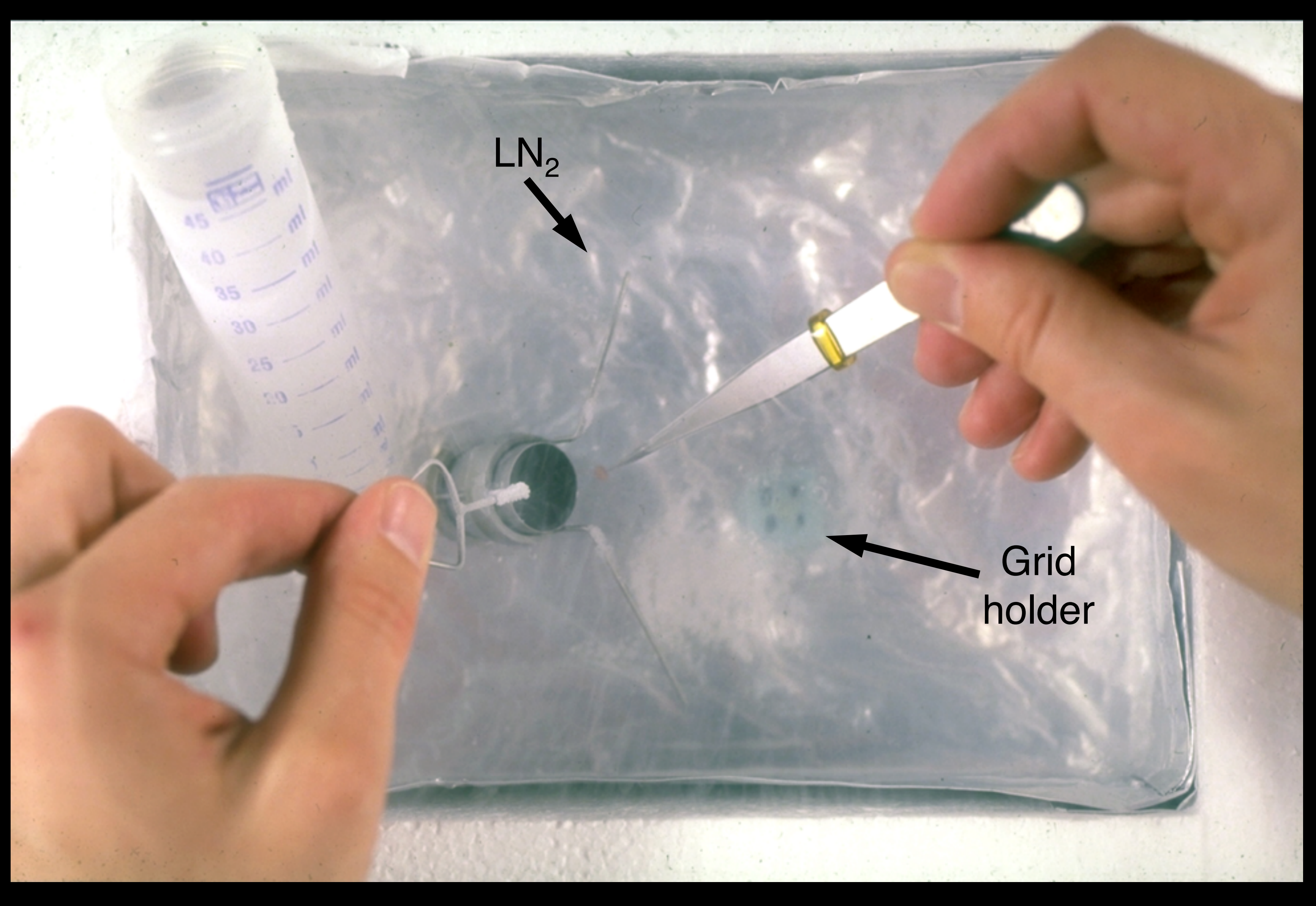
Cryo-EM Procedure

Plunging Grid into Ethane Slush



Cryo-EM Procedure

Transfer Grid Under LN_2 into Storage Box



Cryo-EM Procedure

Quick freezing: Let a robot do it?

Cryo-EM Procedure

Automatic Blotting and Plunge Freezing

FEI Vitrobot

Environmental chamber

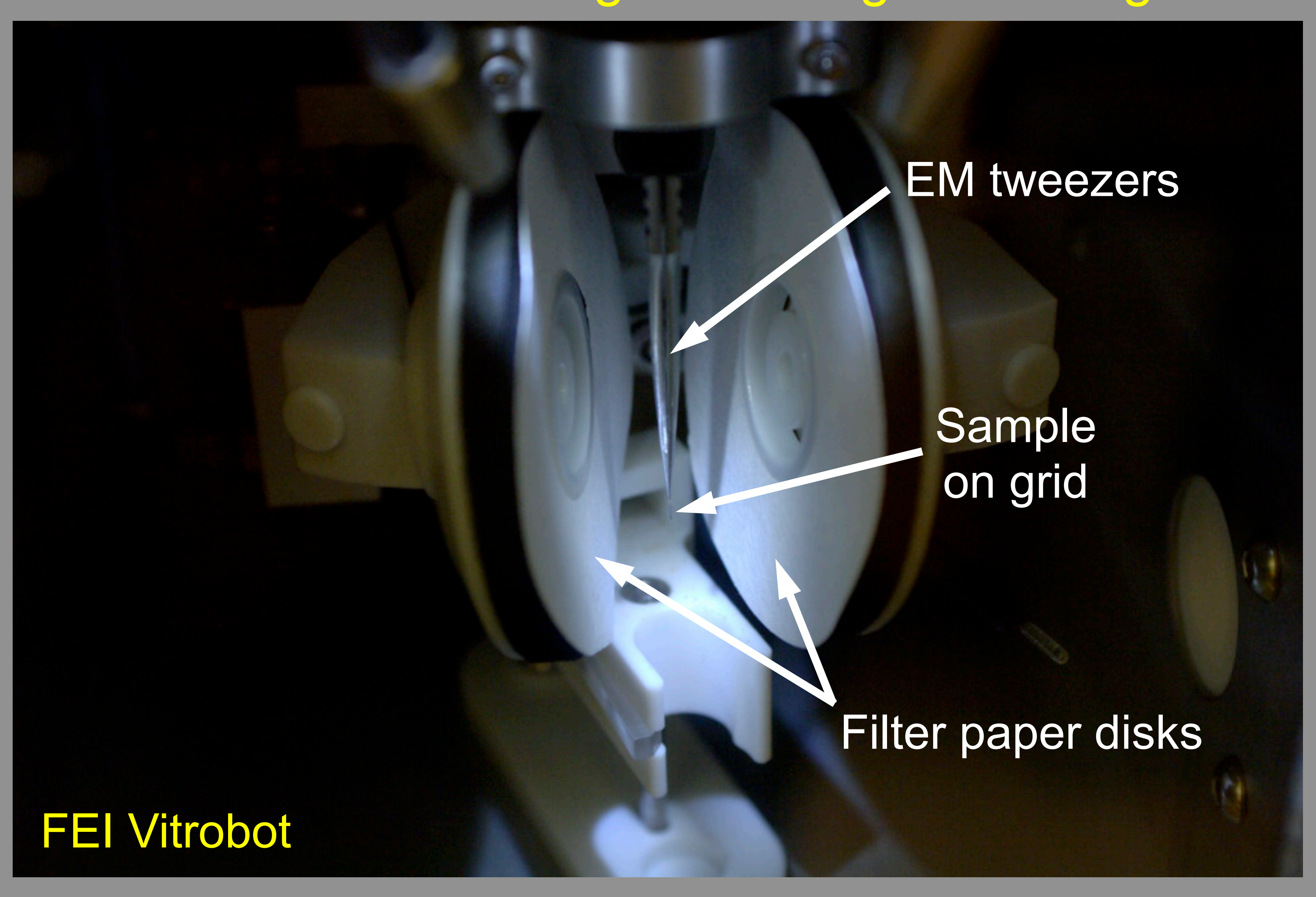
Computer-controlled

Cryo-EM Procedure

Automatic Blotting and Plunge Freezing

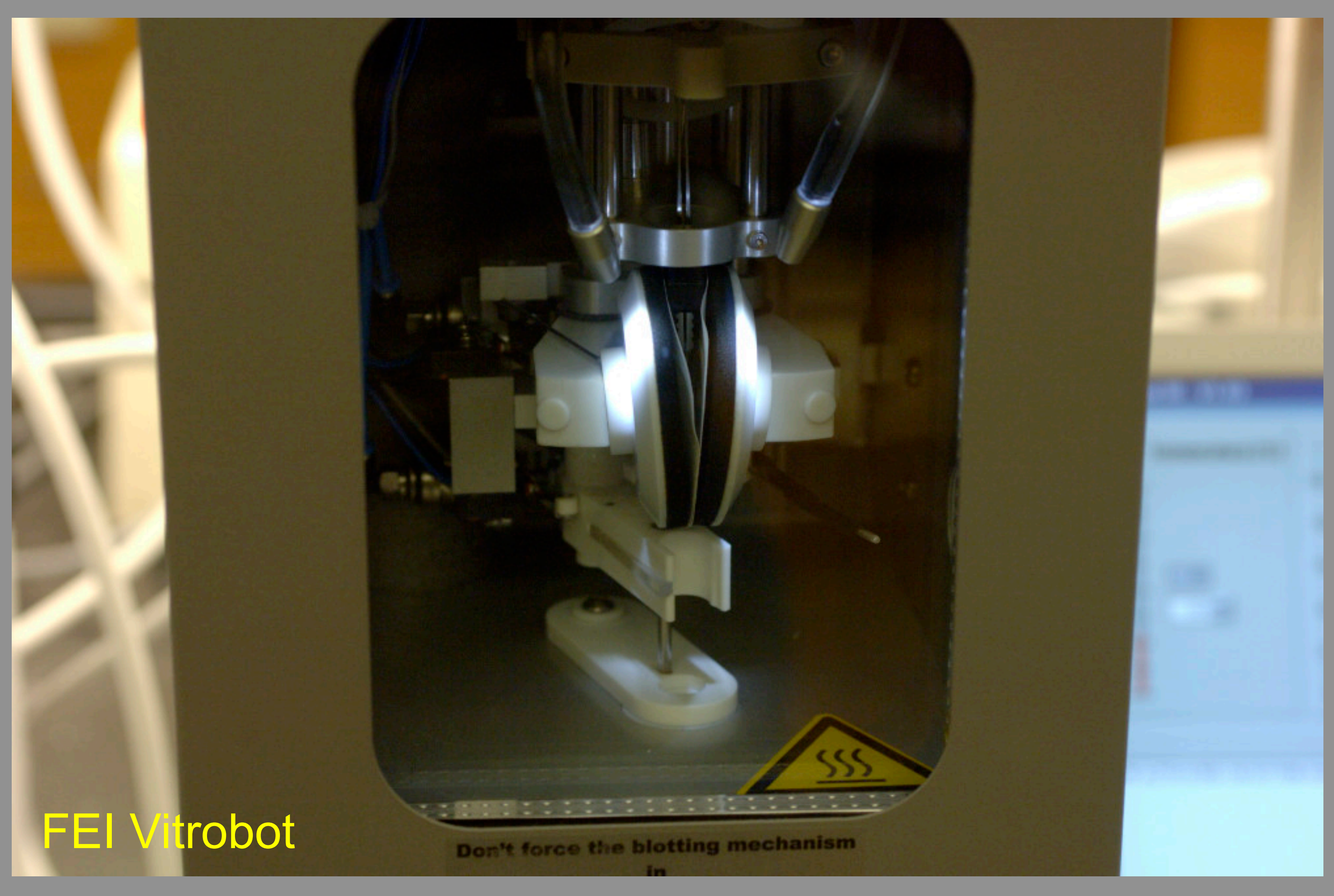
Cryo-EM Procedure

Automatic Blotting and Plunge Freezing



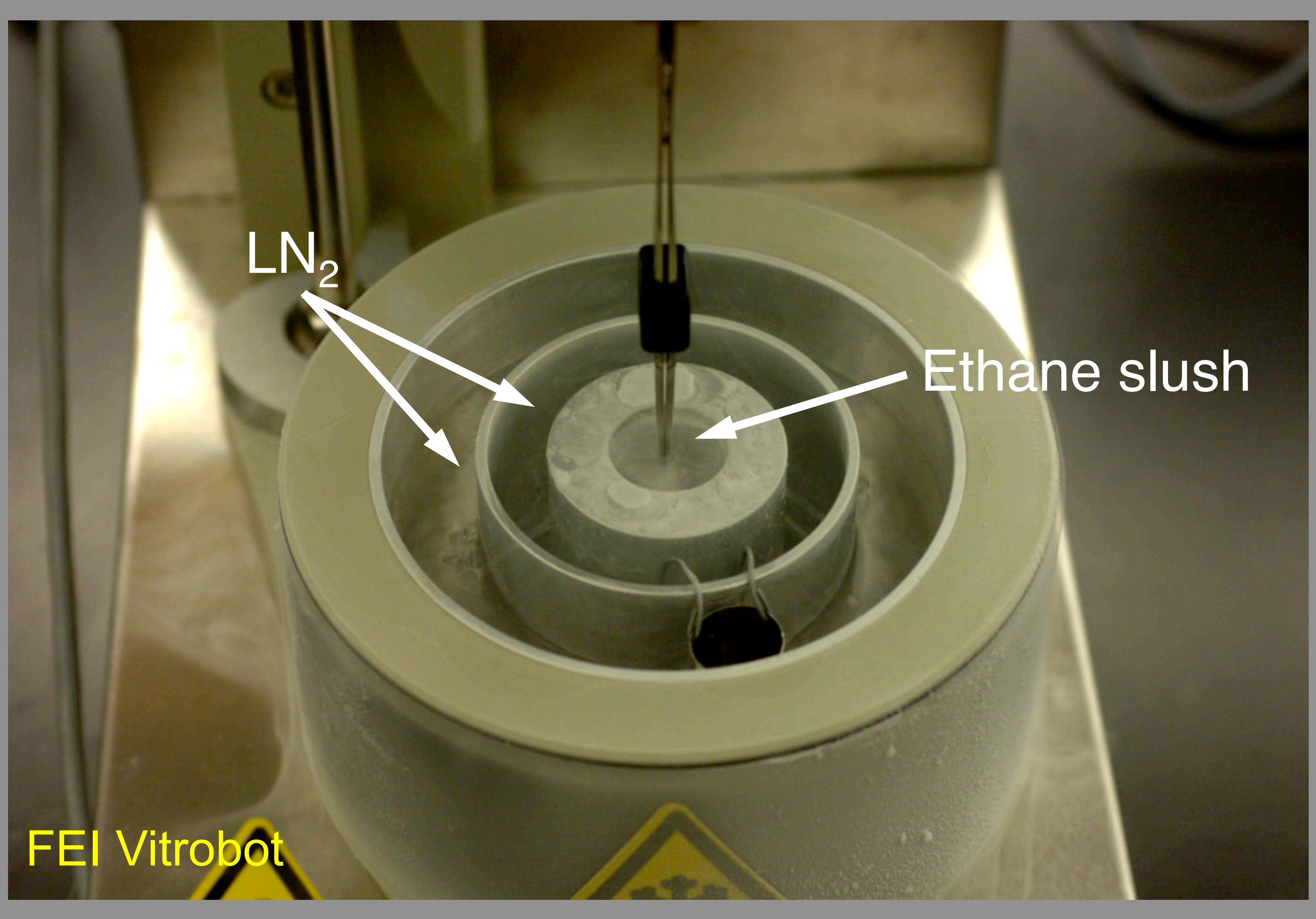
Cryo-EM Procedure

Automatic Blotting and Plunge Freezing



Cryo-EM Procedure

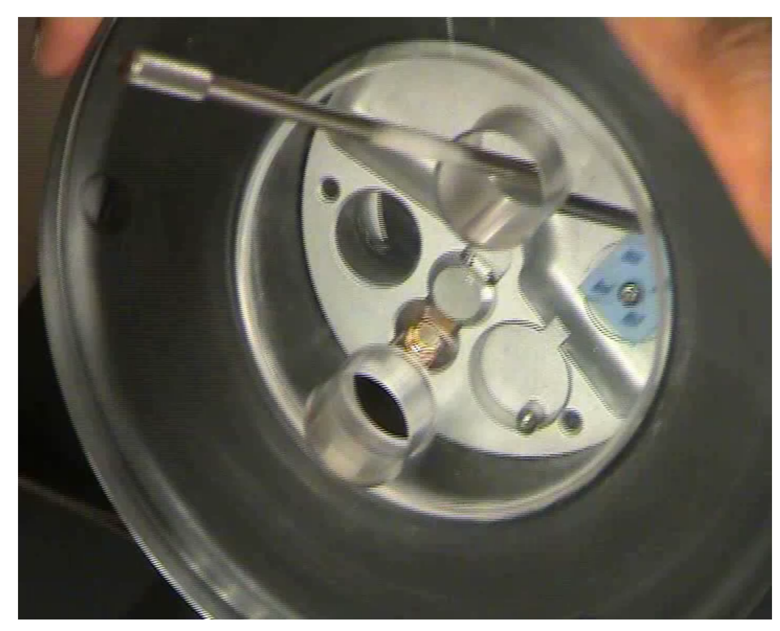
Automatic Blotting and Plunge Freezing



II.A SPECIMEN PREPARATION TECHNIQUES

II.A.6 Unstained and Frozen-Hydrated

Movies posted on Class Web sites



Loading the Gatan 626 cryo-holder

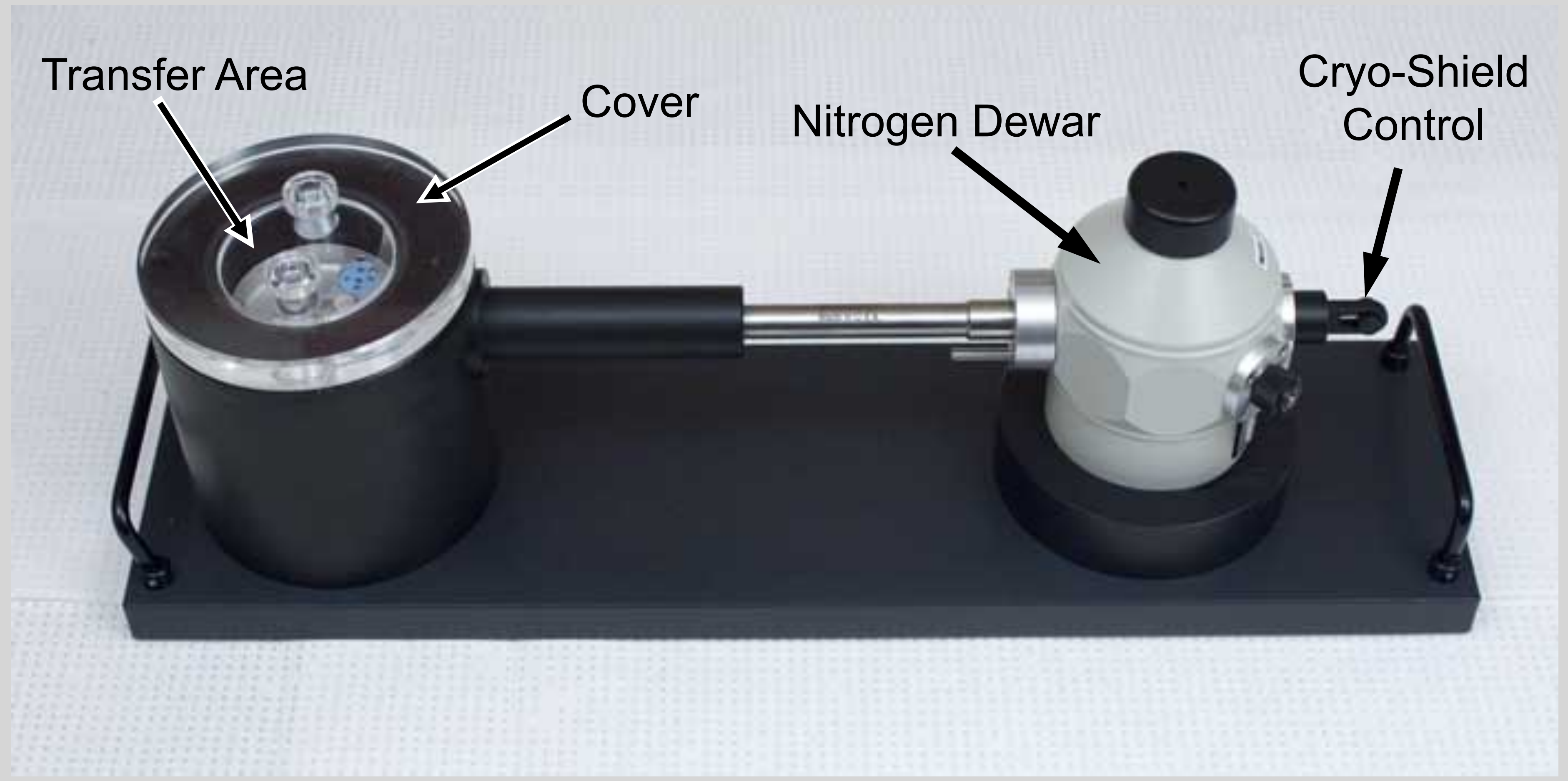
Manual plunge freezing

Cryo-EM Procedure

Time to transfer the grid to the microscope while keeping it *COLD*

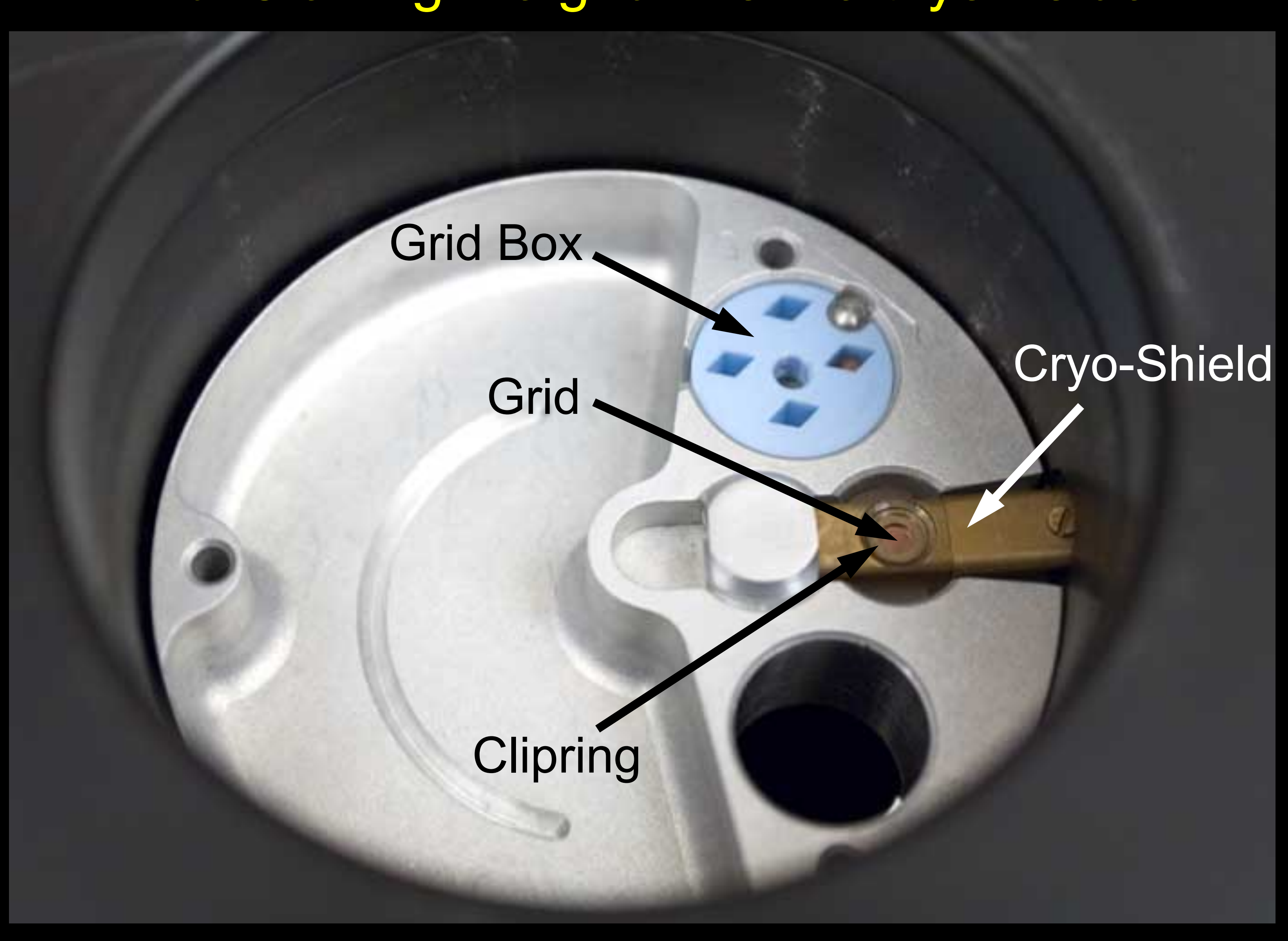
Cryo-EM Procedure

Gatan Cryo-Transfer Workstation and Holder



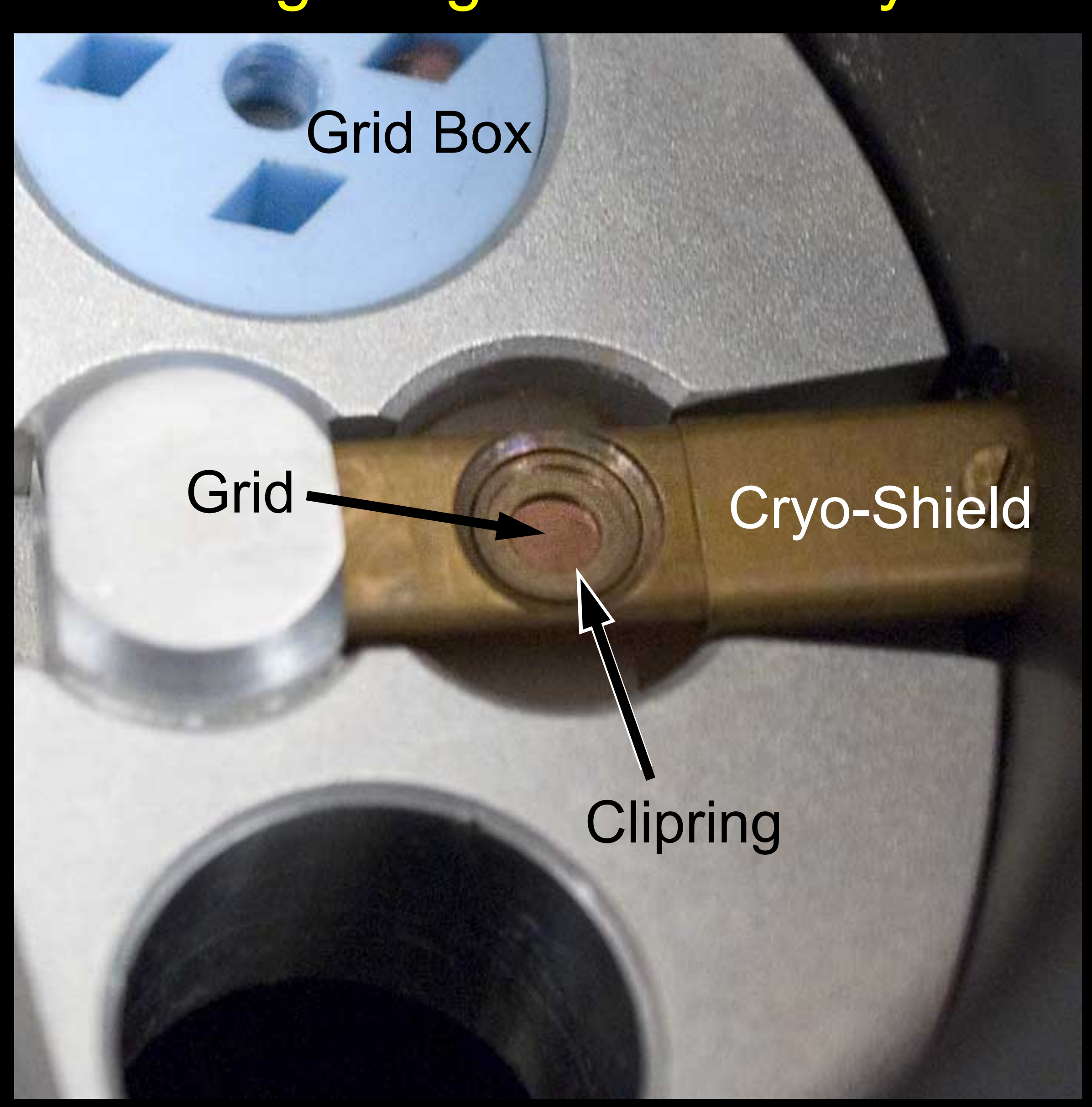
Cryo-EM Procedure

Transferring the grid into the cryo-holder



Cryo-EM Procedure

Transferring the grid into the cryo-holder

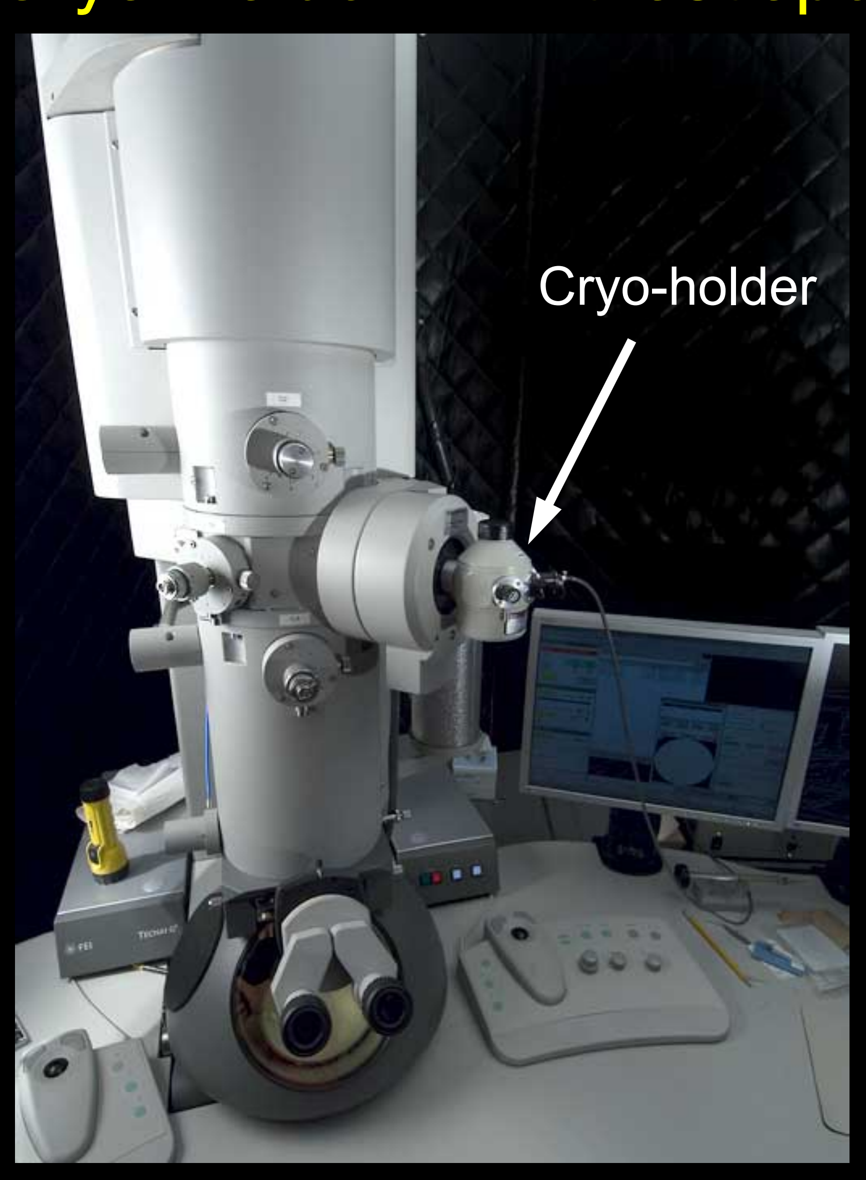


Cryo-EM Procedure

Transferring the grid into the cryo-holder

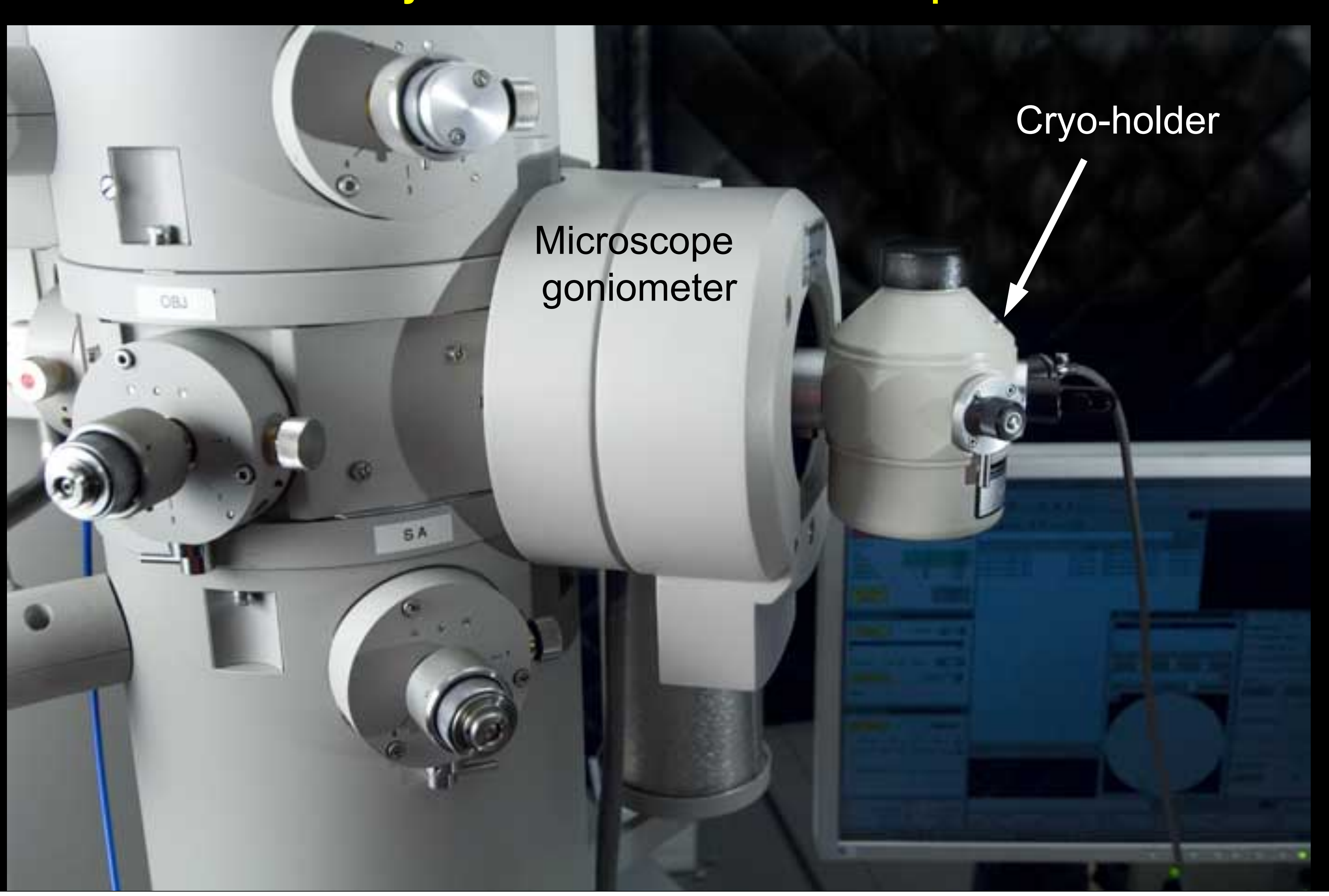
Cryo-EM Procedure

Cryo-Holder in Microscope



Cryo-EM Procedure

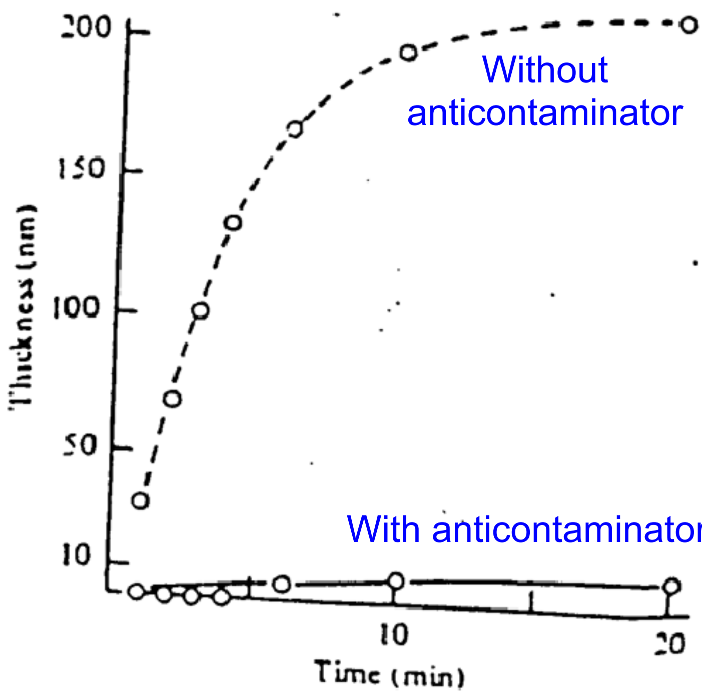
Cryo-Holder in Microscope



II.A.6 Unstained and Frozen-Hydrated

Contamination Problem

Anticontaminator is essential when working with frozen-hydrated specimens



Cryo-EM Procedure

Time to search for some-thing that looks interesting

II.A.6 Unstained and Frozen-Hydrated

Cryo-EM Procedure

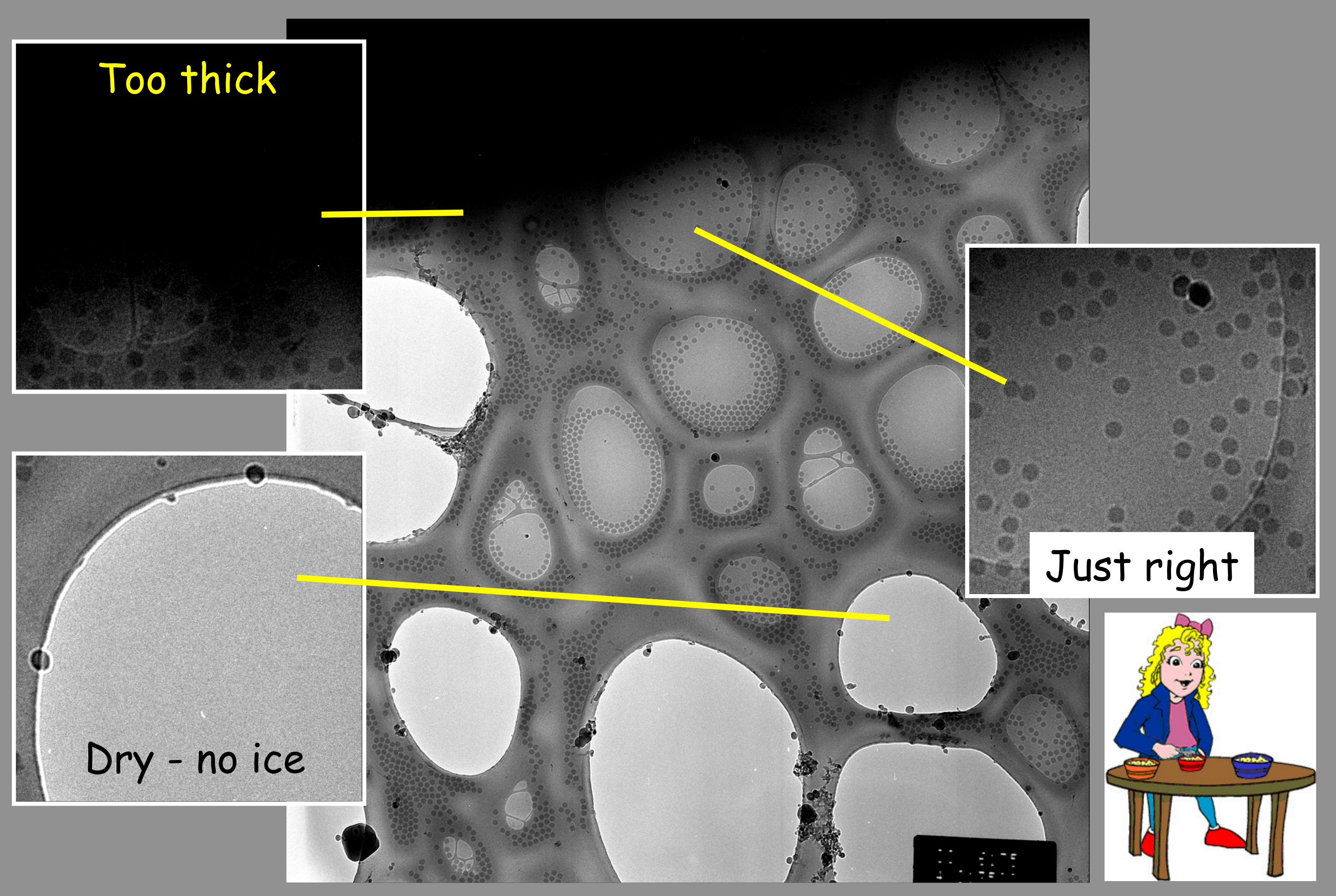
Searching for a "Good" Specimen

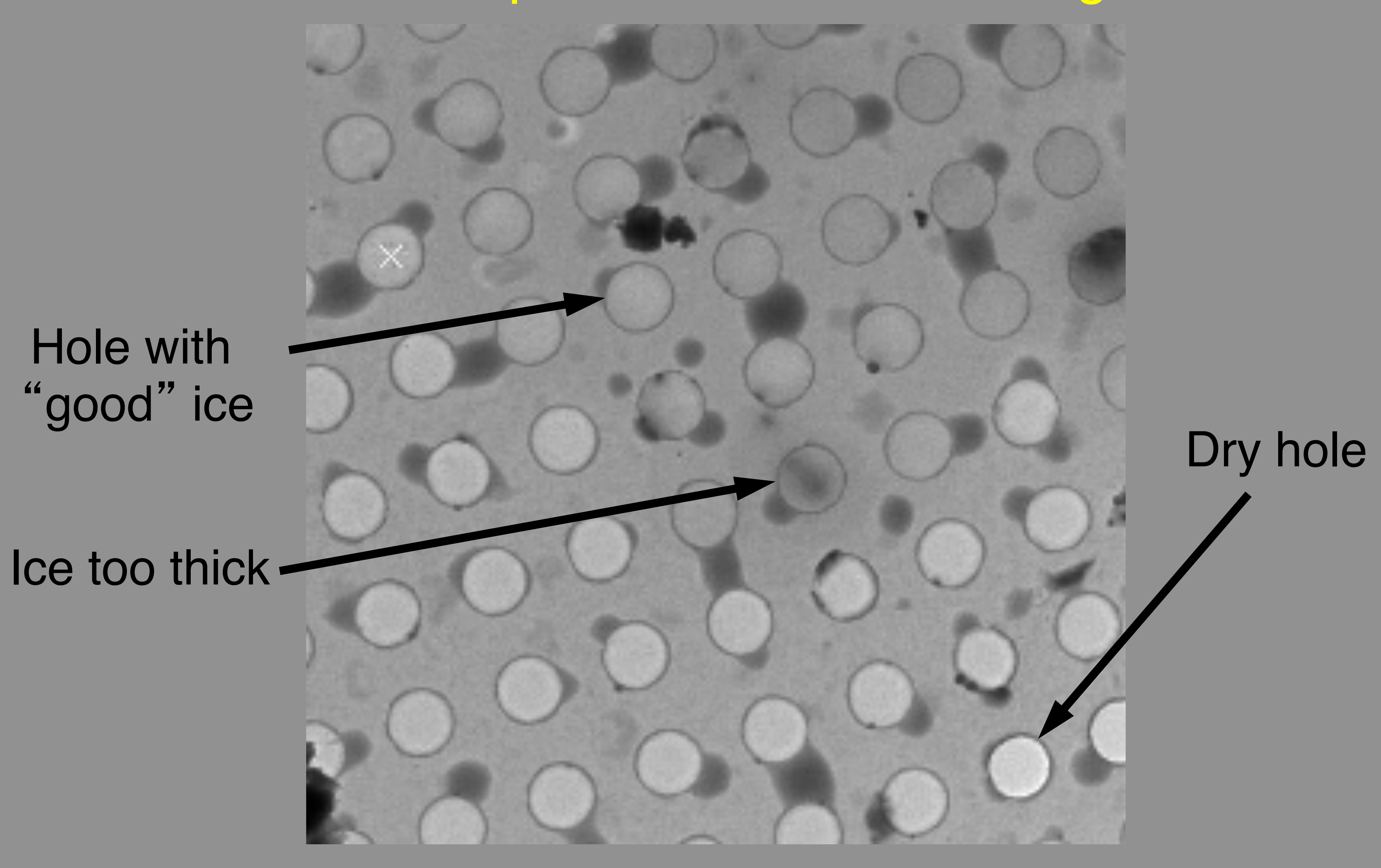
GOAL: Find vitrified sample of **optimum** thickness and concentration

TRICK: Search grid squares at **low magnification** and dose rate ($<0.05\ e^-/Å^2/sec$) to minimize radiation damage as much as possible

Cryo-EM Procedure

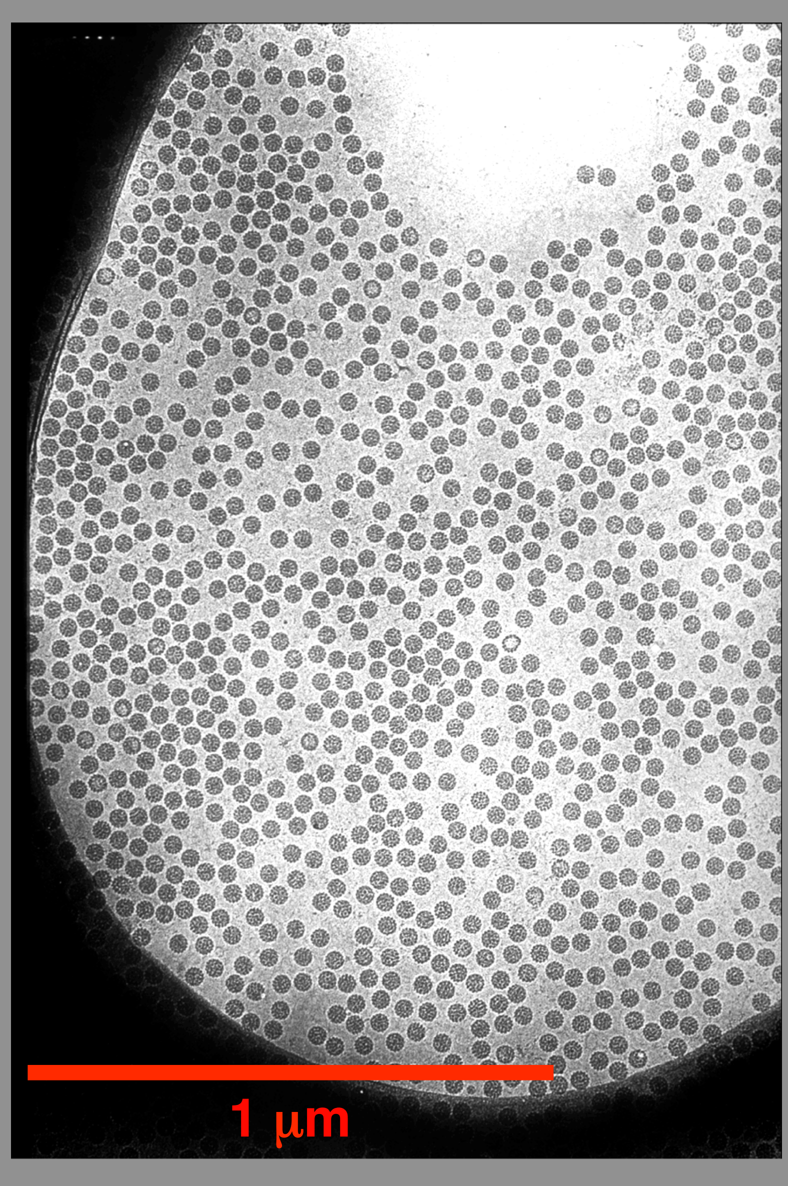
Search for ‘good’ specimen on holey support film




Cryo-EM Procedure
Vitrified specimen on Quantifoil grid
Hole with
“good” ice
Ice too thick
Dry hole
Image courtesy of R. Milligan

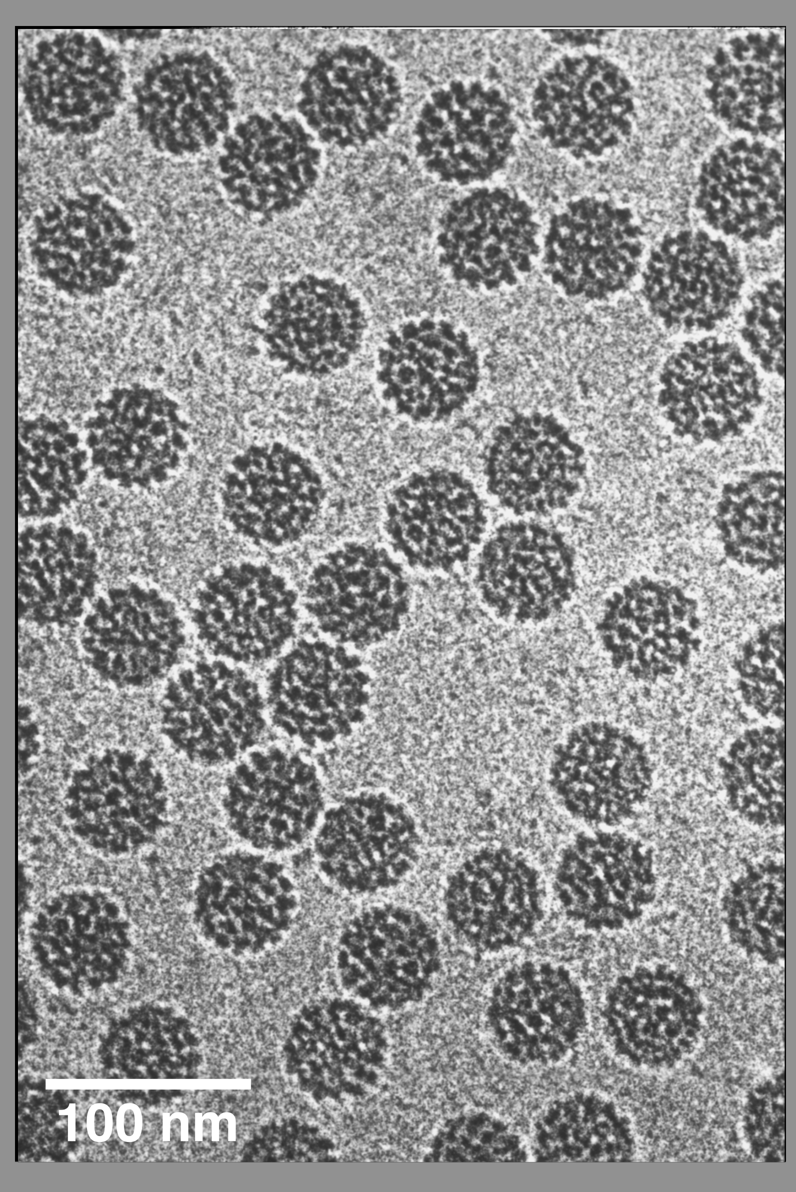
Cryo-EM Procedure

Vitrified SV40 virus specimen on holey carbon film



Cryo-EM Procedure

Vitrified SV40 virus specimen on holey carbon film



Cryo-EM Procedure

Contrast

“Where the heck is my specimen?”

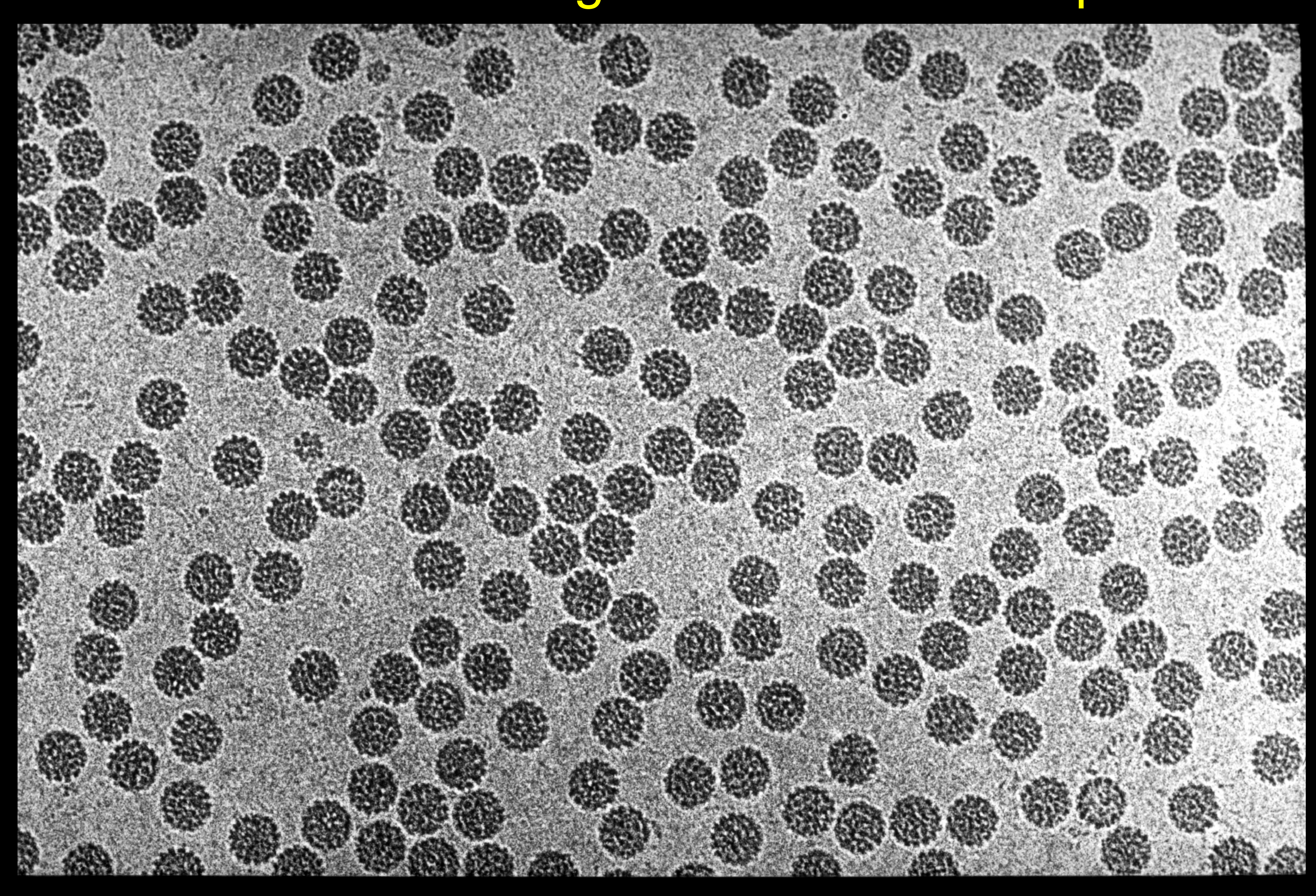
Cryo-EM Procedure

Image of vitrified SV40 specimen

Yes folks, this is what 'raw' cryo-EM images look like...

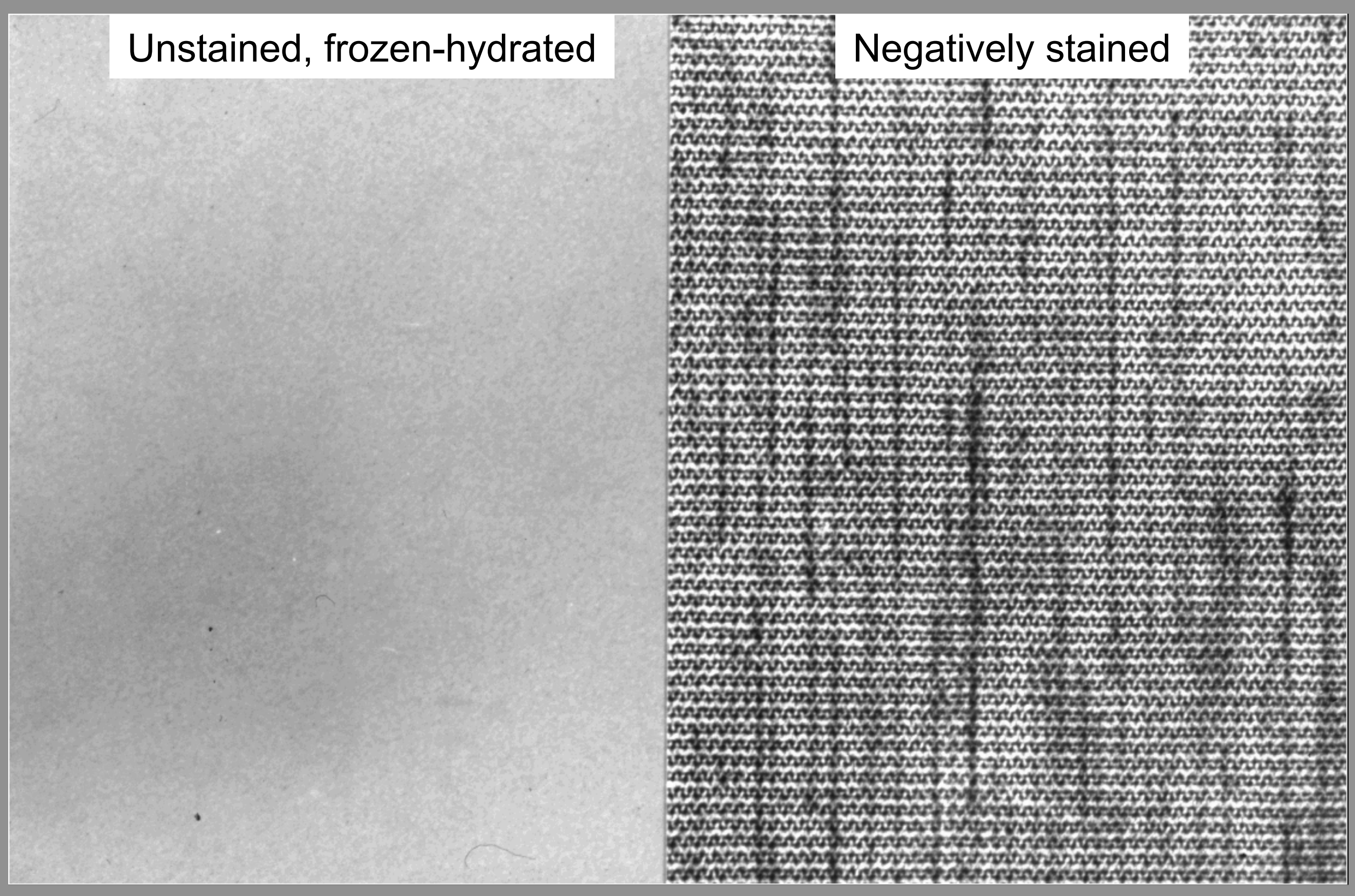
Cryo-EM Procedure

Contrast stretched image of vitrified SV40 specimen



Cryo-EM Procedure

Contrast in unstained and stained 2D catalase crystals



Images courtesy of R. Milligan

II.A.6 Unstained and Frozen-Hydrated

Cryo-EM Procedure

Where does contrast come from?

Aperture:

Mainly from loss of electrons that elastically scatter outside the objective aperture

Interference:

Interference of scattered and unscattered electron waves at image plane caused by (1) spherical aberration in objective lens and by (2) objective lens defocus setting

II.A.6 Unstained and Frozen-Hydrated

Cryo-EM Procedure

Where does contrast come from?

Aperture:

Very minor contribution (<10%) for ‘thin’, unstained specimens

Interference:

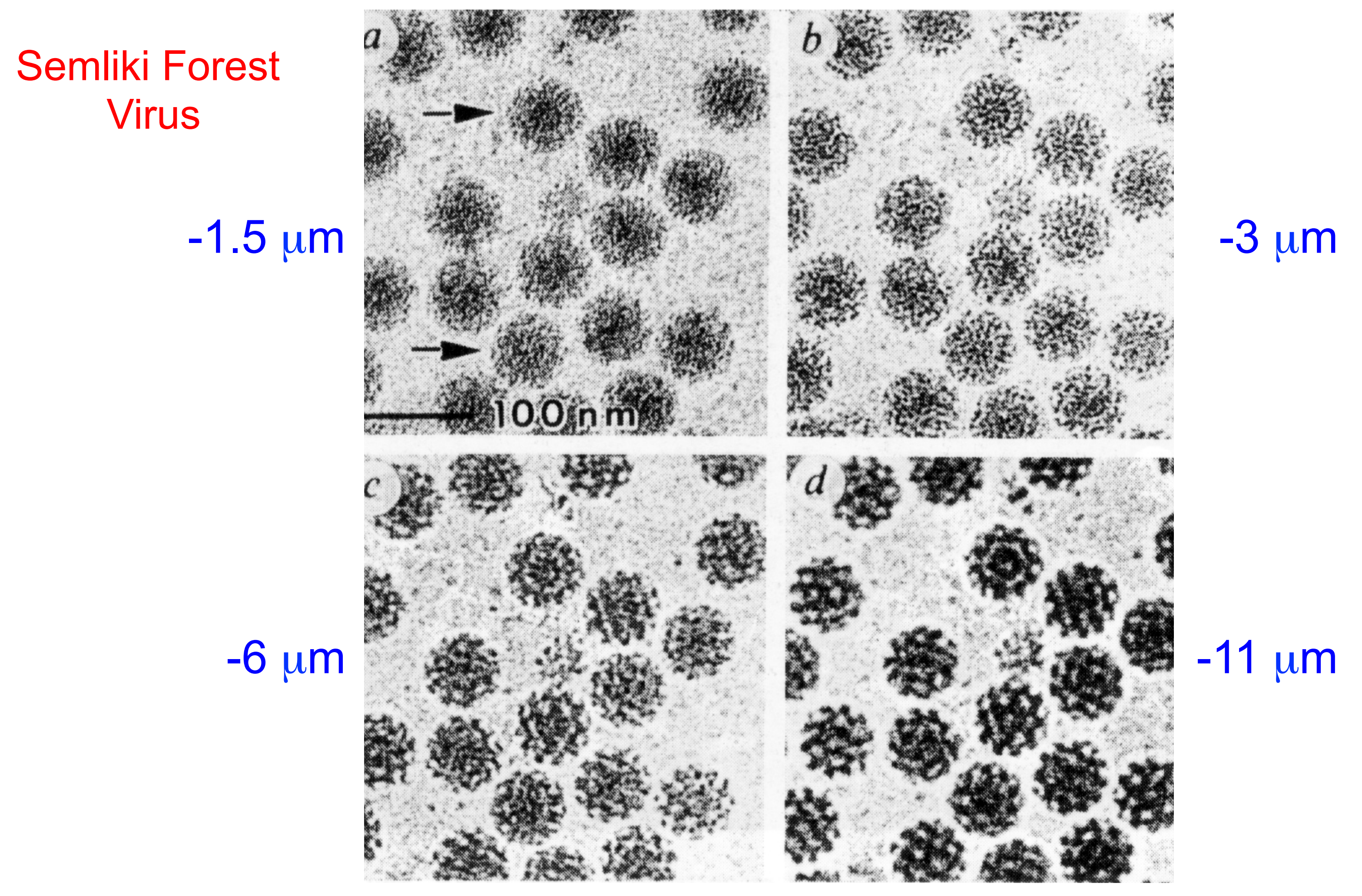
Dominant source is from **judicious under**focusing (1-3 μm) of objective lens

Cryo-EM Procedure

Generating contrast in an unstained specimen

The One and Only Answer: Defocus Phase Contrast

Semliki Forest Virus

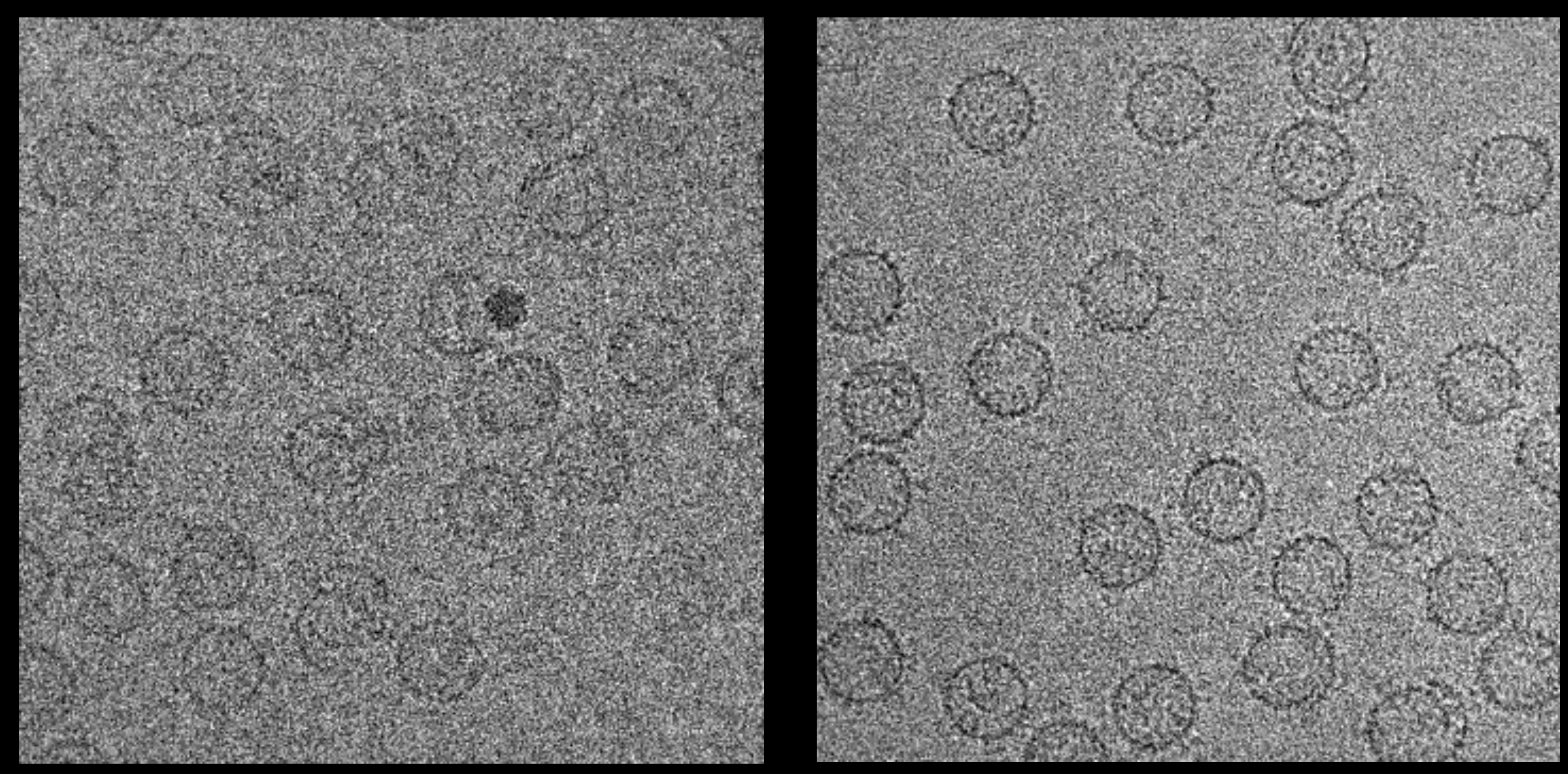


From Vogel et al. (1986) *Nature* **320:533**

Cryo-EM Procedure

Generating contrast in an unstained specimen

The One and Only Answer: Defocus Phase Contrast



Human reovirus at two underfocus settings
(Left image: ~1 μm; Right image: ~3 μm)

Slide not shown in class lecture

II.A.6 Unstained and Frozen-Hydrated

Cryo-EM Procedure

Low Dose Microscopy

There is **not enough time** to visualize let alone focus and record images of biological specimens before they are damaged by the electron beam

Yes indeed, the microscopist is forced to 'shoot in the dark' with unstained, vitrified specimens

II.A.6 Unstained and Frozen-Hydrated

Cryo-EM Procedure

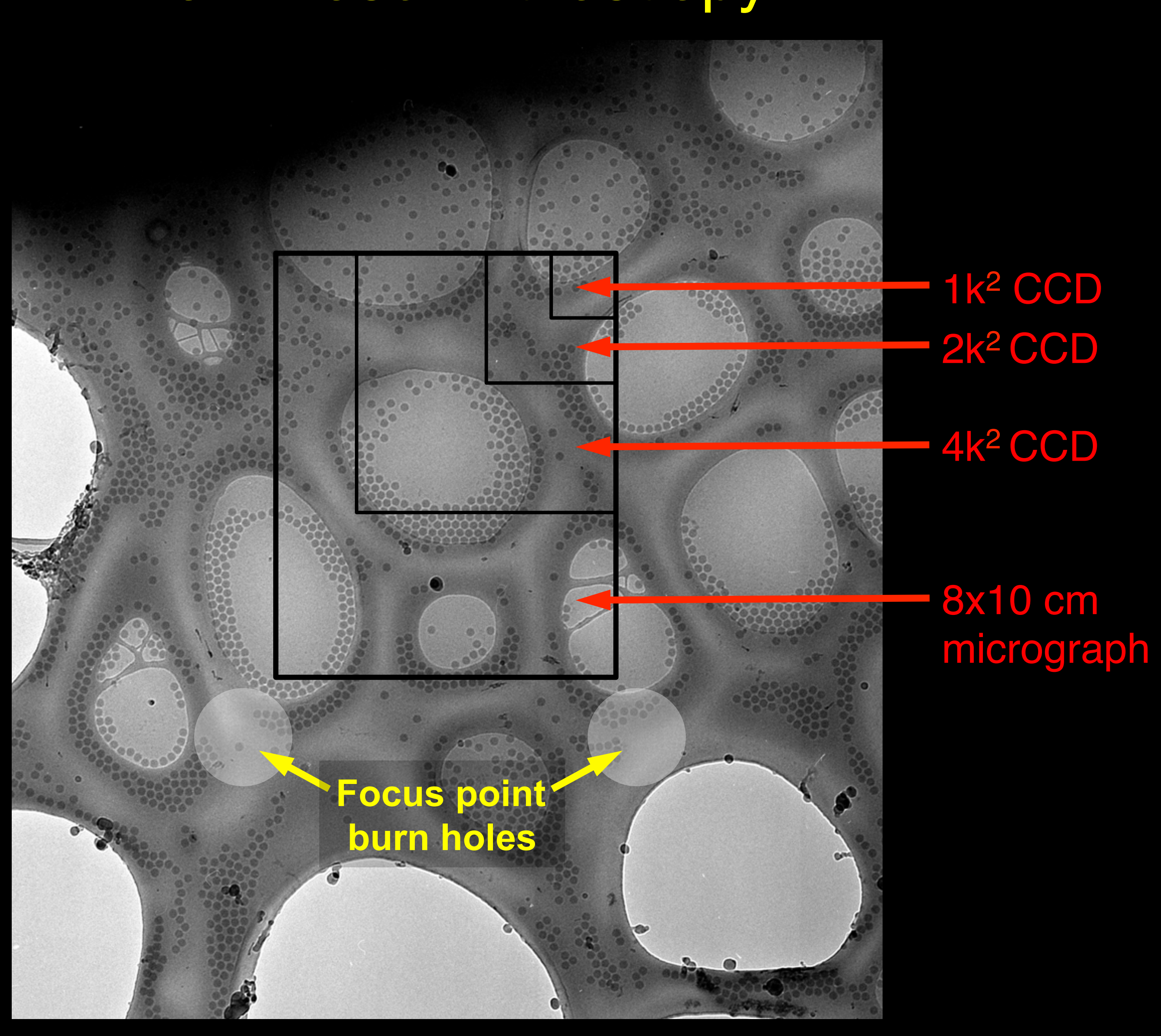
Low Dose Microscopy

GOAL #1: Minimize # electrons used to find, focus, and image specimen (<1e^-/Å^2 for search and focus)

GOAL #2: Use ~10-25 e^-/Å^2 or less to record the final image

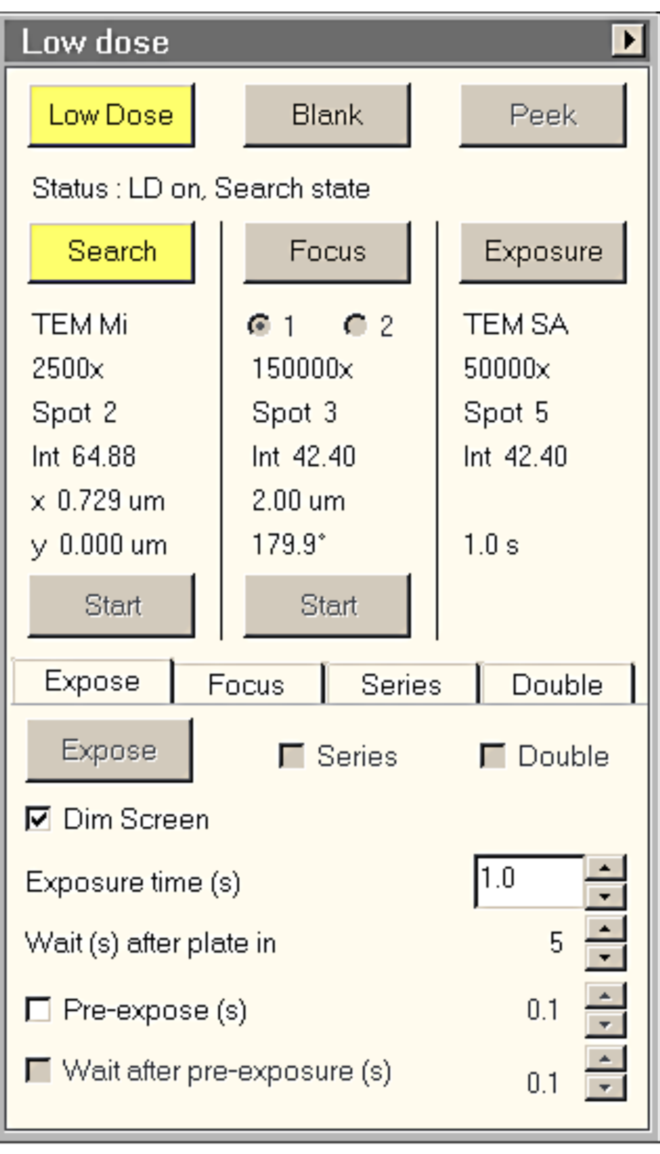
Cryo-EM Procedure

Low Dose Microscopy



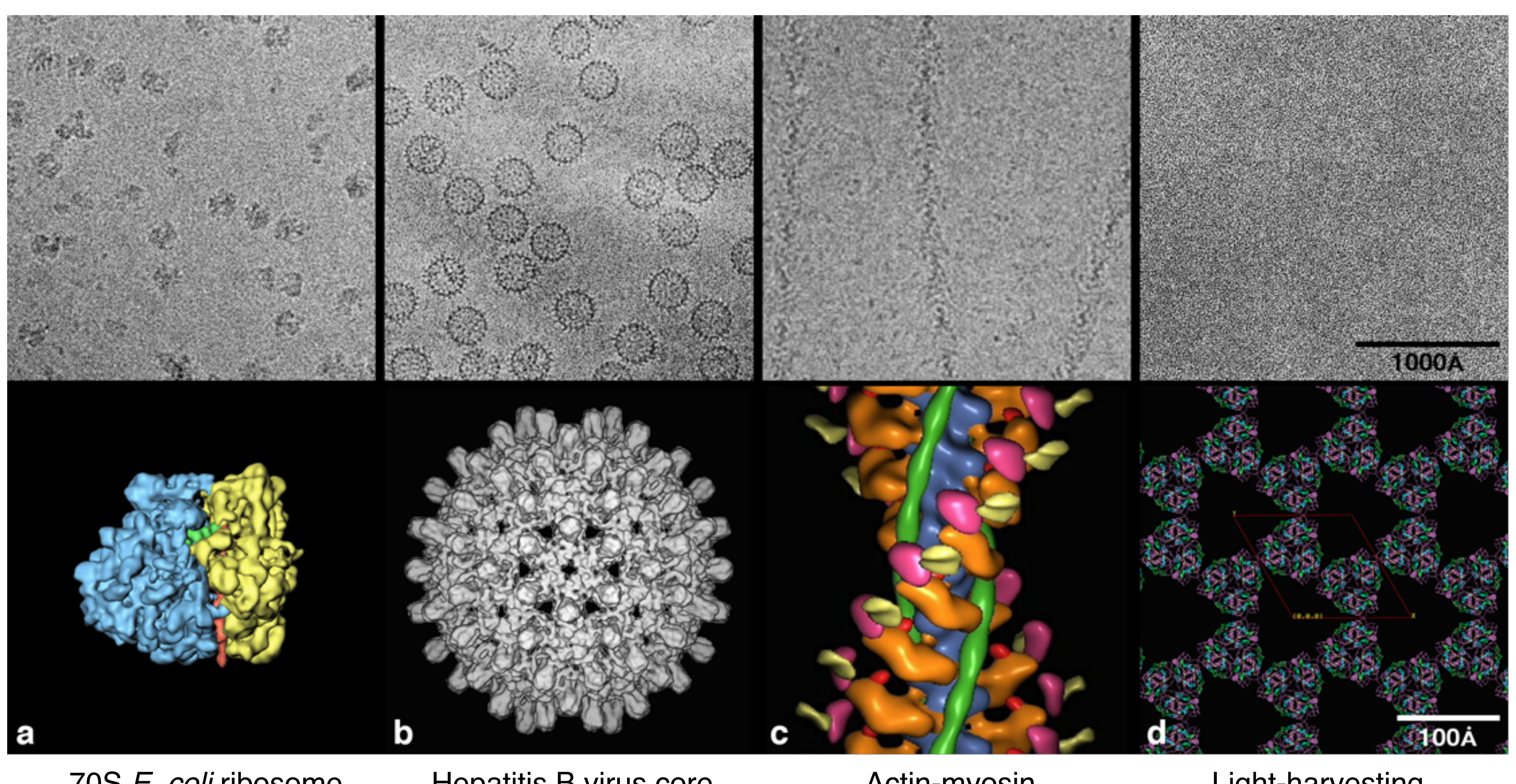
Cryo-EM Procedure

Low Dose Microscopy on an FEI Tecnai TEM



Exposure and magnification parameters are set up for Search, Focus, and Exposure modes.

Electron Cryo-Microscopy of Macromolecules



70S *E. coli* ribosome

Hepatitis B virus core

Actin-myosin filament

Light-harvesting 2D crystal

§ II: The Specimen

II.A. Biological Specimen Preparation Techniques

- II.A.1 Specimen Support Films
- II.A.2 Thin Sectioning
- II.A.3 Negative Staining
- II.A.4 Metal Shadowing
- II.A.5 Freeze Drying/Etching/Fracture
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§ II: The Specimen

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II.B. Radiation Effects

§ II: The Specimen

II.A. Biological Specimen Preparation Techniques

II.B. Radiation Effects

§ II: The Specimen

II.B. Radiation Effects

II.B.1 Introduction

II.B.2 Dose/Dose Rate

II.B.3 1° Effects of Radiation Damage to Biological Samples

II.B.4 2° Effects of Radiation Damage to Biological Samples

II.B.5 Ways to Measure Damage / Critical Dose

II.B.6 Procedures to Reduce Radiation Damage

II.B.7 Relation between Contrast, Resolution, and Damage

II.B.8 Radiation Effects in Negatively-Stained Specimens

Radiation Effects in Frozen-Hydrated Specimens

II.B RADIATION EFFECTS IN BIOLOGICAL TEM

II.B.1 Introduction

BOTTOM LINE

- **Radiation damage** limits usefulness of TEM data **regardless** of initial quality of specimen

- **MAIN limiting factor** in obtaining **HIGH resolution** images of **biological** molecules (**NOT** resolving power of TEM)

- Most **biological** specimens tolerate an exposure of no more than **~100 e^-/nm^2 (1 $e^-/Å^2$)** at **ROOM** temperature and no more than **~1000 e^-/nm^2 (10 $e^-/Å^2$)** at **liquid nitrogen** temperature (-180° C)

§ II: The Specimen

II.B. Radiation Effects

II.B.1 Introduction

II.B.2 Dose/Dose Rate

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§ II: The Specimen

II.B. Radiation Effects

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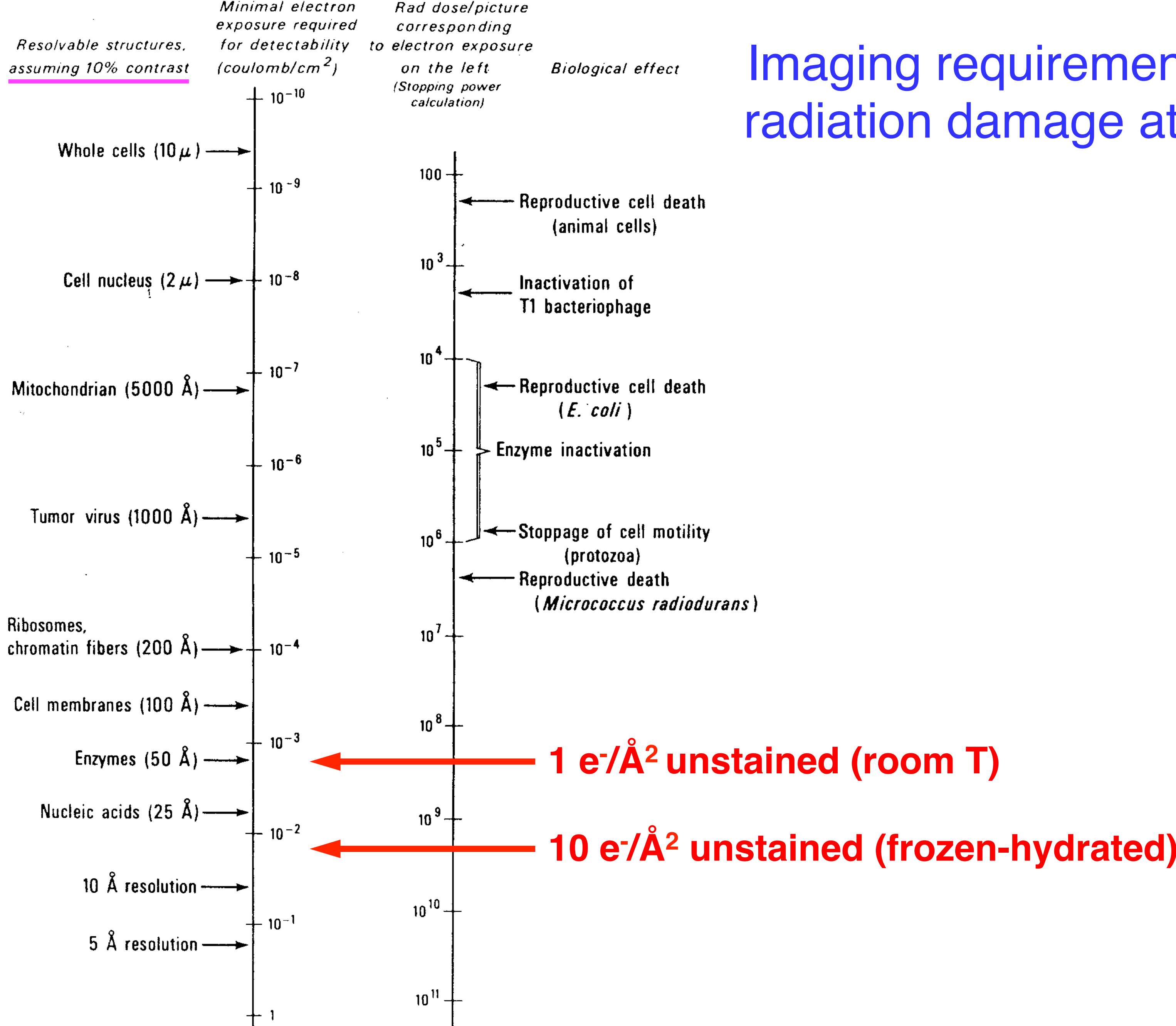
II.B.6 Procedures to Reduce Radiation Damage

II.B.7 Relation between Contrast, Resolution, and Damage

II.B.8 Radiation Effects in Negatively-Stained Specimens

Radiation Effects in Frozen-Hydrated Specimens

Imaging requirements and radiation damage at 1MeV



1 e$^-$/Å^2 unstained (room T)

10 e$^-$/Å^2 unstained (frozen-hydrated)

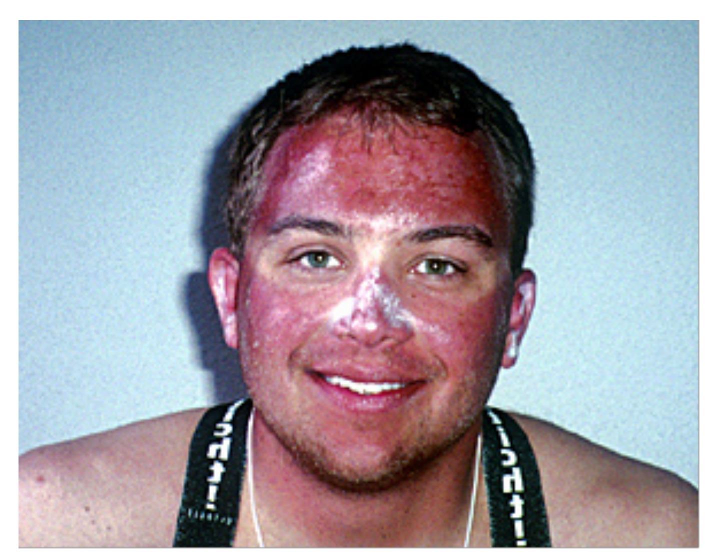
From Glaeser (1975) **12:215**

Slide not shown in class lecture

II.B RADIATION EFFECTS IN BIOLOGICAL TEM

II.B.2 Dose/Dose Rate

Does damage depend on the RATE at which a given electron dose is delivered to the specimen or just on the TOTAL DOSE delivered?



Fast and intense

Slow but steady

II.B RADIATION EFFECTS IN BIOLOGICAL TEM

II.B.2 Dose/Dose Rate

Damage is proportional to **TOTAL DOSE**

TOTAL DOSE = (dose rate) X (exposure time)

Measured as electron flux in the TEM

$coulombs/cm^2$
or e^-/nm^2
or $e^-/Å^2$

(Note: $1e^- = 1.6 \times 10^{-19}$ coulomb)

II.B RADIATION EFFECTS IN BIOLOGICAL TEM

II.B.2 Dose/Dose Rate

Dose leading to **complete molecular disorder** in an **unstained specimen** at **room T** is ~100 e^-/nm^2 at 80 kV (~250 e^-/nm^2 at 500 keV)

But keep in mind: (…ugh, more problems?)

Minimum current density on TEM fluorescent screen needed to **barely see** an object is ~1 $e^-/\mu m^2/sec$ -----> i.e. dose rate at specimen @ 20,000X is 400 $e^-/nm^2/sec$

Hence, **not enough time** to visualize let alone focus, stigmate, and record images of biological specimens before they are damaged

Doesn't sound too promising does it?

II.B.2 Dose/Dose Rate

A Sobering Thought

A two second exposure…

…delivers an amount of energy **at the specimen** approximately equivalent on a **relative** scale to the energy **we** would experience if a **10 megaton hydrogen-bomb** were to explode ~30 meters outside this room!

(10 megatons = 10,000,000 tons of TNT)

§ II: The Specimen

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II.B.4 2° Effects of Radiation Damage to Biological Samples

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II.B.6 Procedures to Reduce Radiation Damage

II.B.7 Relation between Contrast, Resolution, and Damage

II.B.8 Radiation Effects in Negatively-Stained Specimens

Radiation Effects in Frozen-Hydrated Specimens

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II.B RADIATION EFFECTS IN BIOLOGICAL TEM

II.B.3 **Primary** Effects of Radiation Damage to Biological Samples

What causes the damage?

Primary interactions between the electron beam and the specimen:

Excitation

Ionization

Displacement

II.B RADIATION EFFECTS IN BIOLOGICAL TEM

II.B.3 **Primary** Effects of Radiation Damage to Biological Samples

What causes the damage?

Primary interactions between the electron beam and the specimen:

Excitation: raising of atomic electron to higher energy orbital

Ionization: formation of ions or radicals from loss of electrons

Displacement: knock-off of atoms (very rare)

All are essentially **temperature independent**

All occur ***VERY*** rapidly: (**~10^{-14} sec** = 10 femtoseconds)

II.B.3 **Primary** Effects of Radiation Damage to Biological Samples

Ionization caused by **inelastic** interaction of beam electrons with orbital electrons of specimen atoms leads to **bond rupture**

This is the **main cause** of damage to molecular structure

Recall: **Elastic** interactions produce image contrast but **no damage**

Ionization creates **ions or radicals**

Fates of these species are called **"secondary events"**, and **these reactions, not the primary events**, cause material damage to the specimen

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II.B RADIATION EFFECTS IN BIOLOGICAL TEM

II.B.4 **Secondary** Effects of Radiation Damage

Electron irradiation results in one or more of the following:

- Chemical and Physical Changes
- Mass Loss / Cross-linking
- Production of Heat
- Charge Effects
- Contamination and Etching
- Crystal Structure Damage

II.B RADIATION EFFECTS IN BIOLOGICAL TEM

II.B.4 **Secondary** Effects of Radiation Damage

Electron irradiation results in one or more of the following:

→ Chemical and Physical Changes

Mass Loss / Cross-linking

Production of Heat

Charge Effects

Contamination and Etching

Crystal Structure Damage

II.B.4 **Secondary** Effects of Radiation Damage

Chemical and Physical Changes

Ionizing radiation causes lots of molecular changes

C-H bonds very sensitive; C-C bonds are more resistant

Number of C=C bonds increases with dose.

- Molecules acquire more double and triple bonds.
- Leads to bond length/angle changes. (i.e. structure changes)

% C content of specimen typically increases with radiation.
Final product is predominantly carbon

Microtephroscopy

II.B RADIATION EFFECTS IN BIOLOGICAL TEM

II.B.4 **Secondary** Effects of Radiation Damage

Electron irradiation results in one or more of the following:

- Chemical and Physical Changes
- Mass Loss / Cross-linking
- Production of Heat
- Charge Effects
- Contamination and Etching
- Crystal Structure Damage

See lecture notes pp.199-201

II.B.4 **Secondary** Effects of Radiation Damage

Mass Loss / Cross-linking

- Results from **fracture or scission** of specimen molecules

- Scission **alone** would progressively reduce the mass of the specimen in the TEM to nothing!!!

- Stable product we see is either beam resistant or it is getting or has gotten cross-linked

- **Predominant** reaction must be **cross-linking**

- Thus, rate of **mass loss** is **initially rapid but levels off** with continued irradiation, finally reaching a plateau

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II.B RADIATION EFFECTS IN BIOLOGICAL TEM

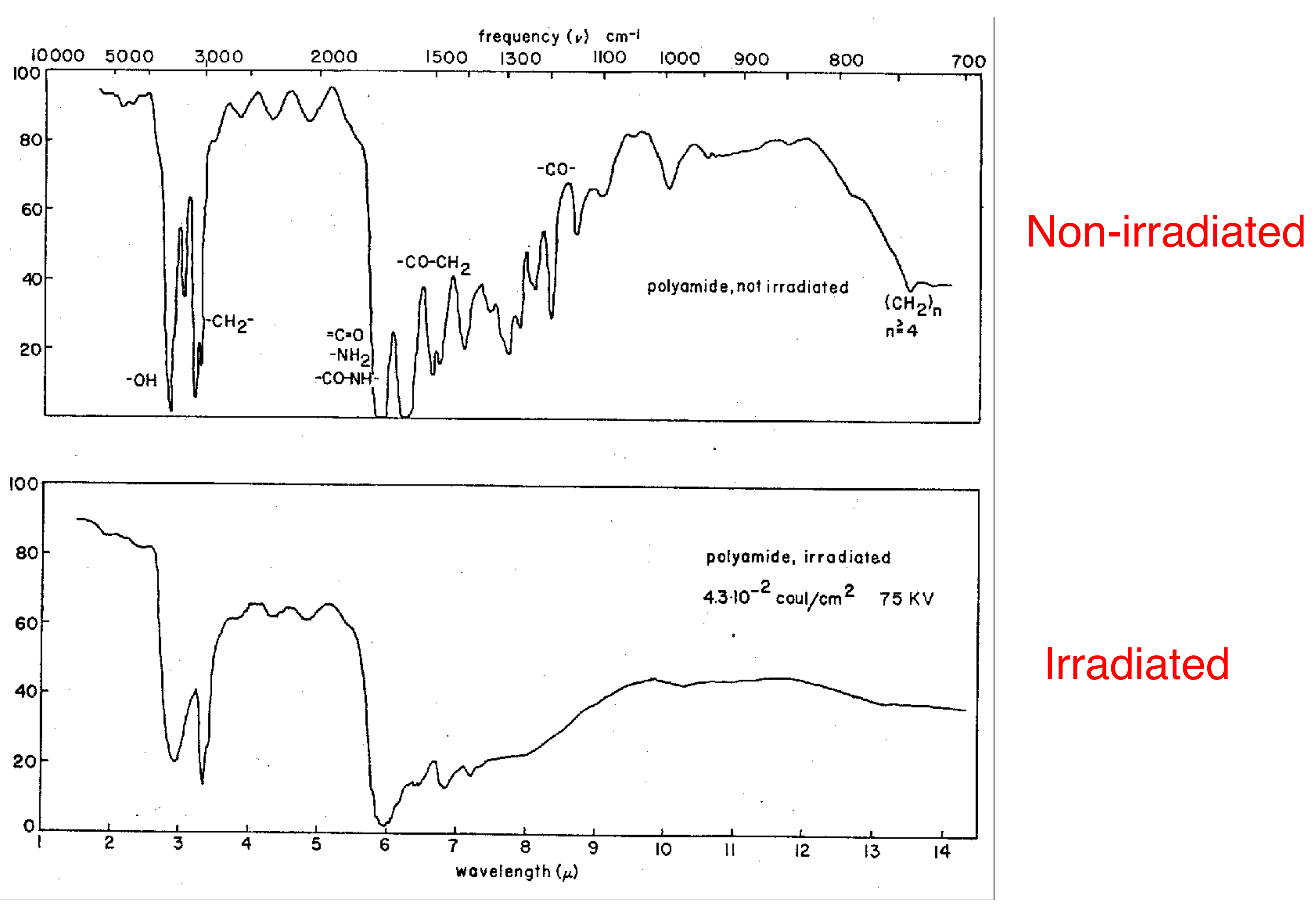
II.B.5 Ways to Measure Damage / Critical Dose

Criteria Used to Measure Radiation Damage

- Total mass loss
- Loss of specific elements
- Loss of crystalline structure
- Changes in the infrared, visible, or ultraviolet spectra (including energy loss spectra)

EXAMPLE: As measured by infrared spectroscopy, protein 2° structure is completely randomized at doses of 60-200 e^-/nm^2

Infrared absorption spectra of non-irradiated and irradiated polyamide



Non-irradiated

Irradiated

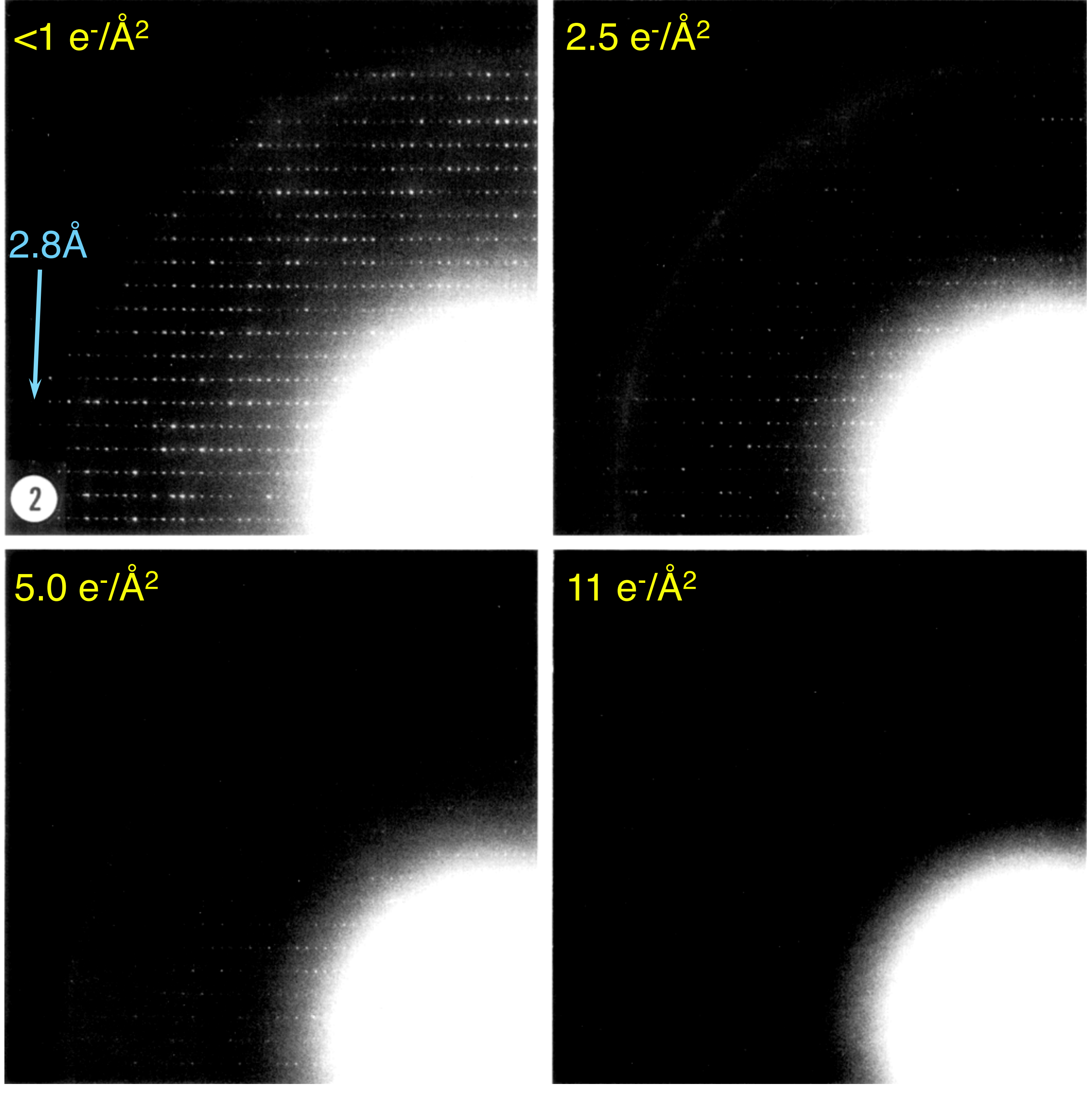
From Bahr, Johnson, and Zeitler (1965) *Lab. Invest.* **14:377**

II.B.5 Ways to Measure Damage / Critical Dose

Loss of Crystalline Structure

Critical Dose:

Dose at which the intensity of a specific spot in an **electron diffraction pattern** falls to **1/exp** (*i.e.* 37%) of its original value at **zero** dose

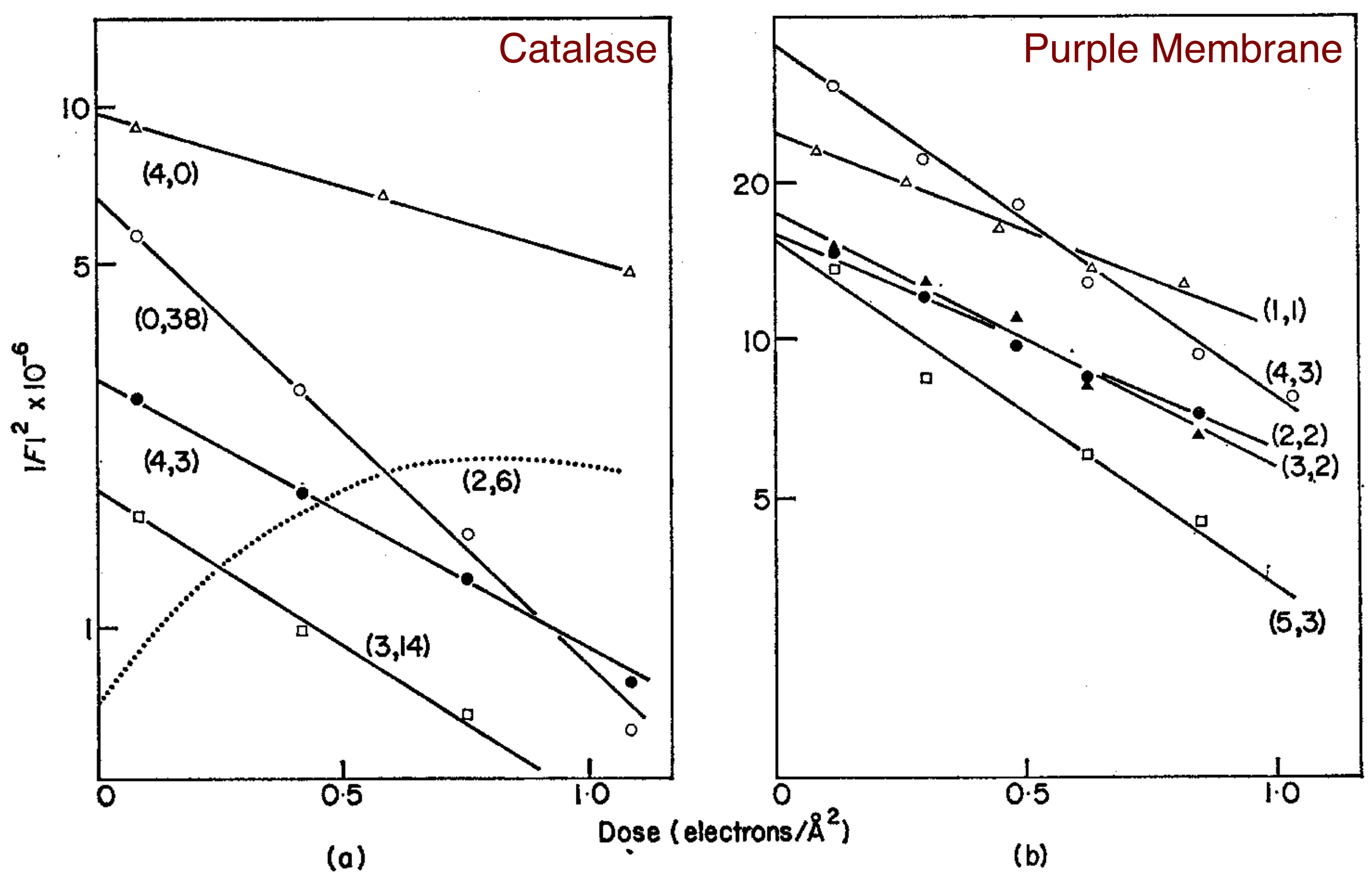


Changes in the electron diffraction pattern of frozen-hydrated catalase crystals resulting from radiation damage

From Taylor and Glaeser (1976) *J. Ultrastruc. Res.* **55:448**

II.B.5 Ways to Measure Damage / Critical Dose

Intensities (on a logarithmic scale) of some typical reflections in electron diffraction patterns, plotted as a function of electron dose



From Unwin and Henderson (1975) *J. Mol. Biol.***94:425**

II.B.5 Ways to Measure Damage / Critical Dose

Loss of Crystalline Structure

Critical Dose:

Dose at which the intensity of a specific spot in an **electron diffraction pattern** falls to **1/exp** (*i.e.* 37%) of its original value at **zero** dose

Fact:

- At 100 keV, critical dose for **unstained protein crystals** at **room T** is ~100 e^-/nm^2 (**1 $e^-/Å^2$**)

II.B.5 Ways to Measure Damage / Critical Dose

Loss of Crystalline Structure

Critical Dose:

Dose at which the intensity of a specific spot in an **electron diffraction pattern** falls to **1/exp** (*i.e.* 37%) of its original value at **zero** dose

Facts:

- At 100 keV, critical dose for **unstained protein crystals** at **room T** is ~100 e^-/nm^2 (**1 $e^-/Å^2$**)

- At 1 MeV (*i.e.* 1000 keV), critical dose is only 2-3 times **higher** than at 100 keV (*i.e.* **~2-3 $e^-/Å^2$**)

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II.B RADIATION EFFECTS IN BIOLOGICAL TEM

II.B.6 Procedures to Reduce Radiation Damage

- Reduce number of electrons ("Low Dose")

- Reduce specimen temperature

- Increase accelerating voltage

- Reduce contamination and etching

- Carbon stabilization of specimens and carbon support films

II.B RADIATION EFFECTS IN BIOLOGICAL TEM

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- Reduce number of electrons ("Low Dose")
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II.B.6 Procedures to Reduce Radiation Damage

Reduce Number of Electrons

Duh! I could have thought of that!

II.B.6 Procedures to Reduce Radiation Damage

Reduce Number of Electrons

Use fewer electrons (reduce exposure) by cutting down:

Exposure time

Beam intensity

OK, so why not use **zero** electrons?

………..we certainly would if we could.

We do try to minimize the number and use as close to zero as possible!!!

II.B.6 Procedures to Reduce Radiation Damage

Reduce Number of Electrons

Basic rule:

Use **minimum magnification** required to reveal detail of a given size (determined by resolution of photographic emulsion or pixel size of CCD or DDD detector)

Minimal exposure (minimum dose) technique:

Focus on a region nearby but not directly on the specimen area of interest

Specimen exposure ***essentially*** **begins** when the beam is shifted onto the specimen and the micrograph is recorded

II.B RADIATION EFFECTS IN BIOLOGICAL TEM

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II.B.6 Procedures to Reduce Radiation Damage

Reduce Specimen Temperature

Chemical bonds broken by electron impact **regardless** of T

- At low T some molecular fragments remain fixed in position

  Though damaged, object still resembles original object at higher irradiation levels than tolerated at room temperature (RT)

- Mass loss greatly reduced (At RT and 1000 e^-/nm^2, loss is 20-80%)

- Trapping of highly reactive radicals at low T reduces structural damage that would otherwise occur owing to chemical reactions

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II.B.6 Procedures to Reduce Radiation Damage

Increase Accelerating Voltage

For a specimen of a given thickness, **faster** (higher voltage) electrons deposit **less** energy (fewer inelastic scattering events), resulting in **less** damage

HOWEVER (i.e. keep in mind):

Reduced damage **offset** by **reduced aperture contrast** at higher voltage since fewer e^- captured by obj. aperture

II.B.6 Procedures to Reduce Radiation Damage

Increase Accelerating Voltage

For a specimen of a given thickness, **faster** (higher voltage) electrons deposit **less** energy (fewer inelastic scattering events), resulting in **less** damage

HOWEVER (i.e. keep in mind):

Reduced damage **offset** by **reduced aperture contrast** at higher voltage since fewer e^- captured by obj. aperture

ALSO (i.e. "what's the catch?"):

Weaker interaction of imaging electrons with photographic emulsion (fewer developed silver grains, therefore ↓ **O.D.** and ↓ **photographic contrast**)

Slide not shown in class lecture

II.B.6 Procedures to Reduce Radiation Damage

Increase Accelerating Voltage

For a specimen of a given thickness, **faster** (higher voltage) electrons deposit **less** energy (fewer inelastic scattering events), resulting in **less** damage

HOWEVER (i.e. keep in mind):

Reduced damage **offset** by **reduced aperture contrast** at higher voltage since fewer e^- captured by obj. aperture

ALSO (i.e. yet another "catch"):

Higher voltage electrons are less well resolved by CCD cameras owing to an increase in 'cross-talk' (*i.e.* spillover of high energy electrons) between adjacent pixels

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II.B RADIATION EFFECTS IN BIOLOGICAL TEM

II.B.6 Procedures to Reduce Radiation Damage

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- Increase accelerating voltage

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See lecture notes p.205

§ II: The Specimen

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II.B RADIATION EFFECTS IN BIOLOGICAL TEM

II.B.7 Relation between Contrast, Resolution and Radiation Damage

As exposure is **reduced**, **statistical fluctuations** from one picture element to another can be **much greater** than the inherent change in density in neighboring portions of the object.

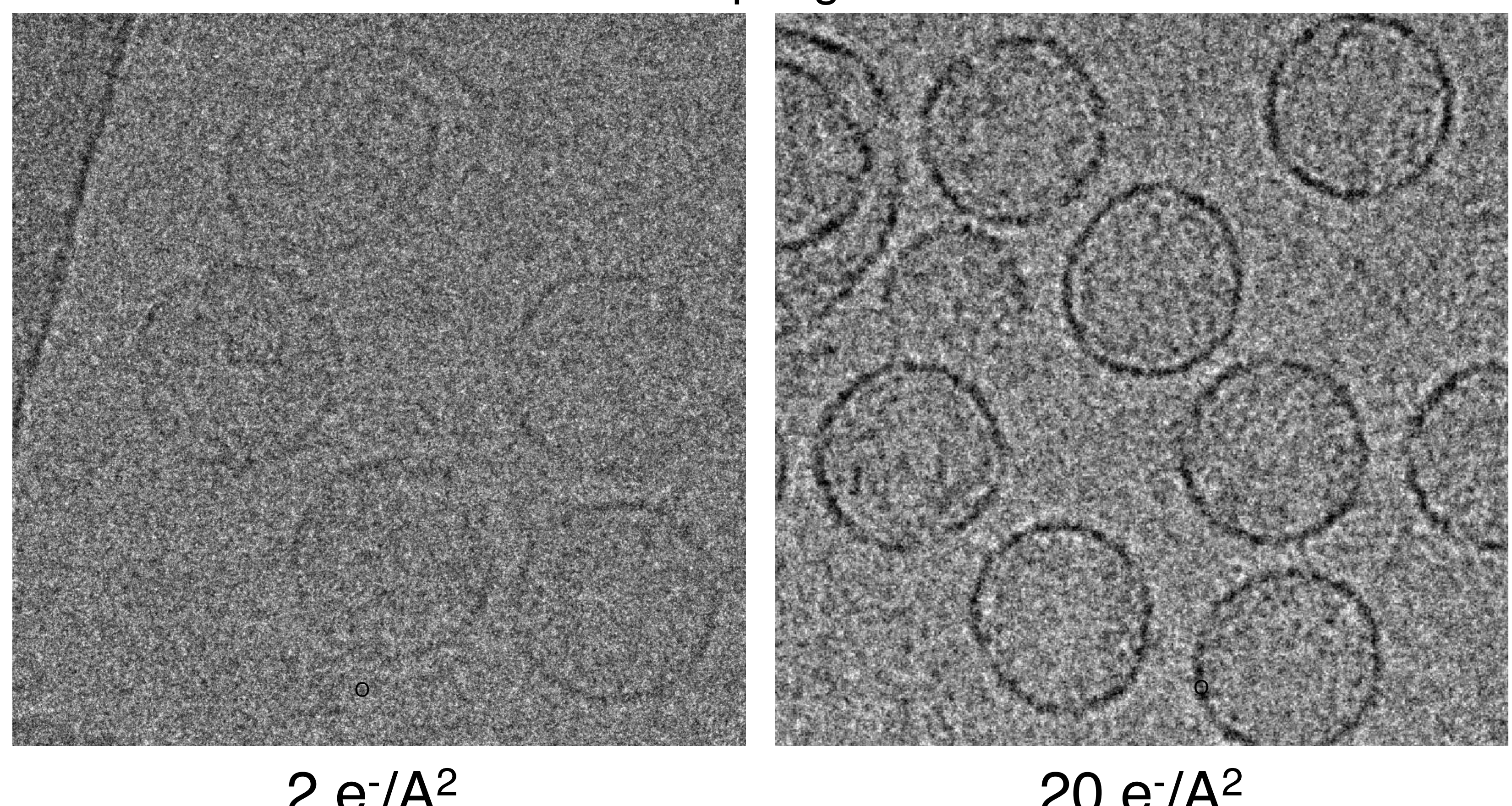
Statistical fluctuations >> inherent contrast

Low dose images exhibit ***REALLY* poor** Signal-to-Noise (S/N) ratios

II.B.7 Relation between Contrast, Resolution and Radiation Damage

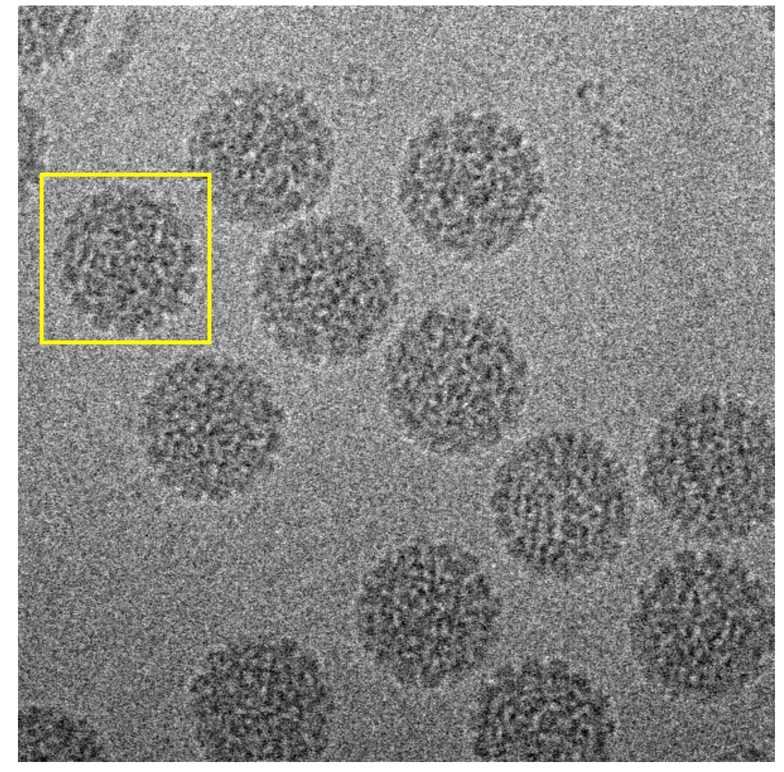
The lower the dose, the higher the noise

Bacteriophage P22



Images courtesy of Dr. Kristin Parent

II.B.7 Relation between Contrast, Resolution and Radiation Damage

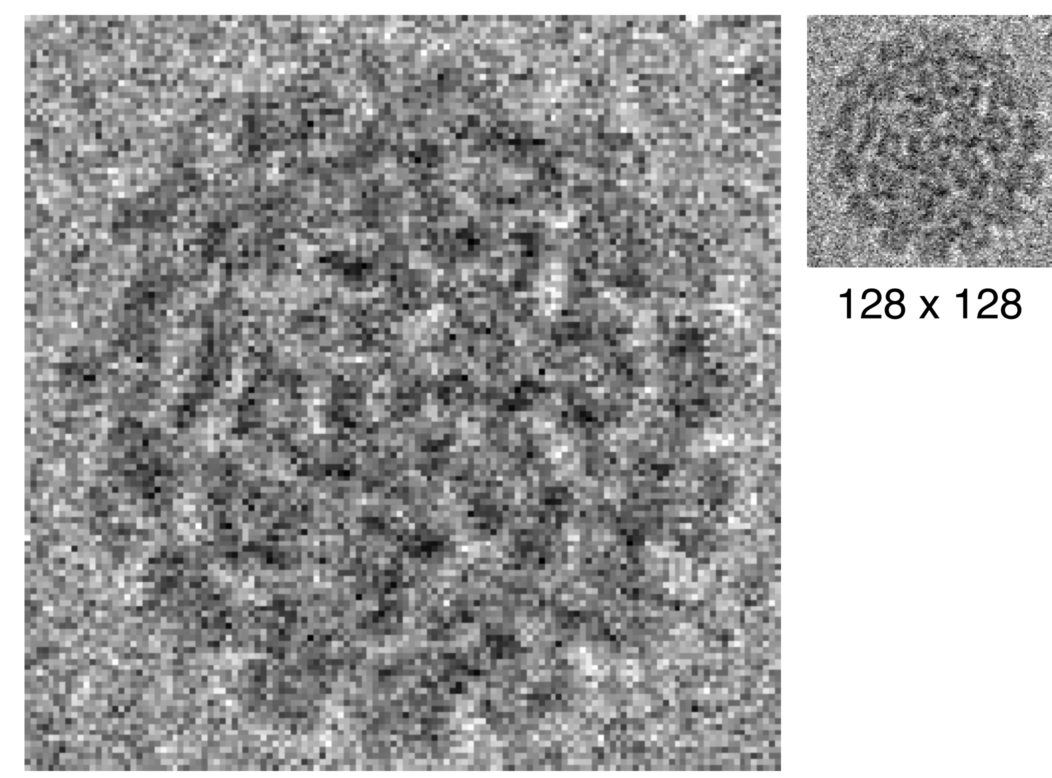


128 x 128

SV40 Virus

Original digital image

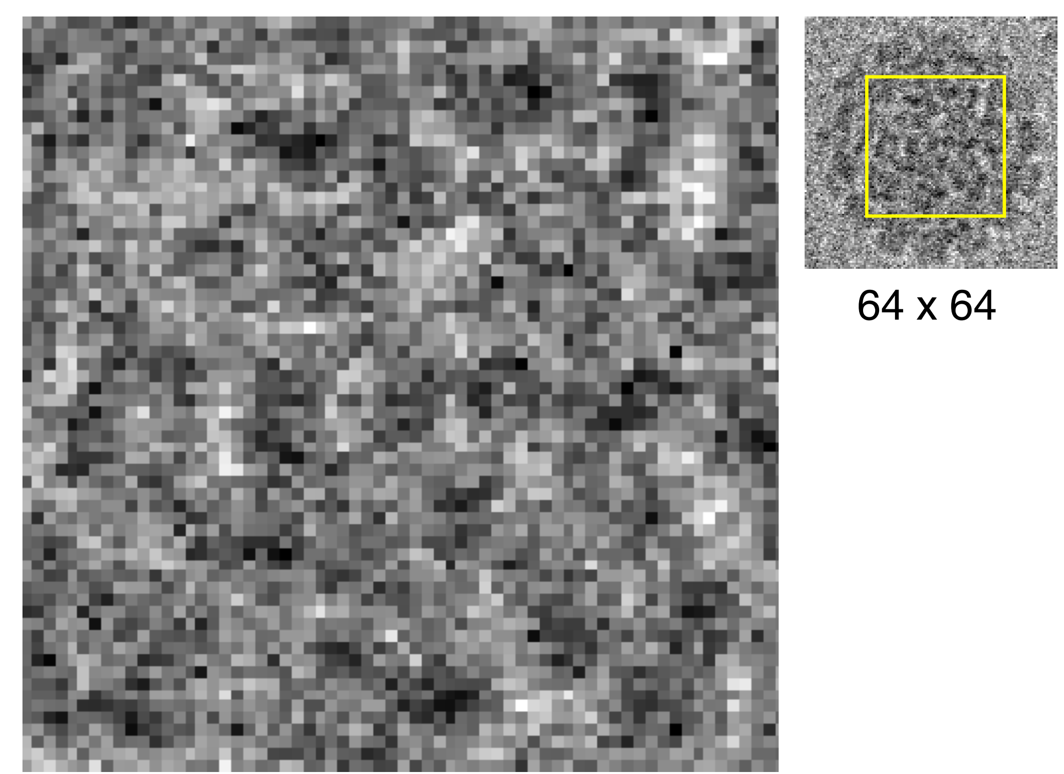
II.B.7 Relation between Contrast, Resolution and Radiation Damage



SV40 Virus

Digital image highly magnified to show individual pixels

II.B.7 Relation between Contrast, Resolution and Radiation Damage



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Digital image highly magnified to show individual pixels

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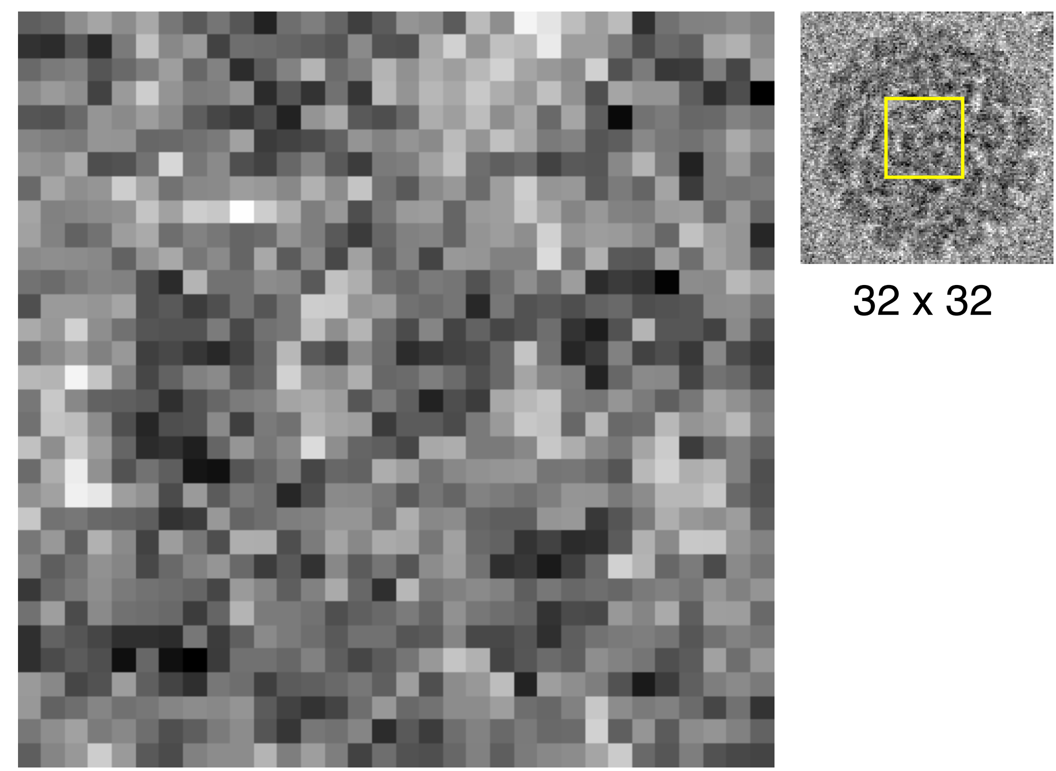

SV40 Virus

Digital image highly magnified to show individual pixels

II.B RADIATION EFFECTS IN BIOLOGICAL TEM

II.B.7 Relation between Contrast, Resolution and Radiation Damage

As exposure is **reduced**, **statistical fluctuations** from one picture element to another can be **much greater** than the inherent change in density in neighboring portions of the object.

Statistical fluctuations >> inherent contrast

Low dose images exhibit ***REALLY* poor** Signal-to-Noise (S/N) ratios

II.B RADIATION EFFECTS IN BIOLOGICAL TEM

II.B.7 Relation between Contrast, Resolution and Radiation Damage

As exposure is **reduced**, **statistical fluctuations** from one picture element to another can be **much greater** than the inherent change in density in neighboring portions of the object.

Statistical fluctuations >> inherent contrast

Reducing exposure **preserves specimen integrity**, but **specific details** can **not** be **observed directly** owing to the noisy quality of the image

To capture **high** resolution details, ***MUST*** resort to **image averaging** techniques

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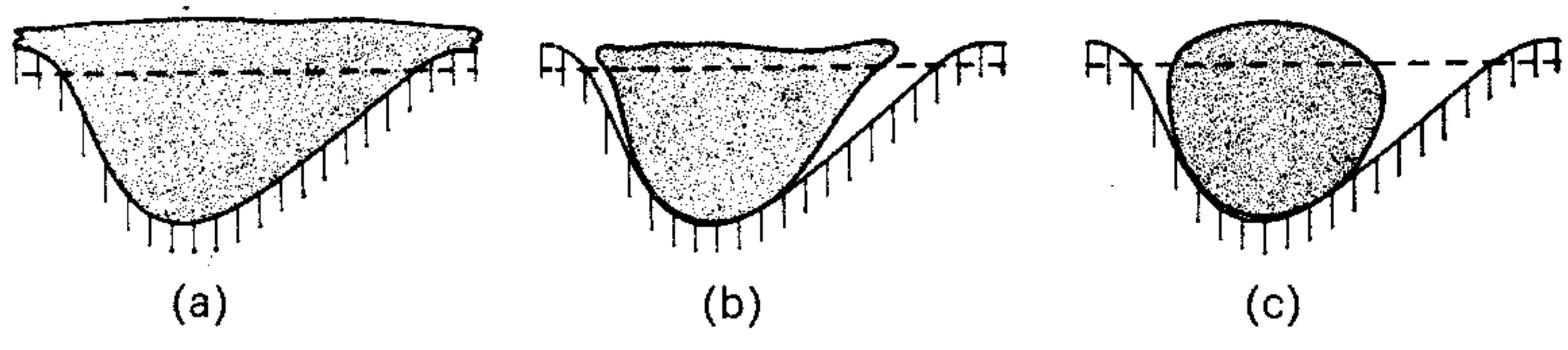
II.B RADIATION EFFECTS IN BIOLOGICAL TEM

II.B.8 Radiation Effects in **Negatively-Stained** Specimens

Suggested influence of an asymmetrical groove in a protein on the radiation changes produced in the stain near the outer surface

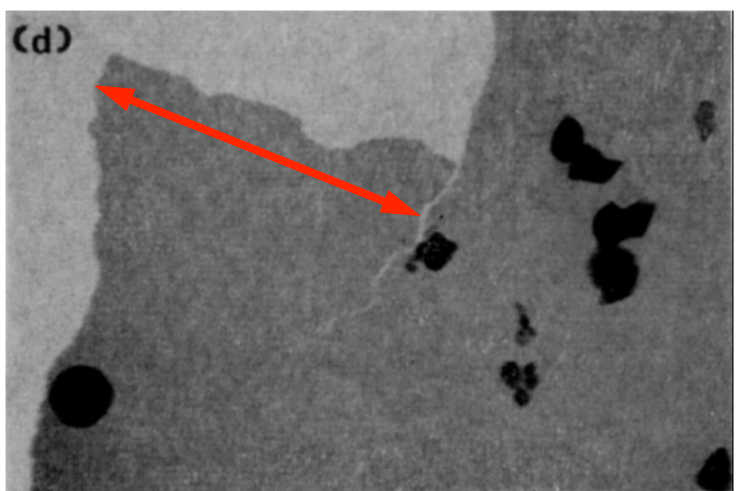


From Unwin (1974) *J. Mol. Biol.* **87:657**

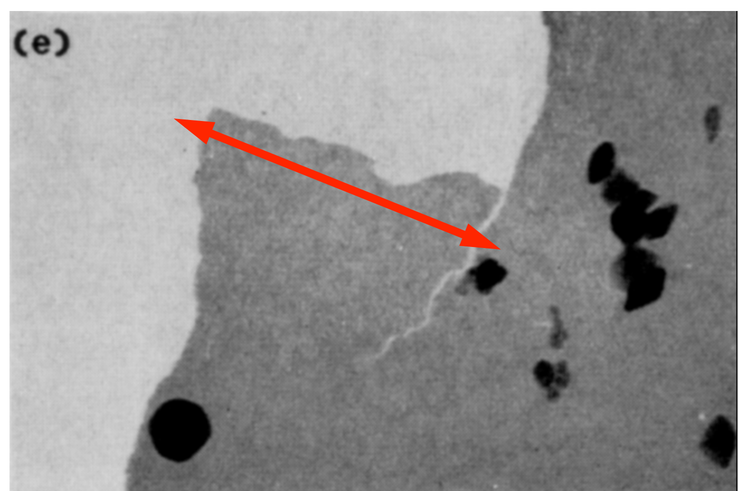
II.B RADIATION EFFECTS IN BIOLOGICAL TEM

II.B.8 Radiation Effects in **Negatively-Stained** Specimens

Radiation induced changes induced in thin films of uranyl formate stain



$16\ e^-/\text{Å}^2$



$625\ e^-/\text{Å}^2$

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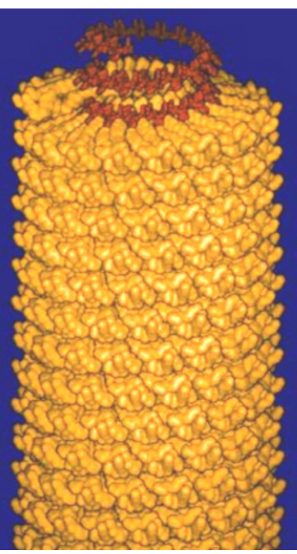
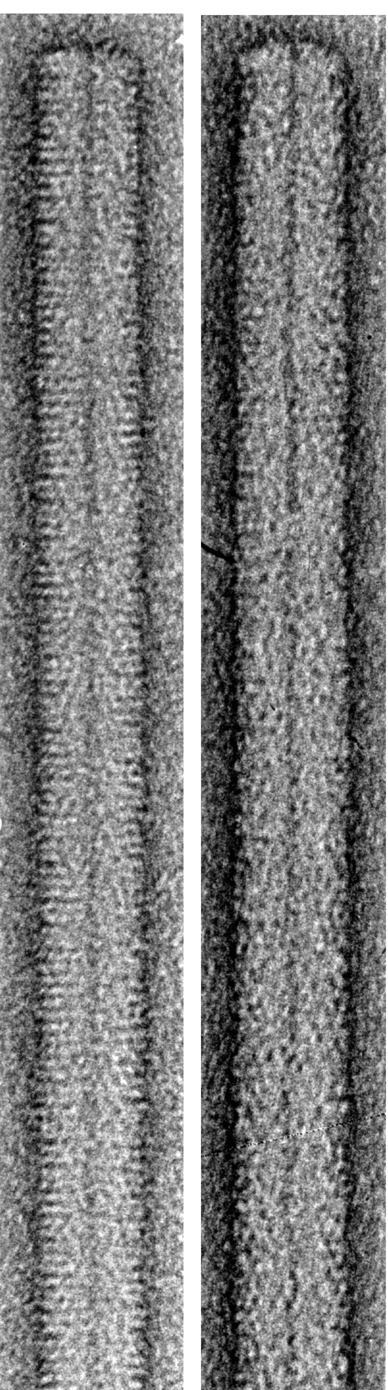
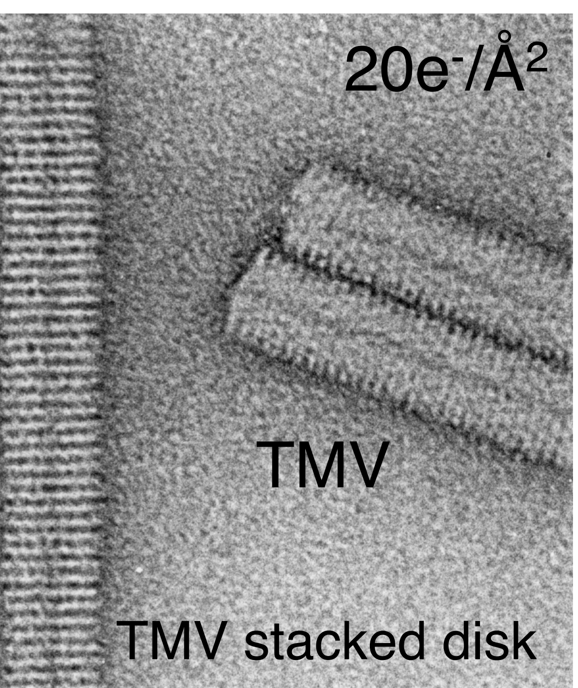
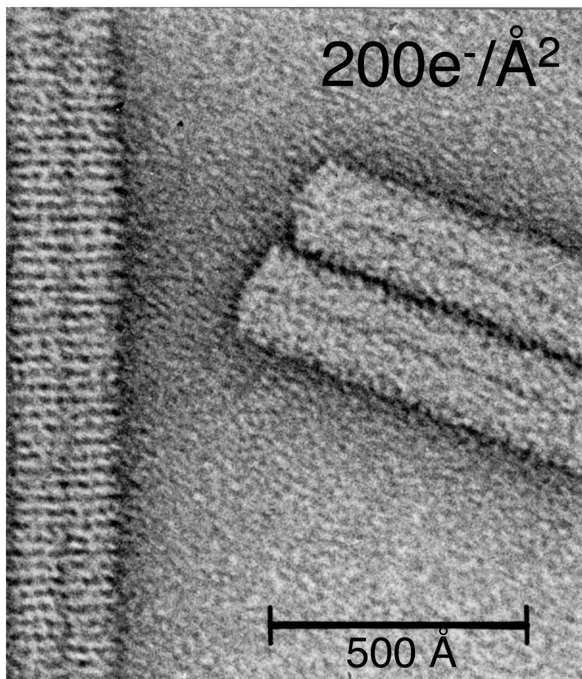
From Unwin (1974) *J. Mol. Biol.* **87:657**

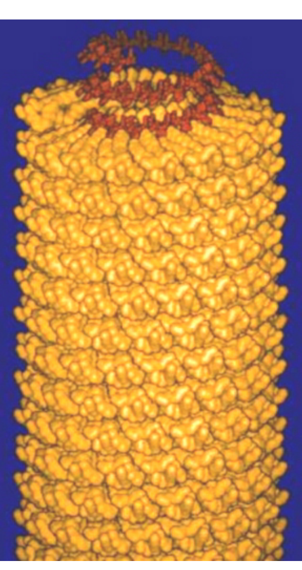

TMV

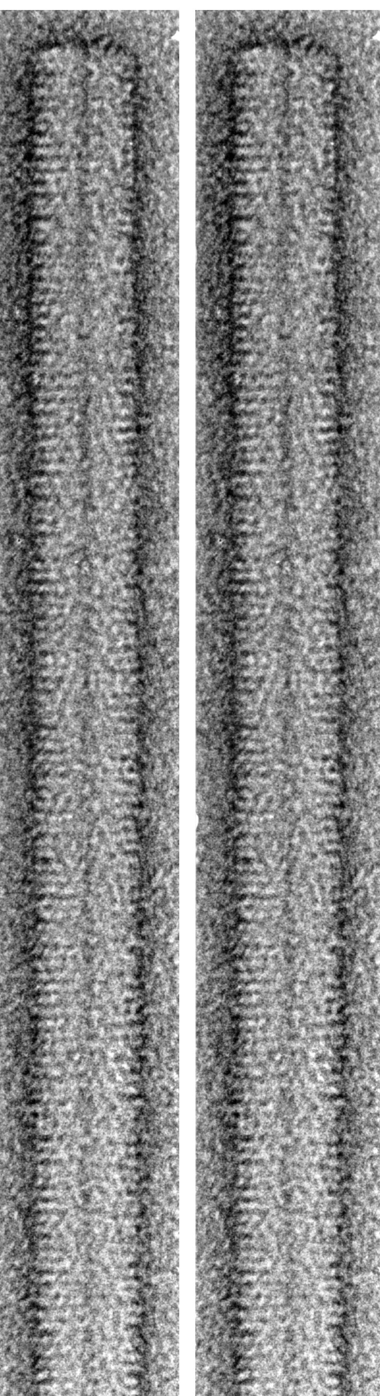


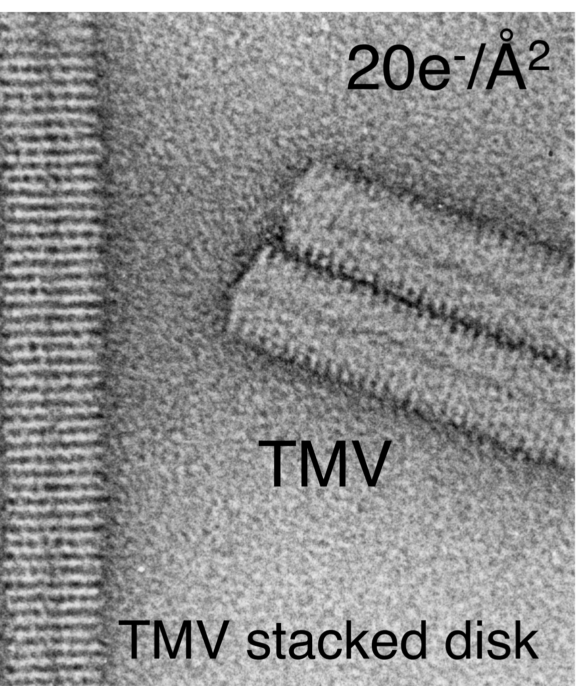

20e-/Å2
TMV
TMV stacked disk

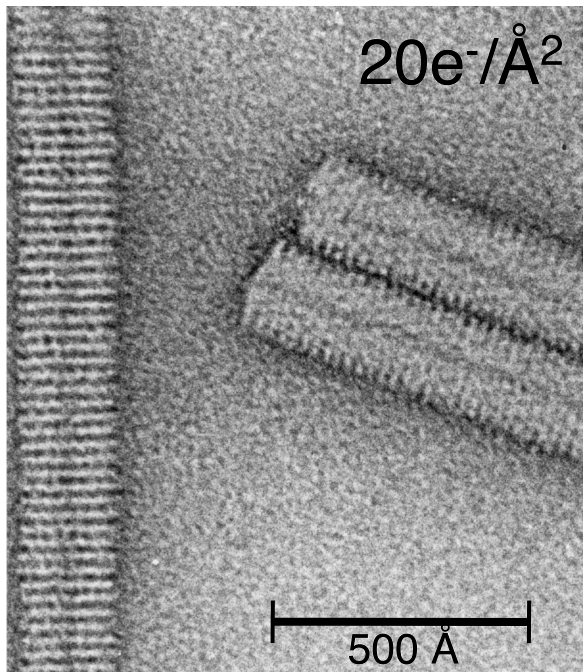

200e-/Å2
500 Å

TMV





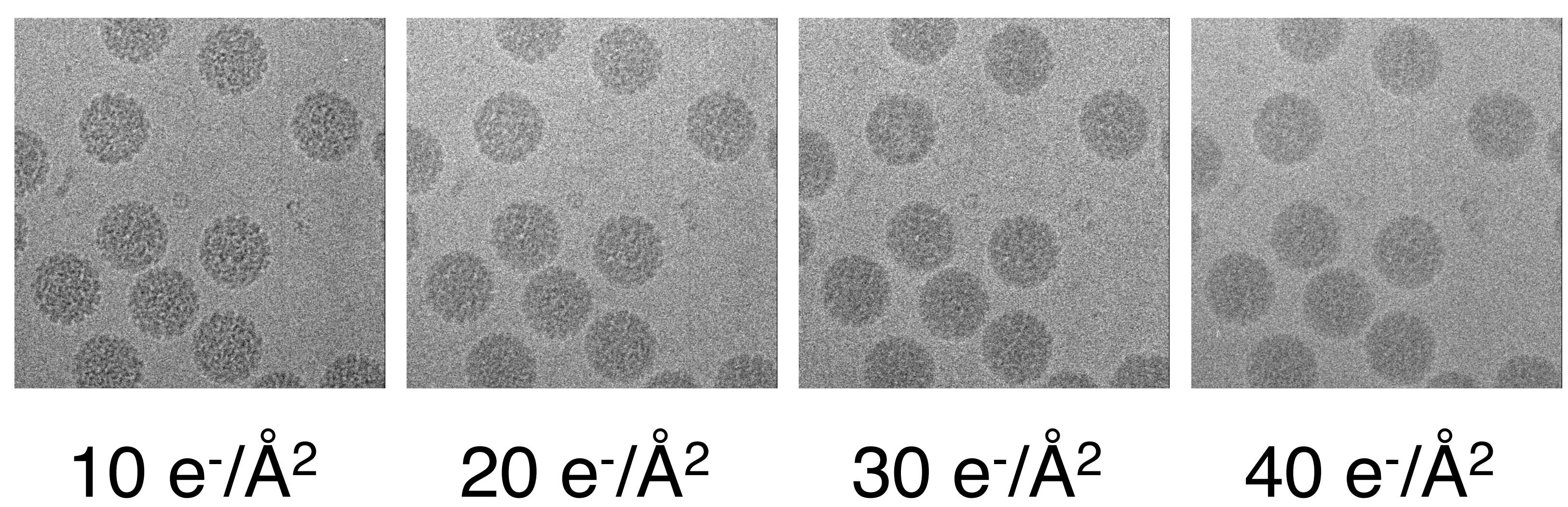




II.B RADIATION EFFECTS IN BIOLOGICAL TEM

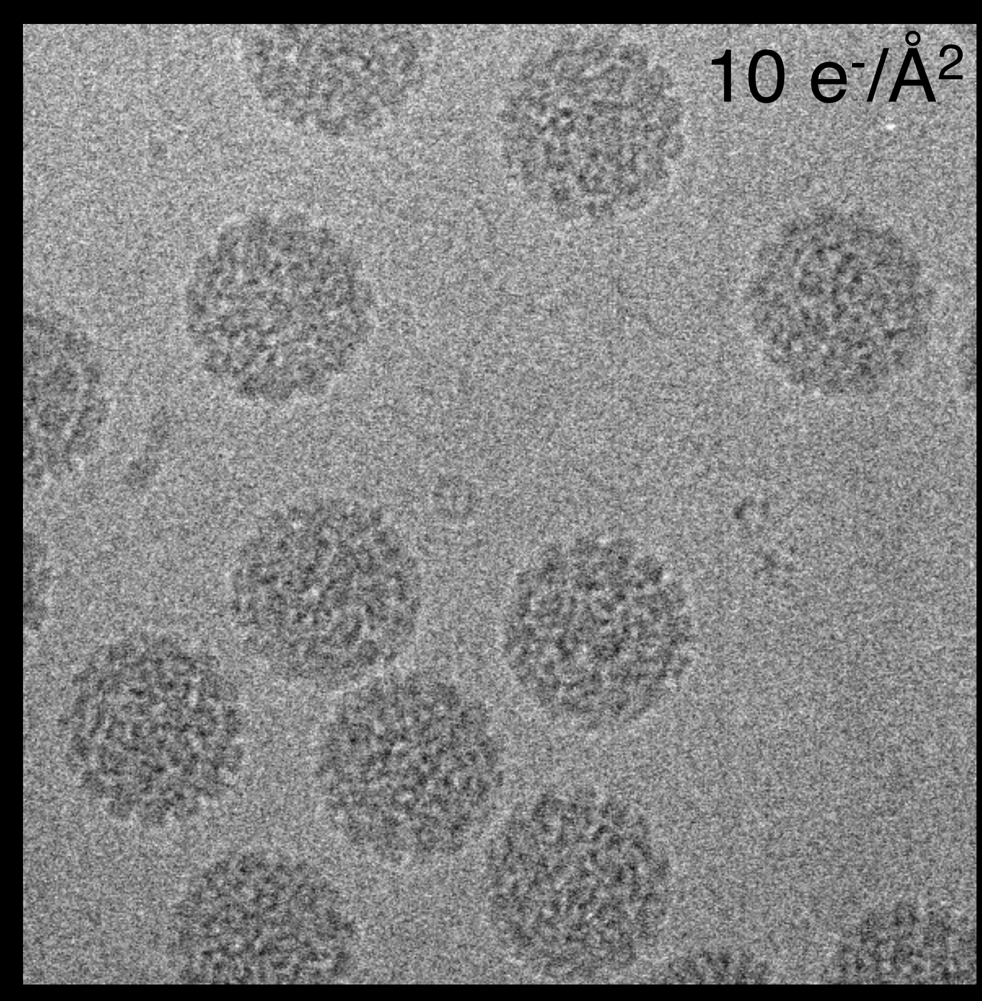
II.B.8 Radiation Effects in **Frozen-Hydrated** Specimens

SV40 Dose Series



Accumulated electron doses

SV40 Dose Series



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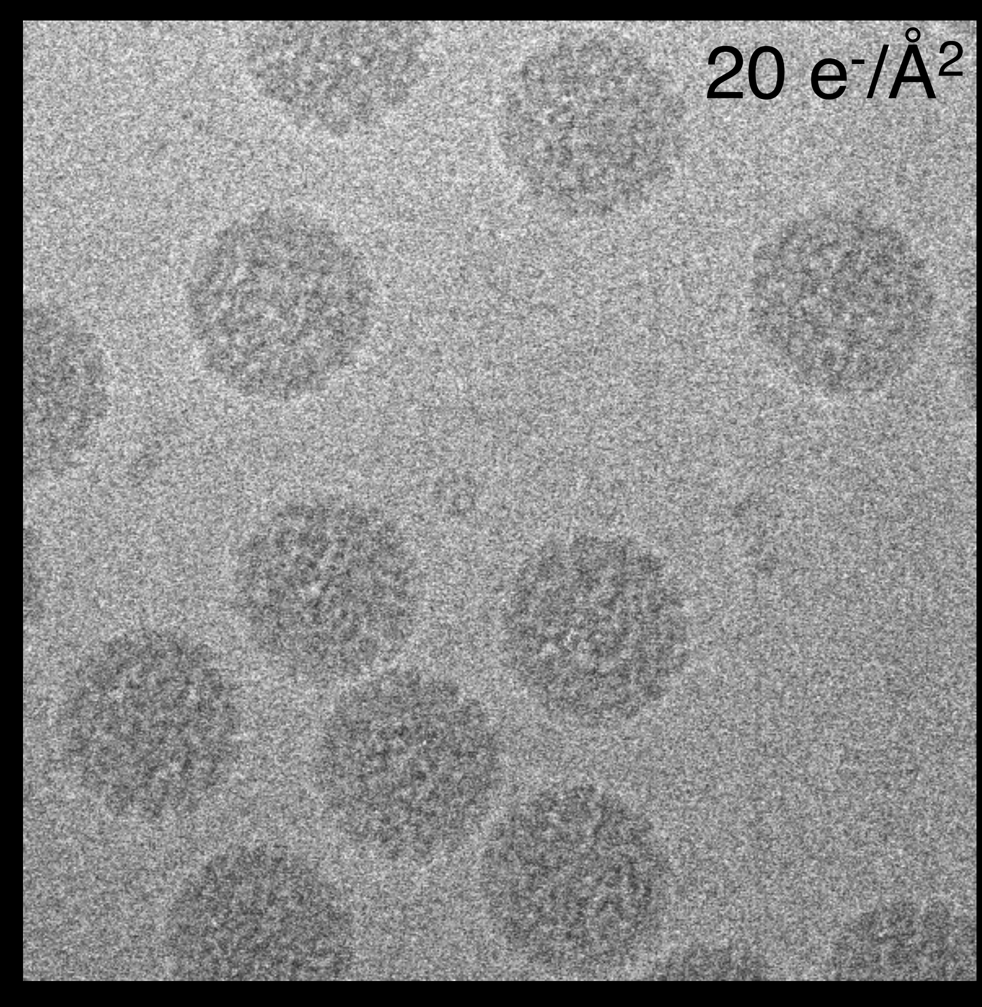

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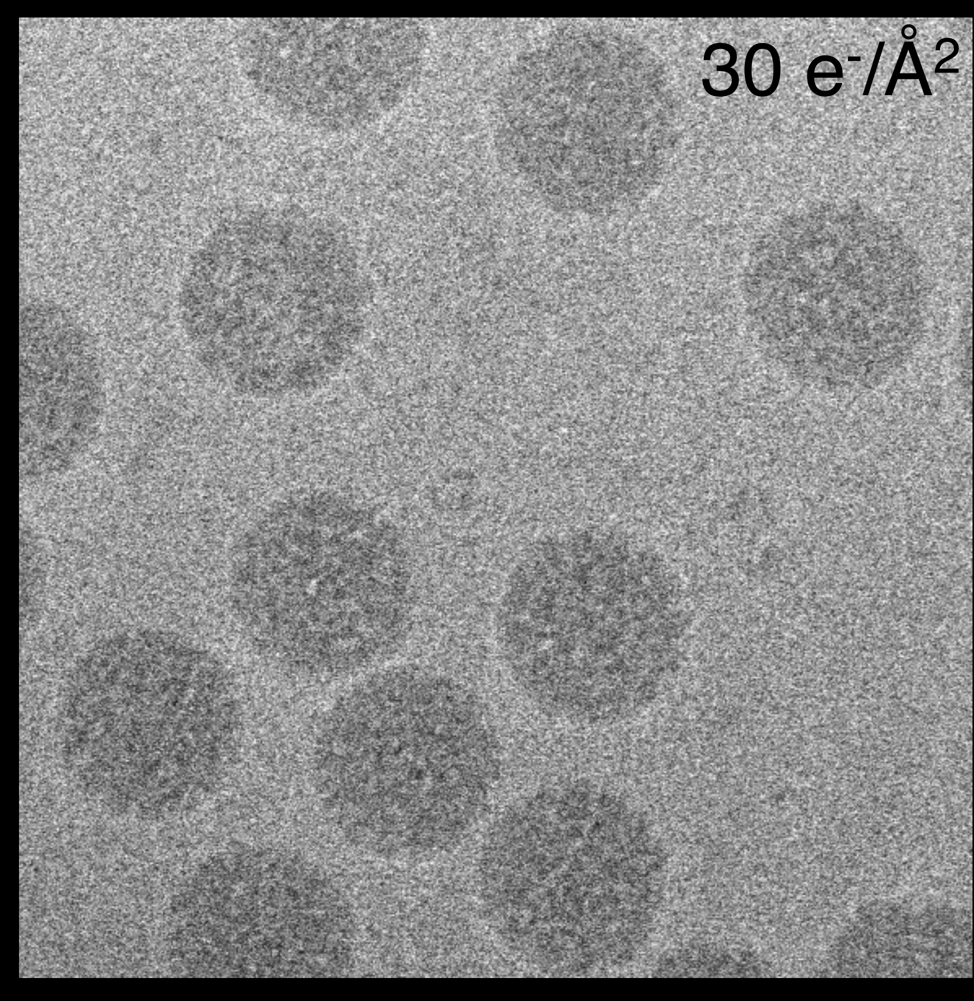

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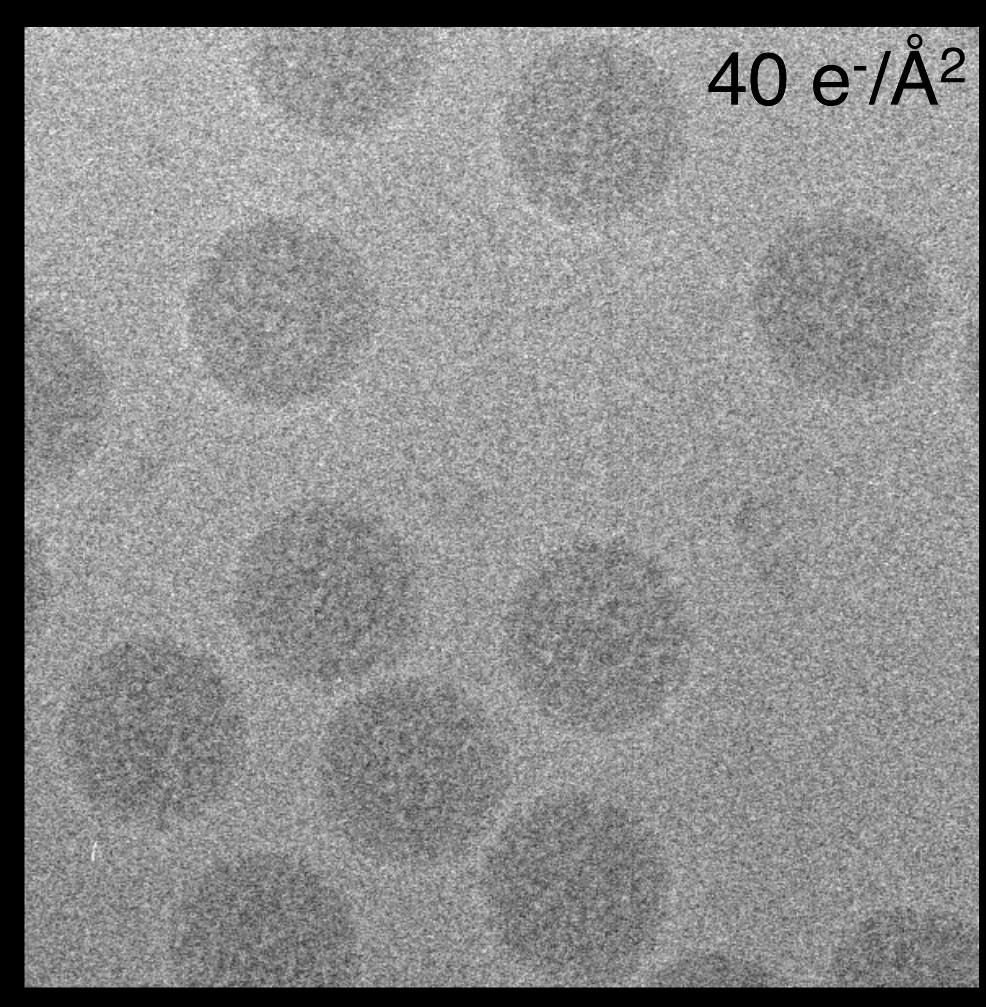
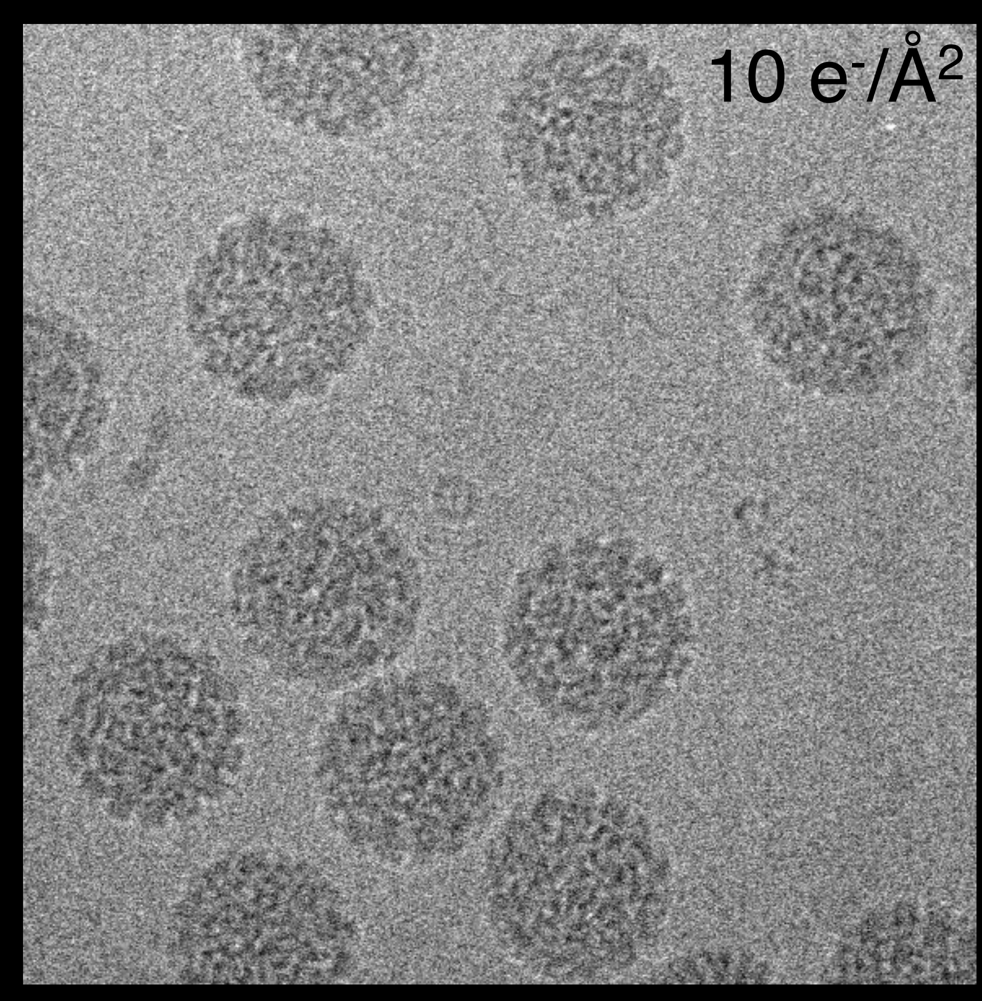


SV40 Dose Series



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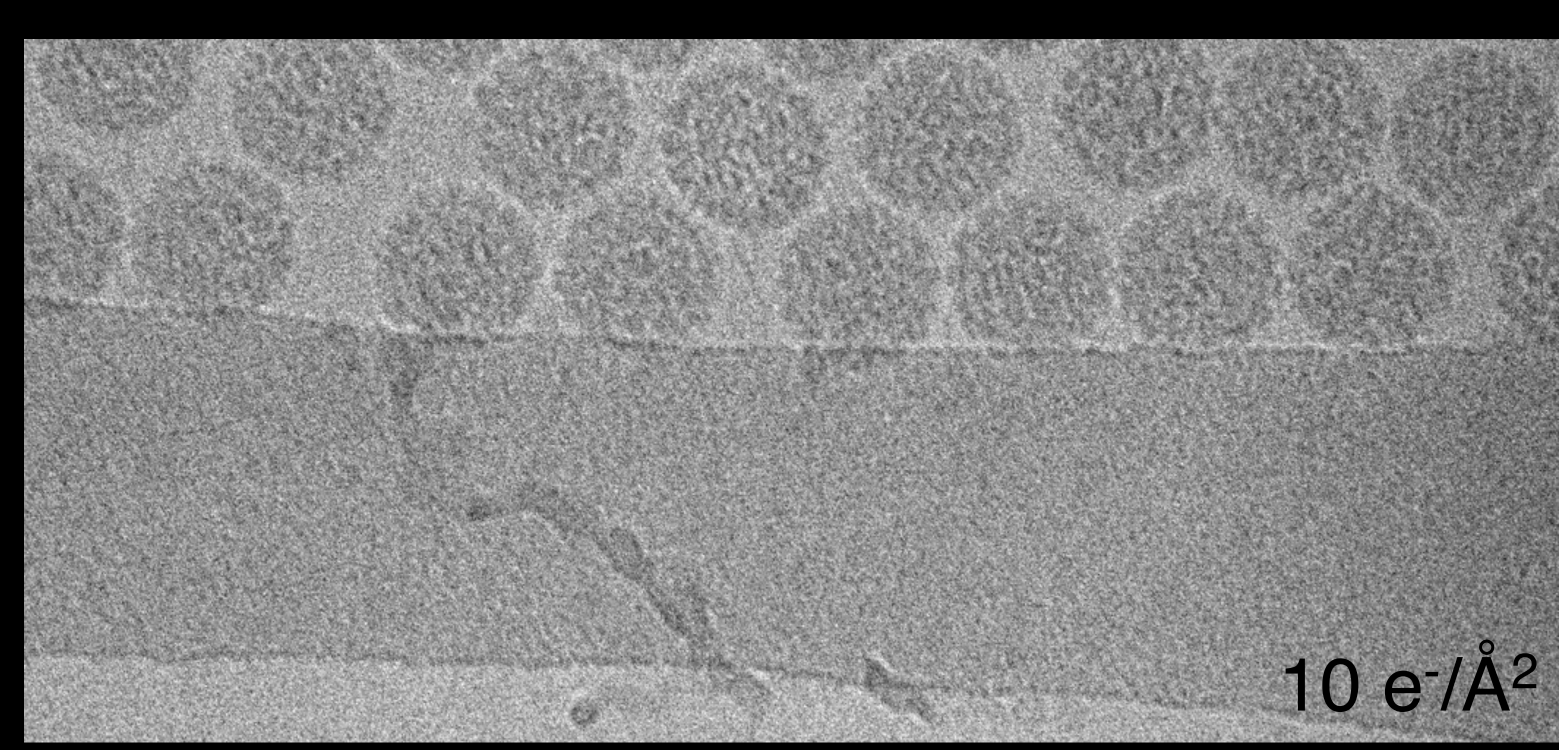

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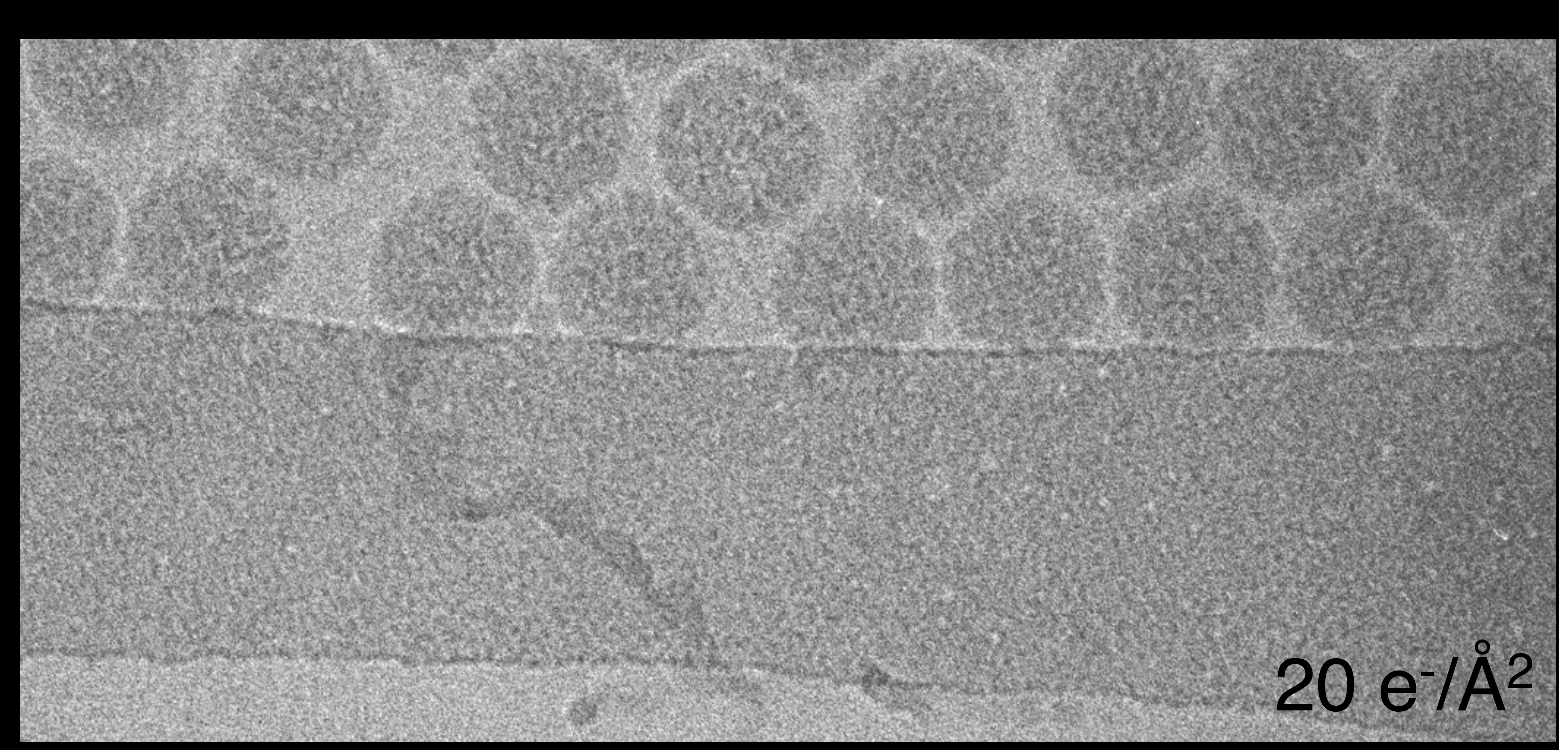

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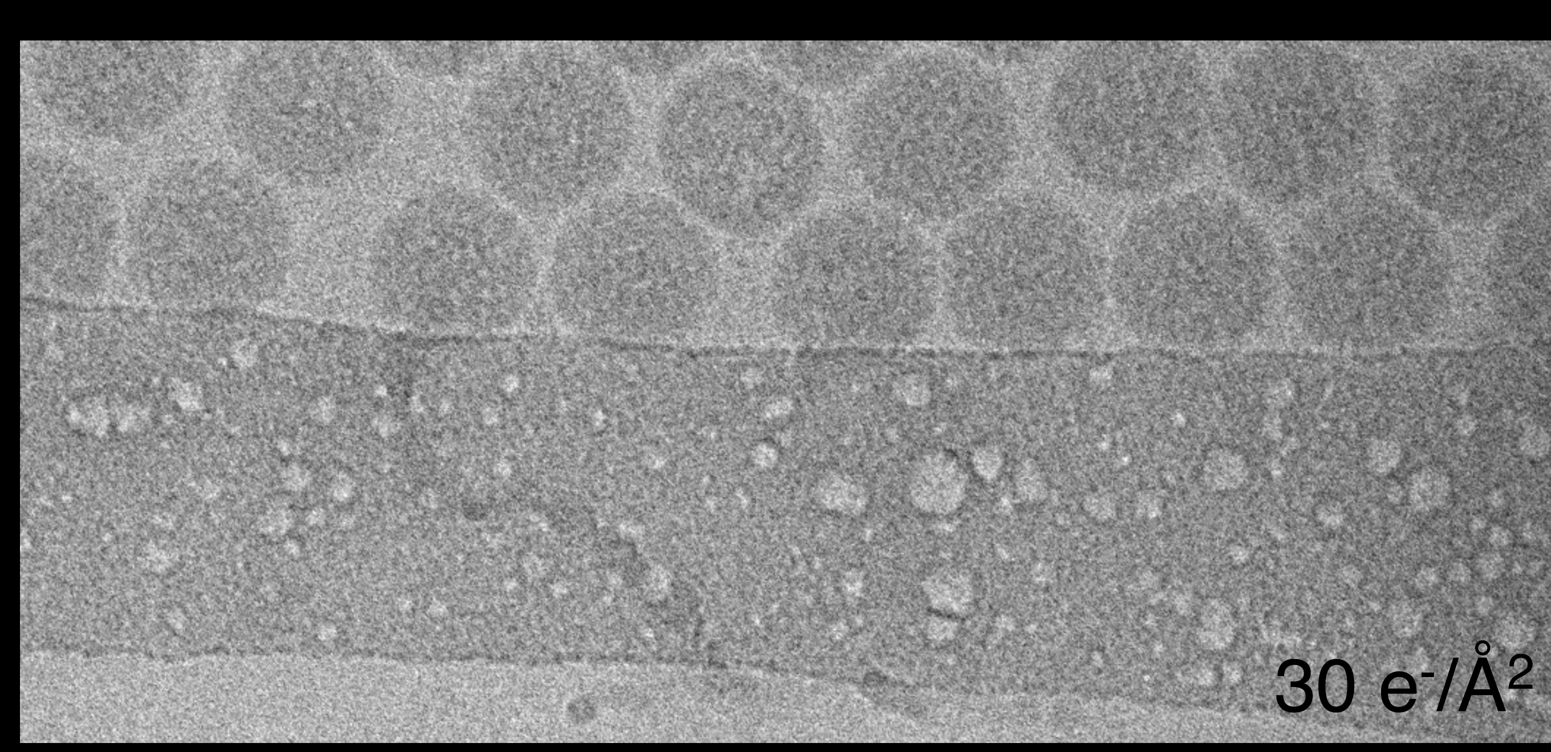

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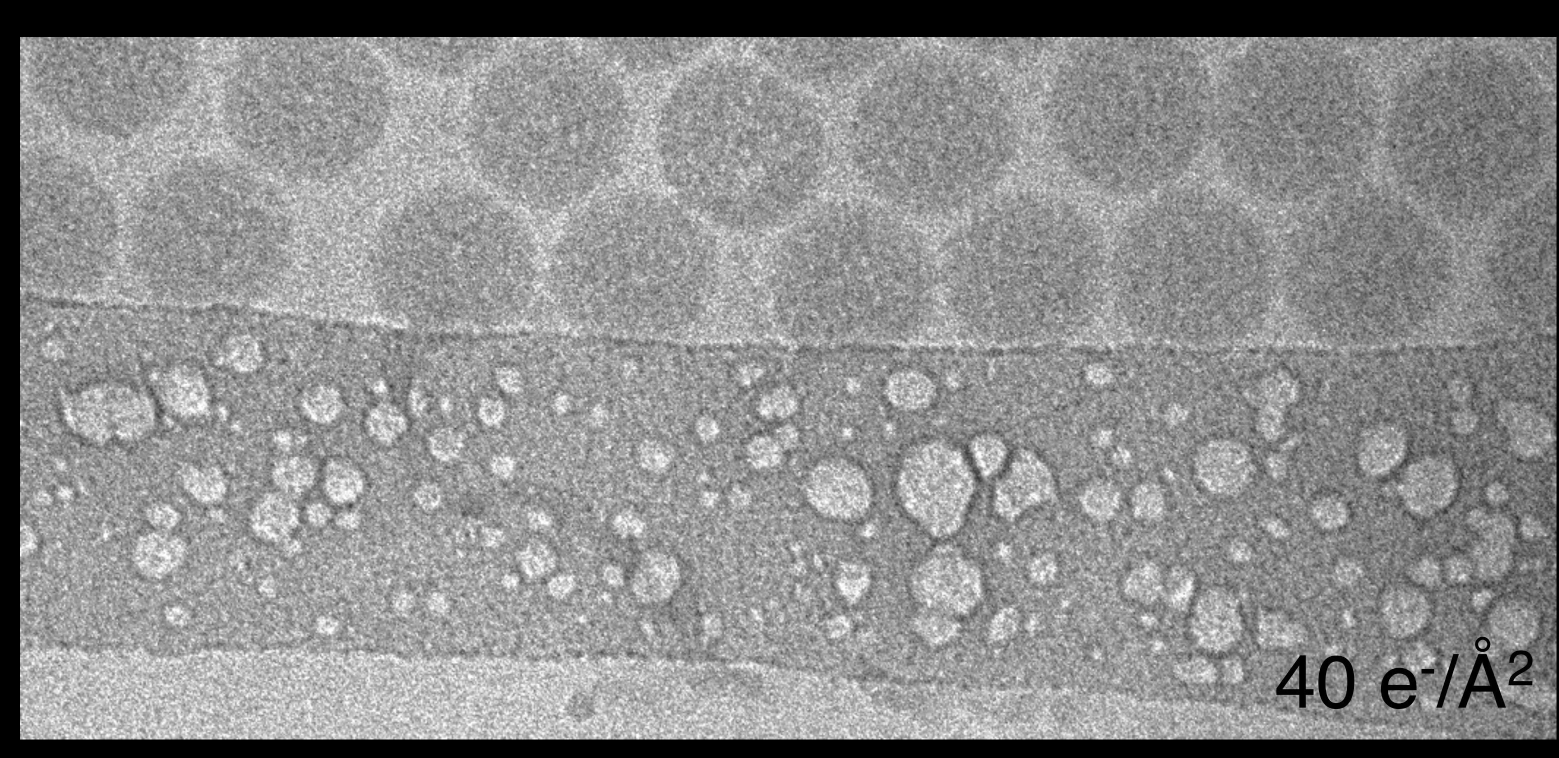

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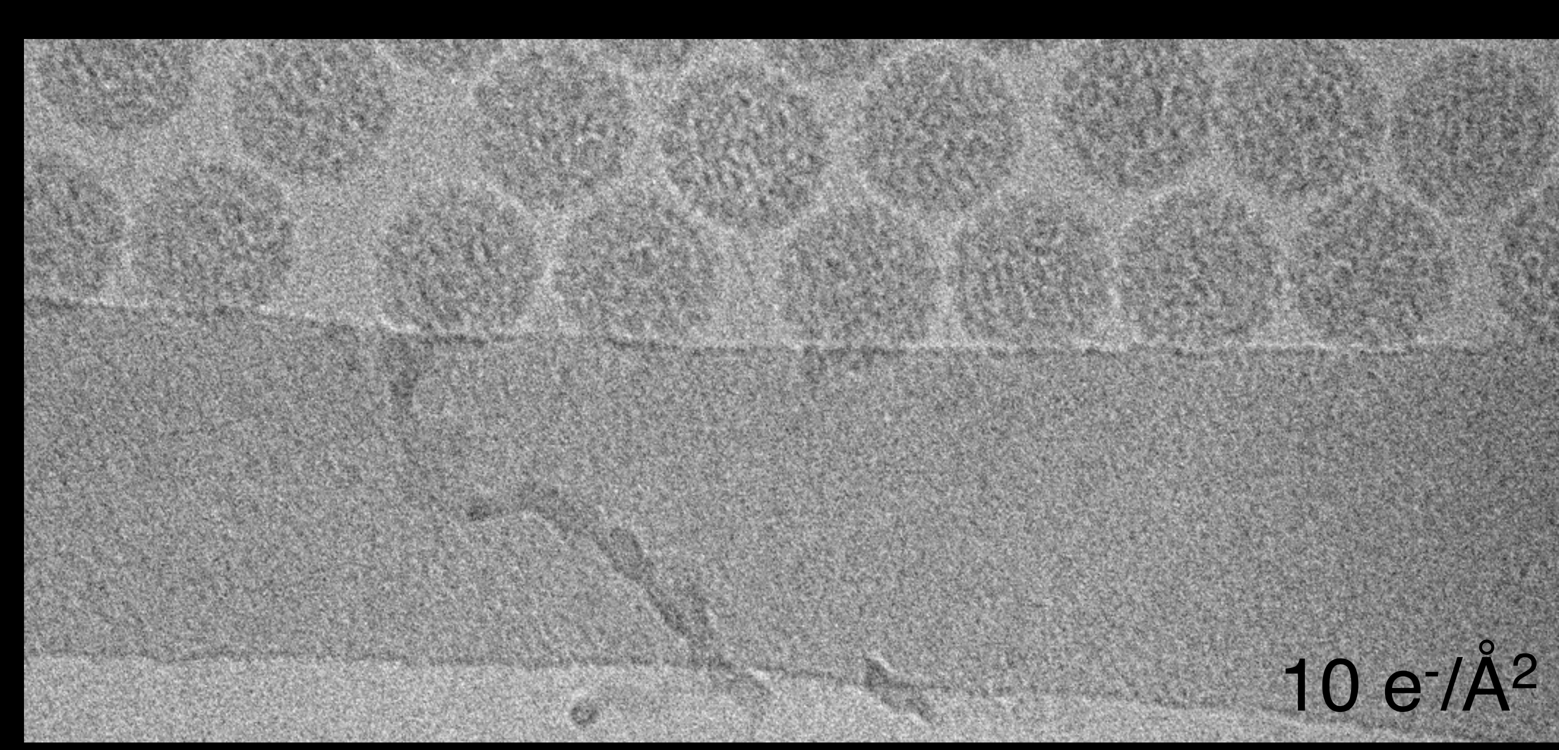

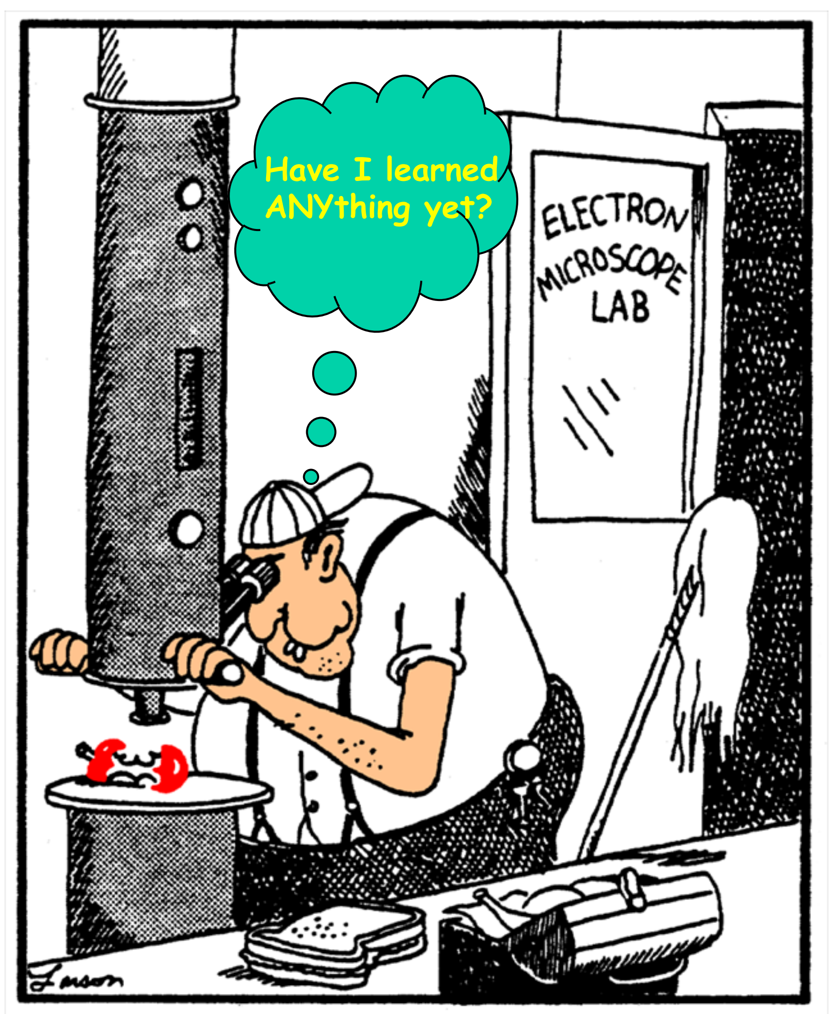

TOPICS

- Principles of TEM
  - Electrons, lenses and optics
- Design of TEM
  - Components top to bottom
- Contrast and image formation
  - Electron scattering from object
- Optimizing TEM performance
  - Alignment assures ‘best’ images
- Operation of TEM
  - “What do all these buttons do?”
- Other modes of TEM
  - Many ways to ‘observe’ specimens
- Specimen preparation for TEM
  - Getting specimen ready
- Radiation damage
  - Less is better
- 3D reconstruction
  - Specimen 3D structure from 2D images

§ I: The Microscope

§ II: The Specimen

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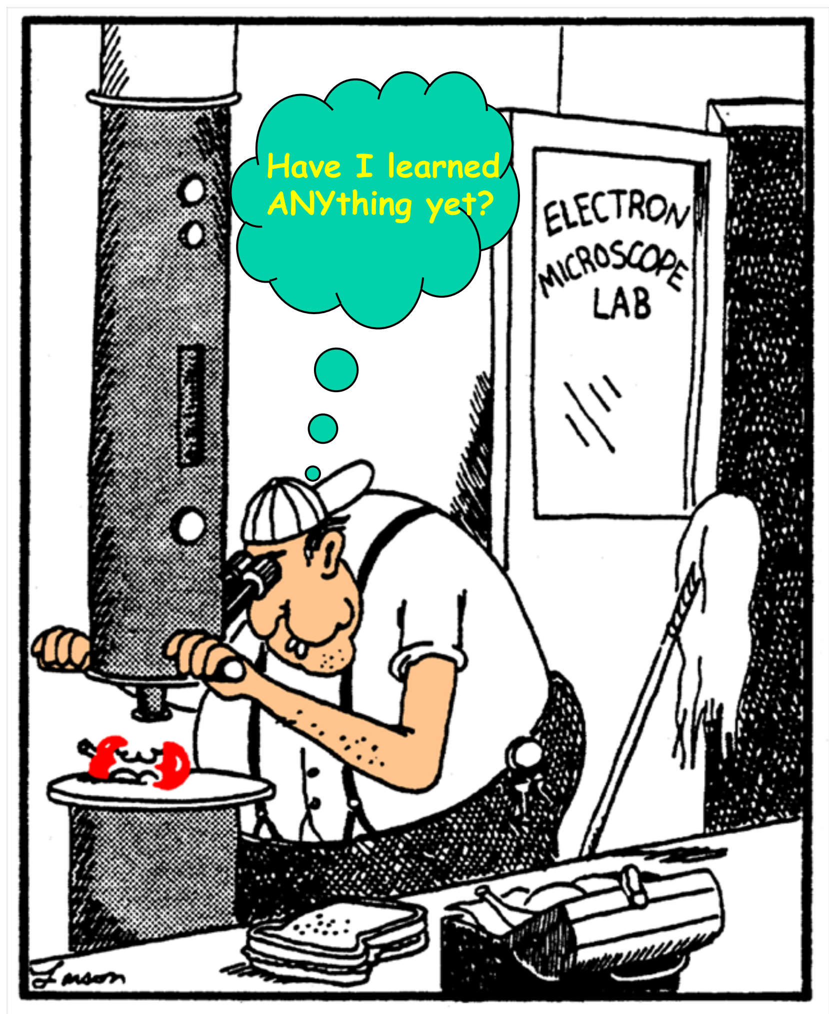
§ II: The Specimen

§ III: The Structure



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