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A FULLY AUTOMATED SYSTEM FOR THE PREPARATION OF SAMPLES FOR CRYO-ELECTRON MICROSCOPY

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ABSTRACT

The preparation of specimens for cryo-electron microscopy is currently a labor and time intensive process, and the quality of resulting samples is highly dependent on both environmental and procedural factors. Specimens must be applied to sample grids in a high-humidity environment, frozen in liquid ethane, and stored in liquid nitrogen. The combination of cryogenic temperatures and humidity-control mandates the segregation of the humidity-controlled environment from the cryogenic environment. Several devices which automate portions of the specimen preparation process are currently in use; however, these systems still require significant human interaction in order to create viable samples. This paper describes a fully automated system for specimen preparation. The resulting system removes the need for human input during specimen preparation, improves process control, and provides similar levels of environmental control. Early testing shows that the resulting system is capable of manipulating samples in an autonomous manner while providing performance similar to existing systems.

INTRODUCTION

Since its introduction, the electron microscope has revolutionized the study of both organic and inorganic structures on the nanoscale. By its nature, electron microscopy allows for the inspection of structures in unprecedented detail; however, samples must be specially prepared for imaging with an electron microscope. The preparation process often involves methods that have deleterious effects on the structure of samples, especially biological ones. Furthermore, radiation damage effected by the necessary use of an electron beam makes detailed examinations of radiation-delicate structures difficult [1].

Cryo-electron microscopy (CryoEM) has repeatedly shown itself as a viable fixing mechanism for biological specimens. CryoEM is a process in which specimens are imaged using an electron microscope while kept under cryogenic temperatures, usually at or below -180°C. Cryogenic temperatures have the effect of protecting a biological specimen from the most degrading effects of the electron beam and the ultra-low vacuums present within the microscope [2].

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Figure 1. TYPICAL CRYOEM GRID

The preparation of samples for CyroEM procedures has been well documented [3] and generally consists of four steps.

- 1. Application of sample to to the imaging substrate
- 2. Removal of excess sample from the substrate
- 3. Vitrification of sample
- 4. Short term storage of sample

The first step in preparing a specimen for CyroEM imaging involves application of the sample to the imaging substrate, also known as a *grid*. A typical grid is a thin copper disc on the order of 3mm in diameter. The grid has a series of perforations, between which the specimen becomes suspended. The diameter of the grid, the size/shape of the holes, and the coating of the grid can be adjusted to fit the needs of the particular imaging study. Figure 1 shows a typical CryoEM grid. The grids are very delicate, and special care must be taken to grip the grid only on its outer circumference to avoid damaging the imaging area. To apply specimen to a grid, the operator grips the grid with a pair of tweezers, and then applies $3-5 \ \mu$ L of an aqueous suspension of the specimen to the central area of the grid using a pipette.

The second step is the removal of excess specimen from the imaging grid. The purpose of this step is to ensure that the specimen forms a thin-film within the perforations and does not bead on the surface of the grid. A thin-film is desirable as it limits electron scattering during the imaging process, resulting in a clearer picture [4]. The removal process is known as *blotting* and involves bringing filter paper into contact with the grid surface. The filter paper absorbs any excess specimen from the grid while a portion of the sample remains suspended within the grid perforations due to adhesive forces. Typically, this step is done in a high-humidity environment to retard evaporation of the thin-film after blotting.

The third step involves the vitrification of the sample. For biological specimens, vitrification usually is conducted in liquid ethane at temperatures between -180 and -175°C. Liquid ethane is used as the freezing cryogen because it is capable of supporting cooling rates of 10^9 K/sec; high enough such that vitreous ice is formed when a thin-film sample is submerged. Crystalline ice is undesirable as ice crystals harm biological structures and cause artifacts in the imaged specimen. Vitreous ice, being amorphous in nature, has none of these characteristics. The liquid ethane is created by condensing gaseous ethane in a liquid nitrogen cooled dewar. The sample is vitrified immediately after blotting by plunging the sample grid into the ethane and holding it there for several seconds.

The final step in preparing a sample for CryoEM studies is placing the sample into short-term storage for transport to the electron microscope. Liquid nitrogen is usually used as the storage cryogen, though any system that maintains the sample's temperature below -140°C could be used [5]. If liquid nitrogen is used, the grid is transferred from the liquid ethane and submerged in liquid nitrogen, where it is then placed into a storage device. The transfer between the liquid ethane and liquid nitrogen must occur very quickly, or the sample will melt.

Commercial systems have been developed to automate portions of the above process [6–8]. These systems are only able to automate the blotting and vitrification processes. As a result, technicians are still required to install a grid in the machine, apply a sample to the grid, and to transfer the vitrified sample to storage. Because human interaction is a vital to the functioning of these devices, they are susceptible to malfunctions precipitated by human error, require extensive training, and can be cumbersome to use even for the most skilled operator. Furthermore, by requiring constant human guidance, these systems hinder the efficient use of two important laboratory resources: time and money.

In our device, we automate all aspects of sample preparation while minimizing the need for human interaction. The system saves time and money, while producing sample grids of a more consistent quality than is possible using other methods. The following report details the major systems of the device, how these systems integrate with one another, and how they function as a whole.

Systems Overview

This section provides information on the hardware used to implement our system. It is divided into subsections detailing each major system of our device.



Figure 2. COBRA ROBOT WITH PNUEMATIC ACTUATOR

Robotic Manipulator For automation of the pick and place tasks, we use an Adept Cobra 600 SCARA robot. Attached to the end effector of the robot is a pneumatic actuator and mating system capable of accepting both a pair of medical tweezers (Dumont #L5) as well as a storage button handling rod (Ted Pella #160-46). Figure 2 shows an image of the robot, while the inset image shows a close-up of the manipulator with both the tweezers are permanently attached to the end-effector, whereas the handling rod is only present during certain operations.

The robot's main purpose is to move the grid between stations so that deposition, blotting, vitrification, and storage can occur in the correct sequence. The robot accomplishes this task in two ways. During deposition, blotting, and vitrification, the robot handles the grid directly using the tweezers. The tweezers are mounted to the robot via a custom bracket and are pneumatically actuated using a parallel pneumatic gripper (PHD #19060-2-001). During the storage process, the robot handles a set of grids indirectly by mating the storage button handling rod with a grid storage button (Ted Pella #160-41) containing up to four grids. The storage button handling rod mates to the pneumatic gripper via a custom bracket as shown in Figure 2.

Vitrification Dewar The vitrification dewar serves two functions: as a vitrification device and as a temporary storage



Figure 3. VITRIFICATION DEWAR SHOWING GRID STORAGE DE-VICE WITH INSTALLED BUTTONS AND ETHANE CUP

vessel. The dewar consists of three parts. An insulated, opentopped, aluminum box is the primary structure. Within this box is a double-walled open-topped cup as well as a structure capable of holding three grid storage buttons. Figure 3 gives a view of the vitrification dewar with double-walled cup as well as a set of three grid storage buttons.

The double-walled cup is designed to condense and hold liquid ethane. During setup, the operator installs three storage buttons into the base of the box and then fills the box with liquid nitrogen until the level is within one inch of the top of the box. A cryogenic temperature sensor (Omega Engineering CY670D-CU) is installed within the ethane cup. When the temperature of the inner wall of the cup reaches -100°C, the operator then condenses ethane within the cup using a gaseous ethane source and a hollow applicator wand.

Humidity Chamber The humidity chamber is a sixwalled acrylic enclosure that encloses the blotting mechanism and the deposition device. The chamber is positioned directly above the vitrification dewar. The humidity chamber is designed such that the end-effector of the Cobra is able to access the chamber from the side, and access the vitrification dewar through the bottom of the chamber. The side door is actuated with a servomotor coupled directly to the shaft of the door, while the bottom door is a sliding mechanism and is actuated by a DC motor through a rack-and-pinion gear set. Label 3 in Figure 4 shows the position of the side door. In the same figure, label 8 shows the position of the vitrification dewar. The sliding door is located directly above the vitrification dewar.

The relative humidity of the chamber is monitored with a Rense HX-748-T-L1 relative humidity sensor. To maintain the desired humidity level, an ultrasonic mister (Ocean Mist DK-24) is integrated into the humidity chamber. When the humidity of



Figure 4. ISOMETRIC AND SIDE VIEWS OF HUMIDITY CHAMBER

the chamber falls below a prescribed value, the ultrasonic mister is engaged until the humidity level returns to the desired level. In Figure 4, label 7 shows the position of the humidity sensor and label 5 shows the position of the humidifier.

Deposition The deposition device is a Chemyx F100 syringe pump with a BD 3mL plastic syringe and hypodermic needle. The Chemyx device sits on a platform outside the humidity chamber while the syringe needle passes through a port on the side of the chamber. The tip of the needle is situated within the humidity chamber such that the Cobra can position the imaging grid within very close proximity to the tip of the needle. Figure 5 shows a view of the Chemyx pump, syringe, and humidity chamber wall.



Figure 5. CHEMYX PUMP SHOWING SYRINGE NEEDLE PASSING THROUGH WALL OF HUMIDITY CHAMBER

In some situations, the volume of sample available may be too small for use with the Chemyx pump. For these situations, a software provision exists to allow the operator to apply sample to the grid manually using a micropipette.

Blotting Mechanism The blotting mechanism is a press-type mechanism that is comprised of two independent halves supported by a common set of rails. Each half is actuated independently using a CreativeWerks LACT2P linear actuator with 2.0" throw and 2.2 in/sec throw rate. Blotting force is monitored using a load cell (Omega Engineering LC302-25) placed in series with the linear actuators and blotting pads. The blotting surface of the mechanism is covered by a soft foam-rubber material. During setup, the operator installs a strip of filter paper such that it lays over the top of the foam rubber blotting surface.

During operation, the Cobra positions the imaging grid midway between the blotting surfaces such that the face of the grid is parallel to the faces of the blotting surfaces. The linear actuators then move the blotting pads towards the grid until they come into contact with each other. The actuators retract once the desired blotting force and time are reached. In Figure 4, label 4 indicates the position of the linear actuator and label 6 shows the position of a blotting pad. Figure 7 shows a more detailed view of the blotting pads.

Temporary Storage After a sample has been vitrified in liquid ethane, it must be stored temporarily before it can be transferred to the electron microscope. In our system, storage takes place in two locations.

Immediately after vitrification, the sample grid is transferred into a storage button that is submerged in liquid nitrogen within the vitrification dewar. Each storage button is capable of holding four prepared sample grids and has a threaded hole designed to mate with the button handling rod.

Once a storage button has been filled, it is transferred to a liquid-nitrogen filled portable storage dewar. Submerged within the storage dewar is a structure designed to hold button handling rods. In order to move the filled button, the Cobra removes a button handling rod from the storage dewar as shown in Figure 2, screws the threaded end of the button handling rod into the full storage button, and then transfers the rod/button unit back to the storage dewar where it is submerged in liquid nitrogen and released.

Controls Control of the system occurs through two separate channels. The Cobra arm is interfaced with a Windows 7 machine through the Adept AWCII controller board and the Adept Compact Controller. Movement of the arm and actuation of the pneumatic gripper is accomplished through a custom Adept V+ script running under AdeptWindows.

Monitoring and control of all other system functions, including the humidity sensors, temperature sensors, and actuation of all doors is accomplished using National Instruments LabView and a USB-6009 DAQ with custom interface circuitry. LabView also coordinates the actions of the Cobra through a software interface with AdeptWindows.

The LabView interface provides the operator with full control of all aspects of the system. User configurable settings include the desired relative humidity, specimen volume applied to grid, blotting pressure, blotting time, time delay between blotting and vitrification, and number of grids desired. The interface also provides basic operational information to the user, which includes current temperatures throughout the system, humidity values, current grid preparation progress, and basic device fault information.

Methods

This section details the process for correctly setting up and running our system. The section not only covers the set-up procedures necessary for the user to perform, but also details all specific steps that occur when the system is running.

Initial Setup The first step when operating the system is calibrating the Cobra robot according the Adept user manual. After this step is completed, the operator loads the controlling Lab-View program and initializes the connection between AdeptWindows and LabView.

The operator then determines the number of sample grids they wish to process, and loads plasma-cleaned grids into the grid-holder box as shown in Figure 6. The operator places a



Figure 6. INSTALLING AN EMPTY GRID INTO GRID-HOLDER BOX

filled and primed syringe containing the desired specimen into the Chemyx pump. The operator places empty grid storage buttons into the vitrification dewar, and places the button handling rods into the storage dewar.

In the LabView interface, the operator specifies the number of grids to be processed, the volume of specimen to be applied to each grid, desired blotting force, desired blotting time, and the delay time after blotting.

The operator fills the water cup for the ultrasonic mister.

The operator then fills both the storage dewar and vitrification dewars fully with liquid nitrogen. The operator should periodically refill these containers as the liquid nitrogen boils off during the cooling process. After the cooling process is complete, the operator should not need to refill the dewars.

The LabView program automatically monitors the temperature of the ethane cup within the vitrification dewar. When the temperature reaches -100°C, LabView will indicate that the ethane cup is cold enough to condense ethane. The user then proceeds to fill the ethane cup using gaseous ethane and hollow wand, refills the liquid nitrogen, and places the vitrification dewar into its prescribed location under the humidity chamber. The operator then indicates to LabView that all preparations have been made and that the program is free to continue.

Sample Preparation After the initial setup process has been completed, the system requires no further human interaction unless a fault condition occurs.



Figure 7. VIEW FROM INSIDE OF HUMIDITY CHAMBER SHOWING GRID IN POSITION FOR BLOTTING

LabView immediately begins to monitor the relative humidity of the humidity chamber, and runs the humidifier as needed to maintain the desired humidity. It should be noted that the system does not begin grid preparation immediately. Instead, Lab-View monitors the temperature of the ethane and begins preparing samples only after the ethane temperature falls between -180 and -175°C, the ideal temperatures for vitrification of a sample. The following sections detail the precise steps the system takes in order to produce a set of samples.

Grid Collection The Cobra picks up a plasma-cleaned grid from the grid-storage box using the pneumatically actuated tweezers.

Deposition LabView opens the side door of the humidity chambers and commands the Cobra to bring the grid into the chamber and within close proximity (0.5-1mm) to the tip of the deposition syringe. LabView then instructs the Chemyx pump to deposit the desired amount of specimen onto the sample grid.

Blotting The Cobra positions the grid midway between the two blotting surfaces, and aligned laterally such that the grid will be blotted using filter paper that has not been used to blot a previous grid. Figure 7 shows a grid in position for blotting.

The linear actuators move the blotting pads towards the grid until they come into contact with each other. The actuators continue to squeeze the grid until the desired blotting pressure and time are reached. At this point, the blotting pads retract fully.

Vitrification After the sample grid has been blotted, the system delays for a user-specified amount of time before vitrifying the sample.

Immediately before the delay time expires, the bottom door



Figure 8. Humidifier Performance Curve

of the humidity chamber is retracted exposing the vitrification dewar. At this point the grid is plunged directly into the ethane cup and held there for several seconds. Afterwards, the grid is quickly transferred from the ethane cup and into a grid storage button submerged in liquid nitrogen.

At this point, if the robot has more grids to process it begins the above process again starting with *Grid Collection*. If all grids have been processed, then the system begins to transfer the grids to temporary storage.

Transfer to Storage The system moves the end-effector to the storage dewar and grasps one of the cooled button handling rods using the pneumatic gripping mechanism. The Cobra then transfers the rod through the humidity chamber and positions it within the vitrification dewar such that the male threaded stud of the grid handling rod mates with the threaded hole of the storage button.

At this point the system delays for 10 seconds to allow the rod to bleed off any heat gained during its transfer between the storage and vitrification dewars. this delay prevents residual heat from the handling rod from melting the vitrified sample. At the end of the 10 seconds, the button handling rod is screwed into the button and the entire assembly is transferred to the storage dewar, submerged in liquid nitrogen, and released.

This process repeats until all storage buttons have been transferred to storage.

Results

Testing of the ultrasonic humidifier and humidity chamber indicates that the system is able to reach 100% relative humidity within roughly 120 seconds, a time that is competitive with other systems. Figure 8 shows the humidity within the chamber as a function of time.

Testing of the grid handling and blotting mechanisms reveal a system that is able to complete its task very quickly. An entire grid can be processed in as little as 20 seconds, depending on the delay time after blotting. This compares favorably with existing systems, as a skilled operator working with a commercial device may take a minute or more to process each grid.

Imaging of processed grids has yet to occur, though no significance difference between our system and existing systems is anticipated.

Conclusion

A fully automated system for the preparation of samples for cryo-electron microscopy has been developed. The system requires minimal human interaction in order to process specimens. Initial testing indicates that initial setup time is comparable between our device and competing systems, however our device is able to process specimens far faster than existing systems. Imaging studies with prepared samples have not yet been completed as of this writing, however no significant difference in quality of images is expected between our system and competing devices.

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