RICE UNIVERSITY

An Automated System for Cryo-Electron Microscopy Sample Preparation

by

Zachary J. Thompson

A THESIS SUBMITTED
IN PARTIAL FULFILLMENT OF THE
REQUIREMENTS FOR THE DEGREE

Master of Science

APPROVED, THESIS COMMITTEE:

Marcia K. O'Malley, Chair
Associate Professor of Mechanical
Engineering and Materials Science

Lydia Kavraki

Lydia E. Kavraki
Noah Harding Professor of Computer
Science and Bioengineering

Pol D. Spanos
Lewis B. Ryon Professor of Mechanical
Engineering and of Civil Engineering

Houston, Texas

November, 2010

Abstract

An Automated System for Cryo-Electron Microscopy Sample Preparation

by

Zachary J. Thompson

Cryo-electron microscopy (CryoEM) is a procedure that has become popular for imaging radiation intolerant structures under electron microscopes. CryoEM involves maintaining the sample at cryogenic temperatures throughout the imaging process. This has the effect of minimizing damage caused by the electron beam, and results in higher quality images than can be obtained through more traditional imaging methods.

The preparation of samples for cryo-electron microscopy studies is currently a labor and time intensive process. Samples must be applied to an imaging substrate under tightly controlled environmental conditions, formed into a thin film, vitrified with liquid ethane, and placed into temporary storage under cryogenic conditions. The grid preparation process is very sensitive to procedural factors, thus the successful creation of viable samples depends on tightly controlling the conditions under which grids are prepared. Several devices which automate portions of the specimen preparation process are currently in use; however, these systems heavily rely on a human operator to function properly.

This thesis describes a system that is capable of fully automating the sample preparation process. The resulting system minimizes the need for human input during specimen preparation, improves process control, and provides similar levels of environmental control. Testing shows that the resulting system is capable of preparing samples without human interaction.

Contents

3 Experimental Setup 30

Illustrations

Chapter 1

Introduction

Cryo-electron microscopy is a process in which specimens are prepared for and imaged under an electron microscope while being maintained at cryogenic temperatures. The sample is prepared (or *fixed*) for imaging in an electron microscope through a process known as vitrification. Vitrification, the most commonly used fixing method, is a technique that involves freezing a sample at such a high rate as to prevent the growth of ice crystals within the specimen. In an aqueous solution, this procedure results in an amorphous ice with embedded specimen. The resulting vitrified sample has physical and chemical properties that closely mimic those of the un-fixed sample[1].

This thesis present the development and design of a system that automates the sample preparation process.

1.1 Background

Since its introduction, the electron microscope has revolutionized the study of both organic and inorganic structures at 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. Traditionally, the preparation process often involved methods that had 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 problematic[2].

Cryo-electron microscopy (CryoEM) has repeatedly shown itself as a viable mechanism for imaging 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 biological specimens from the most degrading effects of the electron beam and the ultra-low vacuums present within the microscope[3].

The preparation of samples for CyroEM procedures has been well documented[4] 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 is 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 1.1 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 applies several micro-liters of an aqueous suspension of the specimen to the central area of the grid using a micropipette or similar tool.

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

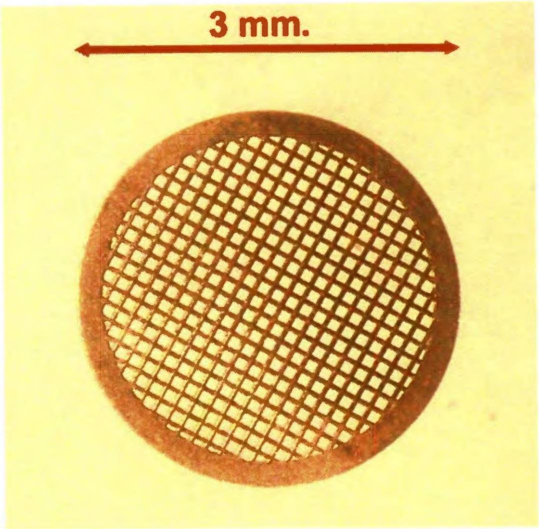


Figure 1.1 : Typical CryoEM imaging grid. This grid is shown sans any carbon coating.

perforations of the grid 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[5]. Thin films also promote the rapid freezing of the sample due to their high surface-area to volume ratio[1]. 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. Typically, this step is done in a high-humidity environment to retard evaporation of the thin-film after blotting. Blotting pressure and time should be monitored to provide control over the amount of sample removed from the imaging grid, allowing for the creation of thin films upon the imaging grid of a predictable and reproducible thickness. Care must be taken to minimize bending of the grid during the blotting process, as this can irreparably damage the grid and render it unfit for introduction into the electron microscope.

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 -10^5 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. 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 bath and holding it there for several seconds. For successful vitrification to occur, the liquid ethane must be maintained at a temperature near its melting point. If the liquid ethane bath is too warm, crystalline ice will form instead of vitrified ice. If the ethane bath is too cold, then it will freeze and the imaging grid will be destroyed during the plunge-freezing process when the grid impacts the frozen ethane.

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 the specimen's devitrification temperature can be used[6]. 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 re-crystalize when its temperature reaches the de-vitrification temperature. For aqueous specimens, this temperature is around -133°C[1].

1.2 Previous Work

The first practical electron microscope was created in the early 1930s by Rusk and Knoll. Even early in its development, the benefits of the electron microscope over traditional optical systems were obvious. The greatest advantage that electron microscopes systems held over competing systems was their vastly superior resolving power: even early systems could resolve details several magnitudes of order smaller than other methods[7].

Interest in the electron microscope steadily increased, and its performance was unmatched in a variety of fields. However the inability to successfully image many biological samples limited the usefulness of the electron microscope to a field that could derive great benefits from its resolving power.

Early electron microscopy studies of biological specimens left much to be desired: imaged specimens often lacked enough contrast to distinguish between structures within the sample. Chemical fixing methods have proven successful at preserving samples for imaging and improving contrast, but chemically fixing the samples oftentimes introduces distortions within the structure of the sample. These distortions make the interpretation of of images produced via chemical fixing methods difficult[4].

Freezing of samples using traditional means also proved problematic. Under most conditions, liquid water freezes to form crystalline ice. During the freezing process, growing ice crystals can impede on delicate biological structures and either distort or damage them[6]. Freezing of samples also results in the separation of the sample into areas containing pure ice and highly concentrated specimen particles that cannot represent the distribution of specimen particles prior to freezing[8]. Furthermore, crystalline ice results in the scattering of the electron beam when a sample is imaged under an electron microscope. Artifacts in the final image caused by the scattering effect limit the ultimate resolution of samples embedded in crystalline ice[9].

A remedy to the problems associated with fixing biological specimens was recognized with the discovery of vitrification of pure water and diluted aqueous suspensions [10, 11]. Specimens that were vitrified maintained physical and chemical characteristics that were closer to those of the un-fixed specime. The key to the creation of vitrified ice is freezing the sample so fast that ice crystals do not have enough time to form: on the order of -10^5 K/sec.

In 1984, Adrian et al. reported the vitrification and imaging of biological specimens under an electron microscope[1]. The procedure outlined in this seminal paper is largely the same procedure used to this day: an aqueous suspension of specimen is applied to an electron microscope grid, the grid is blotted with filter paper to form a thin film of sample within the perforations of the grid, the grid is plunge frozen in liquid ethane that is near its freezing point, the grid is quickly transferred to a liquid nitrogen storage bath, and the grid is transferred into the electron microscope for imaging.

Early on, it was noted that evaporation of the thin film after blotting caused an increase in the concentration of specimen in the image sample, changes to the structure of the sample, and evaporative cooling of the sample [12]. Evaporation of the thin film also results in 'dry grids': sample grids whose sample has mostly evaporated leaving only dehydrated specimen. To combat this problem a variety of solutions were proposed. Some solutions involved plunge-freezing the sample very quickly after the blotting process was completed in an effort to minimize evaporation of the solvent[13]. However, the most common design involved the control of humidity around the grid during and immediately after the blotting process.

Murray and Ward designed a system that passed a stream of humidified air over the grid to prevent evaporation [14]. Most designs, however, entail the creation of a humidity controlled chamber that isolates the grid from ambient conditions during preparation. Methods for maintaining constant humidity levels within the chamber fall into two primary categories: ultrasonic systems and forced evaporative methods.

Systems that operate using forced evaporation rely on the circulation of air over a water saturated porous medium[15, 16], or on the humidification of air bubbles as they pass through a temperature-controlled bath[13]. Ultrasonic systems atomize a water bath by introducing a high-frequency waveform to the liquid. The energy introduced to the water is dissipated when wave peaks at the surface of the liquid break free and are ejected into the air as individual droplets[17]. Several devices have made use of ultrasonic humidification systems [18, 19, 20]

Temperature control during sample preparation allows for control over temperature dependent chemical and biological reactions taking place within the sample, and can minimize micro-convective flow within the sample[15]. Early designs were only capable of maintaining temperatures within the chamber at above-ambient conditions[15, 14]; however, subsequent devices were able to maintain temperatures above and below ambient[13, 18, 19, 21, 22].

While most early systems relied on manual blotting techniques[14, 15], subsequent systems incorporated semi-automatic and automatic systems to improve the consistency of blotting [13, 18, 19, 21]. These systems implemented electromechanical or pneumatic actuators that could blot the imaging grid in a highly controlled and reproducible manner. More recently, designs have been published that attempt to minimize the delay time between blotting and vitrification by utilizing an optimized blotting method[13].

More recent systems have automated other portions of the CryoEM imaging process. Potter et al. described a system capable of automatically loading imaging grids into a transmission electron microscope[23]. Ge et al. have described a system for preparing samples for imaging under a scanning electron microscope[24].

1.3 Commercial Systems

This section presents the basic design element present within two commercial vitrification devices, the Gatan Cryoplunge and FEI Vitrobot.

1.3.1 Gatan Cryoplunge

The Gatan Cryoplunge vitrification device is shown in Figure 1.2. The Cryoplunge consists of a support tower that encloses the pneumatic and electronic controls of the device. On the front of the tower, a small cylindrical glass environmental chamber is present. Within the environmental chamber are pneumatically actuated blotting pads and a passive humidification system. Sensors actively monitor the temperature and humidity of the environmental chamber.

The blotting pads of the Cryoplunge are circular and can be rotated about their axes. During blotting, the grid is positioned midway between the blotting pads and aligned with their outer circumference. The pads are then extended towards one another using pneumatic actuators. After blotting is completed, the operator manually rotates the blotting pads 90° so that fresh blotting paper will be available to the next grid.

Directly below the environmental chamber is the cryogenic workstation. The cryogenic workstation consists of a temperature-controlled ethane cup, a temporary storage area for processed specimens, and a funnel for remotely filling the station with liquid nitrogen.

A pneumatically actuated piston passes through the center of the environmental chamber and aligns with the ethane cup. A door on the floor of the environmental chamber allows the piston to pass through the chamber and approach the ethane cup.

During operation, a pair of electron microscopy tweezers and grid are installed onto the end of the piston. The piston is retracted into the environmental chamber and the grid is aligned with the outer edge of the blotting pads. The operator passes a micropipette through the side port of the chamber and applies a sample to the imaging grid. The system then blots the sample from the grid, opens the bottom door of the environmental chamber, and plunges the grid into the ethane bath at 1.7m/sec[25]. The operator then manually transfers the grid to the storage area and resets the device for the next use. The Cryoplunge is able to process four grids before the blotting paper in the device must be replaced.

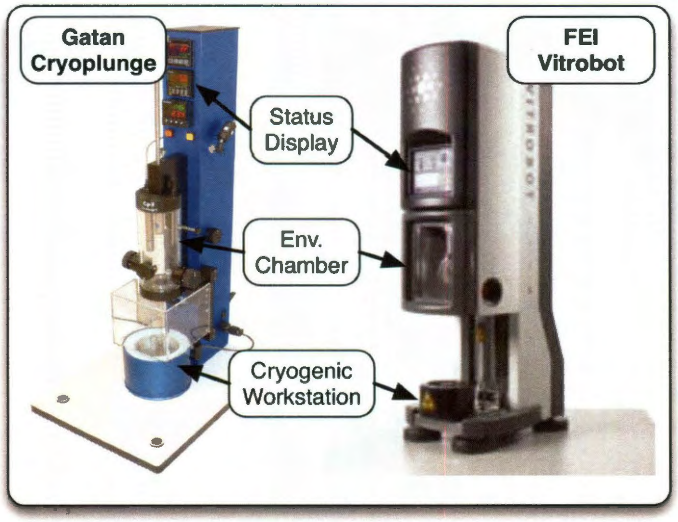


Figure 1.2 : Gatan Cryoplunge (left) and FEI Vitrobot (right) showing major features.

1.3.2 FEI Vitrobot

The FEI Vitrobot vitrification device is shown in Figure 1.2. The Vitrobot consists of a support tower that encloses the pneumatic and electronic controls of the device. On the front of the tower, a stainless steel and teflon environmental chamber is present. Within the environmental chamber is an electrically driven blotting mechanism, ultrasonic humidifier, thermoelectric temperature control device, and sample cup for automatic deposition. Sensors actively monitor the temperature and humidity of the environmental chamber.

The blotting pads of the Vitrobot are circular and can be rotated about their axes. During blotting the grid is aligned with the outer edge of the blotting pad. After blotting is completed, the Vitrobot automatically rotates the blotting pad in preparation for the next imaging grid.

Directly below the environmental chamber is the cryogenic workstation. The cryogenic workstation consists of an ethane cup, a temporary storage area for processed specimens, and a funnel for remotely filling the station with liquid nitrogen. The temperature of the ethane cup is not automatically maintained. The operator manually controls the temperature of the ethane by placing a device onto the cryogenic workstation that transfers excess heat from the ethane bath to the liquid nitrogen or by introducing warm gaseous ethane to the cup.

A pneumatically actuated piston passes through the center of the environmental chamber and aligns with the ethane cup. A door on the floor of the environmental chamber allows the piston to pass through the chamber and approach the ethane cup.

During operation, a pair of electron microscopy tweezers with grid are installed onto the end of the piston. The piston is retracted into the environmental chamber and the grid is aligned with the outer edge of the blotting pads. If automatic sample deposition is desired, the grid is then submerged into a sample bath that is located within the environmental chamber. When manual deposition is desired, the operator passes a micropipette through the side port of the chamber and applies a sample to the imaging grid. The system then blots the sample from the grid, opens the bottom door of the environmental chamber, and plunges the grid into the ethane bath at great speed. The operator then manually transfers the grid to the storage button and resets the device for the next use. The Vitrobot is able to process 18 grids before the blotting paper in the device must be replaced.

1.4 Motivation

The process of preparing samples for cryo-electron microscopy studies is a labor and time intensive process. Even when using the most advanced commercial equipment[16, 26, 27, 28], operators are forced actively participate in the preparation of each individual grid.

Figure 1.3 shows the steps in the grid preparation process that are currently automated and those which require a human operator. Currently, the only processes that are automated with commercial systems are the blotting and plunge-freezing steps. Operators must manually grasp each blank imaging grid with a pair a electron microscopy tweezers, install the tweezers within the plunge freezing device, apply sample to to the imaging grid, transfer the vitrified specimen between the ethane bath and liquid nitrogen storage area, and store the grid in a grid storage button.

Because the operator is intimately involved with the grid preparation process, they are sensitive to the conditions required to successfully prepare a specimen. The sensitivity to the grid preparation process and the lack of reliance of the hard-coded instructions of a machine provide ample room for the operator to improvise and im-

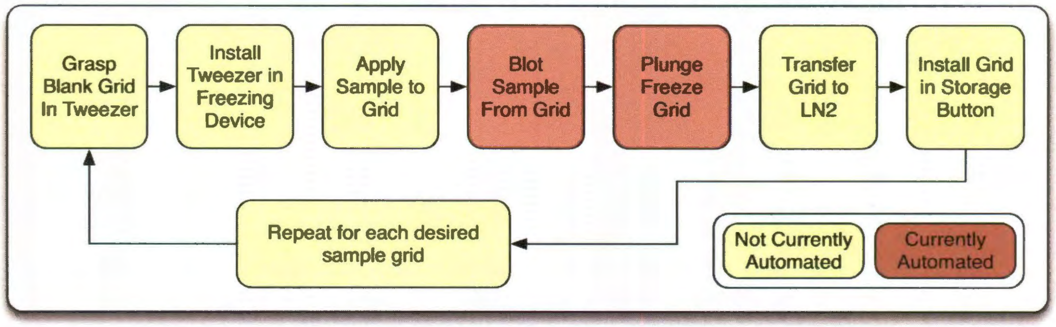


Figure 1.3 : Steps involved in preparation of single grid for imaging in electron microscope. Steps colored red are automated in commercial systems.

prove upon the specific procedures necessary to successfully prepare a given specimen.

A fully automated sample preparation system has the potential to negate this advantage. However, a device or system that completely divorces the user from the method of its operation tends to impart less knowledge about the status of the process to the user. A common example of this phenomenon would be rise in popularity of countertop breadmakers. These devices only require the user to provide the necessary ingredients, while they automate the rest of the breadmaking process. Breadmakers provide minimal feedback to the user about the breadmaking process and generally only provide cryptic feedback on the current status of the machine. Breadmakers only allow minimal modification to the pre-programmed recipe. Because the breadmaker shields the user from the breadmaking process, the operator has no hope of truly understanding the steps necessary to bake a successful loaf of bread. In short, the breadmaker problem involves the insufficient transfer of information between the device and user.

The intention of this project is to automate the grid preparation process without encountering the breadmaker problem. This means that the resulting system should provide information about the current status of the grid preparation process, indicate the steps involved in the preparation process, and allow for flexibility in defining the preparation procedure for a given specimen. The system should also impart as much knowledge as possible about the grid-preparation process to the user.

Commercial vitrification systems solve the breadmaker problem by involving the user in nearly every step of the grid-preparation process. These systems, at the very least, require a highly trained operator to process grids successfully. In essence, these devices sidestep the problem of skill transfer by requiring an expert to successfully operate. Our proposed system does not require an expert human operator, however the machine itself is designed to maximize the amount of information presented to the operator.

Moreover, because human interaction is vital to the functioning of commercial systems, they are susceptible to malfunctions precipitated by human error, require extensive training, and can be cumbersome to use even for the most skilled operator. Inconsistencies between prepared samples can be triggered by small changes in the grid preparation process. These changes are difficult to control when a human operator is a functional unit in the system.

In this thesis, the development of a device capable of automating the preparation of samples for cryo-electron microscopy studies is presented. The system has been dubbed ASPECT: *Automated Sample Preparation Entity for Cryo-TEM.* ASPECT provides flexibility in preparing specimens, is easy to operate, provides extensive information regarding the status of the device, and drastically reduces the need for active human participation in the grid preparation process. The ASPECT device presented in this thesis minimizes inconsistencies in prepared samples by eliminating human input during the sample preparation process.

Chapter 2

Design Rationale

Two commercially available automated vitrification systems inspired the design of the ASPECT system and serve as the benchmark to witch the performance of the ASPECT system is judged. The Cryoplunge and Vitrobot are currently used in laboratories around the world, and their performance characteristics are well understood. These commercial systems only automated a small portion of the grid preparation process as described in section ??. During development of the ASPECT system, an effort was made to improve upon the design philosophies represented by the Cryoplunge and Vitrobot only when significant performance or usability gains were achievable. As a result, the design lineage of some systems in the ASPECT device can be directly traced to subsystems present in the Vitrobot and Cryoplunge devices.

This chapter presents the design rational behind the subsystems of the ASPECT device. For more detailed information on the systems present within the final ASPECT device, please see chapter ??.

2.1 Adept Cobra 600 and Grid Handling

The Adept Cobra 600 is a selectively compliant assembly robotic arm (SCARA) that has proven itself capable of performing a variety of assembly and pick-and-place type procedures[29]. In the ASPECT system, the Cobra transfers the imaging grid between stations where deposition, blotting, vitrification, and storage occur. The Cobra was chosen primarily for its relatively large workspace and capability to operate at a high rate of speed (>100in/sec)[30]. The large size of the Cobra's workspace allows for flexibility when placing other hardware elements of the ASPECT system. The high rate of speed gives the ASPECT device the capability to process grids much faster than competing devices. Furthermore, the positional accuracy of the Cobra is high enough to reliably place the grid at any position within the environmental chamber.

The Cobra was also chosen because of the Adept Technology's good track-record of producing reliable and safe industrial robotics. A custom robotic system could have integrated better with the ASPECT device, but the development of a reliable custom robot was not in the scope of this project and would have hindered the rapid development of ASPECT.

2.2 Grid Capacity

The ASPECT system is designed to process a maximum of twelve grids before a resupply of blotting paper, grid storage buttons, and imaging grids is necessary. This capacity was decided upon after consulting with personnel familiar with the operational characteristics of both the FEI Vitrobot and Gatan Cryoplunge.

The maximum capacity of the Cryoplunge is four grids, and was deemed insufficient for certain imaging tasks. Similarly, the maximum capacity of 18 grids provided by the Vitrobot was rarely taken advantage of. A capacity of 12 grids was determined to represent a happy medium between the two devices, and was settled upon as a design constraint for the ASPECT system.

2.3 Environmental Chamber Access Doors

Areas of the vitrification device containing cryogens must be located where they will not be exposed to the high humidities present within the environmental chamber.

This precaution prevents the formation of frost on structures exposed to the cryogens and the absorption of liquid water into the ethane bath[15].

To facilitate rapid plunge freezing, the ethane bath must still be readily accessed from the environmental chamber. Both the Cryoplunge and Vitrobot systems locate the cryogenic workstation directly below the environmental chamber. The workstation is accessed from the chamber via a sliding-door that opens during the plunge-freezing process.

In the ASPECT device, a similar approach is taken. The cryogenic workstation is located directly below the environmental chamber and separated from the chamber with a sliding door. Before plunge-freezing, the door slides open to expose the ethane cup to the environmental chamber. ASPECT then plunge freezes the sample and transfers the sample to storage before exiting the workstation and closing the sliding door.

A second door on the front of the ASPECT's environmental chamber allows the Cobra's end effector to enter the environmental chamber.

2.4 Automated Sample Deposition

The FEI Vitrobot has the ability to automatically apply sample to the imaging grid. The Vitrobot literally dips the entire grid into the specimen[31]. While this method is functional, it requires a relatively large volume of sample that can be problematic to obtain if the sample is very expensive or rare.

In the ASPECT system, a Chemyx Fusion 100 Syringe pump is used to automatically deposit specimen onto the surface of the grid. This method is preferable to the dipping method as the volume of sample necessary is limited only by the geometry of the syringe. A syringe with a smaller volume requires less sample to function properly.

The ASPECT device also has a manual deposition mode that allows the operator to apply sample directly to the surface of the grid using a micropipette. The manual deposition mode is used when sample volumes are too small to be accommodated by the syringe pump.

2.5 Blotting Mechanism

Besides the automatic grid handling system, the biggest difference between ASPECT and commercial systems is the implementation of a linear blotting mechanism in the ASPECT device.

The grid handling mechanism present in commercial systems consists of a pair of stainless steel tweezers mounted on a pneumatically actuated vertical piston. The piston is only capable of translating the tweezers along its vertical axis. As a result, both the Vitrobot and Cryoplunge must have a mechanism that presents fresh blotting paper to the imaging grid while accounting for translation of the grid solely on the vertical axis.

These systems both solve this problem by implementing a pair of circular blotting pads as shown in Figure 2.1. During the blotting process, the grid is held in a fixed position between the pads so that the grid falls just inside the outer circumference of the blotting pad. The blotting pads are rotated about their axis until unused blotting paper is aligned with the grid. Finally, the blotting pads are extended towards the grid until they make contact with it.

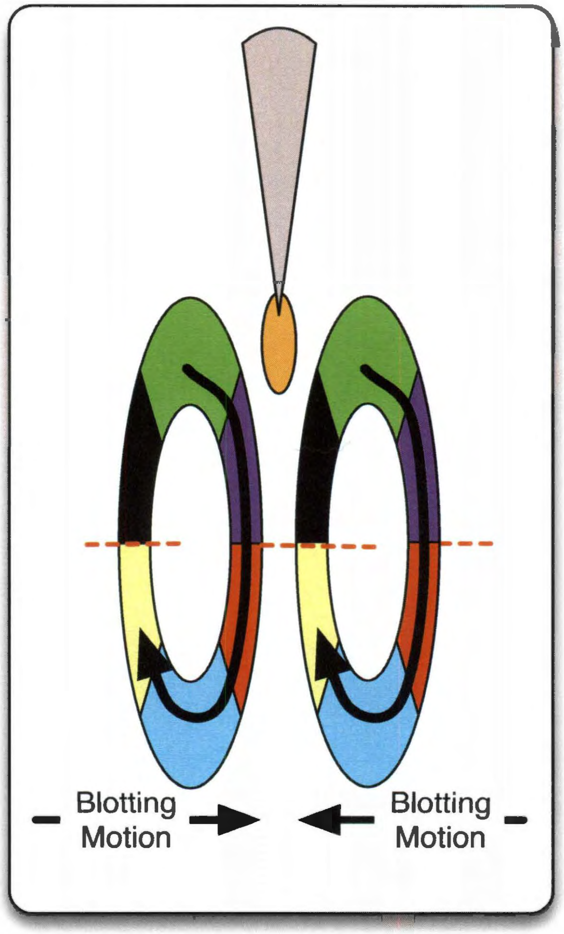


Figure 2.1 : Principles of rotational blotting mechanism. Grid (gold) is held between blotting pads. Pads move towards grid to perform blotting action. Pads rotate about central axis to expose unused blotting (colored sections) paper to grid.

The blotting pads in commercial systems require two actuations, one to blot the grid and another to rotate the pad and present fresh paper to the grid. These additional actuations complicate the control framework for the devices and introduce more points where failure can occur.

The Adept Cobra is not constrained to the vertical axis when positioning the grid within the ASPECT device. As a result, the ASPECT system is not required to place the grid in the same position each time blotting occurs. In the ASPECT system, the blotting pads are linear in nature. Instead of blotting on the circumference of a circular blotting pad, the ASPECT system blots along the length of a linear blotting pad. The grid is placed at a different position along the pads during each blotting cycle. Thus, the pads do not need additional actuations to present fresh blotting paper to the grid. The linear blotting pads in the ASPECT device require only a single actuation to blot the imaging grid, as opposed to two actuations in commercial systems. This design results in a mechanically straightforward and more reliable actuator design as well as a simpler control framework.

2.6 Humidification System

The maintenance of a high relative humidity during the blotting process retards the evaporation of the thin-film from the imaging grid and helps to ensure that the sample is fully hydrated when vitrification occurs. The humidification systems present in commercial systems either rely on the evaporation of water from a porous sponge or on atomizing water into the atmosphere of the environmental chamber.

The humidification system present in the Gatan Cryoplunge is of the passive variety. Air from the environmental chamber is passed over a porous cellulose sponge that has been soaked in water. As the sponge slowly evaporates, the humidity of the environmental chamber increases. Though this system maintains the humidity of the environmental chamber at or near 100%, it has one large drawback: it takes a long time to reach 100% humidity. According to Gatan's system specifications, it takes the Cryoplunge 15 minutes to reach 100% relative humidity[16].

The humidification system present in the FEI Vitrobot operates fundamentally differently than the one in the Cryoplunge. Instead of relying on the passive evaporation of a sponge, the Vitrobot implements an ultrasonic humidification system that actively saturates the air with water vapor. The ultrasonic humdifier in the Vitrobot is capable of humidifying the environmental chamber in less than one minute[20].

Both passive and active humidifications systems were tested with prototypes of the ASPECT system. The performance of both humidification methods in the prototype chambers mirrored the performance of these systems in the commercial devices: ultrasonic humidification systems provided much faster humidification of the environmental chamber. To capitalize on the increased performance of the active humidification system, the final ASPECT environmental chamber was designed to utilize an ultrasonic humidifier.

2.7 Environmental Chamber Temperature Control

Temperature control of the environmental chamber in the ASPECT system is implemented using a thermoelectric temperature control device that was salvaged from a non-functional Vitrobot. Testing of the system revealed that the temperature control device was only able to heat/cool the ASPECT environmental chamber to temperatures within 5°C of ambient. The Peltier effect chip that provides the heating/cooling effect was replaced with a higher wattage unit to compensate for the increased volume and poor insulation of ASPECT's environmental chamber. Subsequent tests with the modified temperature control unit revealed that the ASPECT's environmental chamber could reach temperatures 20°C above ambient and 5°C below ambient.

2.8 Ethane Cup Temperature Control

Ethane cup temperature control systems present in commercial grid preparation systems fall into two broad categories. Passive systems, like the system present in the Vitrobot, rely on the human operator to monitor and maintain the temperature of the ethane bath. Active systems, as implemented in the Cryoplunge system, maintain the temperature of the ethane bath at a user-specified level without the intervention of the operator.

The ethane cup in the Vitrobot is thermally coupled to the liquid nitrogen bath. If left on its own, the ethane bath would freeze and inflict damage to the grid and tweezers during the plunge freezing operation. To maintain the temperature of the bath, the operator periodically allows additional warm gaseous ethane to enter the ethane cup. By introducing this warm gas, the temperature of the entire bath is increased and freezing of the ethane is be postponed.

Active heater systems, similar to those employed in the Gatan Cryoplunge and Electron Microscopy Sciences EMS-002[28], do not require the operator to manually monitor and maintain the temperature of the ethane bath. Instead, these systems actively maintain the temperature of the ethane bath using heating elements.

2.8.1 ASPECT Ethane Temperature Control Evolution

The ethane temperature control system in the ASPECT device was designed to automatically monitor and control the temperature of the ethane bath. The temperature of the ethane bath is monitored using a cryogenic temperature sensor that is submerged within the the bath. A Kapton-film resistive heating element is used to maintain the temperature of the ethane bath and to prevent freezing of the cryogen.

2.8.1.1 Version One

Figure 2.2A shows a cross section of an early design for the ethane cup temperature control support bracket. Fig 2.2E shows a photograph of this design with installed film heater and cryogenic temperature sensor being placed into the ethane cup. The temperature sensor and heating element are mounted onto an acrylic scaffolding and submerged into the ethane bath. The scaffolding was designed to be removable to facilitate cleaning of the ethane cup. A U-shaped cutout in the scaffolding allows the grid to be introduced into the center of the ethane cup without impacting the scaffolding. When deemed necessary, the controller framework energizes the heating element in an effort to maintain the temperature of the ethane bath. This design proved effected at controlling the temperature of the ethane bath, but problems with the design were soon discovered.

The most obvious problem with the early design was a very long time lag between the powering-up of the heating element and registration of a temperature change by the temperature sensor. This problem resulted in an ethane temperature that would not settle at a single value, but would oscillate about the desired temperature with an amplitude of 3-5°C. It is believed that the time lag was caused by insufficient thermal mixing of the cryogen due to the placement of the heating element in the topmost portion of the ethane cup. Buoyancy forces caused the warmed ethane to remain at the top of the ethane cup and resulted in very slow mixing with the cooler ethane at the bottom of the cup. The thermal stratification of the cryogen is also believed to have contributed to the poor vitrification performance of the ASPECT device because the specimen was initially exposed to the warmer ethane at the top of the cup instead of the near-freezing ethane at the bottom of the cup.

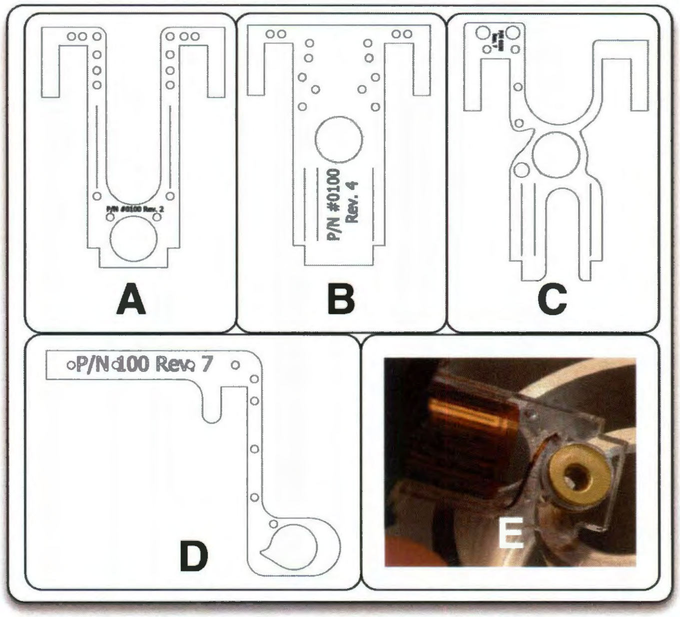


Figure 2.2 : Several designs used to mount the heating element and temperature sensor in the ethane cup. Designs A-C submerge the heating element in the ethane, and contain support structures for the film heater and temperature sensor. Image D represents the final design and only submerges the temperature sensor. In designs A-D the circular hole represents the mount point for the cryogenic temperature sensor. In designs A-C, vertical slits are used to mount the Kapton-film heating element. Image E is a photograph of the structure represented by design A.

A second problem with the initial design of the temperature control hardware was the very delicate nature of the scaffold used to support the heater and temperature sensor. The scaffolding was very brittle, especially at cryogenic temperatures, and was very easy to break when installing into the ethane cup.

2.8.1.2 Version Two

A second design was proposed to address the problems encountered in the first design. This design is shown in Figure 2.2B. This design placed the heating elements at the bottom of the ethane cup, located the temperature sensor above the heating elements, and greatly increased the the cross-sectional area of scaffolding. Because the scaffolding lacks the u-shaped cutout, the grid is held between the scaffolding and the wall of the cup during vitrification. The temperature control performance of this scaffolding was impressive: all of the temperature oscillations exhibited by the previous design were eliminated. Furthermore, the scaffolding was physically much stronger than the previous design and did not break as easily during insertion into the ethane cup.

The second design had two deficiencies. The first involved the gradual buildup of frozen ethane on the walls of the cup after operating for 10-15 minutes. This buildup caused problems when plunge-freezing a grid. If the frozen ethane became to thick, the grid would impact the frozen ethane during the plunging process and become severely damaged.

The more serious problem with the second design was the gradual evaporation of the liquified ethane from the cup. To increase the strength of the newer scaffolding, the U-shaped channel was removed from its design. This removal resulted in the creation of a 'fin' that extended from the ambient conditions above the surface of the ethane bath into the bath itself. During operation, thermal energy was transferred from the ambient atmosphere into the uppermost sections of the ethane bath and and resulted in evaporation of the bath. The heat transfer was severe enough to cause the ethane bath to simmer. Over a period of 5 minutes, roughly 25% of the ethane bath would evaporate.

2.8.1.3 Version Three

A third design for the scaffolding was designed and implemented as shown in Figure 2.2C. This design reintroduced the u-shaped cutout from the first design and the orientation of the heater/sensor elements from the second design. The design also featured a cross-section that was wider than that of the first design, but still narrow enough to minimize heat transfer into the ethane bath.

After several weeks of usage, a flaw inherent to the design of our control system and ethane cup scaffolding became apparent. Under certain conditions, it was possible to provide power to the heating element without the liquid ethane bath being present. When this occurred, it resulted in the rapid heating of the Kapton-film heater and the support scaffolding. Without a means to efficiently dissipate the heat, the resulting high temperatures caused the scaffolding to melt and rendered the heating element inoperative.

2.8.1.4 Version Four

The fourth and final design of the temperature control system solved the overheating problem. Instead of submerging the heating element in the ethane bath, the heating element is wrapped around the external circumference of the ethane cup. The aluminum of ethane cup acts as a sink for the heat produced by the heater. Even with no cryogen present, the heating element does not become hot enough to cause damage to itself or to other elements in the system. The scaffolding within the ethane cup was redesigned to support only the cryogenic temperature sensor as shown in Figure 2.2D. The scaffolding was designed to mount semi-permanently to the vitrification dewar via a support bracket. The semi-permanent nature of this installation allowed the cross-sectional area of the temperature sensor support scaffold to be further minimized.

2.9 Software Control Evolution

The goal of the control framework within the ASPECT system is to present a single user-interface to the operator that provides full control over every subsystem present within the system. The control framework includes both the software and hardware elements necessary to interface with and control the ASPECT device.

Early on, it was decided that National Instruments LabVIEW would be used as the primary development environment for ASPECT. LabVIEW was chosen because of its support for multiple operating systems (Mac OSX, Linux, and Windows) as well as its compatibility with of a wide variety of hardware interface devices. LabVIEW also provides a graphical user interface editor that provides an avenue the operator with an easy-to-use and readily customizable graphical-user-interface.

The desire to present the operator with a single user interface presented some unique challenges. Specifically, the control framework utilized by the Adept Cobra had to be made available for use by the LabVIEW system.

Adept Technology provides a C++ library that allows for the control of Adept robots from devices that do not natively support the Adept V+ operating system. LabVIEW's ability to execute C++ code would have provided an easy avenue for controlling the Cobra from within the LabVIEW environment, however the necessary Adept libraries were too expensive and fell outside the budget for ASPECT. Instead, a variety of hardware and software interfaces were implemented and tested with the ASPECT system.

2.9.1 Version 1 - Human in the Loop

The initial version of the software control framework utilized a human operator as an intermediary between the LabVIEW environment and the Adept system. When LabVIEW indicated to the operator that the Cobra should perform a specific motion, the operator would manually execute the motion using the Adept system.

This control framework was short-lived, and was only used to verify that the LabVIEW code worked as expected.

2.9.2 Version 2 - AutoIT

Subsequent efforts to integrate the LabVIEW environment and Adept systems focused on implementing a direct software interface between the systems. A Windows program, known as AutoIT [32], was chosen to act as an intermediary between LabVIEW and the Adept terminal window. AutoIT is a program that can take control over the Windows environment and perform actions based on scripts that are written by the user. AutoIT scripts have the ability to launch and close programs, move application windows, enter text, and perform many other tasks.

AutoIT scripts were written to perform any action that involved usage of the Adept systems. These actions included executing the Adept terminal window program, powering up the Cobra, calibrating the Cobra, and executing programs on the Cobra.

When the control loop within LabVIEW determined that an action should be performed by the Cobra, a specific AutoIT script would be executed from within the LabVIEW environment. This script would bring the Adept terminal window to the foreground, enter text into the terminal to load and execute the desired program, and return the LabVIEW interface to the foreground.

The AutoIT system showed the feasibility of controlling the Cobra robot from within LabVIEW, however many problems plagued its implementation. The most serious problem was the lack of communication between the LabVIEW environment and the Adept system. Because of the unidirectional nature of the AutoIT interface, LabVIEW had no method to determine whether a command had been received and executed by the Adept system. To further complicate matters, AutoIT did not reliably perform all actions it was commanded to perform. As a result, the ASPECT device oftentimes performed unexpectedly.

2.9.3 Version 3 - Hardware Interface with Soft Emergency Stop

The poor reliability of the AutoIT-based control system highlighted the need for bi-directional and error tolerant communication between the LabVIEW and Adept systems. This need was fulfilled with version three of the interface. This version was hardware based and communication occurred over digital inputs and outputs provided by the Adept system and by a pair of National Instruments USB data acquisition boards.

When a motion was required by the Cobra robot, a digital signal was sent by LabVIEW to the Adept system via digital lines between the systems. Depending on the status of these lines, the Adept system could calibrate the Cobra, move it to various positions within its workspace, or halt the execution of any running program. The hardware interface also allowed the Adept system to relay its status LabVIEW. LabVIEW used this data to verify that the Adept system was performing as expected. In the event of a malfunction, LabVIEW as able to take actions to correct the problem.

The performance of version three of the control system was very good in most circumstances. However, version three relied on software to control the emergency stop functions between the Adept and LabVIEW systems. The software emergency-stop did not reliably stop the system and was rectified in version four of the control system.

2.9.4 Version 4 - Hardware Interface with Hard Emergency Stop

To further increase the reliability of the emergency stop systems, a hybrid hardware-software emergency stop system was implemented. This system does not rely on the emergency stop signal to propagate through the software present in the Adept of LabVIEW systems. Instead, the emergency stop signal is intercepted and processed by custom logic circuitry. If an emergency stop signal is present, this circuitry cuts power to the entire ASPECT system.

Version four represents the most recent control structure present in the ASPECT system. More information on its implementation can be found in sections 3.4 and 3.5.

Chapter 3

Experimental Setup

The ASPECT device is capable of vitrifying specimens for cryo-electron microscopy studies with minimal human interaction. This chapter describes the hardware and software systems that are implemented in ASPECT device.

ASPECT represents the evolution of the commercial systems that are currently on the market. These systems implement a variety of automatic features that endear them to laboratories worldwide, however their overall performance is limited by their utter reliance on the human operator. To overcome this limitation, the ASPECT system has been designed to fully automate all portions of the grid preparation process while requiring little, if any, human interaction.

Our system can divided up into four primary subsystems:

1. Grid Handling
2. Sample Deposition, Blotting, and Vitrification
3. Environmental Control
4. Process Control

Figure 3.1 gives a view of the environmental chamber and vitrification dewar from the ASPECT system. The vitrification dewar is where the vitrification and storage of imaging grids occurs. Images A, D and E in Figure 3.1 show the vitrification dewar positioned underneath the environmental chamber. This is the position of the vitrification dewar during grid processing.

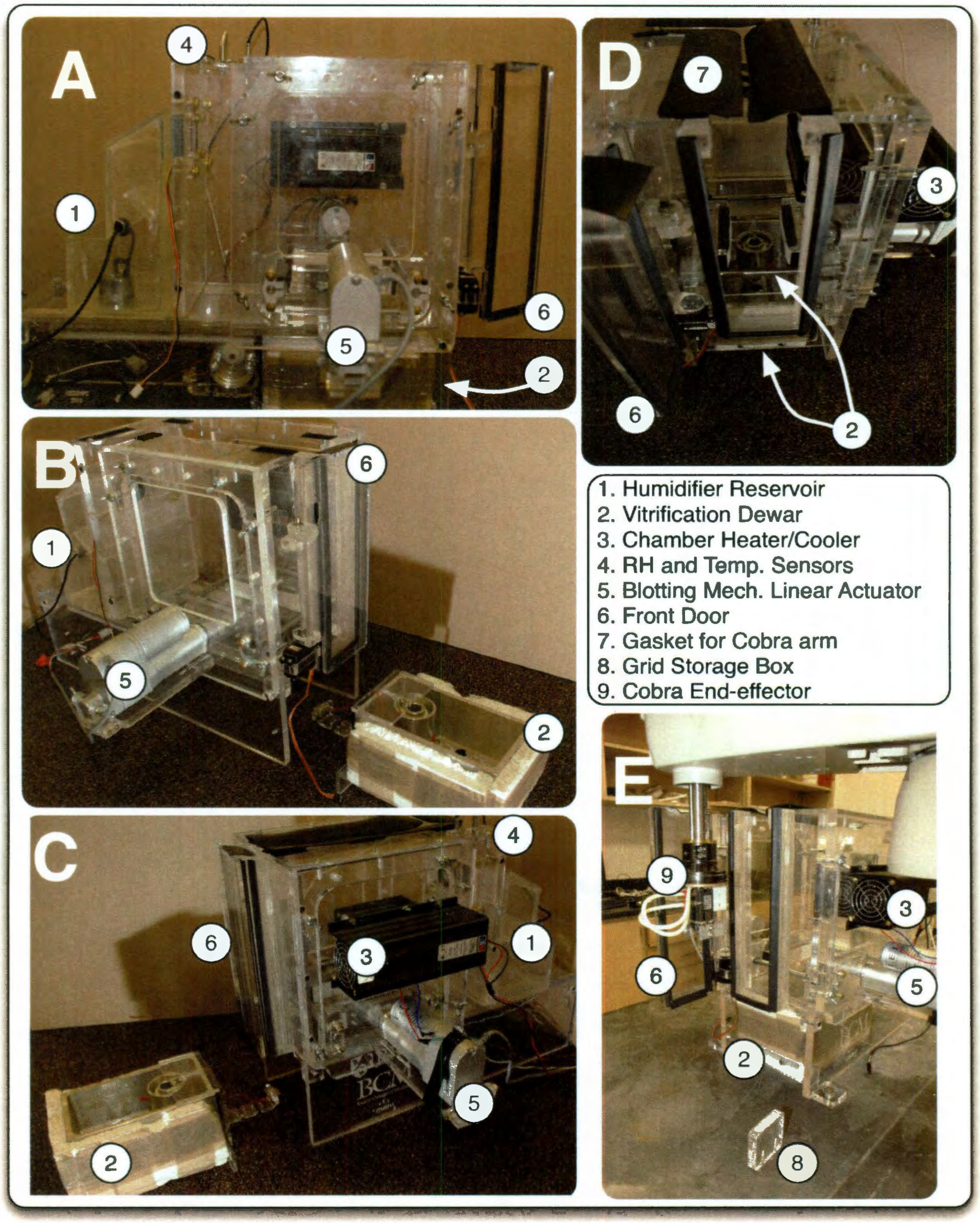


Figure 3.1 : Views of environmental chamber from multiple angles.

The environmental chamber contains systems for maintaining the relative humidity and temperature of the chamber. The chamber also houses the blotting mechanism. A pair of doors on the environmental chamber allows the end-effector of the Cobra to enter and exit the chamber. A pivoting door on the front of the chamber allows the Cobra to enter the chamber to access the blotting and deposition stations. Image E in Figure 3.1 shows the Cobra's end effector poised to enter the chamber through the front door. A sliding door located in the base of the chamber allows the Cobra to access the vitrification dewar.

The remainder of the chapter is dedicated to the discussion of the subsystems that make up the ASPECT device.

3.1 Grid Handling

The heart of the ASPECT device is the grid handling system. The system operates by transferring grids between stations within the device where deposition, blotting, vitrification, and storage occur. A commercially available Adept Cobra 600 4DOF SCARA type robot is utilized to transfer the grid between stations. A PHD #19060-2-001 parallel jaw pneumatic gripper is fixed to the end effecter of the Cobra robot. A pair of Pelco 5044-SV reverse-operation carbon-tipped tweezers is affixed to the pneumatic gripper via an ABS plastic mounting block as shown in Figure 3.2 . The tweezers are oriented between the jaws of the gripper such that the actuation of the gripper causes the tweezers to open and close.

The tweezers chosen feature removable and replaceable carbon-fiber tips. The main benefit of carbon-tipped tweezers is that the tips are replaceable in the event that they are damaged.

The use of carbon tipped tweezers also minimizes conduction cooling of the tweezer body when the tweezer is submerged in a cryogen. This has the effect of preventing the build-up of large amounts of frost on the body of the tweezer that can break off and contaminate the sample.

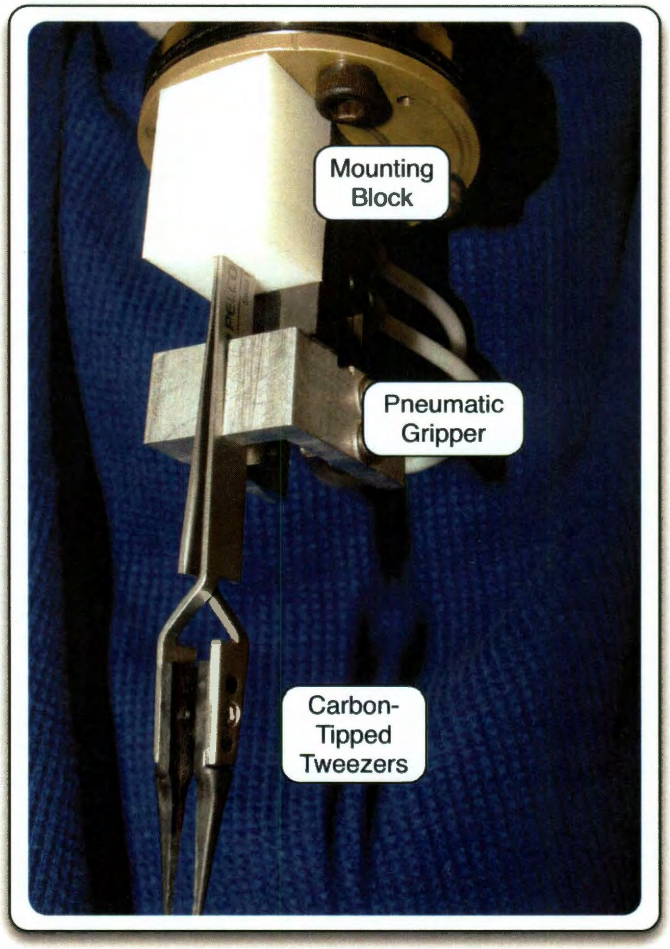


Figure 3.2 : End effector of Cobra showing pneumatic gripper with installed carbon-tipped tweezers.

3.1.1 Grid Storage

Grid storage is divided into three areas within the ASPECT system; blank-grid storage, cryogenic temporary storage, and cryogenic permanent storage.

Blank grid storage takes place in an acrylic grid-storage box that is fixed to the support table. The box can be seen in Figure 3.1E. The box measures 2.0x0.5x2.0 inches. Along the top edge of the box, a series of 12 shallow grooves have been cut. These grooves each accept a single blank imaging grid. When retrieving a grid, the tweezers are opened and centered above the grid. When the tweezers are closed, the grid is gripped on its outer circumference and lifted out of the grid-box. The grooves are arranged in two parallel rows, while the columns are offset from one another as shown in Figure 3.3. This offset prevents damage to neighboring imaging grids as the tweezers open and close during the gripping process. Pink highlights in Figure 3.3 show the area swept by the tweezer tips during the gripping process.

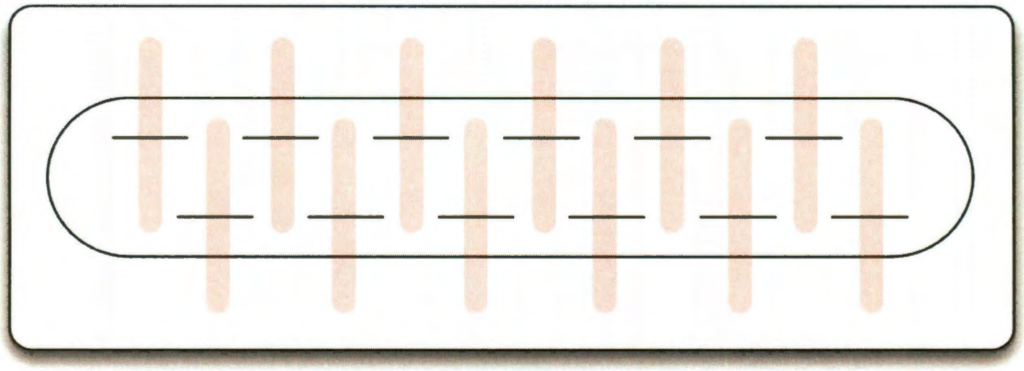


Figure 3.3 : Top view of grid holder box. Horizontal black stripes are holding cells for imaging grids. Pink vertical stripes denote area swept by tweezers during gripping motion.

Cryogenic temporary storage takes place within the outer compartment of the vitrification dewar. The vitrification dewar contains a support mechanism capable of holding three custom designed grid buttons. Figure 3.4 shows a view of the vitrification dewar with a single installed button. Figure 3.5 shows a close up of a button.

Transfer to cryogenic temporary storage occurs immediately after the sample is vitrified. The Cobra robot quickly transfers the imaging grid from the ethane bath and deposits it within one of the diamond shaped cavities in the grid storage button. The vitrification dewar is able hold three buttons at any given time, and each button is capable of holding four prepared grids, thus the system is able to accommodate twelve processed grids at any given time.

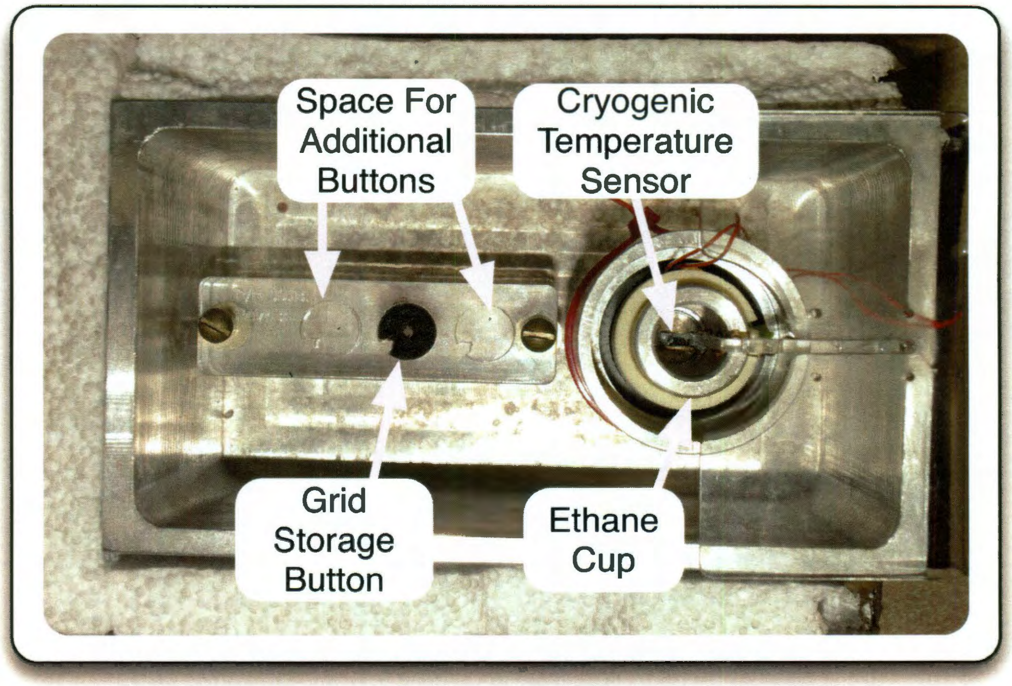


Figure 3.4 : Vitrification dewar showing one installed button.

The grid storage buttons are keyed so that they can only be installed into the vitrification dewar in a single orientation. A secondary key on the storage buttons and their support mechanism prevents the installation of commercially available grid storage buttons into the device. This feature was necessary because the position and orientation of the diamond cavities within commercial buttons varies considerably from one button to the next. Before the grid is inserted into the storage cavity, it must be aligned with the cavity so that the grid does not impact the button during the insertion process. Poor tolerances in commercial buttons resulted in many damaged grids during early testing of the ASPECT device. The design of the secondary key allows ASPECT buttons to be used in any equipment that accepts standard commercial buttons, however commercial buttons cannot be installed into the ASPECT system.

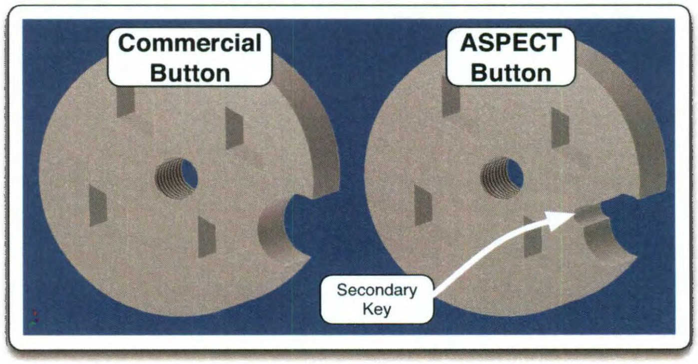


Figure 3.5 : View of grid storage buttons. Commercial button shown on left, ASPECT shown on right

Cryogenic permanent storage takes place in a separate commercially available dewar. The operator transfers the filled grid buttons from the vitrification dewar to the storage dewar using a device known as a 'button rod'. The button rod is a

Teflon rod roughly 6in long. One end of the rod is flared to match the diameter of a grid storage button. A threaded brass stud projects from the flared end of the rod and mates with corresponding hole in the center of the button. The flared end of the rod prevents the grids from escaping the button during transport. The operator mates the flared end of the rod with the submerged button and then transfers the button/rod unit to a permanent storage area, where it is submerged in liquid nitrogen until needed for imaging.

3.1.2 Environmental Chamber Access

The environmental chamber is designed to admit the passage of the Cobra's end-effector with attached tweezer. The access system consists of two electronically actuated doors and a passageway cut into the roof of the chamber that allows the Cobra to move through the environmental chamber.

The front door to the environmental chamber is roughly 12" high and 3" wide. The door pivots 90° to admit the Cobra's end-effector. The door is actuated with a standard-sized high-torque digital hobby servo. The door is removable without tools to ease access to the interior of the environmental chamber. Views of the front door can be seen in Figure 3.1 and 3.7.

The floor door allows the Cobra's end effector to access the vitrification dewar directly from within the environmental chamber. The floor door slides open and closed on a pair of teflon linear rails and is actuated by a DC electric motor via a rack and pinion gear set.

The passageway cut into the roof of the chamber allows the Cobra to translate within the chamber without impacting the chamber. The passageway is sealed with gaskets that allow the Cobra to translate through the chamber while minimizing the escape of atmosphere from the environmental chamber

3.2 Sample Deposition, Blotting, and Vitrification

This section describes the systems involved with deposition, blotting, and vitrification of the specimen.

3.2.1 Sample Deposition

Sample deposition in the ASPECT system occurs either manually or automatically depending on the preferences of the operator and on the demands of the particular imaging study. The operator is able to easily toggle between manual and automatic modes using the ASPECT graphical user interface.

Manual deposition occurs outside of the chamber. After the grid is removed from the grid-storage box, the grid is positioned so that it can be easily accessed by the operator. The operator then proceeds to apply the specimen to the surface of the grid using a micropipette or similar instrument. Once the sample has been applied, the operator indicates to ASPECT system that the specimen has been applied. The system then transfers the grid into the environmental chamber so that blotting can occur.

Automatic deposition occurs within the chamber. A syringe pump is placed outside the chamber and a syringe filled with the desired specimen is installed into the pump. The syringe needle passes through a port on the side of the environmental chamber. During processing, the grid is transferred from the grid-storage box to the environmental chamber where it is positioned 0.1mm from the tip of the syringe needle. The syringe pump then deposits the desired volume of sample onto the imaging grid. The grid is then transferred to the blotting station within the chamber.

3.2.2 Grid Blotting

A photograph of the blotting mechanism is shown in Figure 3.6. The system is located within the humidity chamber and consists of two parallel stainless-steel rails that run between the side walls of the environmental chamber. A pair of support pads are supported lengthwise between the two rails. The support pads are able to translate along the length of the rails. A pair of linear actuators are cantilevered off each wall of the environmental chamber. The actuators pass through the walls of the chamber and attach to one of the support pads. The actuators translate the pads along the length of the rails.

Each support pad mates with its corresponding blotting pad using magnets that are embedded into the faces of each pad. The blotting pads consist of a 4x1x1" piece of acrylic into which magnets have been embedded. One long side of the blotting pad is covered with a soft foam rubber material. Filter paper is stretched across the foam rubber and fastened in place using magnetic clips that attach to the smaller faces of the blotting pads. Section 4.0.7 describes the installation of the blotting pads.

During the blotting process, the grid is placed midway between the fully-retracted blotting pads such that the face of the grid is parallel to the faces of the blotting pads. The pads are extended towards the grid until they come into contact with the grid and squeeze the grid with the desired amount of force. After the desired blotting time has elapsed, the pads are retracted and the grid is transferred into the liquid ethane bath for vitrification.

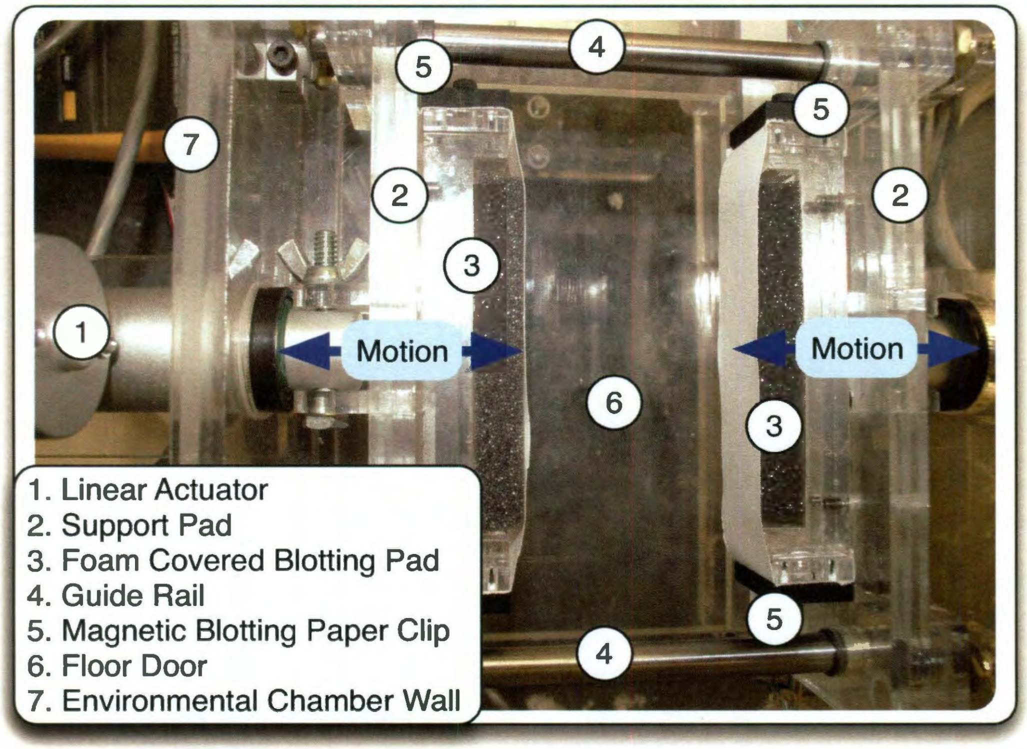


Figure 3.6 : Top view of blotting mechanism showing blotting pads in their fully retracted position

The ASPECT system is also able to control the force applied to the imaging grid during the blotting process. The force applied to the grid is a function of the stiffness of the foam backing material present within the blotting pads and the compression of the foam. The force is controlled by specifying an overlap distance to the blotting pads. An overshoot distance of 0cm will cause the pads to come into contact with each other but will exert no force on an imaging grid held between them. By increasing the amount of overshoot during the blotting process, the operator can modify the amount of force applied to the imaging grid.

A P-controller is used to control the linear actuators during the blotting process. The loop-rate for the P-controller is not bounded, and thus runs as fast as the system can support.

3.2.3 Vitrification

Vitrification takes place in a liquid ethane bath that is located within a liquid nitrogen cooled dewar. During vitrification, the grid is plunged into the ethane bath for several seconds before it is transferred to a liquid nitrogen cooled grid button for storage. The temperature of the ethane is maintained just above its freezing point by a system that is described in section 3.3.3.

3.3 Environmental Control Systems

The ASPECT system contains an environmental chamber that is both temperature and humidity controlled. The conditions within the chamber can be adjusted to meets the needs of the particular imaging study. Typically, a high humidity is desirable to retard evaporation of sample from imaging grids after blotting.

The environmental system consists of an ultrasonic humidifier, thermoelectric heating/cooling apparatus, air circulation fans, and sensors for measuring the relative humidity and temperature within the chamber. Figure 3.1 shows labeled photographs of the environmental chamber from various angles. Figure 3.7 shows a solid rendering of the environmental chamber, with the chamber highlighted in green.

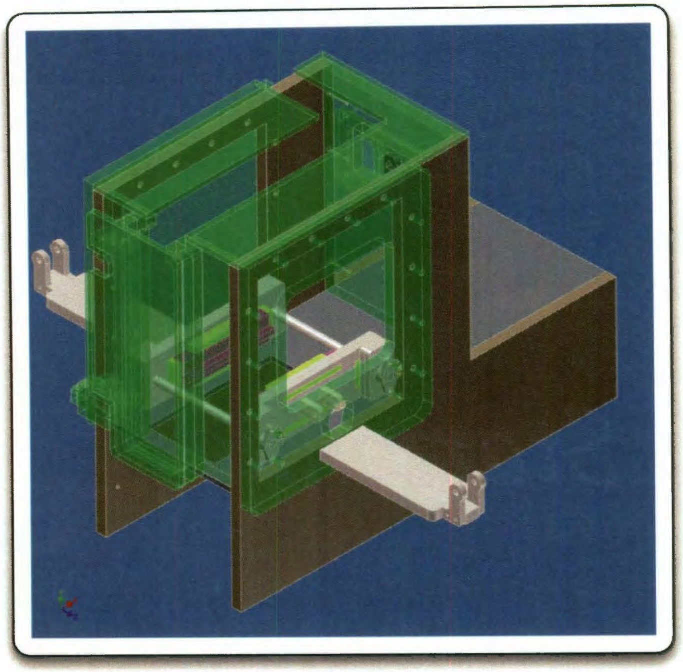


Figure 3.7 : ASPECT system. Environmental chamber highlighted green.

3.3.1 Humidification System

The humidification system consists of an Ocean Mist DK-24 ultrasonic humidifier, water reservoir, ventilation fan, and a Rense HX-748-T-L1 relative humidity sensor.

The ultrasonic humidifier is submerged in a water reservoir as shown in Figure 3.8. A ventilation fan, also shown in Figure 3.8 draws fresh air from the exterior of the chamber, through the water reservoir, and over the ultrasonic humidifier. The humidified air is then forced into the environmental chamber using the same fan.

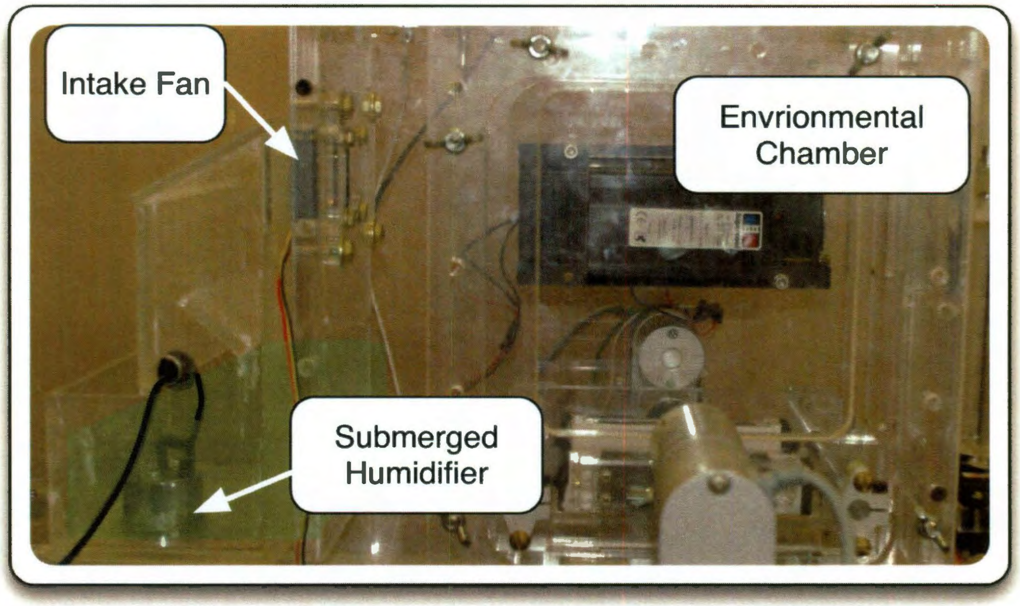


Figure 3.8 : Humidification system. Water highlighted green for clarity.

The humidifier is actuated via a standard electromechanical relay. When the control framework detects that the humidity has fallen below a user-specified level a control signal activates the relay and subsequently the humidifier.

3.3.2 Environmental Chamber Temperature Control

The temperature control system consists of a ThermoCool thermoelectric heating assembly with a Laird Technologies 120W thermoelectric chip. A Carrel NTC008WP00 temperature sensor is used to monitor the temperature within the environmental chamber.

When the temperature of the environmental chamber is within the user-specified range, no power flows into the thermoelectric device and the chamber temperature gradually acclimates towards ambient. When the controller detects that the temperature has fallen outside the user-specified range, current is supplied to the device. The direction of current through the thermoelectric chip determines whether the chamber will be heated or cooled. The change of direction is accomplished using a DPDT power-relay wired as shown in Figure 3.9.

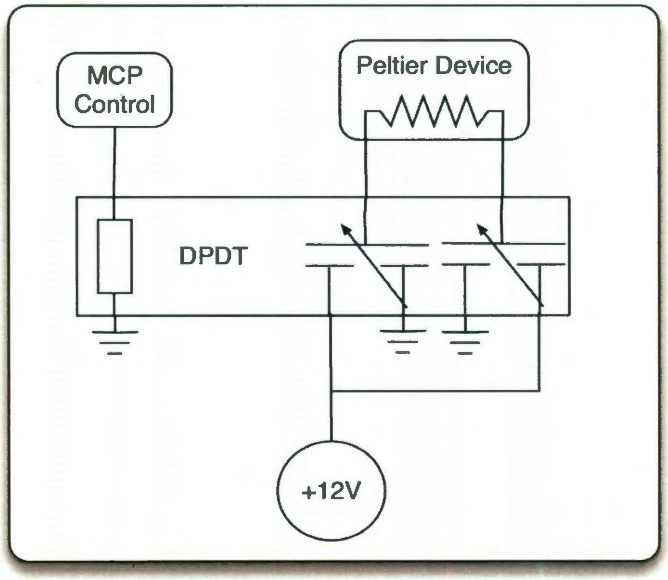


Figure 3.9 : Method for switching current direction through thermoelectric chip.

3.3.3 Ethane Cup Environmental Control

The temperature control system for the ethane cup consists of a 30W Kapton film heating element, Omega Engineering CY670D-CU cryogenic temperature sensor, and an acrylic support bracket. The installed bracket can be seen in Figure 3.4.

The film heater is wrapped around the outer circumference of the ethane cup and aligned with the bottommost edge of the cup. The heater is located at the bottom of the ethane cup to promote thermal mixing of the fluid when power is enabled and to prevent the formation of frozen ethane at the bottom of the cup. The temperature sensor is located at mid-level within the cup and is supported by an acrylic bracket that is rigidly attached to the vitrification dewar. The support bracket is constructed from 1/8" acrylic. A profile view of the support bracket is shown if Figure 3.10. The cross sectional area of the vertical support member was made as small as possible to minimize the conduction of heat into the ethane bath.

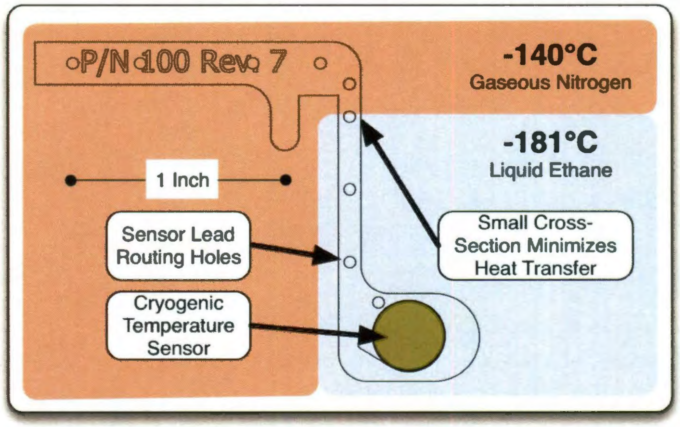


Figure 3.10 : Cross sectional view of cryogenic sensor mounting hardware showing temperature environments.

Temperature control of the ethane bath is accomplished using a PD controller as shown in Figure 3.11. The desired ethane temperature and measured ethane temperature are inputs to the controller. Gains for the PD controller were determined through manual tuning. The output of the PD controller is converted into a PWM waveform. The PWM waveform corresponds to the desired power output of heating element. A correction factor is added to the PWM signal to maintain a baseline output of the heater when the desired equilibrium temperature has been reached.

This correction factor was determined through experimentation, and corresponds to a duty cycle of 15.2% and a thermal output of 5W. The PWM waveform is routed to the film heater via a USB data acquisition card and a high-speed reed relay.

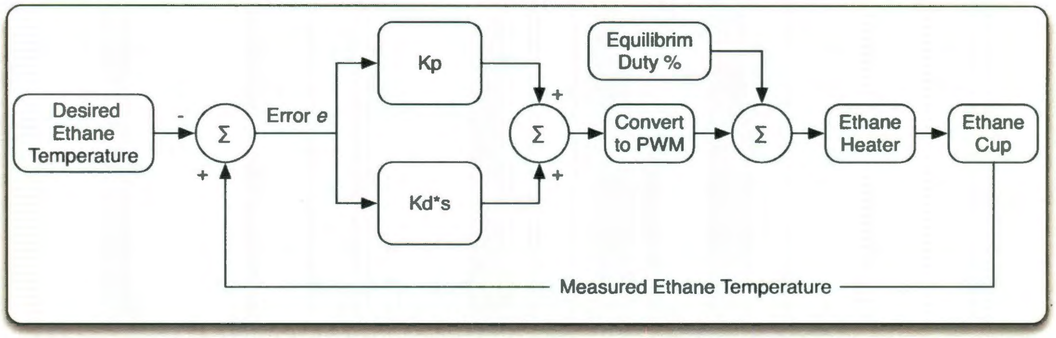


Figure 3.11 : PD Control system for ethane heater.

3.4 Process Control

Figure 3.12 shows a flowchart that illustrates the relationships between the various hardware and software layers present within the ASPECT system.

At its highest level, the control of the ASPECT system is accomplished using a state machine based master control program (MCP) running under National Instruments LabVIEW. The MCP determines what procedure the system must undertake next based on the previous state, input from operators, and on the operational status of hardware and software devices connected to the system. Low level functions within the MCP framework are implemented using hard-coded step sequencing, monitoring algorithms running in parallel with the main control structure, and hardware based emergency stop functions.

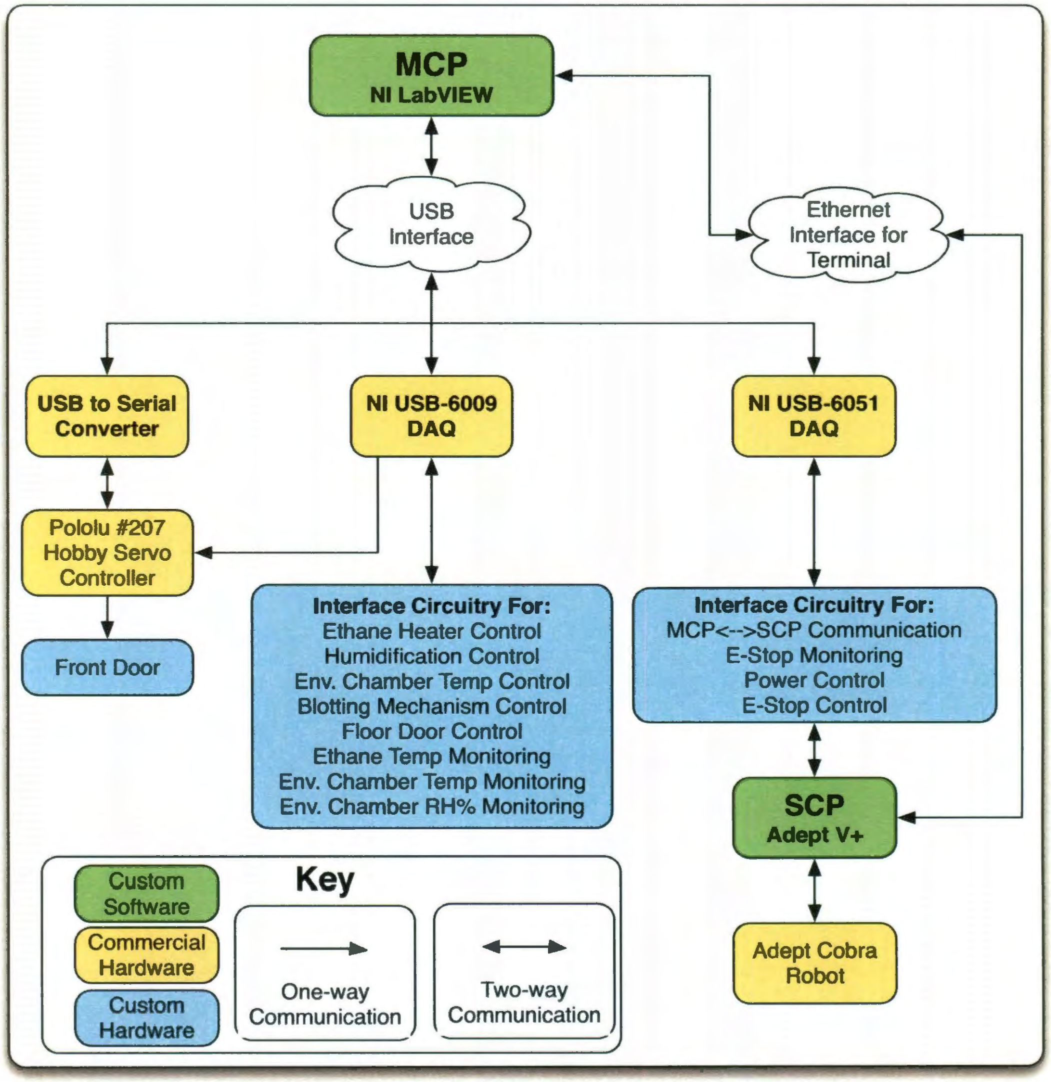


Figure 3.12 : An overview of the hardware/software systems of the ASPECT system.

A slave control program (SCP) runs concurrently to the MCP. The SCP is run on the Adept MV Controller under the V+ operating system[33]. The SCP controls the Cobra robot and receives commands from the MCP over digital I/O lines provided by a National Instruments USB data acquisition device (DAQ).

The MCP communicates with other elements of the system using a pair of National Instruments DAQ boards as well as a Prolific Technologies USB to serial converter. The DAQ boards connect the MCP directly to sensors and actuators within the system and serve as an interface between the MCP and the SCP.

3.4.1 LabVIEW Based Control

The core of our device is Microsoft Windows 7x64 machine running National Instruments LabVIEW. A custom MCP program running under LabVIEW is used primarily as a scheduling and coordinating agent between the various subsystems of ASPECT. All control signals are routed through a pair of National instruments USB data acquisition devices or through a Prolific Technologies USB to serial converter cable. More information on the hardware interfaces utilized within the ASPECT system can be found in 3.4.4

3.4.1.1 Graphical User Interface

The user interface to the device is displayed on the Windows host machine and is implemented using the tools available within the LabVIEW environment. During runtime, the user has direct control over a variety of the parameters of the system. These controls are provided through a tabbed interface as shown in Figure 3.13. Settings are categorized according to their function and placed under the appropriate heading.

1. **Global Controls** Provides controls that must be specified for the system to function. These include the number of grids to be processed, the save path for data storage, and controls that initialize the calibration procedure and begin processing grids.

2. **Timing** Allows for the control of all timing parameters within the system. These include blotting times, delay times between steps, and freezing times.
3. **Environmental** This tab allows the operator to modify parameters that affect the environmental conditions of the environmental chamber and ethane cup. The operator can set desired temperatures and humidities, disable/enable the environmental actuators, and control these actuators manually.
4. **Blotting** Here the user can specify a preference for dual or single sided blotting as well as the blotting force applied to the grid.
5. **Manual Controls** When operating the system in semi-automatic mode, controls here allow the operator to provide various commands to the system.
6. **Debug Controls** These controls are used to control a system in an almost purely manual mode. They are not used during normal operations.

The user interface also provides the operator real-time information on the temperature and humidity levels within the environmental chamber and the temperature within the ethane bath. A secondary display (not shown) informs the user on the current operational status of the robot.

During grid preparation, the MCP records information on the status of the system during the preparation of each grid. This data includes the data/time, grid number, temperature and humidity of the environmental chamber, temperature of the ethane bath, blotting force, and blotting time. In the case of an internal system fault, error information is also written to the file.

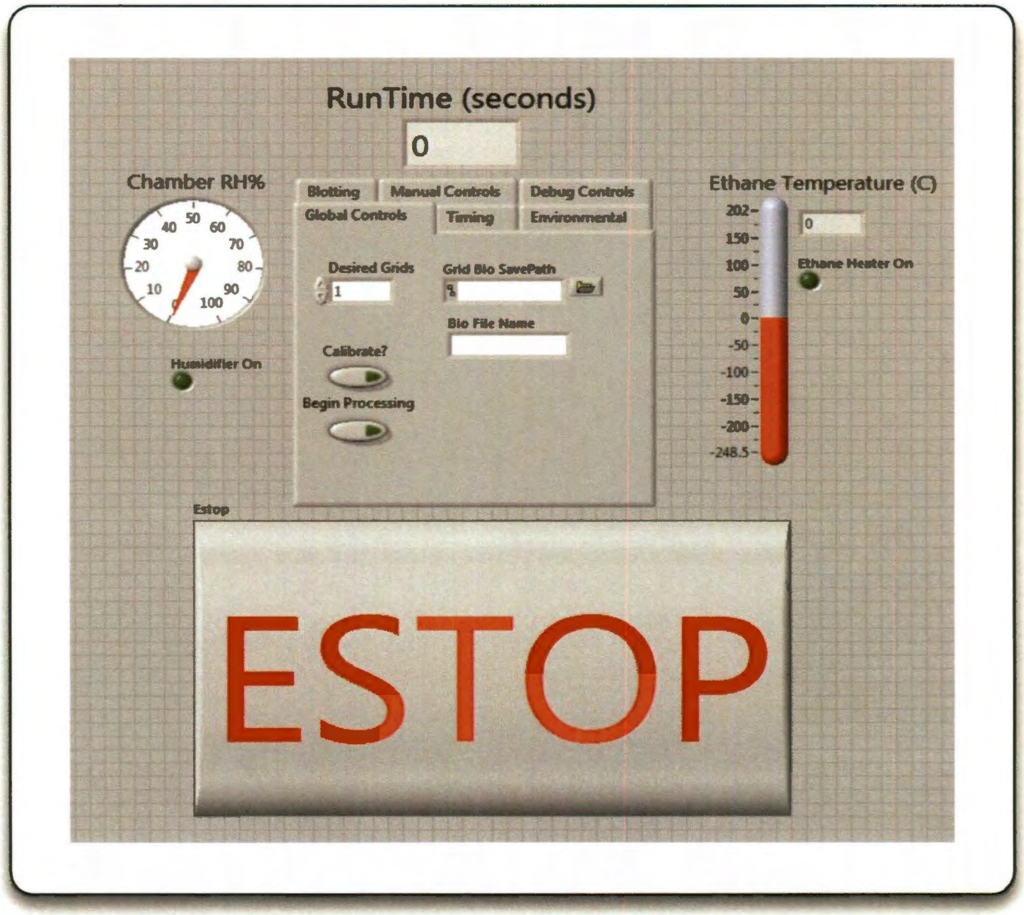


Figure 3.13 : Graphical user interface for ASPECT system

A large emergency stop button is also constantly visible to the user. When activated, this button kills power to the entire system within one-quarter of a second. Further information on the emergency stop system can be found in section 3.5.

3.4.2 Adept V+ Software Control

An Adept MV Controller running the V+ operating environment is used for control of the Cobra robot. The slave control program, SCP, runs on the Adept systems, and is subservient to the MCP running under LabVIEW. Commands are issued by the MCP to the SCP via a hardware interface that is described further in section 3.4.4 .

The Adept V+ operating system and associated hardware allow for the operator to interact with the system in several different ways. A user may launch programs and monitor the status of a running program using the manual control pendant. The user can also interact with the controller through a remote terminal connection. In the ASPECT system, this terminal connection is provided over an ethernet connection to the same Windows computer running the MCP program. This connection allows the user to create, launch, and monitor programs on the Adept system from a familiar Windows interface. The ethernet interface also allows flexibility when physically locating the host PC with respect the the Adept system.

During operation of the ASPECT system, the user has minimal interaction with the Adept terminal. Before a grid can be processed, the user must power up the Cobra, calibrate the robot, and launch the slave control program (SCP) on the Adept system. These text commands are issued through the Adept terminal. After these commands are issued, the MCP has control over the Adept system and the user interacts only with the graphical user interface provided by the MCP.

The SCP continually monitors the digital input lines provided by the MV Controller. When the MCP issues a command over these lines, the SCP launches a new program that corresponds to the specific command issued by the MCP. These subprograms contain to specific movements that the Cobra should perform during the operation of the ASPECT system. Before beginning any robot movements, these sub-programs check to ensure that any movements performed by the Cobra will not impact structures within the workspace. This feature is implemented by assigning a set of safe-zones within the Cobra's environment to each sub-program. If the Cobra's position falls within a safe-zone, then the the sub-program continues to execute and the Cobra is free to move to its commanded position. If the sub-program is executed and the Cobra's position falls outside one of these safe-zones, then an error code is reported, the SCP closes, and an emergency-stop is issued.

During run-time, the SCP provides feedback to the user through the terminal window. This output includes the current status of the robot, what motion is currently being performed, and the status of various software safety interlocks.

3.4.3 MCP $\leftrightarrow$ SCP Communication Protocol

The MCP and SCP programs communicate with one another over a parallel interface as depicted in Figure 3.14.

Lines 0-1 are outbound lines from the MCP that instruct the SCP to begin execution of a subprogram, verify that the Cobra falls into a safe-zone, or report the position of the Cobra to the MCP (not currently implemented).

Lines 2-7 are outbound lines from the MCP that specify the desired subprogram the SCP. These lines are in binary-coded-decimal format, and code for a maximum of 39 possible subprograms. Lines 2-5 are also used for control of ASPECT emergency stop system (see section 3.5).

Lines 8-10 are inbound lines from the SCP that communicate status of the Adept system to the MCP. These lines are used to indicate to the MCP when a subprogram has begun execution, and when that subprogram has finished execution. The MCP uses this information to coordinate the motions of the Cobra with the operations of other devices within the ASPECT system.

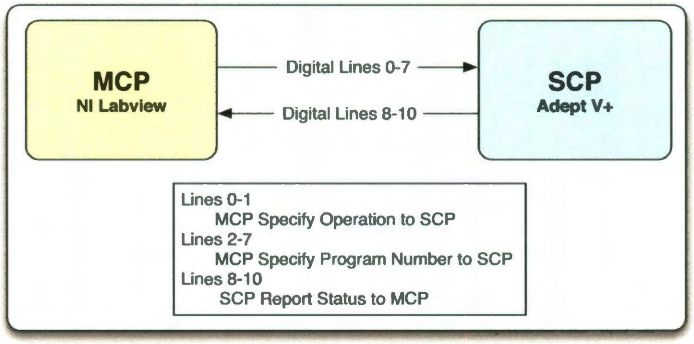


Figure 3.14 : Bidirectional communication protocol between MCP and SCP programs.

3.4.4 Hardware Interace Elements

This section describes the hardware devices that provide interface the MCP and SCP to each other and to other elements of the system.

3.4.4.1 National Instruments Interface Devices

A pair of National Instruments USB data acquisition devices are the primary interface between LabVIEW and the rest of the system. A USB-6009 provides both digital and analog I/O. A USB-6051 provides additional digital I/O lines. A Prolific Technology USB to serial converter is also utilized. Figure 3.12 gives a high-level overview of the tasks each device performs.

Analog input lines on the USB-6009 are used by the MCP to monitor the temperature and humidity sensors within the system and to to monitor the position-sensing potentiometers installed within the blotting mechanism. Digital output lines on the USB-6009 are used with supporting circuitry to control the blotting mechanisms, floor-door, ethane heater, ultrasonic humidifier, and Peltier cooling device.

Digital I/O lines on the USB-6051 DAQ are used to communicate with the Adept hardware, provide e-stop functionality, and to power on the system.

3.4.4.2 Adept Technologies Hardware Devices

An Adept Technologies MV controller with installed AWCII interface card provide the operating environment and digital interface options to the Cobra robot. The AWCII board interfaces with an Adept CIP-2 interface panel and manual control pendant.

The MV Controller is a self contained cabinet that contains all the hardware and software necessary to interface with the Cobra robot. The MV Controller runs a version of the V+ operating system that has been customized by Adept. User designed programs are run within the V+ system. In the ASPECT device, the MV controller runs the SCP program as described in section 3.4.2.

The AWCII is an expansion card that installs into the MV Controller chassis. The AWCII expands the input/output capability of the MV Controller by providing additional serial, parallel, and ethernet connectivity options. ASPECT utilizes both the parallel and ethernet options for communication between Adept supplied hardware and other elements in the ASPECT system. The ethernet interface is used in conjunction with Adept DeviceNET to provide a terminal interface to the operator on a Microsoft Windows based host machine. The parallel port interface provides several dozen digital input/output lines that are the primary communication channel between the MCP and SCP programs. More information on the hardware/software interface between LabVIEW and V+ can be found in sections 3.4.1 and 3.4.2.

The CIP-2 interface panel plugs into the AWCII expansion board. The CIP-2 provides a hardware emergency stop button,acts as a breakout box for the digital I/O lines, and controls the power state of the Cobra.

The Adept manual control pendant plugs into the CIP-2 interface panel. The pendant allows for full control of the Cobra in manual mode, provides an additional emergency stop button, and can act as an interface device for programs running on the MV controller.

3.4.4.3 Miscellaneous Interface Devices

A Prolific Technologies USB to DB9 serial converter is used to interface the Windows PC with a Pololu #207 hobby servo controller. The servo controller is used to control the servo that actuates the front door.

3.4.5 Circuit Elements

Custom circuitry interfaces the sensors and actuators of ASPECT system to the National Instruments DAQ boards. This section describes the functionality of these circuit interfaces.

3.4.5.1 Power Supplies

A standard off-the-shelf ATX computer power supply is used to provide power to the system. A switchable 12V rail provides primary power and is rated at 15 amps. A constantly powered 5V rail provides power to circuit elements necessary to enable primary power and to the circuit elements that provide emergency stop functionality.

5 and 7.5 Volt Supplies: 5V and 7.5V rails are implemented with a pair of LM317T voltage-regulators. The source voltage for these regulators is the 12V ATX rail. The 5V and 7.5V rails are subsequently used to power all circuitry present in the ASPECT system.

Constant Current Sources: Both the cryogenic temperature sensor and environmental temperature sensor are powered by constant current sources of 10μA and 100μA respectively. These current sources are implemented using an LM234Z constant current source.

Power Enable: The contacts of a pair of 5V SPST-NO electromechanical relays are placed in series with the "Power On" line of the ATX power supply as shows in Figure 3.15. Both relays must be in the closed position for the 12V line of the ATX power supply to become active. One relay is interfaced to the USB-6051 DAQ through a PNP darlington power transistor. The actuator signal for relay 3 is inverted before being fed to the relay. In order to close both relays the digital line connected to the NPN transistor must be driven high while the inverted digital line must be driven low. This design minimizes the chances that the system is unintentionally powered up. The power for these relays is supplied by a constantly energized 5V line from the ATX power supply. Please see section 3.5 for more information on electrical power control in the ASPECT system.

Digital IO Buffers All outbound communication lines from the NI DAQ boards to the Adept CIP-2 interface panel are routed through non-inverting hex buffers. Theses buffers allow the low-current output of the NI DAQ devices to drive the more current hungry optocoupled input circuitry present within the CIP-2 interface panel. Outbound signals from the CIP-2 are also routed through non-inverting buffers.

H-Bridge Control H-bridge logic is used to control the DC motors around which the blotting mechanisms and floor-door are based. A ST Microelectronics

L298N dual bridge driver provides power to both blotting mechanisms. An NTE 1716 DC motor driver powers the floor door. Two digital control lines for each motor specify its power state: off, brake, rotate clockwise, rotate counter clockwise. These control lines are provided by the USB-6009 DAQ.

3.5 Safety Systems

The Adept Cobra is a powerful robot and is capable of moving at very high velocities. In the ASPECT system, the Cobra is run at 80% of its maximum speed, and can cause serious injury to operators if appropriate safety precautions are not taken. Because the Cobra operates in close proximity to its human operators, a robust emergency stop system is required to minimize injury to personnel and damage to system components in the event of a system failure. Furthermore, to prevent operators from unintentionally powering up the ASPECT system, a hybrid hardware/software safety interlock system has been implemented. Figure 3.15 gives an overview of the ASPECT emergency stop, power, and safety interlock systems.

3.5.1 Emergency Stop

In the event of a system malfunction, an emergency stop system disconnects power to the entire ASPECT system. The emergency stop system consists of two hardware buttons located on the Adept CIP-2 interface panel as well as on the manual control pendant. A software emergency stop button has been implemented within the MCP program. All hardware emergency stop buttons conform with OSHA Category 1 requirements[34]. The software emergency stop button does not conform to any software emergency stop standard because none exists.

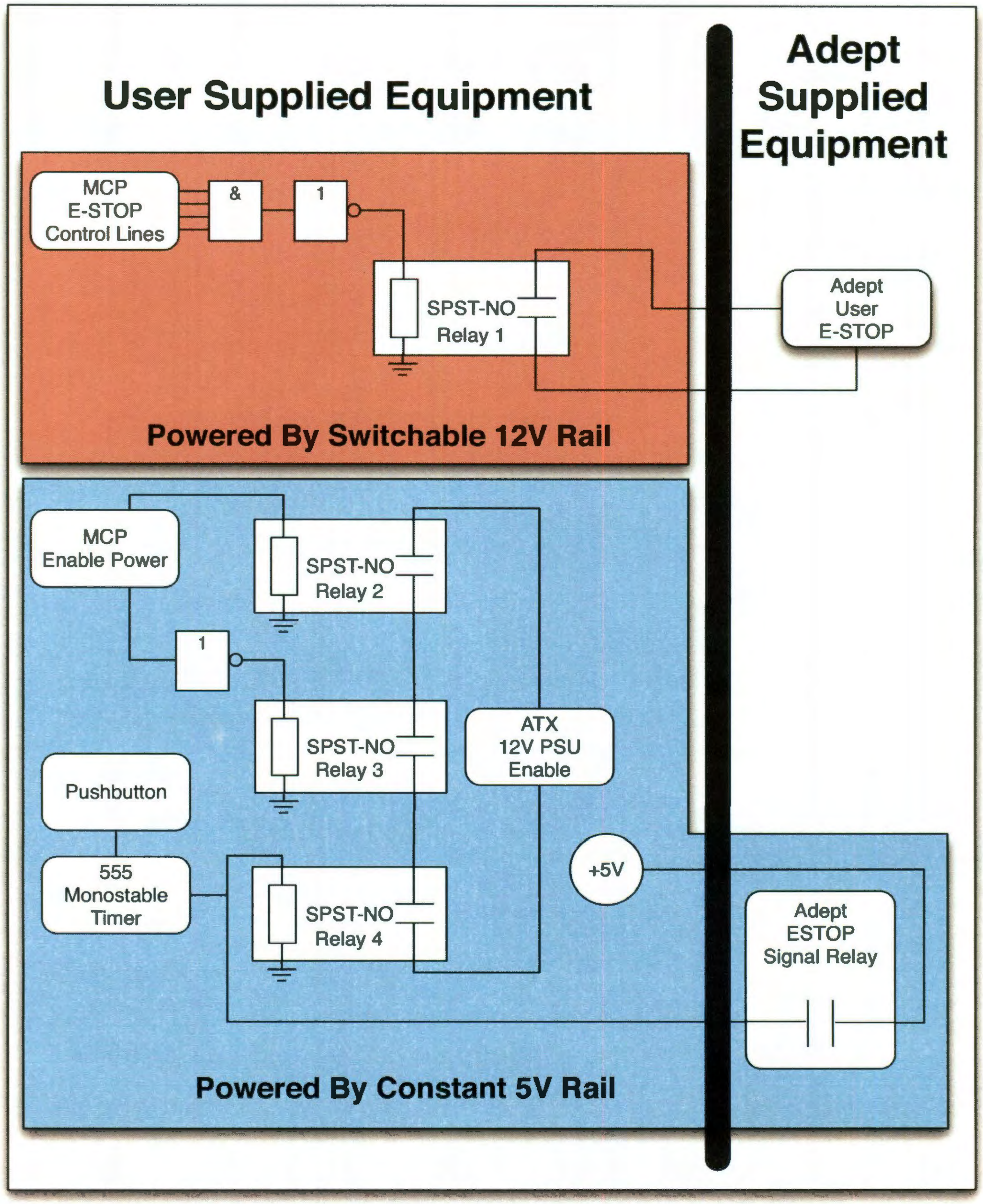


Figure 3.15 : Emergency stop and safety interlock systems. 12V rail is not powered until relays 2-4 are closed.

The Adept MV Controller provides two emergency stop lines for use by user-supplied equipment. During normal operation these lines are closed. To issue an e-stop signal, at least one of these lines must be broken. An open e-stop line immediately disconnects the power from the entire Adept system. In the ASPECT device, one of these lines is used to issue e-stop commands via the emergency stop button located in the MCP program.

When the e-stop button is depressed, the MCP signals 1-1-1-1 on the first four digital lines that are normally used to specify sub-program selections to the SCP. These digits were selected because they code to a value greater than 9 in the binary-coded-decimal format utilized by the SCP program. Under normal operating conditions, a 1-1-1-1 input would cause errors if introduced to the SCP. However this problem is of little importance because in an emergency situation all programs running on the Adept system are automatically killed.

The 4-bit signal is routed through a four-way AND logic gate as shown in Figure 3.15. The signal is then inverted and used to trigger an electromechanical SPST-NO relay via a NPN darlington transistor. This logic has the effect of closing the relay when the 1-1-1-1 signal is not present. When the 1-1-1-1 signal is present, the relay opens and trips the emergency stop condition to the Adept system.

The Adept MV controller provides a single unpowered line that is designed to signal external user-supplied devices when an emergency stop condition has occurred. Under normal operating conditions, the Adept e-stop signal relay maintains the continuity of this line. When an e-stop occurs, or when the system exits high-power mode, the relay opens and breaks the continuity of any circuit attached to this line. In the ASPECT system, this line is used to provide e-stop functionality to all non-Adept supplied equipment. The line is powered with the persistent 5V line from the

ATX power supply. The coils of SPST-NO relay are placed in series with the 5V power supply and the Adept e-stop signal relay. The contacts of relay 4 are placed in series with the ‘Power On’ line from the ATX power supply. When the Adept system receives an e-stop signal, the Adept e-stop signal relay opens and disconnects power to relay 4, thus opening its contacts and breaking the continuity of the ‘Power On’ line. When the continuity of this line breaks, the ATX power supply enters standby mode and powers down the 12V rails upon which the remainder of the system relies on for power.

3.5.2 Timed Interlock

The integration of the emergency stop systems and power systems presents a problem when the operator wants to power up the system. When the system is powered down, all of the relays powered off of the 12V rail are in the open position. This means that the e-stop line triggered by the MCP (relay 1 in Figure 3.15) is indicating to the Adept system that an e-stop is present. Because the Adept system is receiving an e-stop command, the e-stop signal relay is open, which prevents relay 4 in Figure 3.15 from enabling power to the 12V rails. In short, the system is stable if relay 1 and relay 4 are either both on or both off. A situation in which only one relay is on quickly results in that same relay also transitioning to the off state. To successfully power on ASPECT, a condition must be created in which the states of relays 1 and 4 are not coupled to one another. Furthermore, this condition must only be temporary in nature so as to not effect the emergency operation of these relays.

To allow for powering of the system, a 555 timer circuit in monostable mode is used to trigger relay 4. When a temporary switch is depressed, the timer circuit closes relay 4 for a period of 20 seconds. Once this relay is closed, the operator can power on the remainder of the system. If the system has not been fully powered within the twenty second window, then relay 4 will open as the timer output falls to ground and the ASPECT system will power off. When the system is fully powered, the e-stop monitoring relay is closed and relay 4 is powered even when the output of the timer circuit goes low.

3.5.3 DAQ Hi/Lo Power Requirement

To further reduce the occurrence of situations in which the ASPECT system can be inadvertently powered up, the outputs of two lines from the USB-6051 DAQ must be driven high and low before the system can be powered on. The high signal is routed through a darlington NPN transistor to power relay 2 as shown in Figure 3.15. The low signal is inverted before powering relay 3 in the same manner. The contacts of relays 2 and 3 are placed in series with ATX 'Power On' line. Only when the contacts of relays 2-4 are closed does the 12V rail of the power supply become energized.

3.5.4 Power-up Procedure

To successfully power up the ASPECT system, the user must understand how the emergency stop system interacts with the power supply. The system cannot be completely powered on until all emergency stops have been cleared, the timed interlock has been enabled, and the Adept Cobra has entered high-power mode. The following procedure describes how to successfully power up the system

1. Execute MCP Program in LabVIEW to clear e-stop conditions controlled by MCP
2. Trigger timer circuit to temporarily power up system

3. Enable high power mode within Adept system to close the emergency stop signal relay

Once these steps have been completed, the ASPECT system is fully powered up and ready to begin processing grids. The system will remain powered-up until an emergency stop command is issued or until the system has complete processing a set of grids.

Chapter 4

System Operations

This chapter describes the setup and operation of the ASPECT device. The chapter is written in the chronologically beginning with the steps that should be undertaken first to successfully utilize the ASPECT system.

4.0.5 Sample Selection

The decision to manually or automatically deposit samples on the grid is determined partly by the volume of sample that is available. Sample volumes of greater than 0.25 mL can utilize the automatic deposition mode. For samples with smaller volumes, a manual deposition mode is available. In this mode, the user manually deposits sample to the grid using a micropipette.

By default, automatic sample deposition is selected. The operator needs to specify the desired sample volume under the 'Blotting' tab in the MCP interface. A button under the 'Manual' tab of the MCP allows the operator to select manual deposition.

4.0.6 Grid Cleaning

Before use, all imaging grids must be cleaned to remove any impurities and to maximize the hydrophobic qualities of grid's carbon coating. Grids are washed in acetone to remove any surface impurities, and then plasma cleaned. During plasma cleaning, the grids are enveloped in a plasma for several seconds. At the end of this process, the grids are removed from the device and are ready for use.

4.0.7 Blotting Paper Installation

Whatman #1 blotting paper is cut into rectangular strips 1" wide and 6" long. These strips are installed onto the removable blotting pads using magnetic clips as shown in Figure 4.1. The completed blotting pad assembly then clips magnetically onto the support as shown in Figure 4.1. The process is then repeated for the second blotting pad.

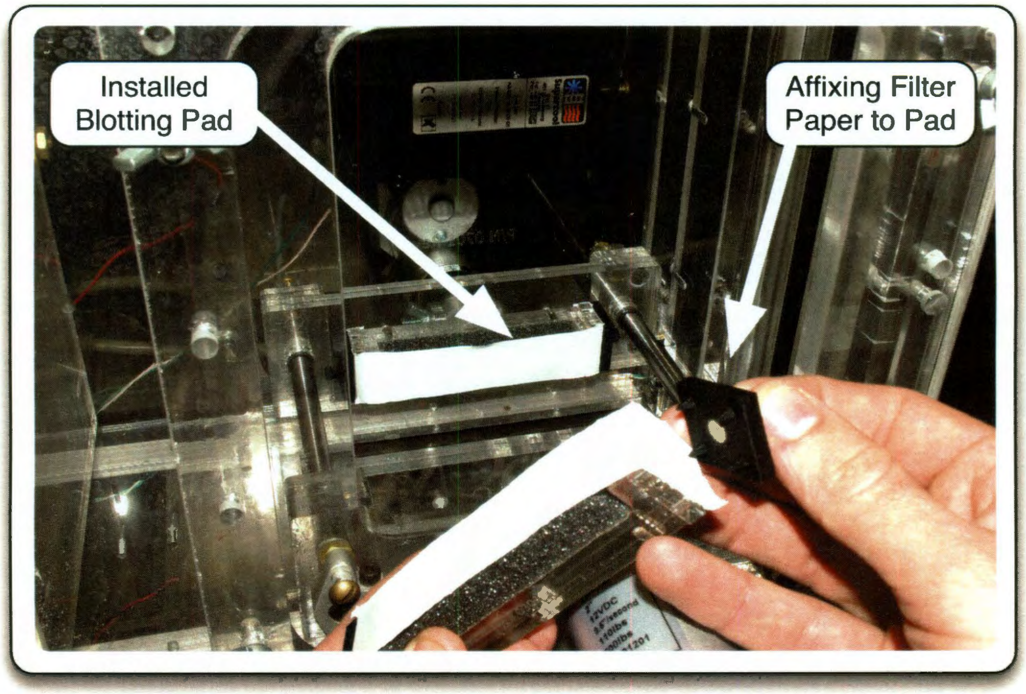


Figure 4.1 : Installing blotting paper. Foreground shows clipping blotting paper onto blotting pad. Background shows blotting pad installed into ASPECT.

4.0.8 Humidifier Setup

The ultrasonic humidifier is placed within the empty humidifier reservoir. The reservoir is the then filled with water until the level of the water is at least 1cm above the highest point of the humidifier. The reservoir is then placed in its designated position at the rear of the device such that the reservoir's vent is aligned with the inlet port of the environmental chamber. Figure 3.8 show the humidifier installed on the environmental chamber, the water has been highlighted green for clarity.

4.0.9 Blank Grid Installation

Blank imaging grids are installed into slots cut into the grid storage box as shown in Figure 4.2. The box is capable of holding a maximum of twelve grids.

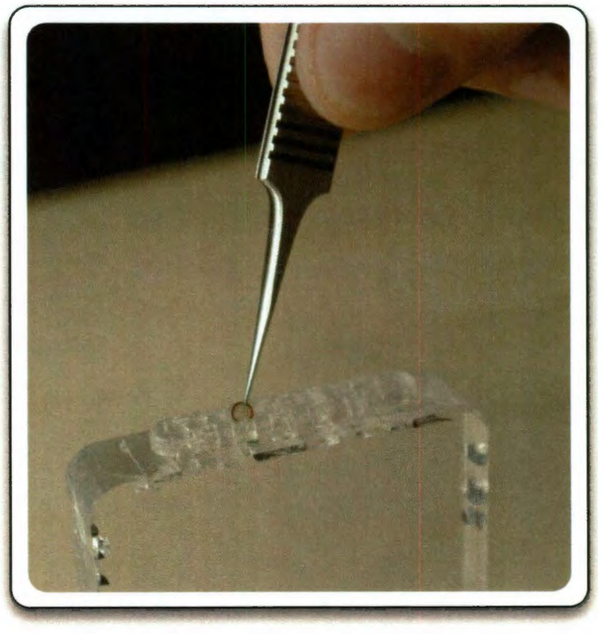


Figure 4.2 : Installing a grid into the grid-storage box.

4.0.10 Dewar Cooling

The outer section of the vitrification dewar is filled with liquid nitrogen. The nitrogen is periodically replaced as it boils off. The dewar is fully cooled when the vigorous boiling action of the liquid nitrogen has subsided to a simmer The cooling process typically takes 5-10 minutes.

4.0.11 Power Up

The ASPECT system should be powered up as described in section 3.5.4.

The Cobra robot should be calibrated and moved to its home position by executing the 'calib' program from the Adept terminal interface or manual control pendant.

4.0.12 Ethane Condensing

The desired temperature of the ethane should be specified within the MCP program, typically -181°C. When the ethane cup temperature reaches -160°C, the ethane cup can be filled.

To fill the cup the operator inserts a hollow glass application wand into the ethane cup as shown in Figure 4.3. The wand is connected to a pressured regulated ethane source at 5-10 PSI. The operator opens the ethane check valve and allows the gas to flow into the cup. When the gas touches the cooled walls of the cup, it immediately condenses into a liquid. The operator continues to fill the cup into the liquid is just below the lip of the cup. When the cup is full, the operator shuts off the ethane flow and removes the wand.

Figure 4.3 : Condensing ethane into ethane cup. Liquid nitrogen has been omitted for clarity.

4.0.13 Storage Button Installation

A storage button is threaded onto the mating end of the button handling rod. The button is submerged in liquid nitrogen for several minutes or until it reaches equilibrium temperature. The button is then inserted into the button stand that is located within the vitrification dewar as shown in Figure 4.4. The button handling rod is then unscrewed from the button and removed. The user should install as many buttons as necessary to hold the desired number of processed grids.

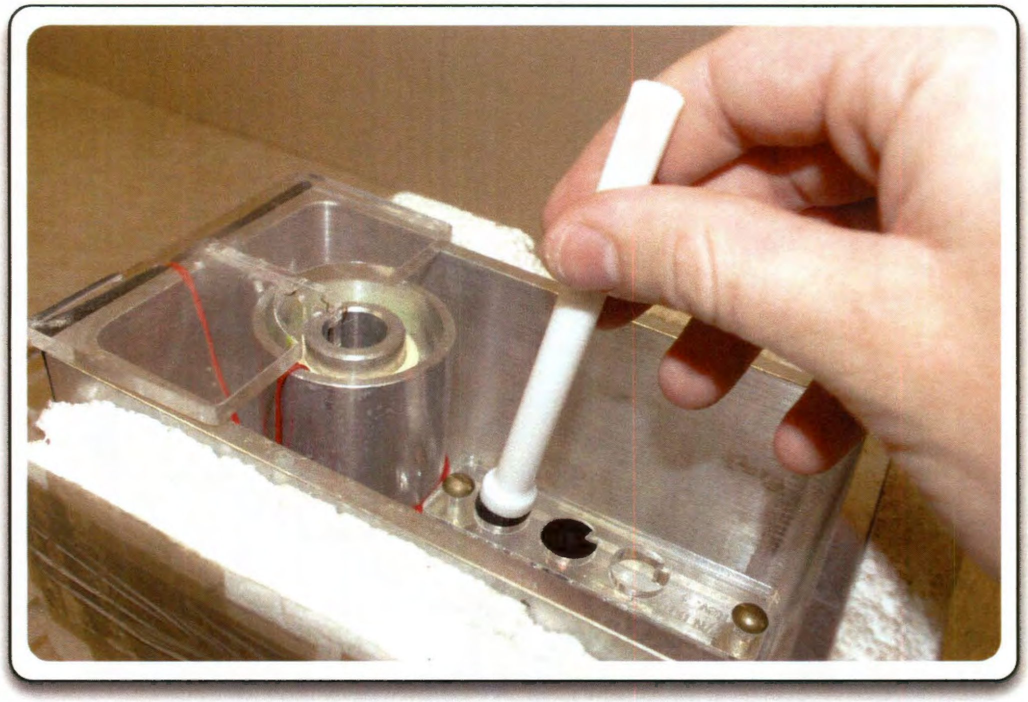


Figure 4.4 : Installing grid storage buttons into vitrification dewar with button handling rod. Liquid nitrogen has been omitted for clarity.

4.0.14 Dewar Installation

The liquid nitrogen is topped off and the vitrification dewar is slid under the environmental chamber until it contacts the bump stops under the chamber.

4.0.15 Specify Grid Preparation Settings

Within the MCP program, the operator specifies the conditions under which the grids will be processed. These conditions include:

1. Ethane Temperature

2. Environmental Chamber Temperature
3. Environmental Chamber Relative Humidity
4. Blotting Time
5. Blotting Force
6. Sample Volume
7. Total Number of Grids to Process
8. Specify One or Two Sided Blotting
9. Specify Manual or Automatic Sample Deposition
10. Specify Path for Results File
11. Specify Various Delay Times

4.0.16 Sample Application

When the desired parameters have been set, the operator executes the SCP program through the Adept terminal interface. The operator begins processing grids by clicking the 'begin' button within the MCP user interface. If the operator has selected automatic sample deposition, then the system immediately begins to process grids. If manual sample deposition is desired, then the Cobra removes a grid from the grid storage box and waits for the operator to apply a sample using a micropipette. When the operator indicates to the MCP that the sample has been deposited, the system proceeds to blot, vitrify, and store the grid.

4.0.17 Blotting, Vitrification, and Storage

After a sample has been applied to the grid, the Cobra transfers the grid into the environmental chamber and places the grid in position for blotting. The blotting pads are extended until they come into contact with the grid at the desired force level. After the user-specified blotting time has elapsed, the blotting pads retract and the sliding door separating the environmental chamber and vitrification dewar retracts. The grid is then plunged into the liquid ethane for five seconds, and then quickly transferred from the ethane bath into the liquid nitrogen. The grid is then placed into an empty slot within a grid button. The Cobra then exits the environmental chamber and begins to process any remaining grids.

4.0.18 System Power Off

When all grids have been processed, the Cobra is transferred to its home position. The MCP then issues an emergency stop command to power-off the entire system.

Chapter 5

System Performance

The performance of the ASPECT system is dependent on a variety of factors, not the least of which is the skill of the operator in determining the optimum sample preparation parameters. Early in the design process, a concerted effort was made to provide the operator with a large latitude in selecting these parameters, thus maximizing the range of sample preparation conditions available.

This section presents the performance of the ASPECT system. The section does not present an in-depth investigation of the ideal sample preparation parameters, as these are highly dependent on the sample being tested.

5.1 Environmental Control

The creation and maintenance of a stable environment is vital to successful preparation of of electron microscopy grids. Our system is able to maintain stable environmental conditions in both the environmental chamber and within the ethane bath.

5.1.1 Environmental Chamber Humidity Control

The humidification system of the ASPECT device is capable of maintaining the humidity of the environmental chamber at levels high enough to retard evaporation of the thin film after blotting. Figure 5.1 shows the relative humidity within the chamber as a function of time during the processing of a single grid. The graph shows that the humidification system is able to keep the chamber humidity at a consistently high level throughout the preparation process. Shading on the figure indicates when the doors of the chamber have been opened to admit the passage of the Cobra's end-effector. The graph shows that the system is able to maintain a high relative humidity within the chamber regardless of the status of the doors. No drop in humidity was detected.

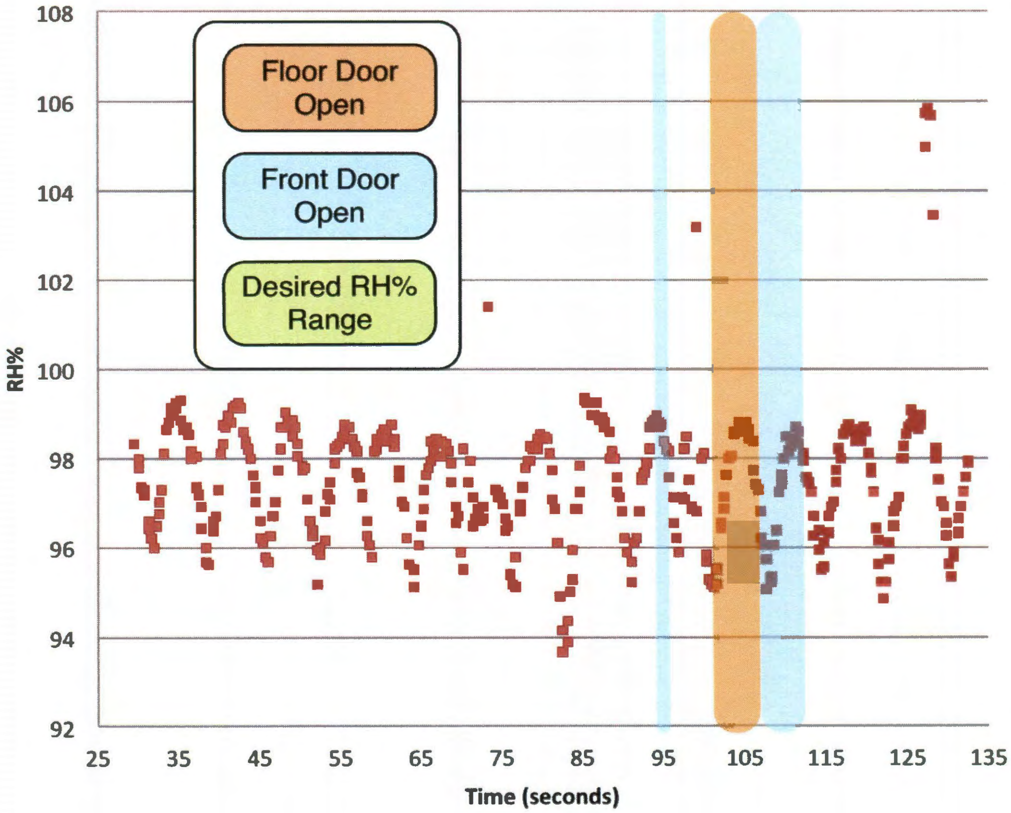


Figure 5.1 : Relative humidity within ASPECT system while processing single grid.

5.1.2 Ethane Bath Temperature Control

Accurate temperature control of the ethane bath is vital to the successful vitrification of a specimen. The goal of the system is to maintain the ethane bath at the lowest possible temperature so as to maximize the cooling rate of the submerged specimen. Figure 5.2 shows the performance of our system at maintaining the temperature of the ethane bath. The programmed ethane temperature is -181.4°C, just above the freezing point of ethane at standard pressure. The graph shows that the system is able to maintain the ethane temperature to within 0.5°C. Tweezers are introduced into the cup in the section that is highlighted green. The data indicates that the introduction of a tweezer into the ethane bath causes no discernible increase in the ethane temperature.

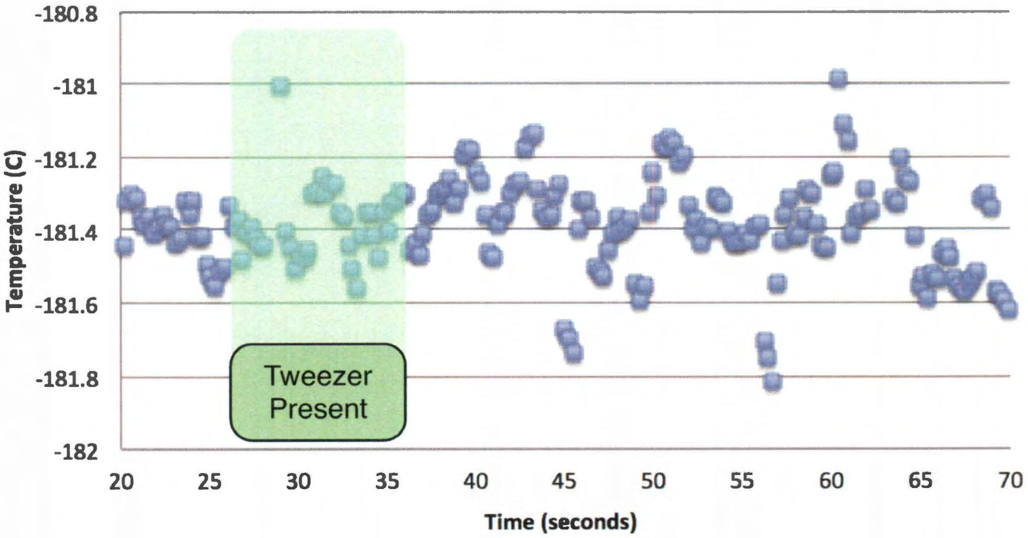


Figure 5.2 : Temperature of the liquid ethane bath during processing of single grid

5.2 Grid Handling

5.2.1 Damage

When properly calibrated, the system does not physically damage the grids. Damage can occur, however, during some emergency-stop situations, when specific hardware fails to function properly, or when the operator fails to set-up the ASPECT system properly. Taken together, grid damage from all sources affects less than 10% of grids, with the vast majority of damage being precipitated by human error. This rate is comparable to rates attained when preparing grids using traditional methods[35].

5.2.2 Vitrified Specimen Transfer Time

Once vitrified, a sample will revert to its crystalline form if its temperature rises above the devitrification temperature[36]. For aqueous samples, this temperature is around -140°C. The transfer time is defined as the amount of time that the grid is not submerged in a cryogen during transfer between the liquid ethane bath and liquid nitrogen baths. Highly trained operators using commercially available systems can transfer the grid between the ethane and nitrogen baths in as little os 0.4 seconds. A Sony high-speed video camera was used to film the ASPECT system performing the same motion. It was found that the system is capable of transferring the grid between the ethane nitrogen in only 0.0875 seconds: a 4.5 fold improvement over manual transfer . The drastically reduced transfer time minimizes the chance that the specimen devitrifies due to exposure to heightened temperatures during transfer.

5.2.3 Single Grid Processing Time

A stopwatch was used to time the ASPECT system processing a single grid in manual deposition mode. Blot time was set to one second, vitrification time was set to five seconds, and all other user selectable delays were set to zero seconds. The ASPECT system was able to fully process and store a grid in 27 seconds. Trained professionals can process complete grids in as little as 90 seconds, but average 3-5 minutes per grid[35]. The ASPECT system processes grids between 4.5 and 15 times faster than competing methods.

5.2.4 Button Processing Time

The ASPECT system was timed while processing a full button (4 grids) in manual deposition mode. Blot time was set to one second, vitrification time was set to five seconds, and all other users selectable delays were set to zero seconds. The ASPECT system was able to process an entire button one one minute and forty-three seconds. A trained operator will spend 15 minutes preparing an entire button using a traditional system[35]. The ASPECT system provides 7.5 fold improvement over competing methods.

5.3 Vitrification Results

As of this writing, the ASPECT system has yet to successfully vitrify a specimen. All imaged specimens have contained crystalline ice.

The presence of crystalline ice indicates that either the cooling rate of the grids is not high to support the formation of vitreous ice, or that the specimens devitrify during the time between vitrification and imaging.

The temperature of the ethane bath has been verified to be cold enough to vitrify samples, thus current work is focusing on identifying heat sources within the system that could cause devitrification of the samples. Two likely culprits have been identified.

The first possibility is that the grids devitrify during transport to the electron microscope at Baylor College of Medicine. During transport the grid-storage buttons are affixed to button handling rods and submerged in a shallow bath of liquid nitrogen. It is possible that any sloshing of the liquid nitrogen during transport temporarily exposes the grids to the ambient conditions of the storage dewar. This slight increase in temperature may lead to devitrification of the samples. To prevent this, all future samples will be stored in a dewar with a much larger volume of liquid nitrogen. This will ensure that the buttons remain submerged during transport.

The second, and more likely, possibility is that the use of carbon-tipped tweezers prevents the sample grids from vitrifying. All current grid preparation systems use stainless steel electron microscopy tweezers. Because of its low specific heat, the stainless steel cools very rapidly when it is exposed to the liquid ethane, this prevents the tweezers from conducting heat into the imaging grid. The carbon tweezers have a specific heat that is three times that of stainless steel. It is believed that the tweezer tips retain enough heat when submerged in liquid ethane to prevent the vitrification of the sample grid. The heat from the tweezers is transferred to the grid and either prevents vitrification in the ethane bath, or devitrifies the sample during transfer to the grid storage button. To test this hypothesis, the carbon-tipped tweezers in the ASPECT device are being replaced with stainless steel tweezers.

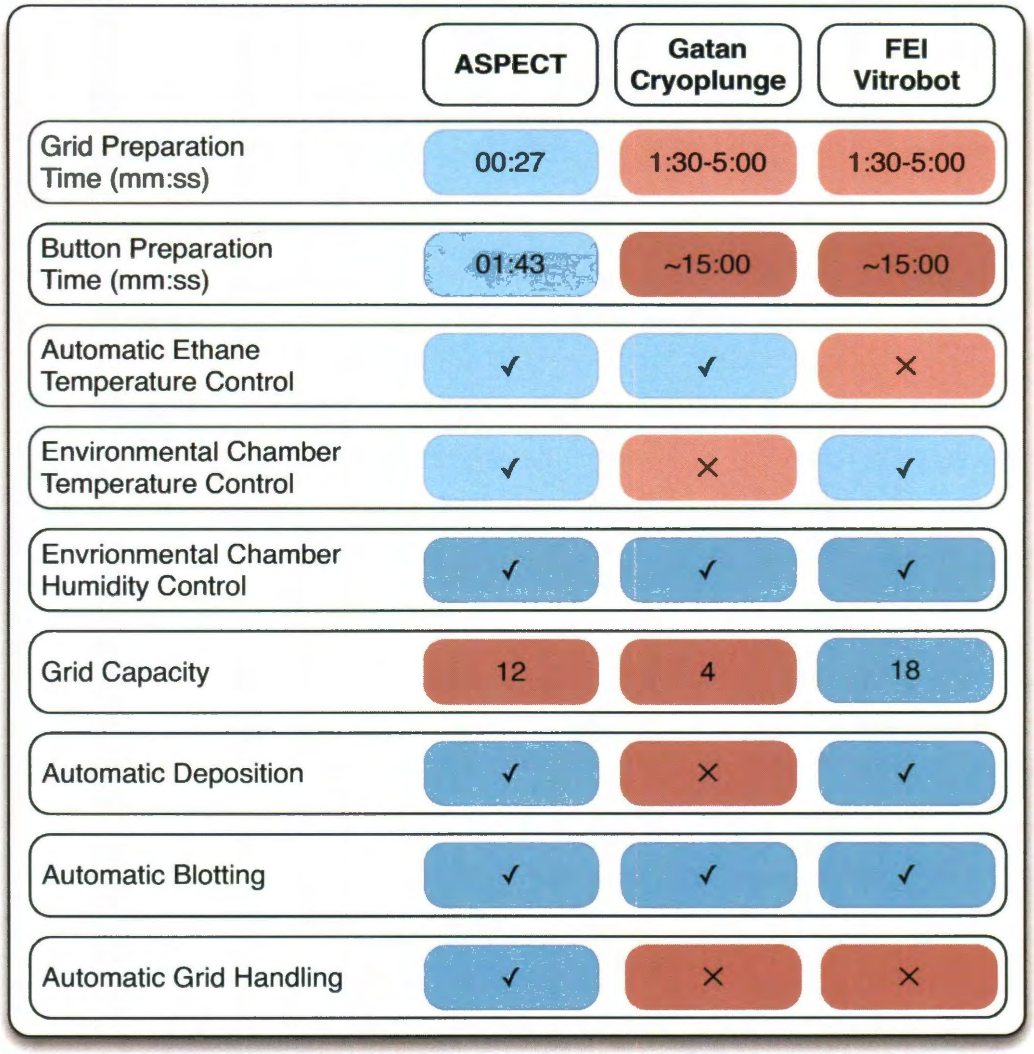
Chapter 6

Conclusion

The preparation of samples for cry-electron microscopy studies is a well understood process, and countless devices and procedures have been developed to assist researchers in preparing these samples. Current grid preparation methods have one major drawback though, they are highly dependent on a well-trained human operator to be successful. The necessity of highly trained operators narrows the potential user pool of this technique. Furthermore, inconsistencies in the prepared grids can be a problem if the operator is unable to follow the grid preparation procedure faithfully.

In this project, the goal was to design a device capable of preparing samples for cry-electron microscopy studies without the need for human interaction. The resulting device, ASPECT, has proven itself capable of automating all aspects of the grid preparation process. The performance of each subsystem present within the ASPECT device meets or exceeds the performance levels of similar subsystems from current commercial grid preparation systems. Figure 6.1 gives an overview of the performance of the ASPECT system compared with two other common commercial systems.

Though the performance of the individual subsystems of the ASPECT device indicate that the system should be capable of vitrifying samples of similar or better quality than competing systems, to date all imaging grids produced with the ASPECT system contain crystalline ice instead of amorphous ice. The apparent inability of the ASPECT system to successfully vitrify samples is currently under investigation, and several potential fixes are currently being implemented.



	ASPECT	Gatan Cryoplunge	FEI Vitrobot
Grid Preparation Time (mm:ss)	00:27	1:30-5:00	1:30-5:00
Button Preparation Time (mm:ss)	01:43	~15:00	~15:00
Automatic Ethane Temperature Control	✓	✓	×
Environmental Chamber Temperature Control	✓	×	✓
Envrionmental Chamber Humidity Control	✓	✓	✓
Grid Capacity	12	4	18
Automatic Deposition	✓	×	✓
Automatic Blotting	✓	✓	✓
Automatic Grid Handling	✓	×	×

Figure 6.1 : Performance overview of ASPECT system compared to commercial grid preparation systems.

The future outlook of the ASPECT system and similar devices is bright, though. By demonstrating that a fully automated grid preparation system is possible to design and operate, the work presented in this thesis raises the possibility that the preparation of technically demanding specimens will be much simpler in the future.

For example, time-resolved studies involve the imaging of a chemical or biological reaction as it progresses. . A fully automated system, such as ASPECT, would also be able to vitrify several of these grids in short order, and produce a time-lapse series of images detailing the progression a specific reaction.

The ASPECT system has been designed to prepare samples for cryo-electron microscopy studies. Though the ASPECT system has yet to produce a vitrified specimen, the performance of the various subsystems within ASPECT device indicate that the system will be able to successfully produce samples in the future. In short, the ASPECT system shows promise as a method for preparing samples for electron microcopy.

Bibliography

[1] M. Adrian, J. Dubochet, J. Lepault, and A. W. McDowan, "Cryo-electron microscopy of viruses," *Nature*, vol. 308, pp. 32–36, March 1984.

[2] R. M. Glaeser, "Limitations to significant information in biological electron microscopy as a result of radiation damage," *Journal of Ultrastructure Research*, vol. 3, pp. 466–482, August 1971.

[3] J. Dubochet, F. P. Booy, R. Freeman, A. V. Jones, and C. A. Walter, "Low temperature electron microscopy," *Annual Review of Biophysics and Bioengineering*, vol. 10, pp. 133–149, 1981.

[4] M. A. Hayat, *Principles and Techniques of Electron Microscopy: Biological Applications.* Cambridge, UK: Cambridge University Press, 4 ed., 2000.

[5] J. Dubochet, M. Adrian, J. Chang, J. Homo, J. LePault, A. W. McDowall, and P. Schultz, "Cryo-electron microscopy of vitrified specimens," *Quarterly Review of Biophysics*, vol. 21, pp. 129–228, 1988.

[6] L. Terracio and K. G. Schwabe, "Freezing and drying of biological tissues for electron microscopy," *The Journal of Histochemistry and Cytochemistry*, vol. 29, pp. 1021–1028, 1981.

[7] E. Ruska, "Ernest Ruska autobiography." On the WWW. URL `http://nobelprize.org/nobel_prizes/physics/laureates/1986/ruska.html`.

[8] G. E. W. Wolstenholme and M. O'Conner, eds., *The Frozen Cell: A Ciba Foundation Symposium.* J. & A. Churchill, 1970.

[9] Y. Talmon, "Imaging surfactant dispersions by electron microscopy of vitrified specimens," *Colloids and Surfaces*, vol. 19, pp. 237–248, August 1986.

[10] P. Brüggeller and E. Mayer, "Complete vitrification in pure liquid water and dilute aqueous solutions," *Nature*, vol. 288, pp. 569–571, December 1980.

[11] J. Dubochet and A. W. McDowall, "Vitrification of pure water for electron microscopy," *Journal of Microscopy*, vol. 124, pp. 3–4, 1981.

[12] M. Cyrklaff, M. Adrian, and J. Dubochet, "Evaporation during preparation of unsupported thin vitrified aqueous layerf for cryo-electron microscopy," *Journal of Electron Microscopy Technique*, vol. 16, pp. 351–355, 1990.

[13] S. U. Egelhaaf, P. Schurtenberger, and M. Müller, "New controlled environment vitrifiction system for cryo-transmission electron microscopy: design and application to surfactant solutions," *Journal of Microscopy*, vol. 200, pp. 128–139, November 2000.

[14] R. W. J. Murray, "Principles for the contruction and operation of a device for rapidly freezing suspension for cryo-electron microscopy," *Journal of Electron Microscopy Technique*, vol. 5, pp. 279–284, 1987.

[15] J. R. Bellare, H. Davis, L. Scriven, and Y. Talmon, "Controlled environment vitrification system: An improved sample preparation technique," *Journal of Electron Microscopy Technique*, vol. 10, pp. 87–111, 1988.

[16] L. Malenson, "Cryoplunge$^{\mathrm{TM}}$3: Gatan's versatile and affordable plunge-freezing instrument." On the WWW. URL `http://www.gatan.com/resources/knowhow/kh19-cryoplunge.php`.

[17] R. J. Lang, "Ultrasonic atomization of liquids," *The Journal of the Acoustical Society of America*, vol. 34, pp. 6–8, January 1962.

[18] S. Trachtenberg, "A fast-freezing device with a retractable environmental chamber suitable for kinetic cryo-electron microscopy studies," *Journal of Structural Biology*, vol. 123, pp. 45–55, 1998.

[19] P. M. Frederik, P. H. H. Bomans, P. F. J. Laeven, and F. J. T. Nijpels, "Device for preparing specimens for a cryo-electron microscope." European Patent Specification, June 2006.

[20] P. M. Frederik and D. H. W. Hubert, "Cryoelectron microscopy of liposomes," *Methods in Enzymology*, vol. 391, pp. 431–448, February 2005.

[21] B. Battersby, J. C. W. Sharp, R. I. Webb, and G. T. Barnes, "Vitrification of aqueous suspensions from a controlled environment for electron microscopy: an improved plunge-cooling device," *Journal of Microscopy*, vol. 176, pp. 110–120, November 1994.

[22] Y. Fink and Y. Talmon, "The flow thru controlled environment vitrification system," in *Proceeding of the 13th International Congress on Electron Microscopy*, vol. 1, p. 37, 1994.

[23] C. S. Potter, J. Pulokas, P. Smith, C. Suloway, and B. Carragher, "Robotic grid loading system for a transmission electron microscope," *Journal of Structural Biology*, vol. 146, pp. 431–440, June 2004.

[24] H. Ge, J. Suszynski, H. T. Davis, and E. Scriven, "New controlled envrionment vitrification system for preparing wet samples for cryo-SEM," *Journal of Microscopy*, vol. 229, no. 1, pp. 115–126, 2007.

[25] Gatan, "Cryoplunge 3 (cp3)," December 2009.

[26] "Vitrobot mark IV." On the WWW. URL `http://www.fei.com/products/sample-prep/vitrobot.aspx`.

[27] "Automatic plunge freezing with bare grid technique: Leica em gp." On the WWW. URL `http://www.leica-microsystems.com/products`.

[28] E. M. Services, "Ems-002 rapid immersion freezer." On the WWW. URL `http://www.emsdiasum.com/microscopy/products/equipment/plunge_freezer.aspx`.

[29] S. Y. Nof, ed., *Handbook of Industrial Robotics.* John Wiley & Sons, 2 ed., 1999.

[30] Adept Technology, Inc., *Adept Cobra 600 Robot Instruction Handbook*, May 1998.

[31] F. Company, "Vitrobot mark IV: Cool science," 2007.

[32] J. Bennett, "Autoit." On the WWW. URL `http://www.autoitscript.com/autoit3/index.shtml`.

[33] Adept Technology, Inc., *V+ Language User's Guide: Version 12.1*, September 1997.

[34] Occupational Safety and Health Standards, U.S. Department of Labor Wahington, D.C. 20210, *29 CFR 1910.147, the Control of Hazardous Energy (Lockout/Tagout) - Inspection Procedures and Interpretive Guidance*, September 1990.

[35] C. Fu, "Competing systems grid handling perfromance." Personal Correspondence, November 2010.

[36] J. Dubochet and J. Lepault, "Cryo-electron microscopy of vitrified water," *Journal de Physique*, vol. 45, pp. C7.85–C7.94, September 1984.