A Unique BSL-3 Cryo-Electron Microscopy Laboratory at UTMB

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Abstract

This article describes a unique cryo-electron microscopy (CryoEM) facility to study the three-dimensional organization of viruses at biological safety level 3 (BSL-3). This facility, the W. M. Keck Center for Virus Imaging, has successfully operated for more than a year without incident and was cleared for select agent studies by the Centers for Disease Control and Prevention (CDC). Standard operating procedures for the laboratory were developed and implemented to ensure its safe and efficient operation. This facility at the University of Texas Medical Branch (Galveston, TX) is the only such BSL-3 CryoEM facility approved for select agent research.

Introduction

Studying the three-dimensional (3D) structure of viruses and bacteria is important to understand the key pathogenic components of the agents and disease mechanisms. Determining how viruses and bacteria function and interact with each other, as well as studying their pathogenic interactions with host cells, may reveal their “Achilles’ tendons” and allow researchers to develop new and improved vaccines and therapeutics.

Recently, cryo-electron microscopy (CryoEM) has become a powerful tool to study viruses and bacteria in nearly native states. CryoEM allows the preservation of the native state of the agents by extremely rapid freezing of suspensions in buffered solutions. The cooling rates are so fast that water does not have time to crystallize, leaving the agents embedded in a solid matrix of a frozen buffer and ensuring that biological macromolecules maintain their native conformations. The only drawback to CryoEM is that it usually maintains the viability of macromolecules and of the pathogenic organism itself, which can pose a biosafety hazard. Although the volumes of the pathogens imaged are less than one nanoliter, the very high concentrations needed (up to \(10^{12}\) infectious units per ml for some viruses) can result in potential exposure to lethal doses, especially for agents transmitted by aerosol. Therefore, ensuring investigator and community safety is a challenge that requires special engineering, facility design, personal protective equipment (PPE), training, and administrative controls to include standard operating procedures (SOPs).

Before the W. M. Keck Center at the University of Texas Medical Branch (UTMB) became operational, no other BSL-3 facilities were equipped with high-end CryoEMs. Therefore, standards for construction and SOPs for handling specimens to obtain maximum image resolution yet ensure safety were developed.

Results

Imaging Equipment at the W. M. Keck Center

The W. M. Keck Center for Virus Imaging was developed within the UTMB Center for Macromolecular Systems CryoEM Imaging (CMSI) facility. This CryoEM facility was designed to include both BSL-3 and BSL-2 containment laboratories. While the CMSI is used to study viruses, bacteria, and non-infectious macromolecules, the Keck Center focuses on high-resolution imaging of viruses at BSL-3. To the authors’ knowledge, it is the only facility in the world with this capability. Although live viruses recommended for handling at BSL-3 have been imaged in other facilities (Neuman et al., 2006), the microscopy was not conducted at BSL-3.

The CMSI houses three electron microscopes (EM): a 100 keV JEM 1010 EM (at BSL-2), a 200 keV JEM 2100 CryoEM (at BSL-2), and a state-of-the-art 200 keV JEM 2200FS CryoEM (at BSL-3) (JEOL, Tokyo, Japan). The BSL-3 EM has the brightest currently available electron source (field emission gun), an in-column electron energy filter (omega type), a large-format CCD camera for full-scale digital imaging, and an oil-free vacuum system with a turbo-molecular pump (Figure 1). The microscope can be controlled remotely via computer network connections. To demonstrate this capability, the microscope has been operated successfully from a control room outside the containment laboratory and from more distant locations including Chicago (IL), Tallahassee (FL), and Atlanta (GA). The CryoEM Center is a fully-equipped laboratory capable of studying a wide range of biological samples including thin plastic sections of cells for studying cellular structure and whole tissues for analyzing diagnostic specimens. On the other side of the spectrum, cell organelles, protein complexes, and single virus particles can be studied under BSL-3 or BSL-2 containment.
BSL-3 Entry/Exit

The containment was designed following recommendations for BSL-3 facilities in the *Biosafety in Microbiological and Biomedical Laboratories (BMBL)* (U.S. Department of Health and Human Services). These include an anteroom with secured interlocking doors and physical separation from the rest of the Center, negative air pressure, and a downstream clean change room with PPE storage. A hands-free operated sink with an eyewashing station and a chemical safety shower are located in the change room.

Access to the BSL-3 laboratory is restricted to authorized researchers and support personnel who have undergone appropriate training and background checks by the U.S. Department of Justice for select agent access. Required PPE includes a seamless-front gown, double gloves, and a respirator. The respirator is selected based on the risk assessment, which is performed with the assistance of personnel from the Department of Environment Health and Safety (EHS). Persons exiting the laboratory leave their gowns and respirators in the change room and are required to thoroughly wash their hands with soap before leaving containment.

Design of the Room for CryoEM

Electron microscopes and CryoEMs, in particular, are very sensitive to environmental conditions. Variations in air temperature and pressure, air movement across the microscope column, acoustic noise, electromagnetic fields, vertical and lateral vibrations, cooling water temperature, and other parameters greatly affect microscope performance and achievable resolution. Therefore, special considerations were given to the design and optimization of the environment of the BSL-3 containment laboratory within the guidelines of the BMBL. The room temperature was kept constant with drift of less than 0.5°C per hour, which was difficult to achieve considering the required movement of air through the facility. The walls and the ceiling were specially designed with resilient channels embedded in three-layered drywall to dampen acoustic noise from outside sources. A drop ceiling was installed to act as an air damper to ensure slow, laminar air flow. The air exhaust grilles were lowered and distributed around the perimeter of the room to minimize air movement around the microscope. The air ducts in the facility incorporated inner, acoustic-dampening liners and flexible joints (couplings) to minimize vibrations originating from the building’s air handlers, and acoustic transmittance was also minimized by the shape and lengths of the ducts. The microscope had a special suspension (IDE-ADV 2000, Integrated Dynamic Engineering, Randolph, MA) installed to protect it from floor vibrations and was enclosed in a Helmholtz cage (Figure 1) that cancels electromagnetic fields from the external sources (IDE-Mk4Ac/Dc, Integrated Dynamic Engineering, Randolph, MA).

**Figure 1**
JEOL 2200FS CryoEM in BSL-3 containment at the W. M. Keck Center for Virus Imaging at UTMB. The microscope is enclosed in a Helmholtz cage to suppress stray electromagnetic fields that might compromise its performance.
Noise, air movement, abrupt pressure changes, and electromagnetic fields changes that would disrupt the optimal functioning of the microscope were further reduced by preventing the opening of doors during microscopy sessions. To maximize safety and minimize the effects of personnel movement on its performance, the microscope was usually controlled remotely during data collection.

Although the amount of infectious substances was nominal, less than a microliter, so that the chance of considerable spillage requiring whole-room decontamination was minimal, if necessary that still could be achieved by dismantling the drop ceiling and using a gaseous decontamination procedure either with vaporized hydrogen peroxide or chlorine dioxide gas.

The use of select agents added an extra layer of complexity to the operation of the CryoEM facility. Experiments were carefully scheduled because select agents were not authorized to be stored in the facility. Also, a UTMB security officer was present during the times when select agents were present, and only personnel approved for select agent access were authorized to enter the BSL-3 laboratory. Due to these security requirements, all select agent experiments were completed during regular work hours (Monday-Friday, 8:00 a.m. to 5:00 p.m.). Select agents were transferred to the Center from approved storage facilities in other campus locations following rigorous protocols, and then returned to the storage facility after microscopy.

**Specimen Preparation**

CryoEM has the advantage of studying “live” agents that are immobilized in a solid matrix—a frozen aqueous solution. These agents are still potentially infectious when thawed, and therefore research personnel must be protected at all times from possible exposure. SOPs for the CryoEM operation include specimen preparation to ensure the safety of research personnel. All agents were transported into the BSL-3 facility appropriately packaged in double containers that were never opened outside a BSL-3 laboratory. CryoEM grids were prepared in a ducted BSC following general BSL-3 practices, and researchers were required to wear appropriate PPE including a respirator while working with BSL-3 agents. Containers were opened at least 20 minutes after bringing them into the BSC to allow for recovery of the laminar air flow. All of the necessary tools and chemicals were placed inside the BSC in advance to minimize air disturbances. All manipulations of the agent were performed inside the BSC without withdrawal of the investigator’s hands.

To preserve biological samples for CryoEM, they must be flash-frozen in a cryogen (typically liquid ethane or propane) with an extremely high cooling rate (> $10^5$-$10^6$°C/second). A small volume, approximately 2-3 μL, of a purified agent suspended in buffer was applied to an EM grid (Figures 2A and 2B). The excess liquid was blotted with filter paper (Figure 2C), and the grid was plunged into the cryogen. This procedure led to water vitrification, a process in which water hardens like glass.

**Figure 2**

Specimen preparation for CryoEM. A, A 3 μL sample applied to an EM grid held in forceps. B, Detailed view of the grid in forceps with pipette tip for specimen application and the shield protecting the researcher. C, Removal of excess liquid with filter paper. D, A shield surrounding the forceps to protect the investigator from injury. E, EM grid in forceps, ready for specimen application. F, Cryo-box with four slots for EM grids (arrow). A transparent lid secured with a screw in the center protects the grids from ice contamination when the box is exposed to humid air.
preventing the formation of ice crystals and thereby preserving the agents’ near-native structures. This method left the agents immobilized in a solid water matrix, reducing the risk of aerosol exposure as long as the grids remained frozen. Although the volume on the grid was extremely small and the likelihood of producing infectious doses of aerosols was very limited, this risk could not be excluded given the unique conditions required for grid preparation. After freezing, the grids were stored in liquid nitrogen and were never allowed to warm above -150°C.

To properly flash-freeze the sample in the BSC, a special, pneumatically operated cryo-plunger was developed (Figure 3). After plunge-freezing, the grids were transferred to a pre-cooled (liquid nitrogen) plastic cryo-box that had a separate slot for each grid as well as a lid to protect the grids from frost during exposure to humid air (Figure 2F). After filling the storage box with grids and securing the lid with a screw, it was transferred to a dewar filled with liquid nitrogen for short-term storage and transported from the preparation laboratory into the microscope room.

The cryo-plunger resided in the BSC (Figure 3) and was surface-decontaminated after each session with CAVICIDE (Metrex Research Corporation, Romulus, MI). Inside the BSC, all of the tools, bottles, solutions, stands, and other materials used during the session were also surface-decontaminated. In case of an accident, after the evacuation of all personnel for at least 30 minutes, the spill would be covered with absorbent material and flooded with CAVICIDE or freshly prepared 10% chlorine bleach, and the investigator would immediately leave containment. The Center Director, Principal Investigator (PI), and EHS would then be notified of the accident. After 30 minutes, the researcher would return to the containment laboratory with respiratory protection to clean the spill using standard procedures for infectious agents.

Frozen EM grids containing samples were stored in a liquid nitrogen dewar in the sample preparation room or were immediately used for imaging. The specimen transfer to the microscope was performed in a dedicated cryo-transfer station filled with liquid nitrogen. The microscope’s anti-contamination device was pre-cooled with...
liquid nitrogen well before the specimen transfer to ensure a low ice contamination rate during the microscopy session. The frozen grids were brought to the station in a dewar with the lid securely latched. A cryo-specimen holder was pre-cooled with liquid nitrogen, and the cryo-grid box was then quickly transferred from the dewar into the station. The lid was loosened and a grid was inserted into the holder, secured with a clip ring, and protected from humid air with a cryo-shield. The station was then transferred to the microscope countertop and the holder was inserted into the specimen airlock adjacent to the column, pre-evacuated with the rotary and diffusion vacuum pumps, and then inserted inside the microscope column for imaging (Figures 4A and 4B).

**Decontamination Process**

Once the imaging session was complete, the cryo-holder was removed from the microscope and placed into the pre-cooled cryo-transfer station. The grid (still frozen) was removed and immediately immersed in CAVICIDE. A new grid was then inserted into the holder and transferred to the microscope for a new imaging session.

For decontamination, the cryo-holder was transferred while still cold into the vacuum pumping station, where it was warmed under high vacuum and maintained at approximately 100 °C for at least 10-15 minutes to ensure proper decontamination of the tip that contacted the grids. In some cases the grids remained in the holder for decontamination, followed by immersion in CAVICIDE.

The cryo-transfer station and all tools used during the transfer of frozen grids to and from the cryo-holder were decontaminated by spraying and wiping with a 70% w/w ethyl alcohol solution or immersing in CAVICIDE. Used cryo-grid storage boxes were stored in a 70% w/w ethyl alcohol solution for at least 1 hour and thoroughly dried before the next use. The cryo-transfer station and other tools used for specimen transfer were held in an oven at 60 °C for at least 1 hour before the next use. Based on the thermal stability assays of all agents used in the facility, this treatment should be capable of inactivating any residual virus.

**Potential Incidents Affecting Safety**

One of the major biosafety concerns was microscope contamination in case of an accident involving the thawing of a grid inside the microscope column or in the airlock during specimen transfer and pre-pumping. The
cryo-grid was clamped in the holder by a clip-ring but, if not secured properly, could be dislodged resulting in poor thermal contact with the holder. The grid could also fall from the holder and contact warm parts of the microscope column, resulting in instant thawing and the potential release of the agent into the microscope column. Although the volume of the agent suspension was small, it would be difficult to locate a grid or droplet inside the microscope and perform decontamination using recommended protocols for infectious agents.

Initially, gaseous decontamination of the CryoEM was considered because it would be the most thorough method to implement in the event of a grid thaw or some other incident in the microscope room with the potential to generate an aerosol. However, consultation with the JEOL Company and empirical testing of several microscope components indicated that any approved method would damage expensive instrumentation components. In addition, standard chemical surface decontamination procedures typically implemented in a BSL-3 environment would not be possible within the microscope column because the addition of liquids would compromise the ultra-high vacuum system. Also, most chemicals used for decontamination are corrosive to the microscope components even after thorough removal with a secondary cleaning solution. In addition, the required disassembly of the microscope column before decontamination would be unrealistic. Finally, a prolonged downtime would result if re-evacuation of the vacuum system followed a long exposure to ambient humidity.

An additional hazard that was considered was a vacuum leak that could cause airflow within the vacuum system to aerosolize a thawed agent. That, in turn, could create a hazard when servicing the microscope. To address this risk, heat decontamination of the microscope column and vacuum lines was implemented prior to any service. Fortunately, the microscope column could be warmed to ≥60 °C as part of the normal “bake-out” procedure used to improve the vacuum inside the column, and that temperature was shown experimentally to inactivate all of the enveloped BSL-3 viruses under study (data not shown). As part of the “notice of use” biosafety protocol for each agent to be studied in the BSL-3 facility, a “kill curve,” or experimental thermal stability study of each agent, was required to be performed by the project’s PI according to guidelines developed by the UTMB Institutional Biosafety Committee. These requirements were designed to demonstrate that the 10⁶ plaque-forming units of the virus, the maximum amount that could be contained on a frozen grid, could not survive at 60 °C for longer than 1 hour on a dry surface similar to that in the microscope column or vacuum system. The inside of the microscope could then be safely decontaminated using bake-out at 60 °C for an extended period of time (at least 24 hours).

Although the major components of the microscope could be decontaminated using this protocol, some parts of the vacuum system did not have any standard provisions for raising their temperature. We modified the vacuum system of the microscope by replacing the standard rubber hoses with stainless steel bellows and mounting heaters and temperature controllers to achieve the necessary temperature for heat decontamination (Figure 5). Several decontamination scenarios were developed depending on the hypothetical contamination event. In the case when no abnormal procedures or operations occurred before the JEOL service engineer visited, a routine 24-hour bake-out was performed without heating the vacuum lines. In the event that a grid containing an agent was lost or thawed inside the column, full decontamination of the microscope, including a complete shutdown and activation of the vacuum system heaters, was specified. The full decontamination process was successfully tested by measuring the temperatures throughout the system, but was never activated because no accidents occurred. The exhaust of the mechanical vacuum pumps was HEPA-filtered to prevent any possible hazardous discharge from the pumps themselves. The pumps were serviced by CMSI-trained personnel (mostly during routine maintenance oil changes), and used oil was collected and incinerated.

**Waste Disposal**

All waste generated in containment was either: (1) collected into biohazard containers, autoclaved, and discarded into regulated medical waste containers, which were then incinerated as part of UTMB’s waste management process, or (2) was decontaminated chemically using CAVICIDE, 70% alcohol, or freshly prepared 10% bleach. Autoclaving was done using standard protocols; autoclave tape was used as an indicator of proper temperature during the process, and biological bacterial spore inactivation tests were used with every autoclave cycle to ensure full decontamination.

**Structures of Agents Studied in the Center**

Several agents were studied in the Center, including western equine encephalitis virus (WEEV), an alphavirus that causes highly debilitating and often fatal disease in humans and horses (Smith et al., 2009). WEEV is classified as a National Institutes of Allergy and Infectious Diseases (NIAID) high-priority Category B biothreat agent. This virus was successfully imaged in the Center and its structure reconstructed in 3D at 1.5 nm resolution (Figure 6). To the authors’ knowledge, this was the first BSL-3 virus ever imaged in BSL-3 containment using CryoEM.

Another example of 3D reconstruction obtained in the Center is the structure of Rift Valley fever virus (RVFV), a prototypical bunyavirus associated with major disease outbreaks in livestock and humans throughout Africa and the Arabian Peninsula. RVFV is classified as a
**Figure 5**

Modifications to the JEOL 2200FS CryoEM. **A.** Thermocouples (arrows) attached to the microscope to monitor the temperature during the heat decontamination cycle. **B. and C.** Fragments of vacuum lines wrapped with aluminum foil (B) and heat-insulating foam. Heaters are mounted inside the foam to maintain a temperature of 60°C during “bake out” for decontamination of the system.

![Image](image1.png)

**Figure 6**

A CryoEM image of western equine encephalitis virus (WEEV) particles embedded in vitreous water. The sample was held in a cryo-specimen holder inside the JEOL 2200FS cryoEM at -184°C and imaged with 200 keV voltage using a large format CCD camera (16 Mpxl). The scale bar is 100 nm.

![Image](image2.png)
NIAID priority Category A biothreat agent. Work from the W. M. Keck Virus Imaging Center produced the first three-dimensional reconstruction of RVFV from electron microscopy images (Figure 7) (Freiberg et al., 2008; Sherman et al., 2009). This structure provides a detailed model for many bunyaviruses and will aid in the design of antiviral drugs, diagnostics, and effective vaccines.

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References


