UTMB The University of Texas Medical Branch

ESTABLISHMENT OF A CRYO-ELECTRON MICROSCOPY LABORATORY UNDER BSL-3 CONDITIONS

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Overview

- **Introduction:** - W. M. Keck Center for Virus Imaging
  - Basics on electron microscopy (EM)/cryo-EM
- **W. M. Keck Center for Virus Imaging:**
  - construction plan
  - sample freezing and storage, waste handling
  - cryo transfer
  - decontamination protocol of microscope
- **Summary**
- **First results**

W. M. Keck Center for Virus Imaging

- **W. M .Keck Center for Virus Imaging = BSL-3 cryo-EM lab**
- **Cryo-Electron Microscopy Center for Macromolecular Systems (UTMB)**
- **Manager: Dr. Michael Sherman**

mbsherma@utmb.edu

- **Funding: W. M. Keck Foundation, Kleburg Foundation, and UTMB**
- **First U.S. laboratory of its kind in a BSL-3 containment environment (approved for select agent work and functional since spring 2008)**

Research program

- **Structural imaging of pathogens presenting a threat to the US and other regions of the world**
- **Structural studies:**

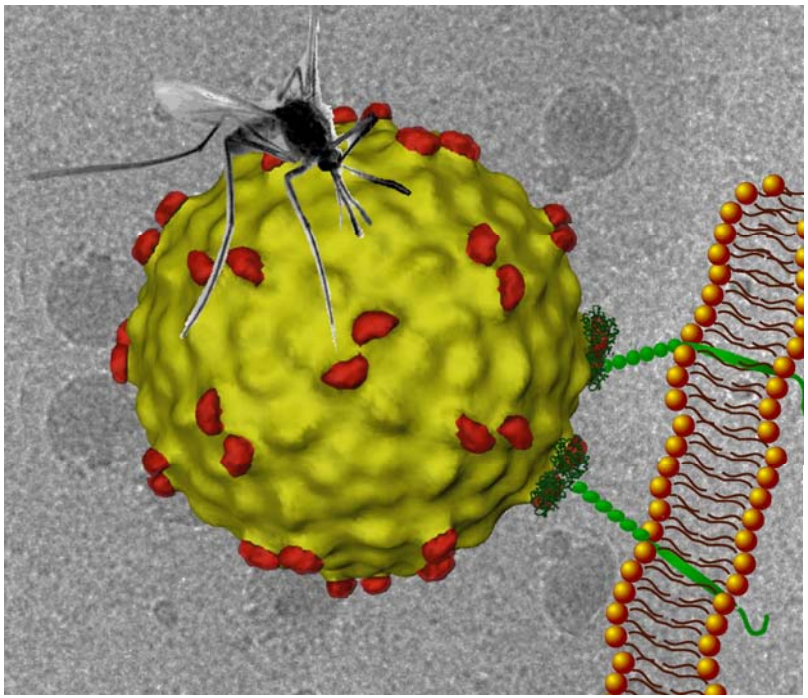
  → assembly of pathogens

  → development of potential vaccines

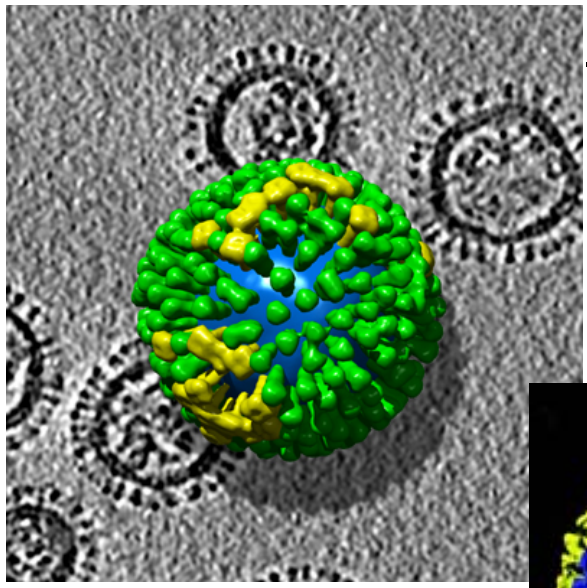
- **Research program focuses on RNA viruses:**

1) **encephalitis (e.g. alphaviruses [VEEV, WEEV] and flaviviruses [WNV, JEV])**
2) **hemorrhagic fever (e.g. bunyaviruses [RVFV], arenaviruses [Junin virus], flavivirus [Dengue virus])**
3) **acute respiratory disease (e.g. hantaviruses, SARS)**
4) **influenza (avian influenza)**
5) **Hepatitis C**

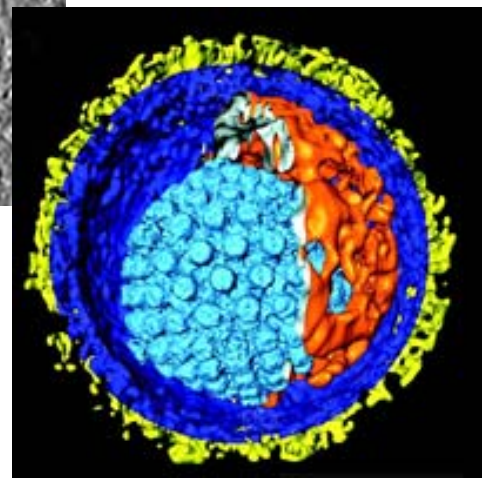
Examples of infectious agents solved by cryo-EM



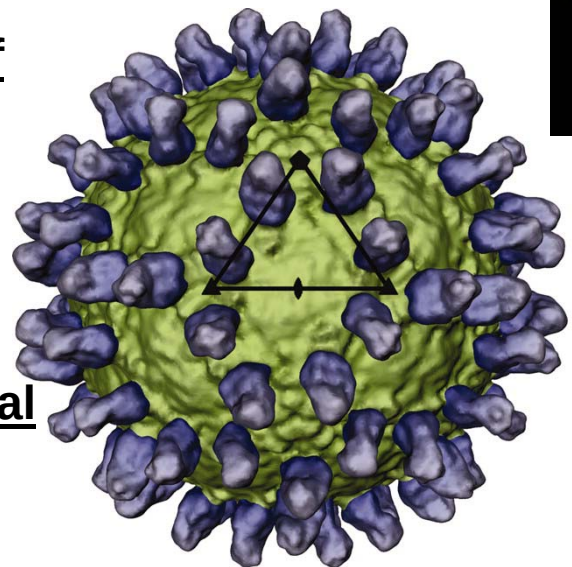
DENV in complex with the carbohydrate recognition domain of DC-SIGN
Pokidysheva et al., Cell 2006



Influenza H3N2
Harris et al., PNAS 2006



Herpes simplex virus
Grunewald et al., Science 2003



WNV in complex with the Fab fragment of a neutralizing monoclonal antibody
Kaufmann et al., PNAS 2006

Background electron microscopy

JEM-2200FS TEM

JEM: JEOL Electron Microscope

TEM: Transmission Electron Microscope

- Magnification: up to x2,000,000 (light microscopes: up to x2,000)
- Need for special rooms, since microscopes are very sensitive to:

→ vibrations,

→ acoustics,

→ air flow,

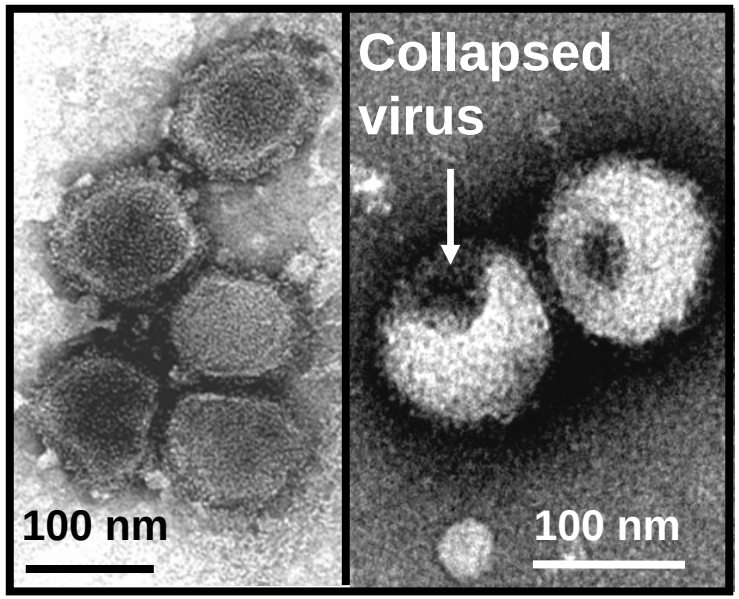
→ external magnetic fields,

→ pressure,

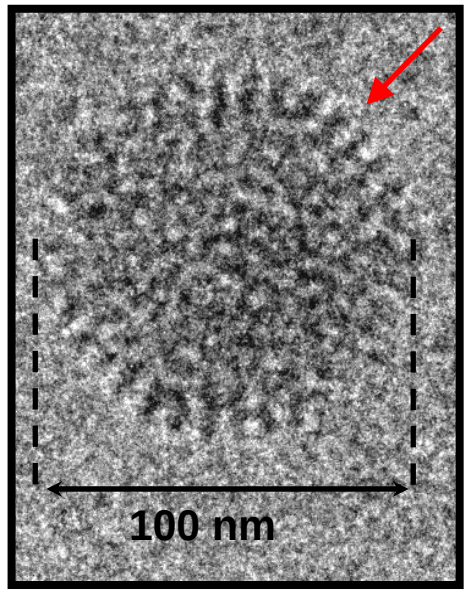
→ temperature fluctuations

What is cryo-Electron Microscopy (cryo-EM)?

e.g., Rift Valley Fever virus vaccine strain MP-12

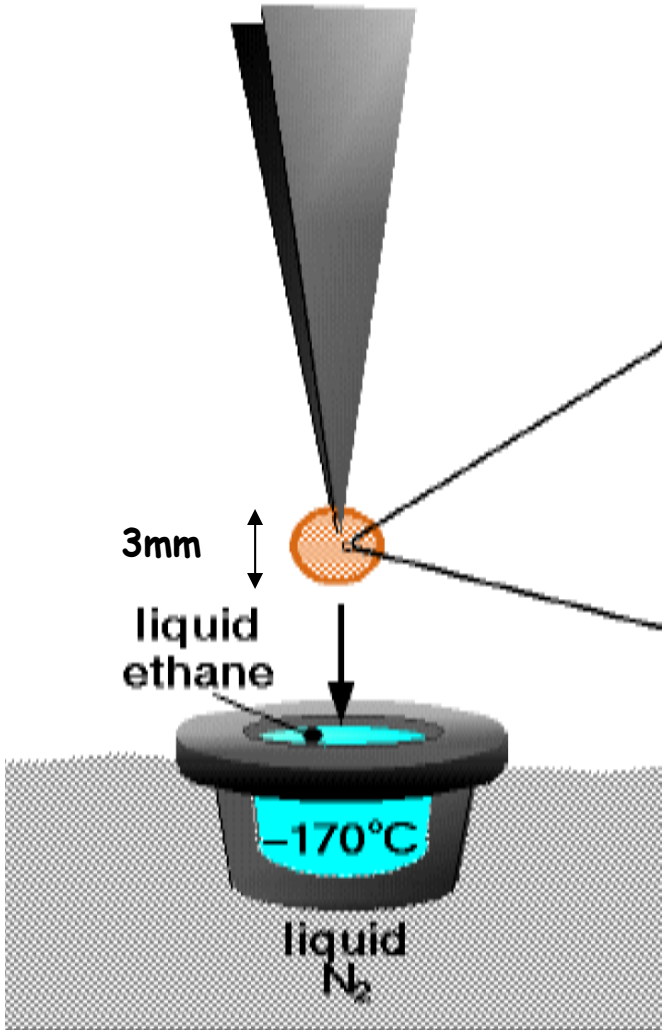


Surface spikes

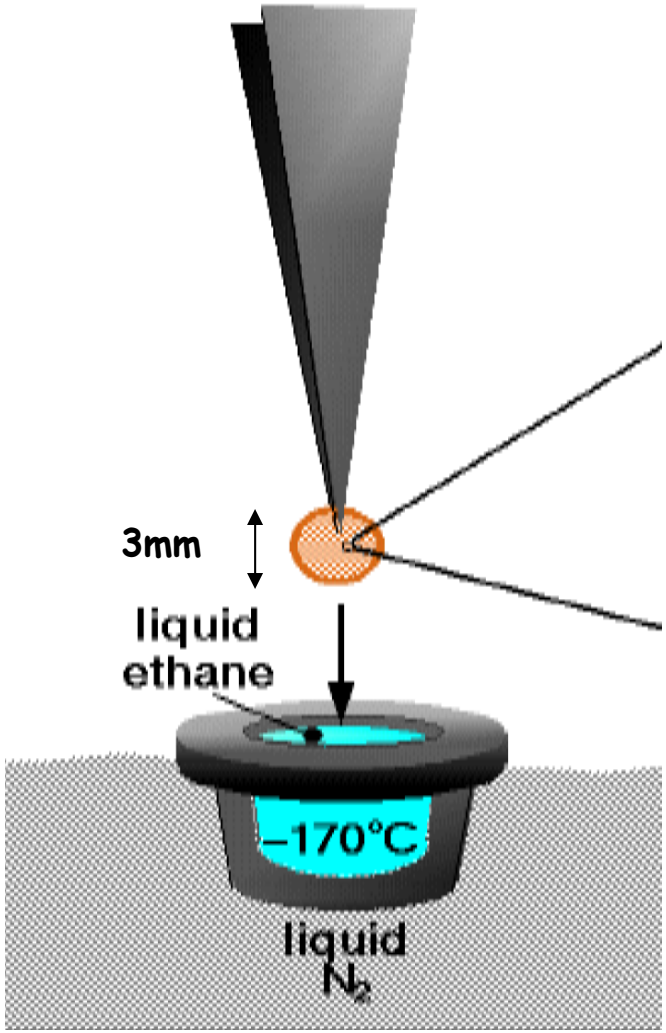


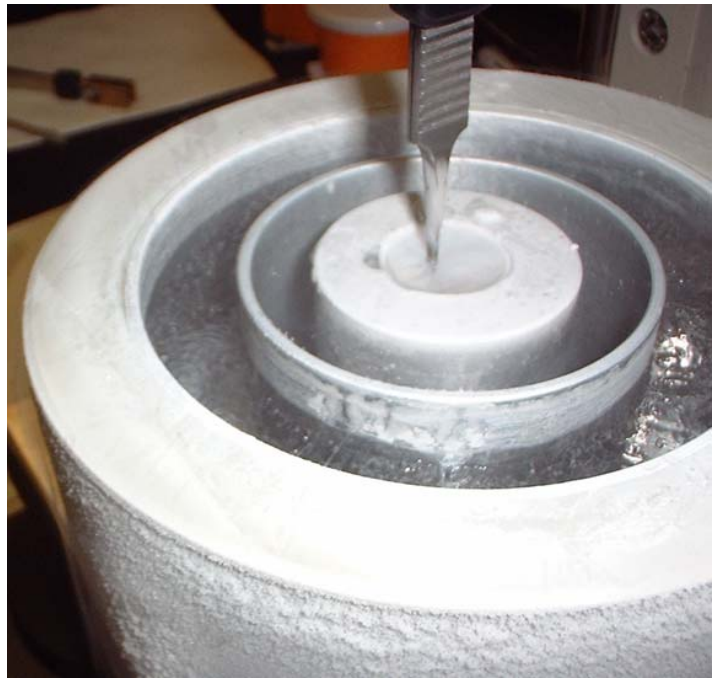
method	Negative staining	Cryo-EM
stain	heavy-metal stain	vitrified in amorphous ice (non-crystalline ice)
condition	often distorted due to dehydration	native-like hydrated structure
structure	limited detailed information	detailed information

Plunge freezing of specimen

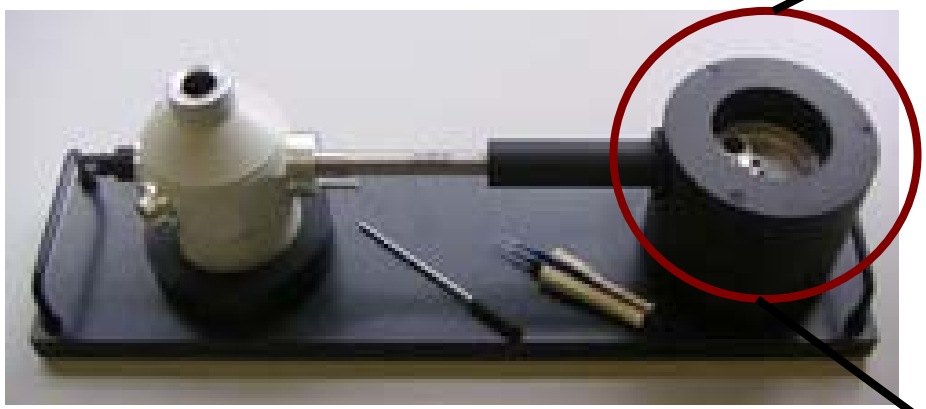


Plunge freezing of specimen

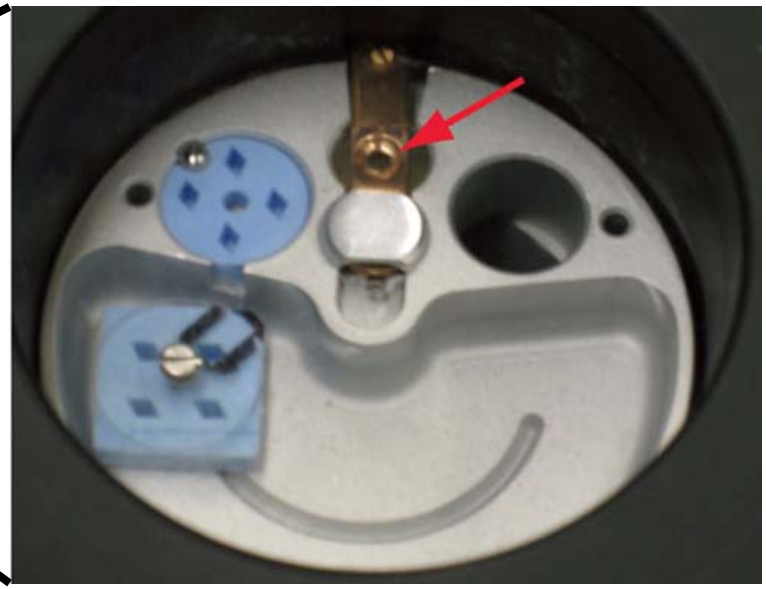




Plunge freezing of specimen



cryo transfer station



Plunge freezing of specimen

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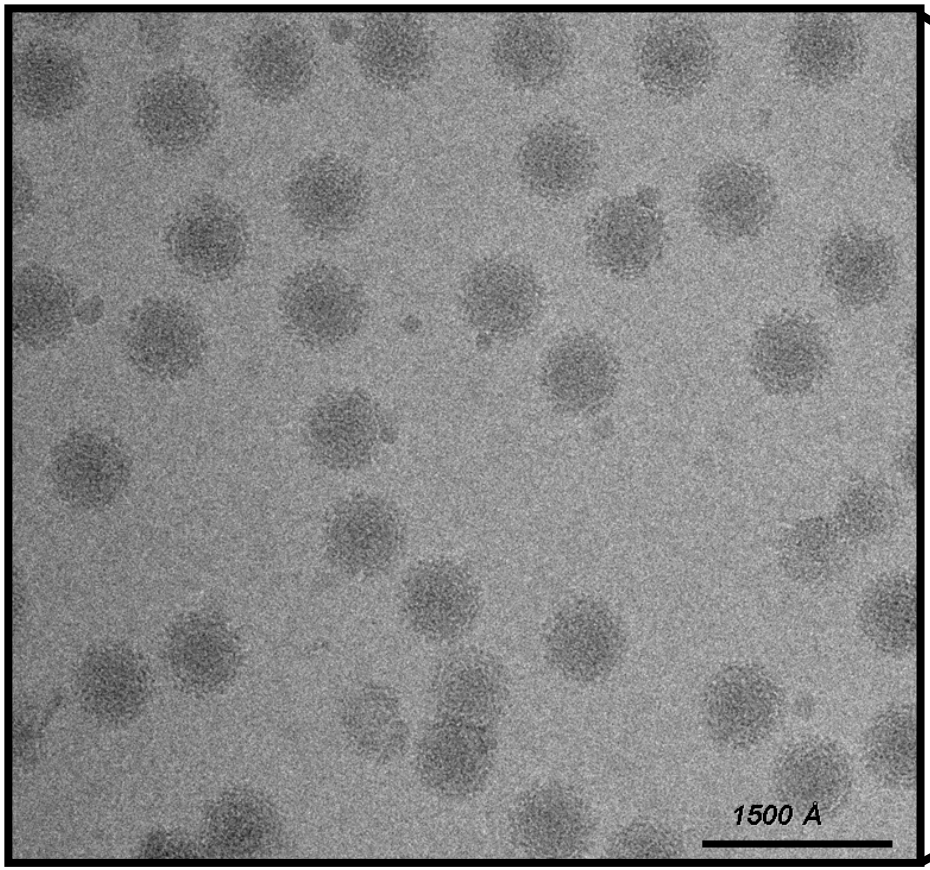
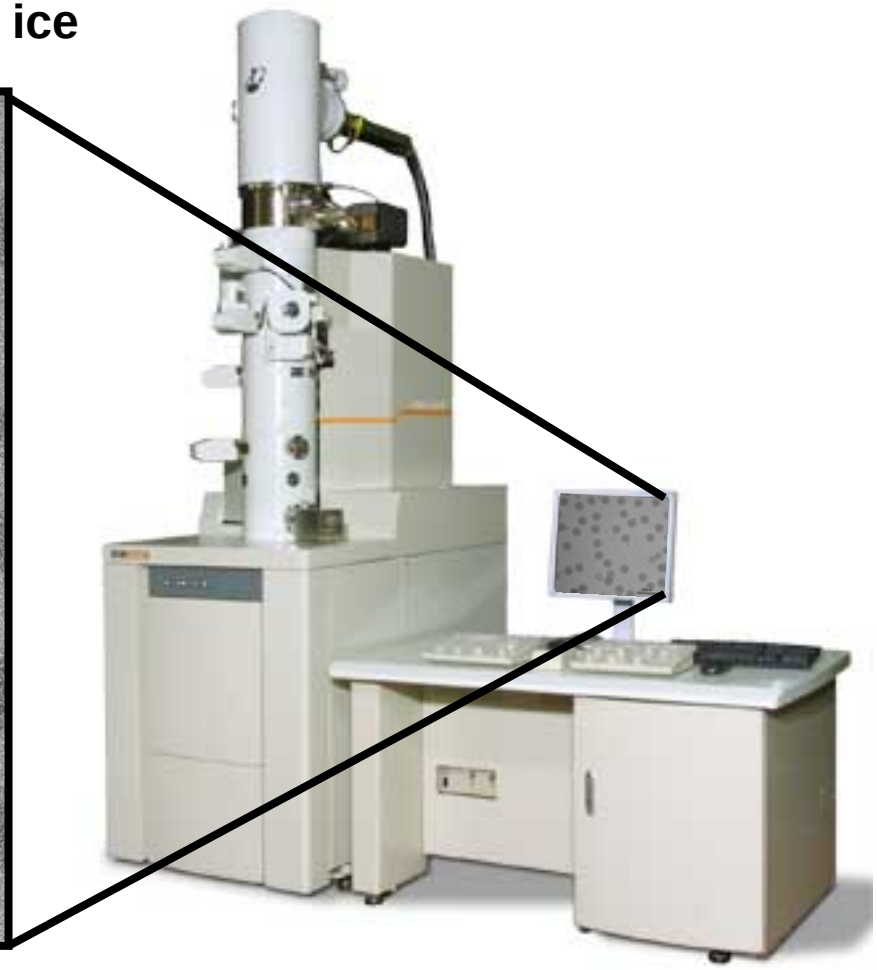
Virus particles embedded in amorphous ice





JEM-2200FS TEM

Flow diagram in Cryo-EM

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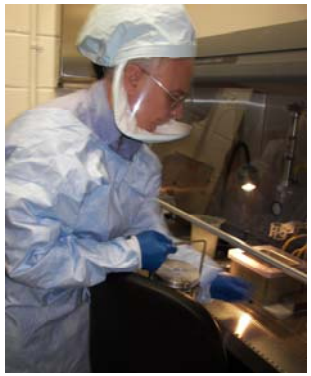
Virus purification

BSL-3

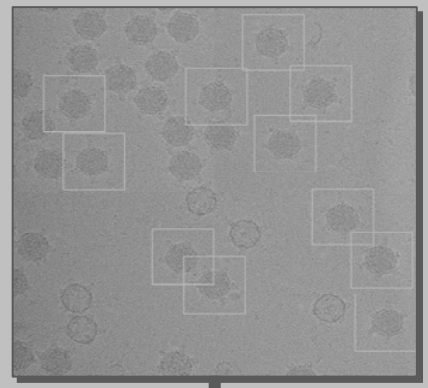
Cryo-EM sample preparation



Imaging

W. M. Keck Center BSL-3

Data Collection



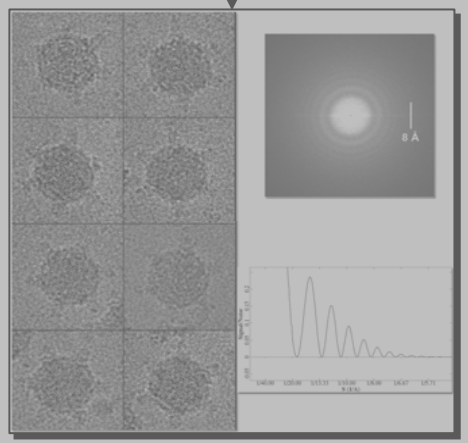
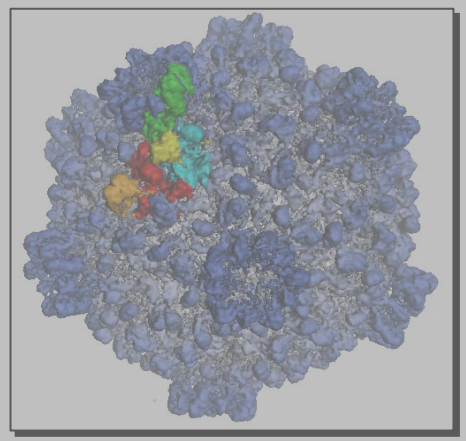
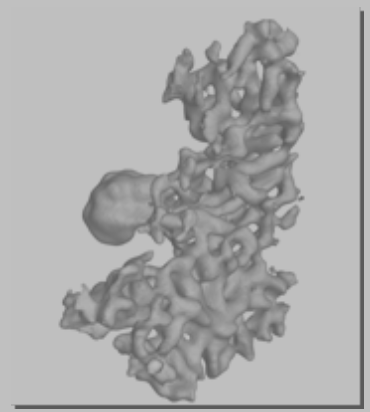


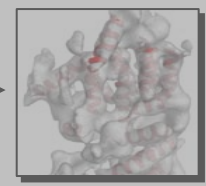
Image Processing

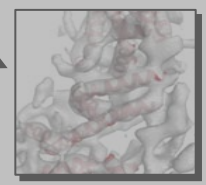


Reconstruction



Structural Analysis





Annotation

modified from Chiu *et al* (2006) JEOL News

Ground plan Cryo-EM suite

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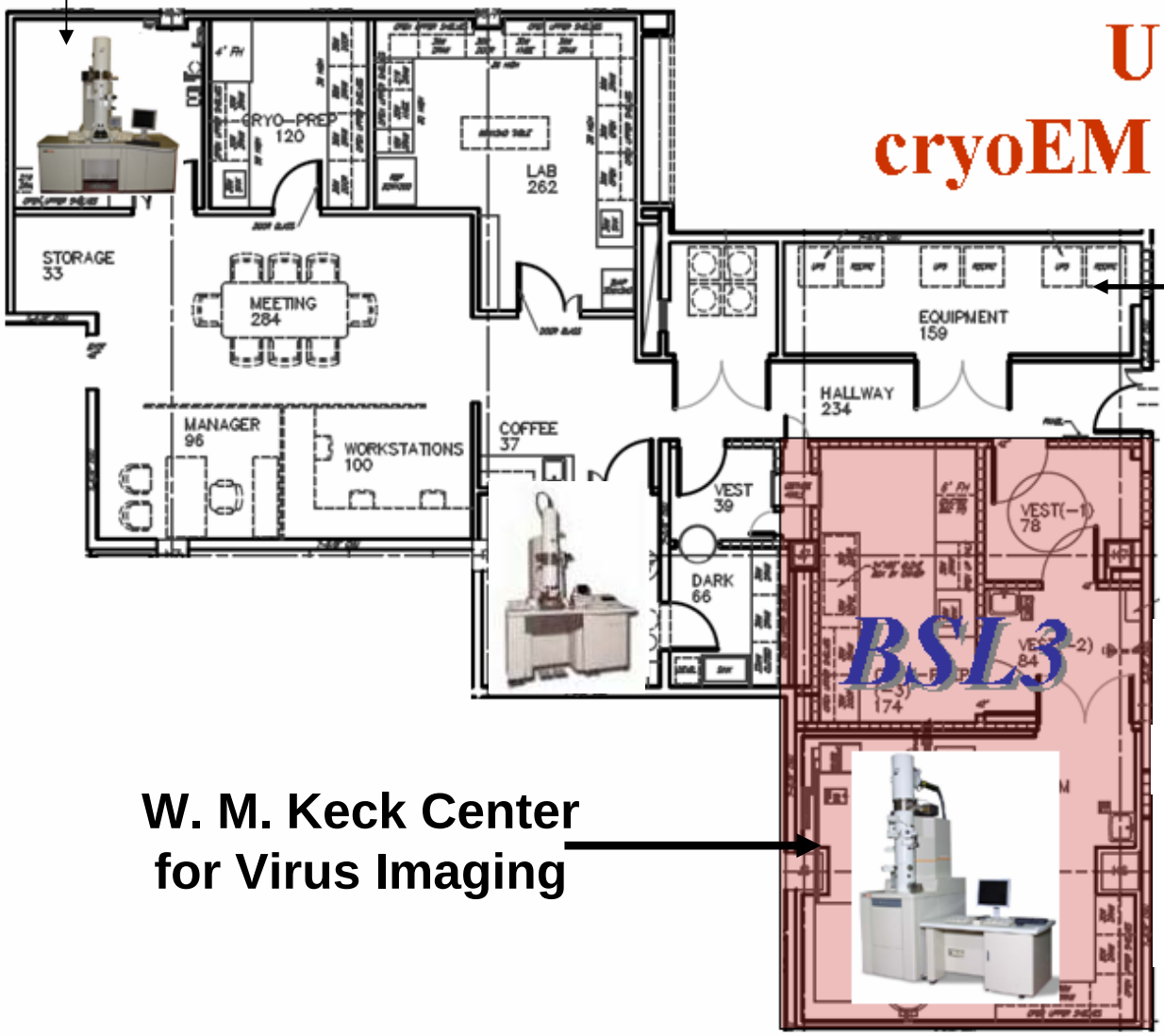
BSL-2



UTMB
cryoEM core facility

Remote control room

3 TEMs:

- 200 keV JEM 2200FS (BSL3)
- 200 keV JEM 2100
- 120 keV JEM 1010

W. M. Keck Center for Virus Imaging

Ground plan Cryo-EM suite

UTMB
yoEM core facility

Remote
control room

BSL3

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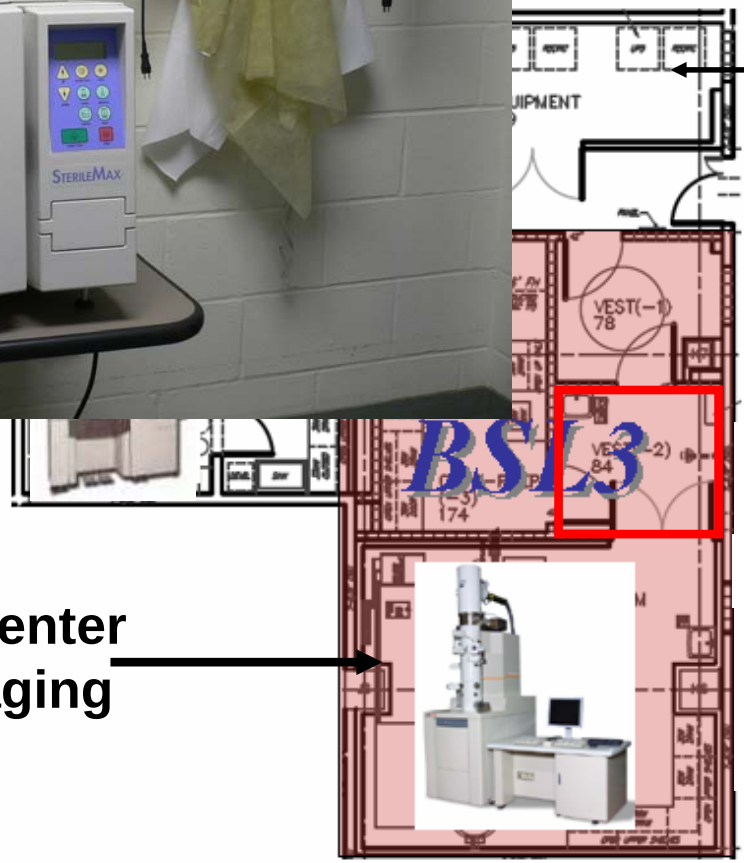

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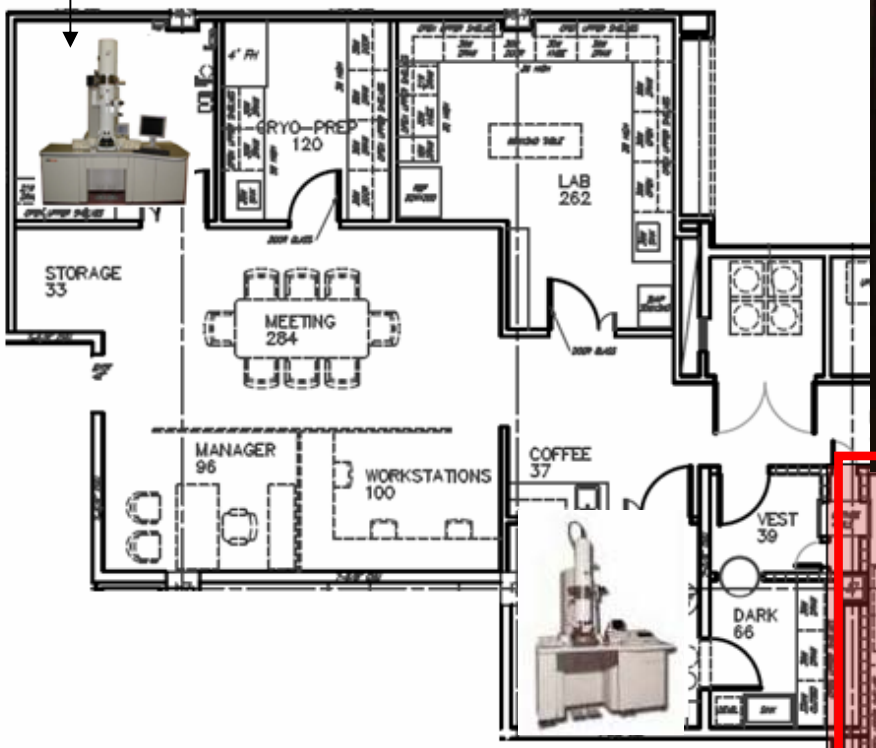

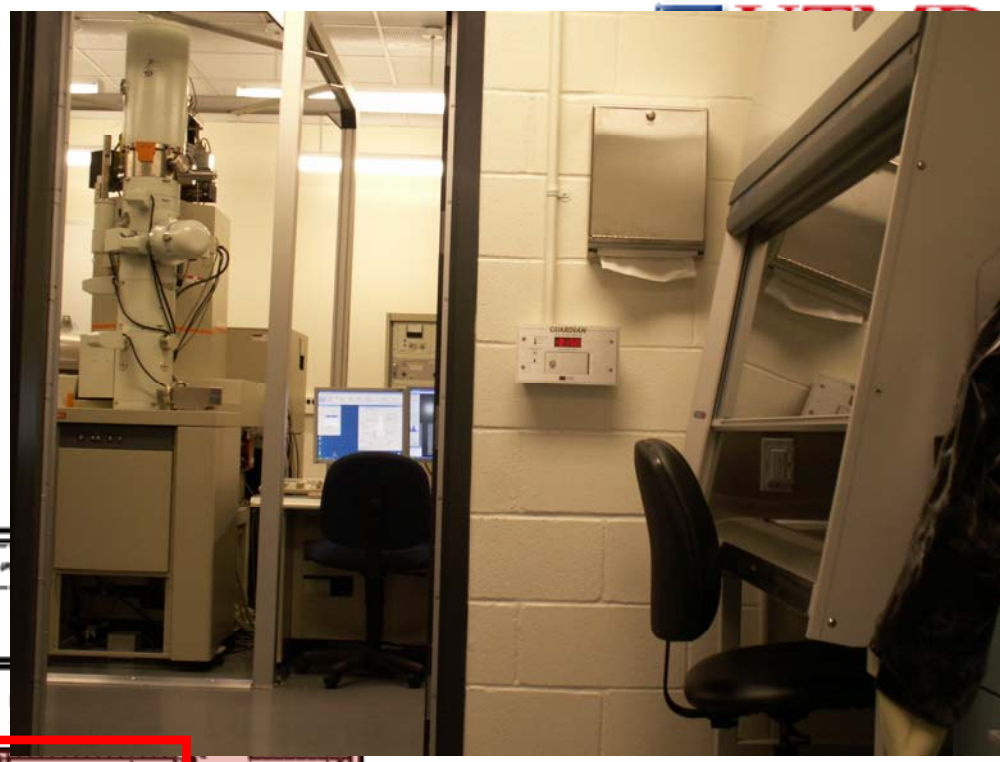

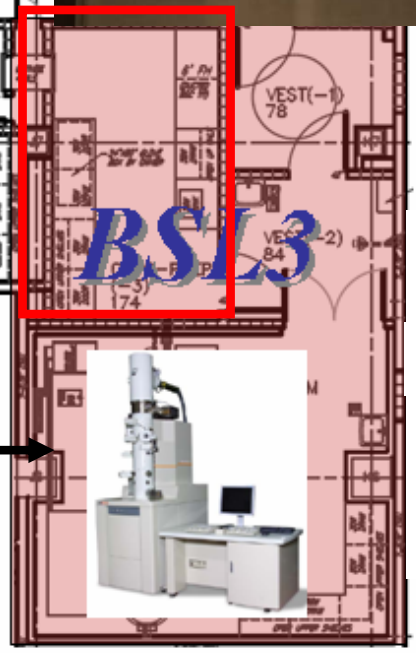

W. M. Keck Center for Virus Imaging

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Ground plan Cryo-EM suite

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UTMB
cryoEM core facility

Remote control room

3 TEMs:

- 200 keV JEM 2200FS (BSL3)
- 200 keV JEM 2100
- 120 keV JEM 1010

Standard Operating Procedures (SOP)

- **Basic general BSL-3 procedures apply, exceptions are:**

Entry Procedures

Exit Procedures

Personnel Practices

General Laboratory Procedures

Respiratory Protection

Procedures for Centrifugation

Biological Safety Cabinet (BSC) Protocol ←

Waste Handling and Disposal Procedures

Equipment Repairs/Service ←

Storage, Packaging and Shipping **of Infectious Substances** ←

Removal of Biological Material from the BSL-3 Suite ←

Removal of non-biological material from the BSL-3 Suite

Preparation of infectious agents

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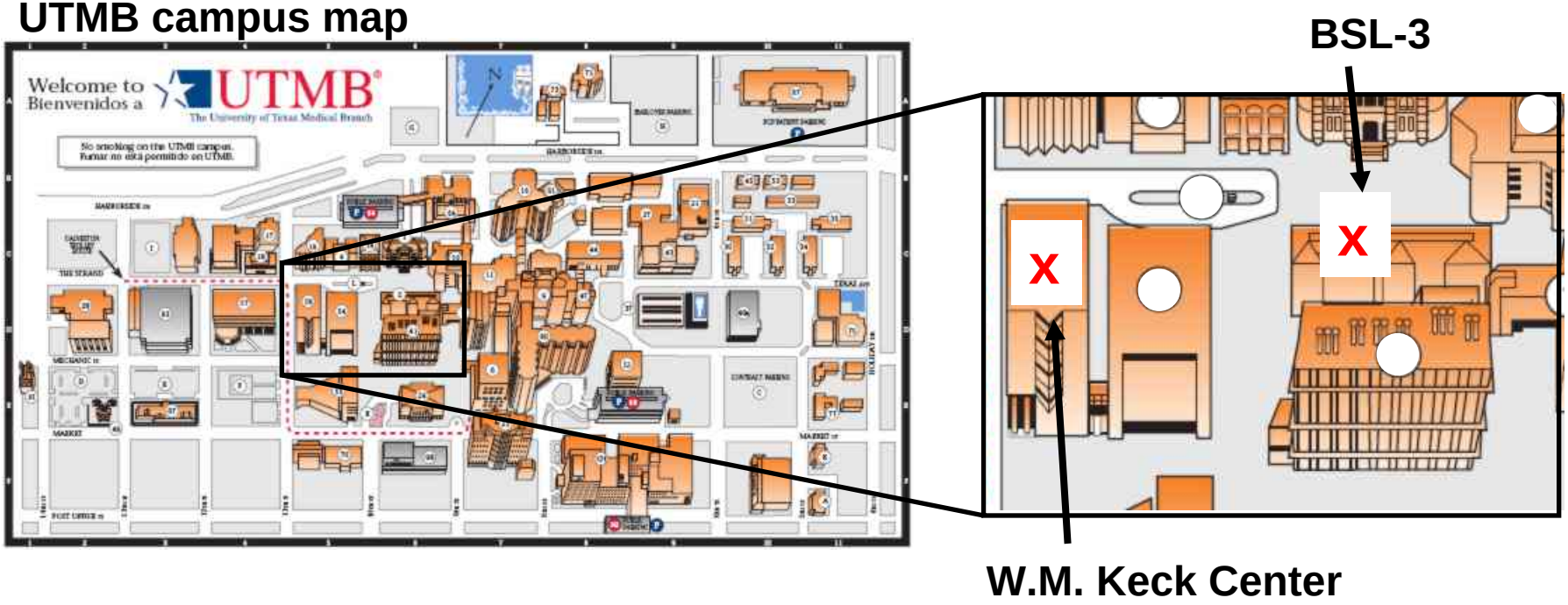
UTMB campus map



- **No containment work (growth, purification, and concentration) is performed in W. M. Keck Center**
- **Transport of agents according to the UTMB Institutional Biosafety Committee and Federal Guidelines**
- **Police escorted transport of select agents from BSL-3 to Keck Center**
- **Entry/Exit procedure protocols**

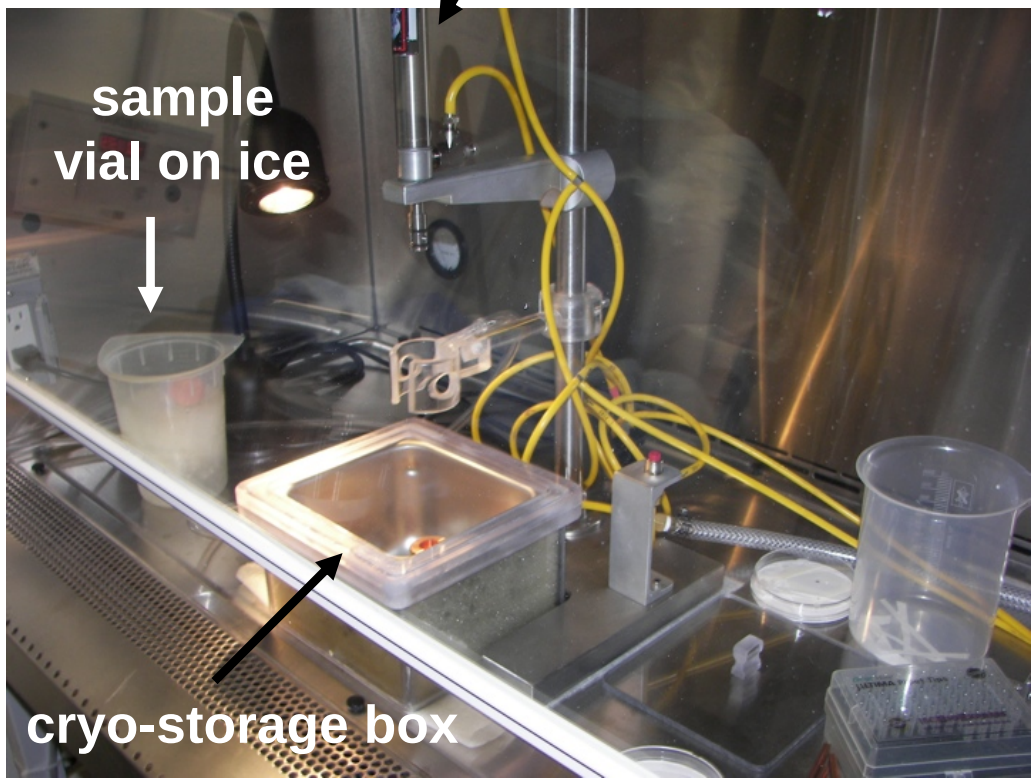
Select agent storage

- No storage of select agent after an imaging session has ended
- Specimen volume brought to the facility should be prepared in such a matter that no leftover material is to be removed
- EM grids containing non-select agent may be stored in liquid nitrogen dewars for further studies

Cryo-preparation

Personnel protection:

gown, gloves, respirator



Cryo-preparation – cont‘

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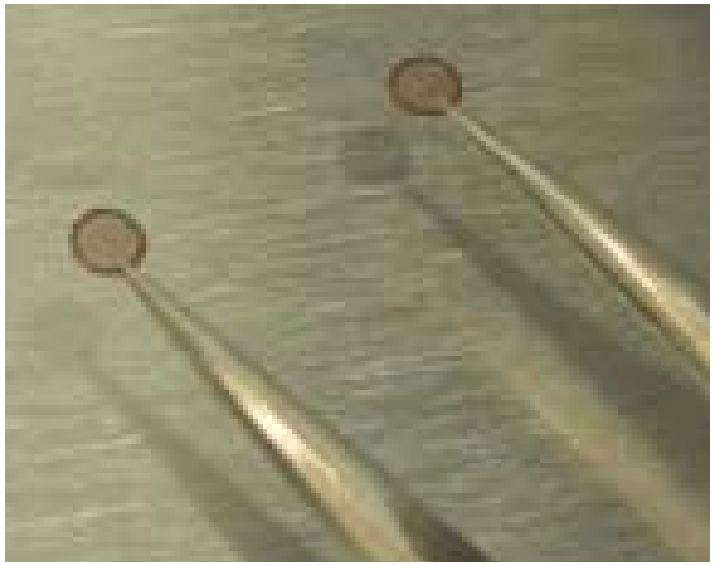
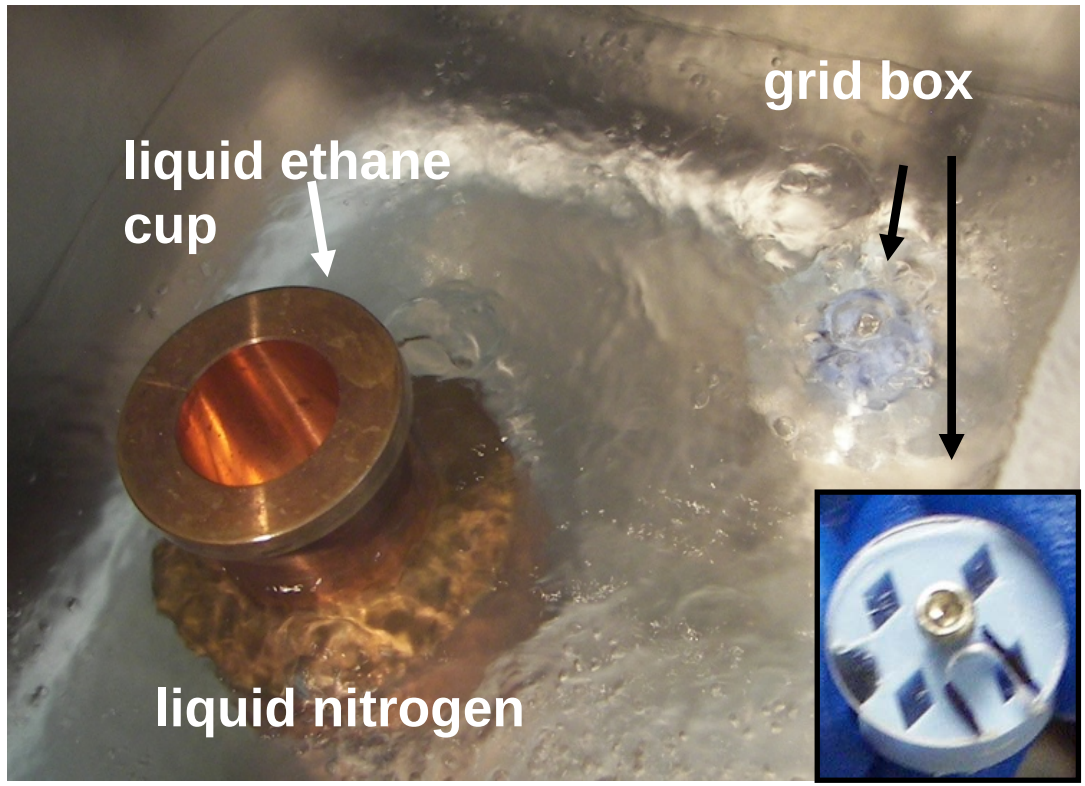
tweezers with EM grids

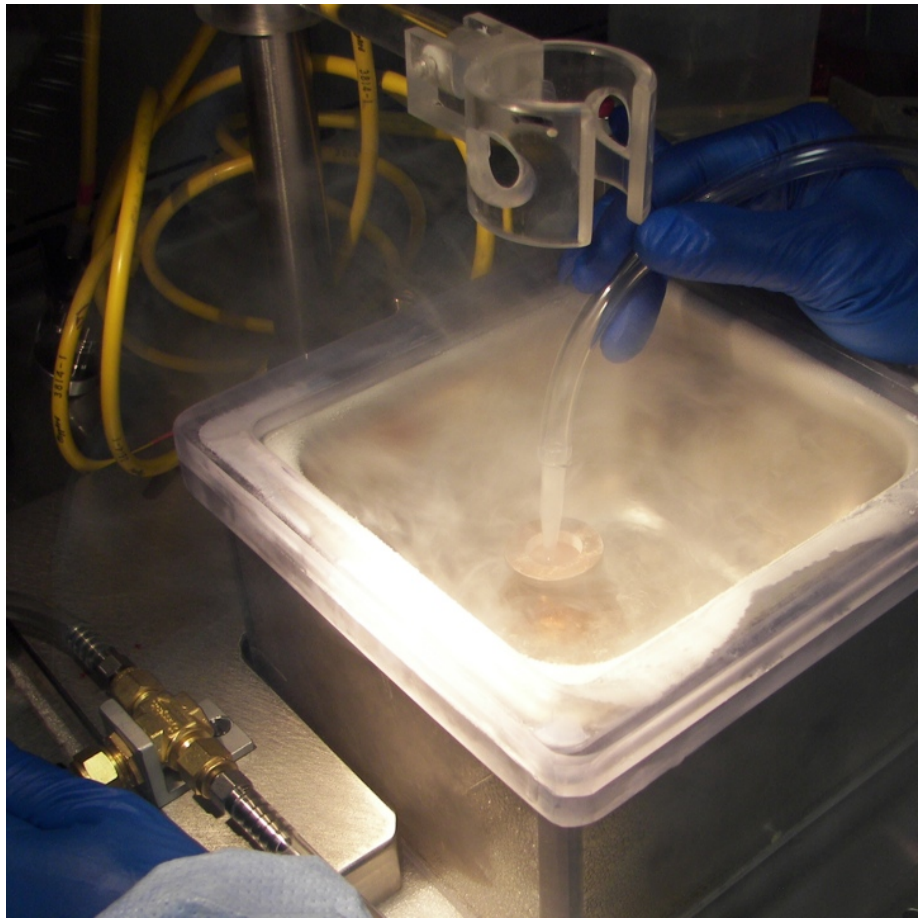


grids are very small (3 mm in diameter)
→ sharp tweezers are needed to manipulate grids



Cryo-preparation – cont‘

gaseous Ethane



Cryo-preparation – cont‘

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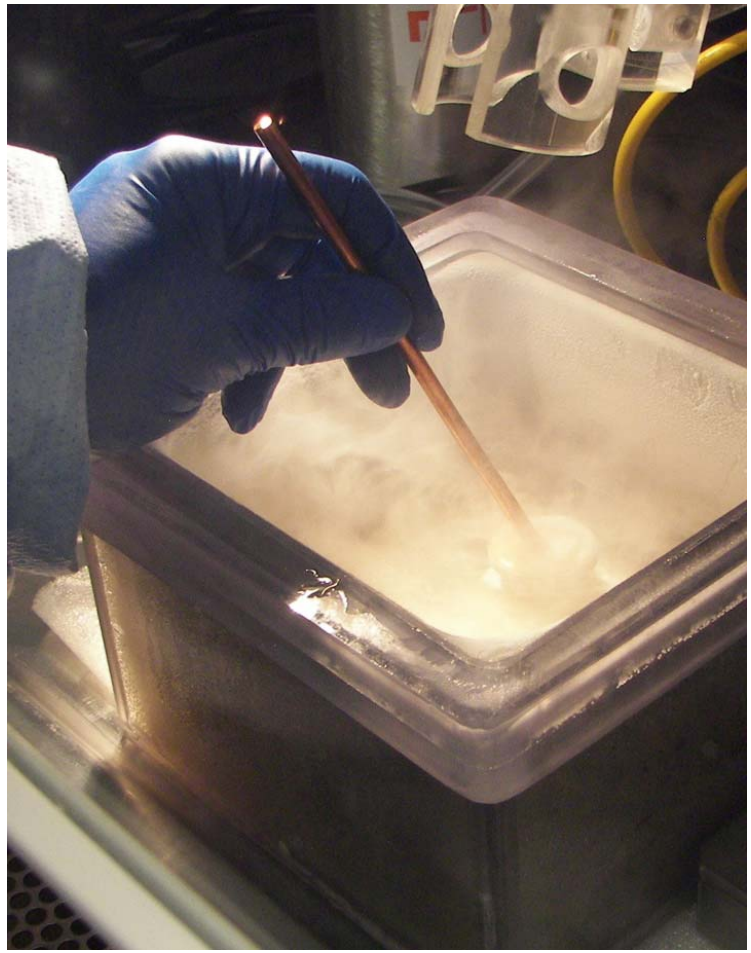
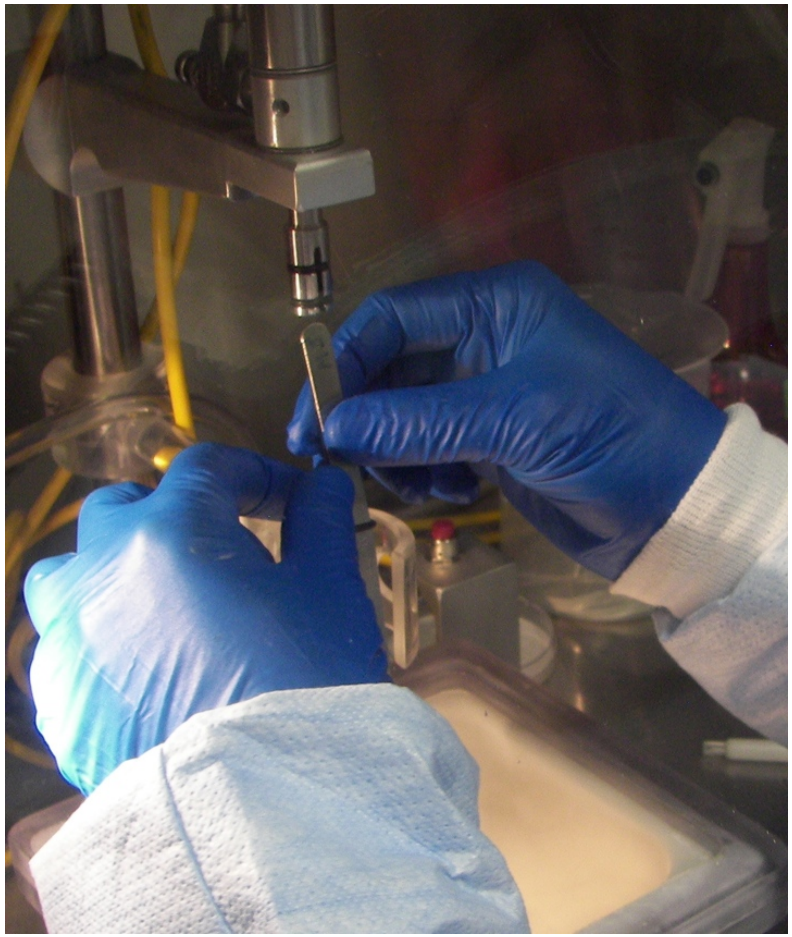
Ethane (g) $\xleftrightarrow{-88.6°C}$ Ethane (l) $\xleftrightarrow{-181.76°C}$ Ethane (s)

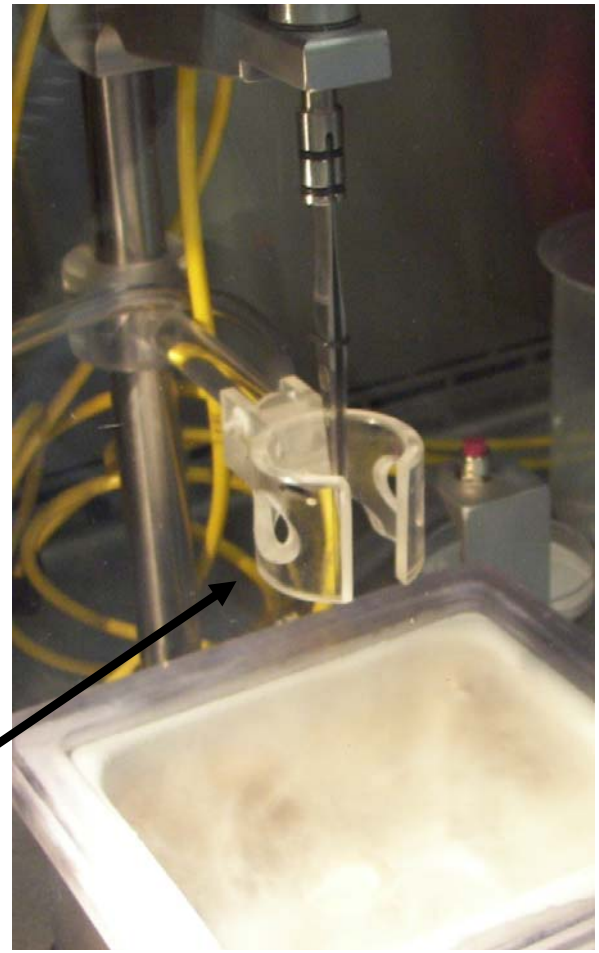
Condensation Freezing

gaseous Ethane



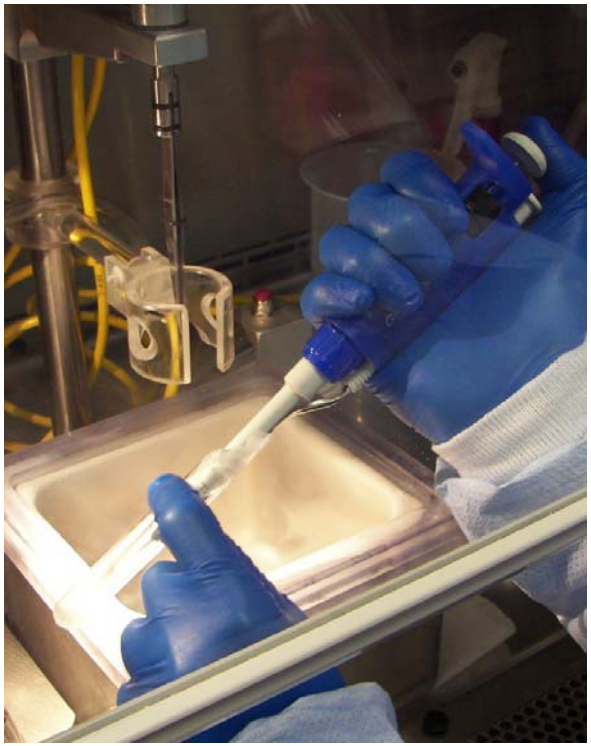
Cryo-preparation – cont‘

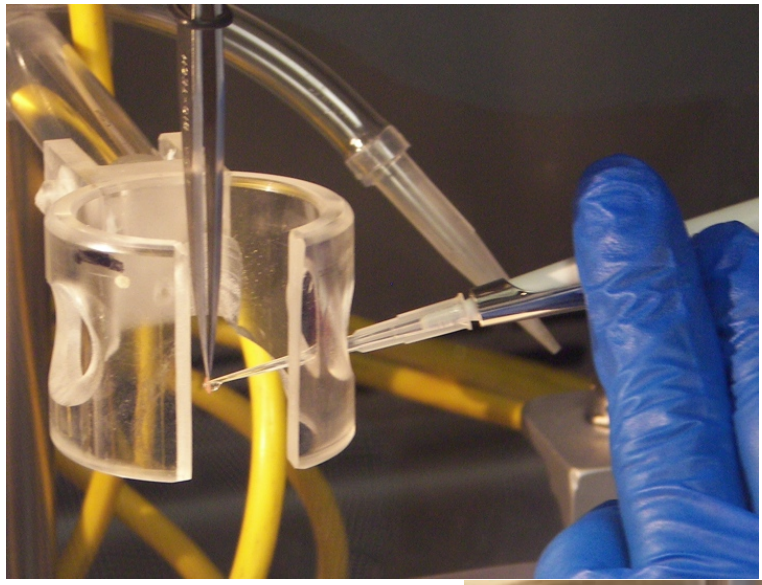


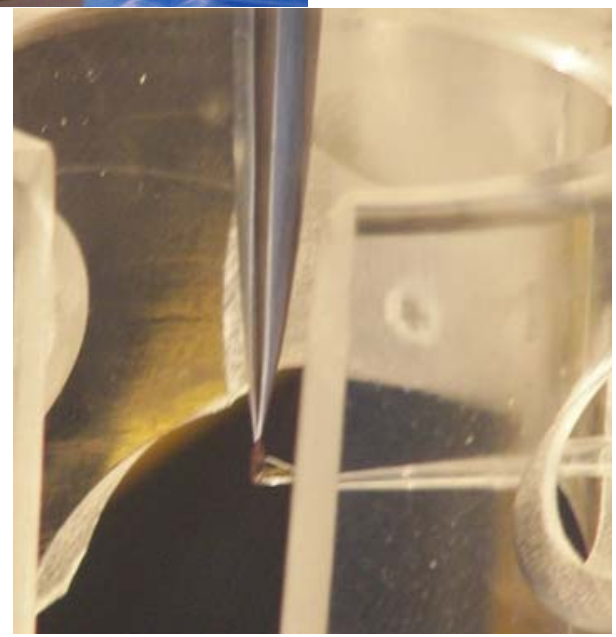


protecting plastic shield

Cryo-preparation – Sample application







3.5 µl drop of sample

Cryo preparation – Plunge freezing

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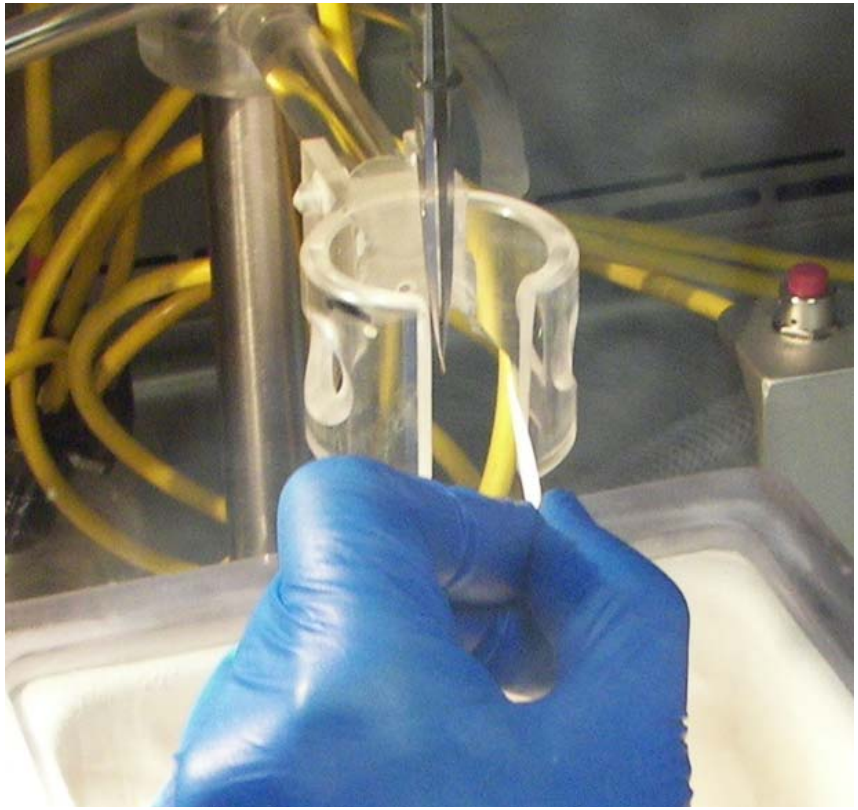
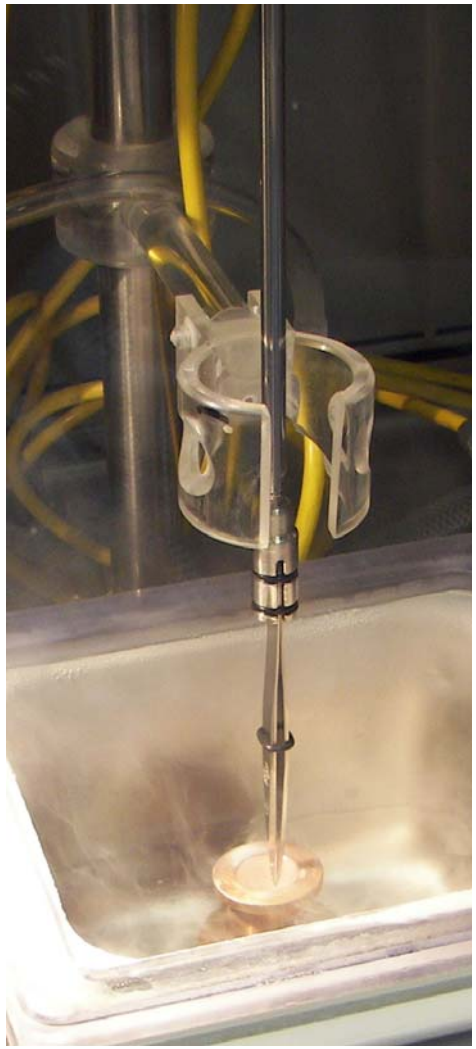
blotting with filter paper



guillotine-like motion of plunger

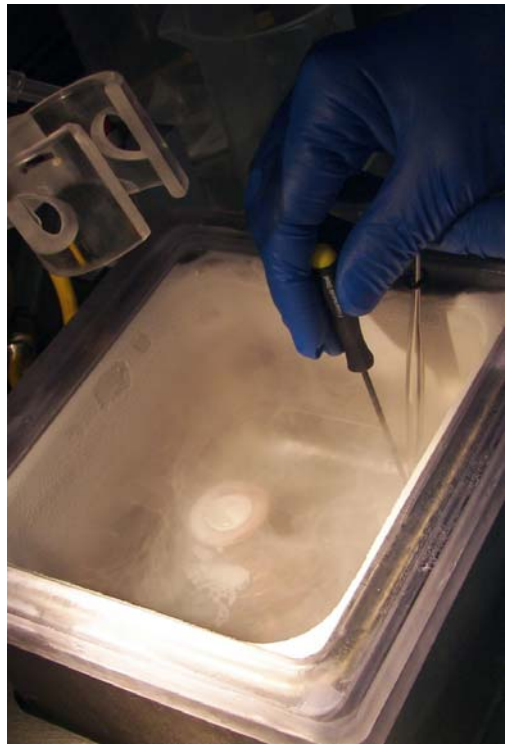


plunge freezing (triggered by foot paddel)

Cryo preparation – EM grid transfer

careful removal of
tweezer from plunger

transfer of EM grid into
cryo-storage box



Note: grid has to be maintained at liquid nitrogen temperatures during all times

Cryo preparation – Grid box transfer

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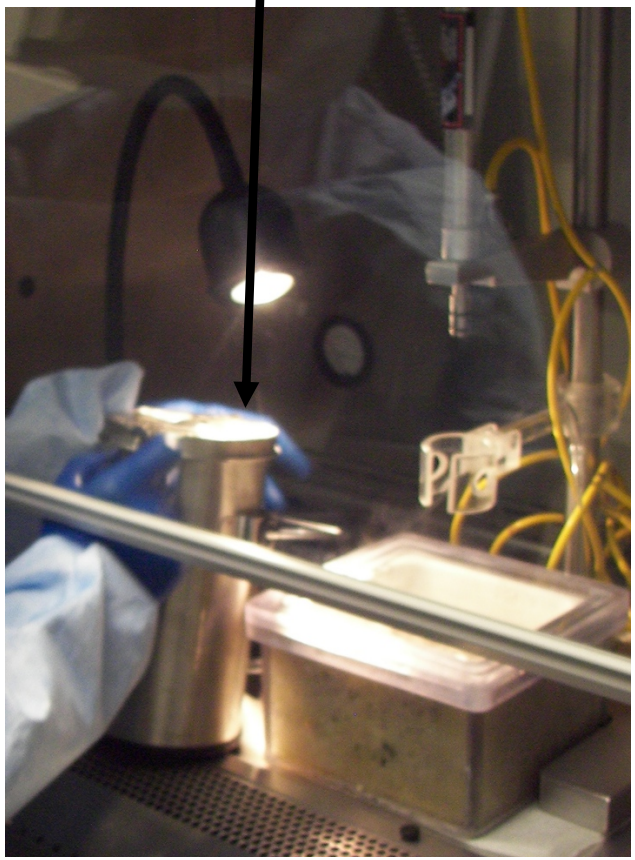
transfer dewar with liquid nitrogen



storage or imaging

Note: frozen grids containing the agent embedded in a solid ice matrix cannot produce any aerosol unless thawed

Decontamination

- **Once transfer dewar is removed from the BSC:**

  → Transfer liquid nitrogen from cryo-box to a styrofoam box and place inside BSC

  → BSC, plunger, tweezers, etc. will be decontaminated using Cavicide

- **All waste and liquids will be autoclaved according to procedures described in BSL-3 cryo-EM SOP**

Accidental warm-up / Spill

- **Under no circumstances will transfer boxes be thawed or warmed to a temperature higher than -150 ºC**
- **Accidental warm-up:**

  → Decontamination of grids and cryo-storage boxes in Cavicide
- **Grid dropped outside of BSC, investigator will :**

  → Cover grid and any spilled liquid with absorbent material and flood with 10% bleach or Cavicide

  → Immediately notify EHS and facility director and leave affected area

  → Wait 30 minutes, return to clean the spill

EM grid transfer into microscope

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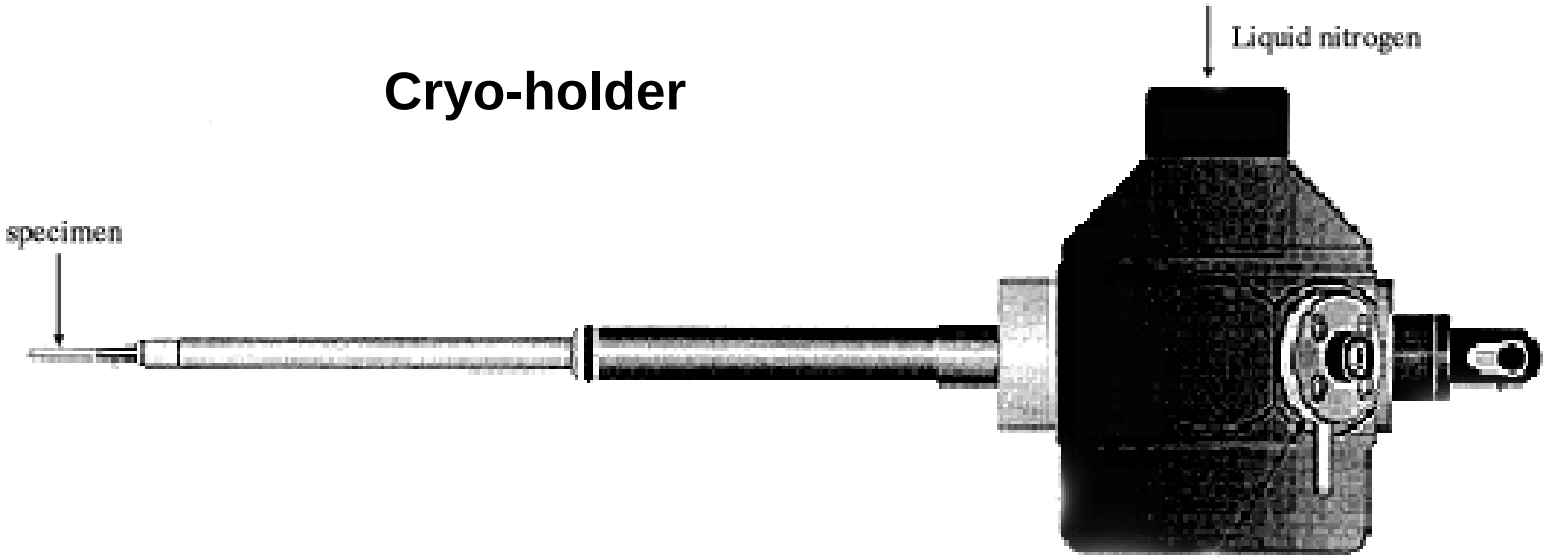
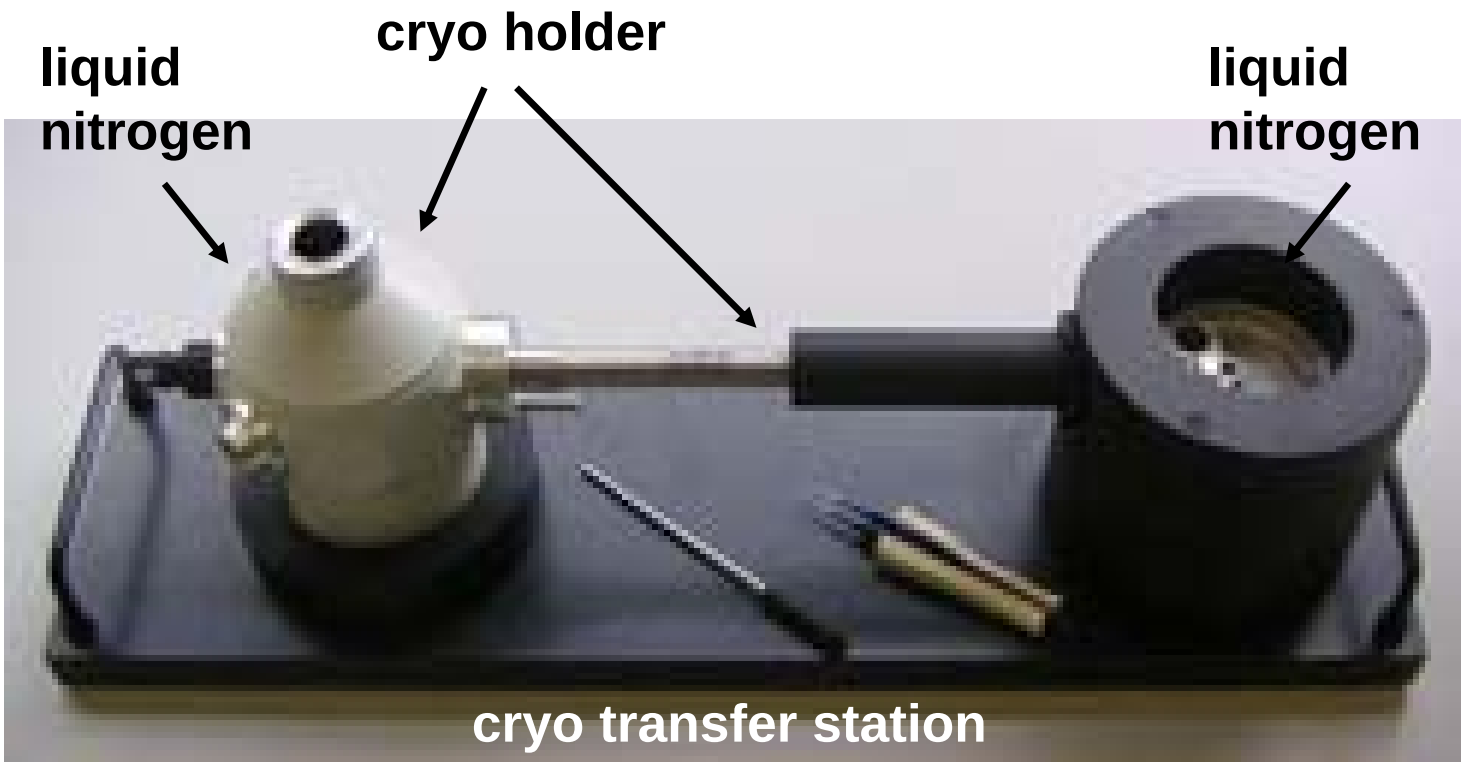
Cryo-holder

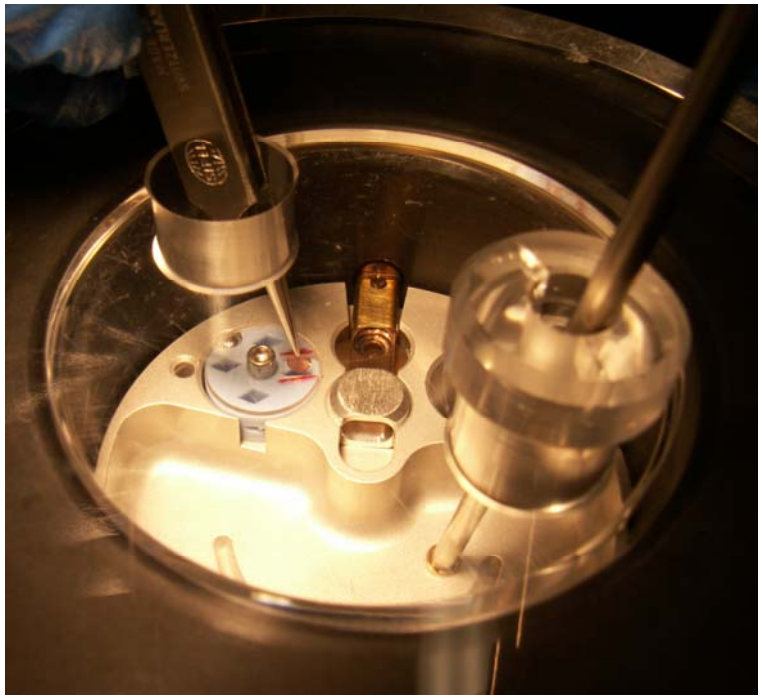


EM grid transfer into microscope

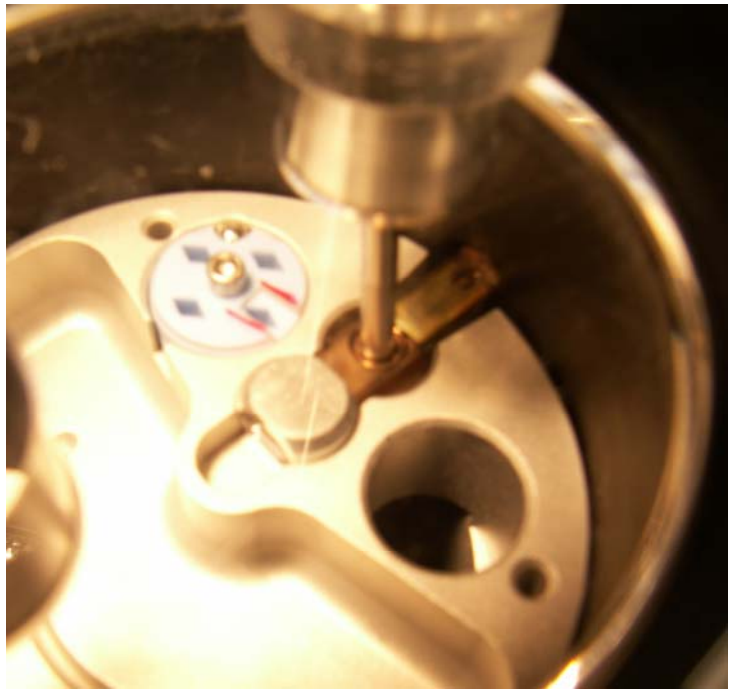


Note: Only manipulation of a frozen sample that will not be done within a BSC, so a respirator will be required of all investigators

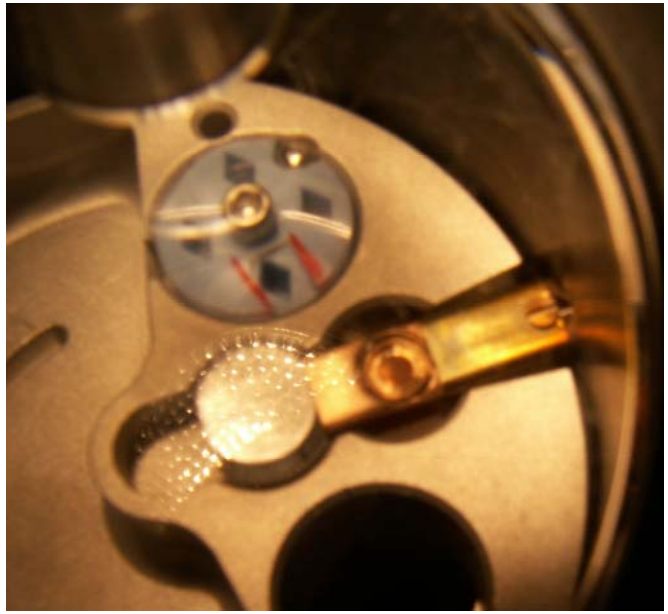
EM grid transfer into microscope – cont‘



grid with frozen agent will be inserted into the holder

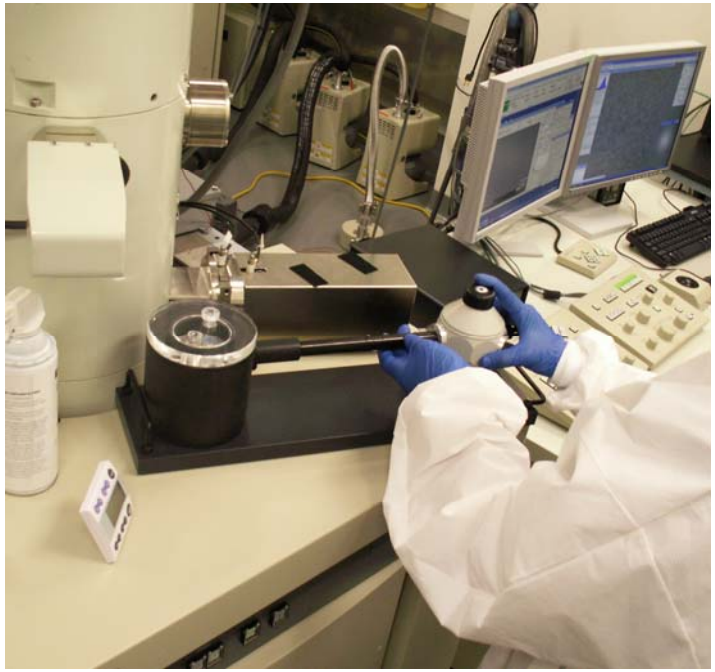


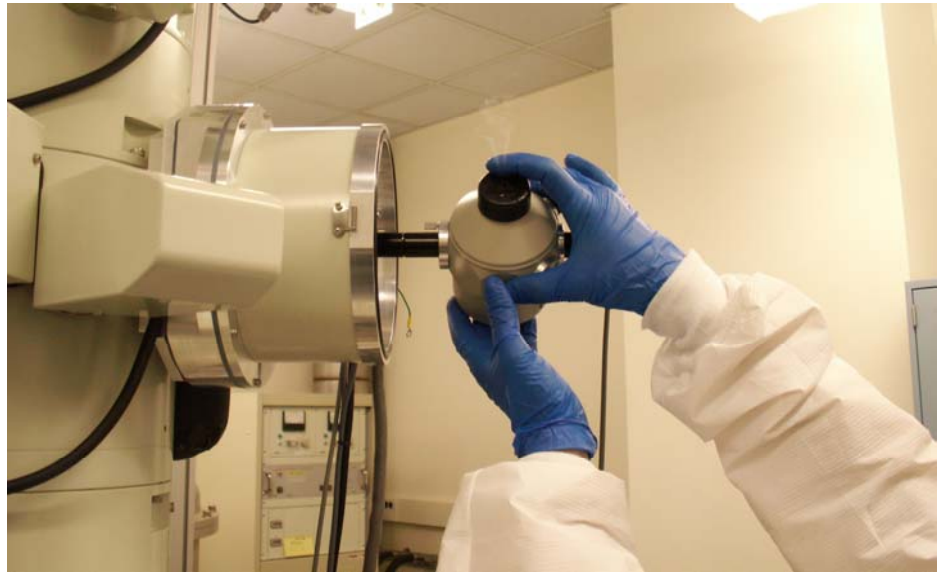
grid secured with a clip ring

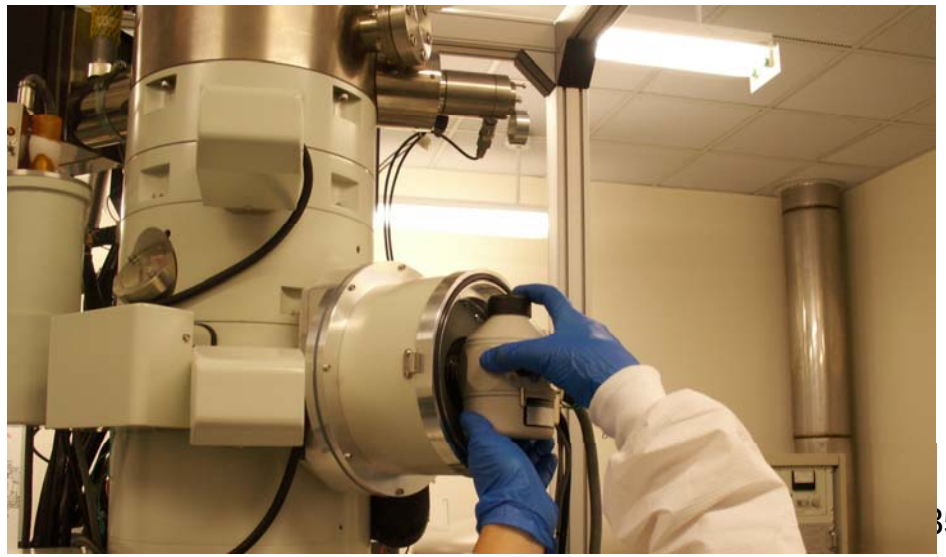


cryo shield protects specimen against damage

Cryo-holder transfer into microscope

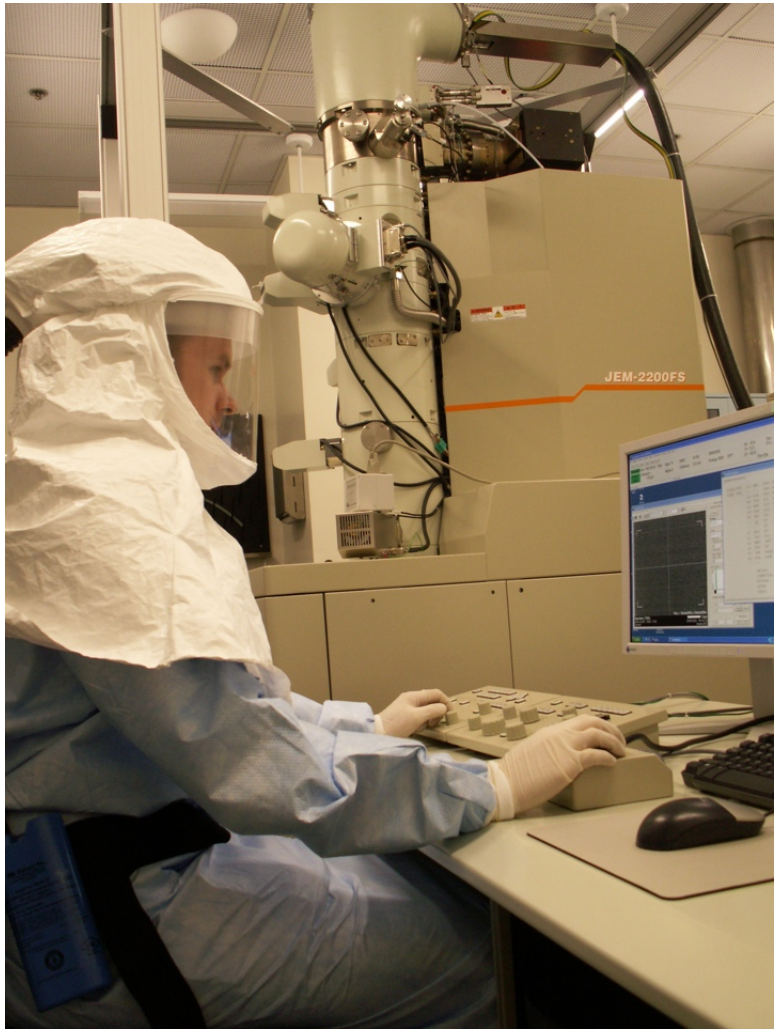






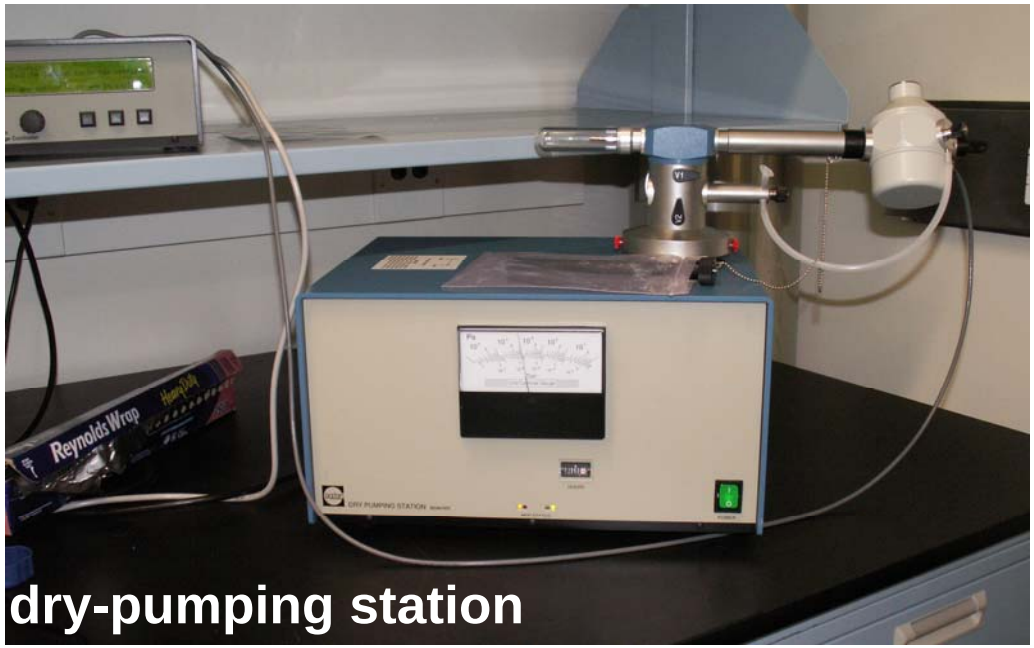
fast but careful holder transfer into the microscope

Imaging



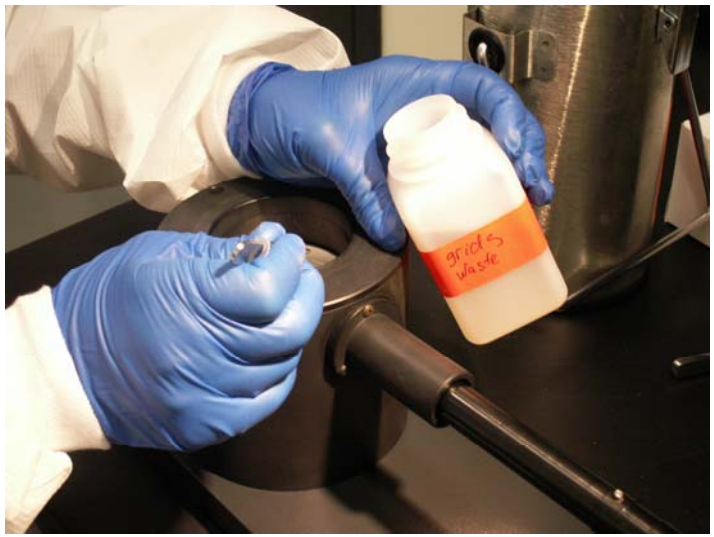
imaging from inside BSL-3 or outside (remote control room)

Decontamination after imaging session



dry-pumping station

- cryo transfer holder: decontamination at 90ºC for min. of 10 min in dry-pumping station
- holder will be held in dry-pumping station until next use



grid will be disposed in Cavicide or a 10% bleach solution

Safety issues associated with microscope

- **Decontamination of microscope necessary for:**

  → shutdown of microscope for service/repair

  → thawed frozen sample

  → loss of grid inside scope (worst-case scenario)

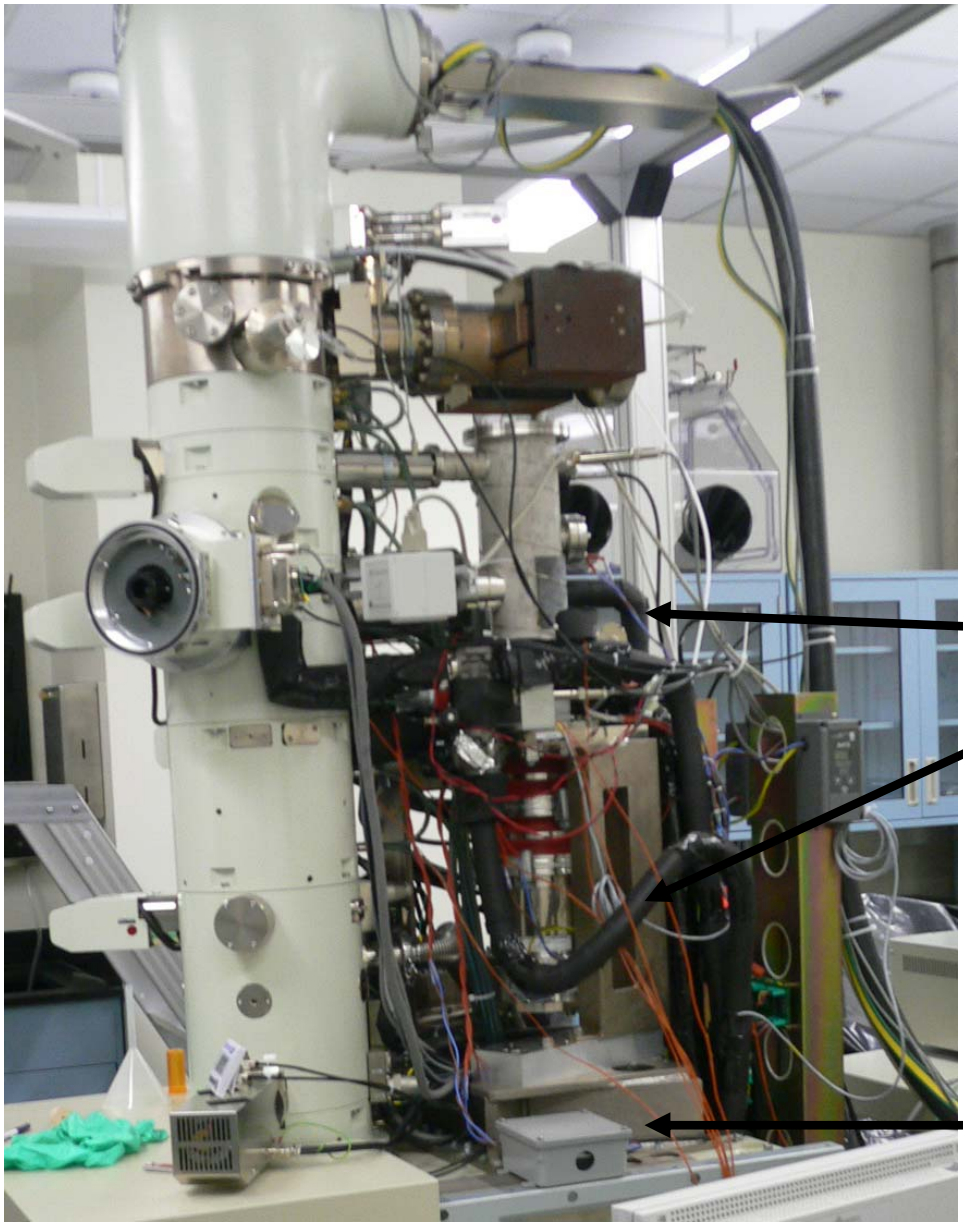
- **Virus inside microscope column and pumping tubes**

- **Standard bakeout procedure of microscope column at 60°C**

  → Modified to initiate additional bakeout of vaccum lines and valves

  → "Full-system" bakeout for min. 24 hrs at 60°C

Some modifications of JEM-2200FS for bakeout

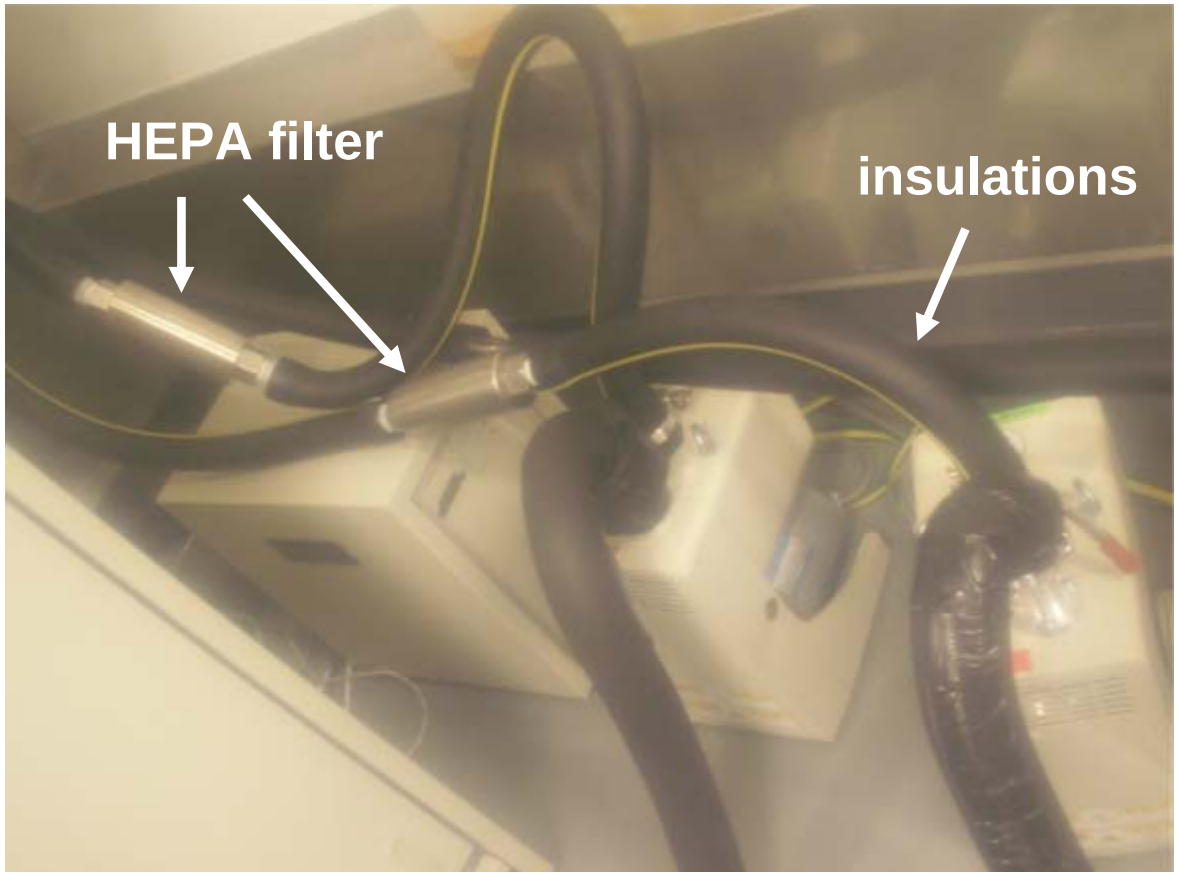


insulated pumping tubes

temperature controller

Modifications on pumping equipment

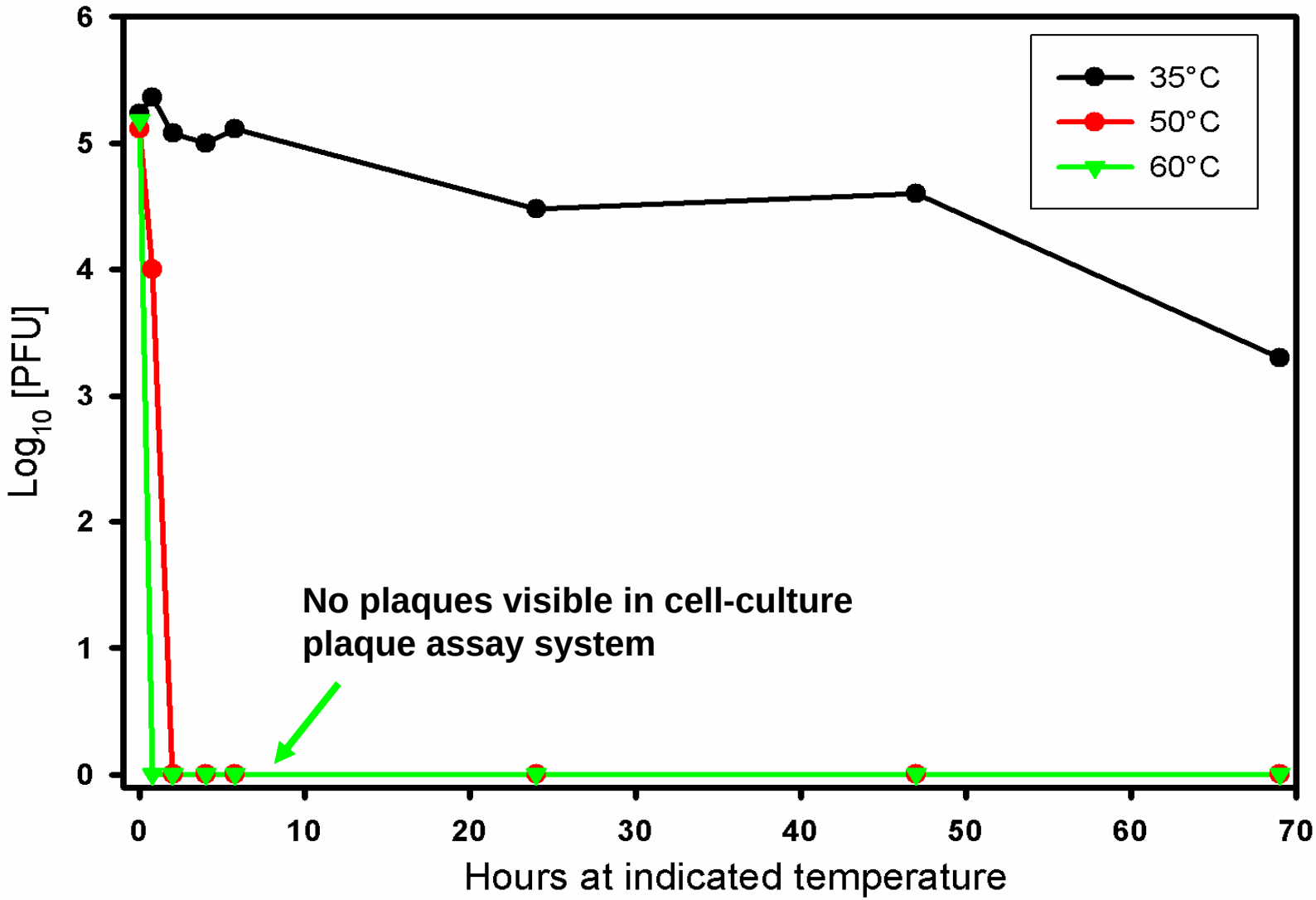
- **Pumping tubes insulated for bakeout**
- **HEPA filter on exhaust side of pump**



Heat inactivation curve of BSL-3 agent

UTMB

e.g. RVFV vaccine strain MP-12



Summary

- **Modifications in SOP to safely handle BSL-3 agents for preparing EM grids used for structural studies by cryo-EM**
- **Established heat decontamination protocol for microscope**
- **Heat inactivation curves of agents are necessary**
- **Equipment modification for usage inside BSL-3**

- **Only functional virus imaging BSL-3 cryo-EM facility worldwide**
- **Similar facilities under construction:**

  → Oxford Particle Imaging Centre, UK (S. Fuller)

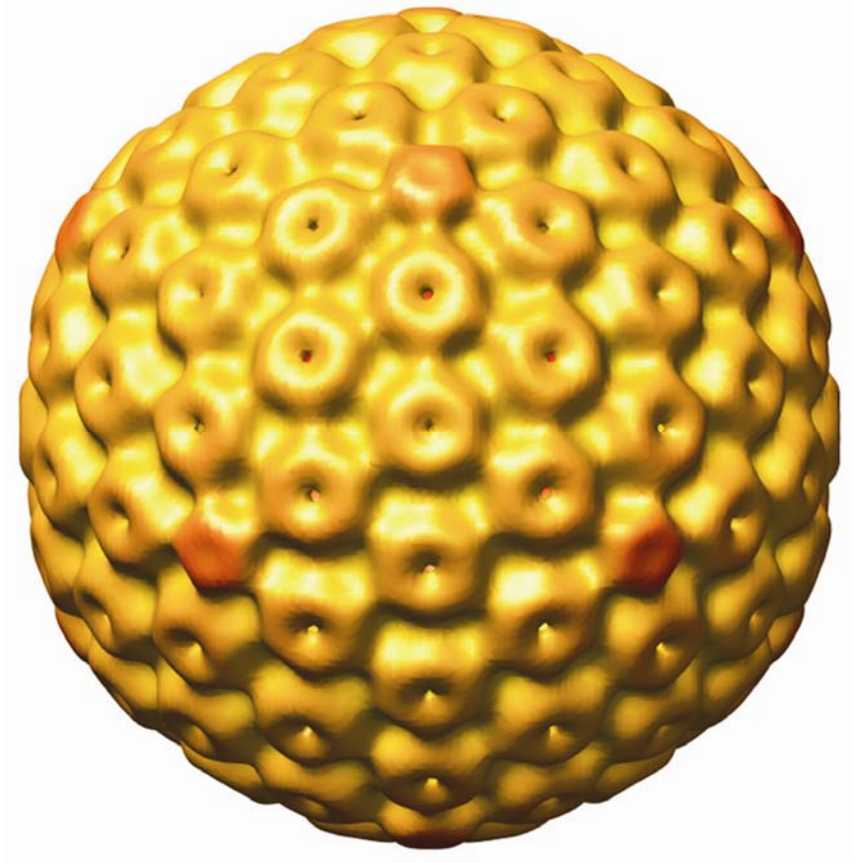
  → Purdue University, Indiana, USA (M.G. Rossmann, R.J. Kuhn)

First results

- **RVFV vaccine strain MP-12 (family *Bunyaviridae*) – a BSL-2 agent**

Frozen-hydrated RVFV MP-12 particles

3D reconstruction of RVFV MP-12

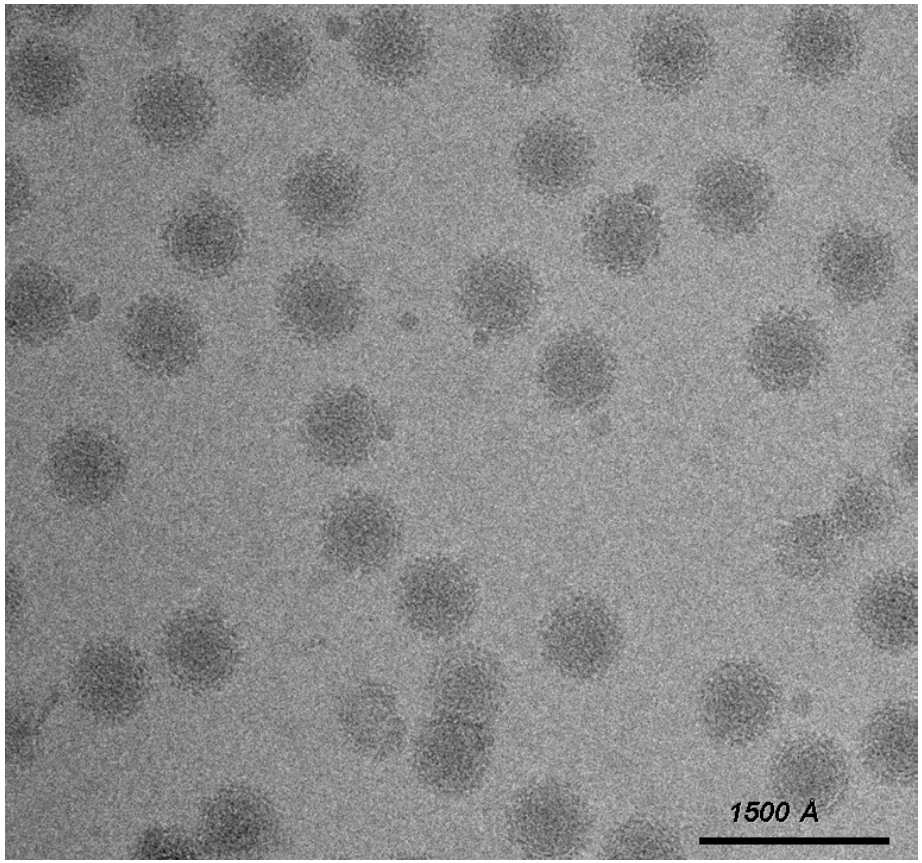


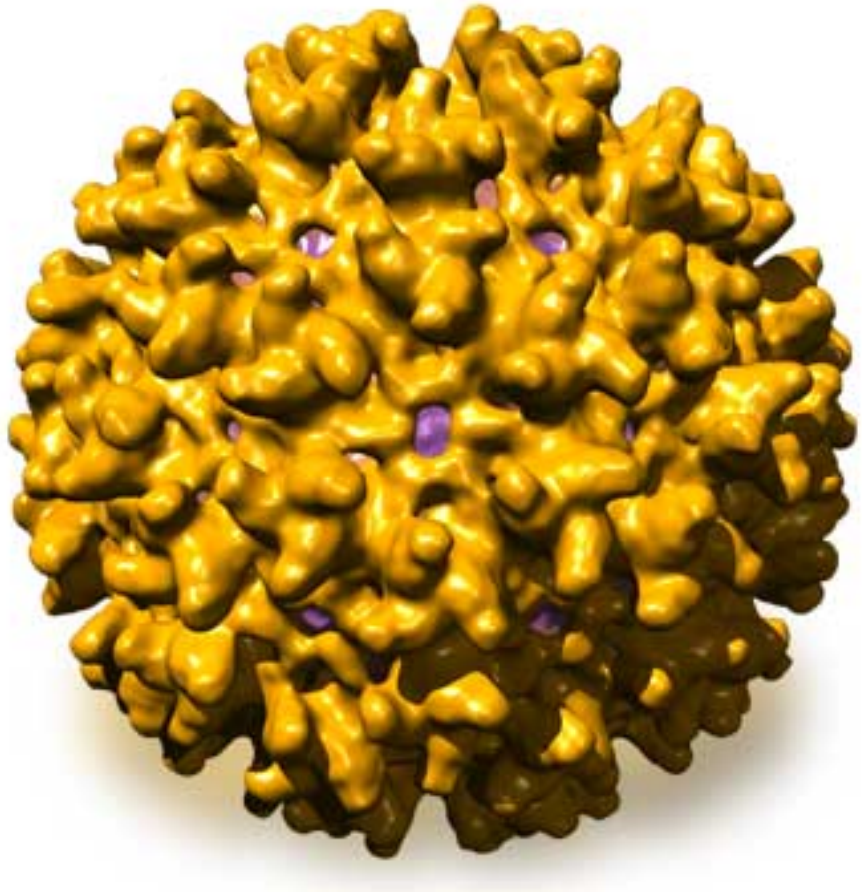
Freiberg *et al.*, JVI, 2008

First results

- **Western Equine Encephalitis virus (family *Alphaviridae*)**

Frozen-hydrated WEEV particles





Preliminary 3D structure of WEEV; first BSL-3 agent studied (MB Sherman, SC Weaver, UTMB, unpublished results)

Acknowledgments

- **Foundations:**

  W. M. Keck Center for Virus Imaging (Scott Weaver, Stanley Watowich, Wah Chiu)

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- **UTMB:**

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