

Cryogenic Freezing System



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Abstract

One aspect of current genetic research is focused on the molecular mechanisms of the electrical mechanisms involved with the nervous system; in particular the synaptic regions in the human brain. *Drosophila melanogaster** (fruit flies) were chosen as the model organisms due to the correlation between their nervous system and that of the human brain. In order to advance the research, an improved means of sample preparation is required so that the specimen can be analyzed with an electron microscope. Using cryofixation* to prepare the samples would allow for the specimens to be thinly sliced and decrease the amount of preparation artifacts*, which can distort morphology* and cause interpretation errors. Commercially available cryofixation units are expensive and contain extra side features not desired by the client. This project addresses the design and construction of a personalized cryofixation unit can be built in house for a fraction of the cost.

Introduction

In genetic research, *Drosophila melanogaster* (fruit flies) are used because of their genetic simplicity. The genetic make up of *Drosophila melanogaster* consists of four chromosomes, which allows for easy determination of the location of the mutated gene. Due to the short life cycle of the species, multiple generations can be obtained within a few weeks.

Problem Statement

The goal of this project is to design a device that is able to rapidly freeze biological specimens with maximum vitrification*, thus preserving the morphology of the mutant samples.

Currently, Professor Barry Ganetzky is involved in research focused on the synaptic connections in *Drosophila melanogaster*. Utilizing cryofixation techniques would allow the client to look at his samples using transmission electron microscopy, thus improving the resolution from the confocal laser-scanning microscope he now uses. The commercial units available for this type of sample preparation are expensive, around \$10,000 and up per unit, due to the low demand and unnecessary features. The client believes modifications can be made to the conceptual designs of the commercial units and a personalized unit could be built in-house for a

* For definitions see the glossary in Appendix B

fraction of the cost. The device needs to include two main mechanisms for freezing the flies; a spray mechanism and an immersion tank. The pressure and temperature of the delivered secondary cryogen needs to be regulated, so the sample is frozen quickly (~10ms) while still preserving its morphology. The selection of primary and secondary cryogens will be based on safety concerns and performance qualities.³

Background Information

Current Research

Professor Barry Ganetzky is currently doing research on the molecular mechanisms of electrical signals in the nervous system; in particular the human brain synapses. Isolating mutants of *Drosophila melanogaster* is an important method used in genetics because it directly correlates to human brain synapses. The mutants that are isolated usually illustrate particular behavioral abnormalities such as temperature sensitivity. Genes that encode key proteins, which direct the electrical impulses in neurons and send these signals across synapses, can be detected by isolating the mutant samples. Specific genes that have been located on chromosomes are cloned in order to determine the gene and its corresponding encoded protein on the molecular level. The mutant samples that can be used in research exhibit particular characteristics. These mutants show a disturbance in the development and morphology of synapses between an incoming motor axon and the neuromuscular junction*. The mutants also provide a basis for the determination of the unknown mechanisms that control size, shape, and other synaptic characteristics. By performing molecular studies on mutant *Drosophila melanogaster*, affected proteins and their specific roles can be identified.³

It is hypothesized that wild types of synaptic terminals are involved in the development of the synapses. (Figure 1) Comparing the mutations of the wild type exposes an increased number of synaptic boutons*, showing that mutations within the gene cause changes to occur in synaptic structures such as extended branches. Increased resolution of wild type synaptic terminals* is desired in genetic research.³

* For definitions see the glossary in Appendix B

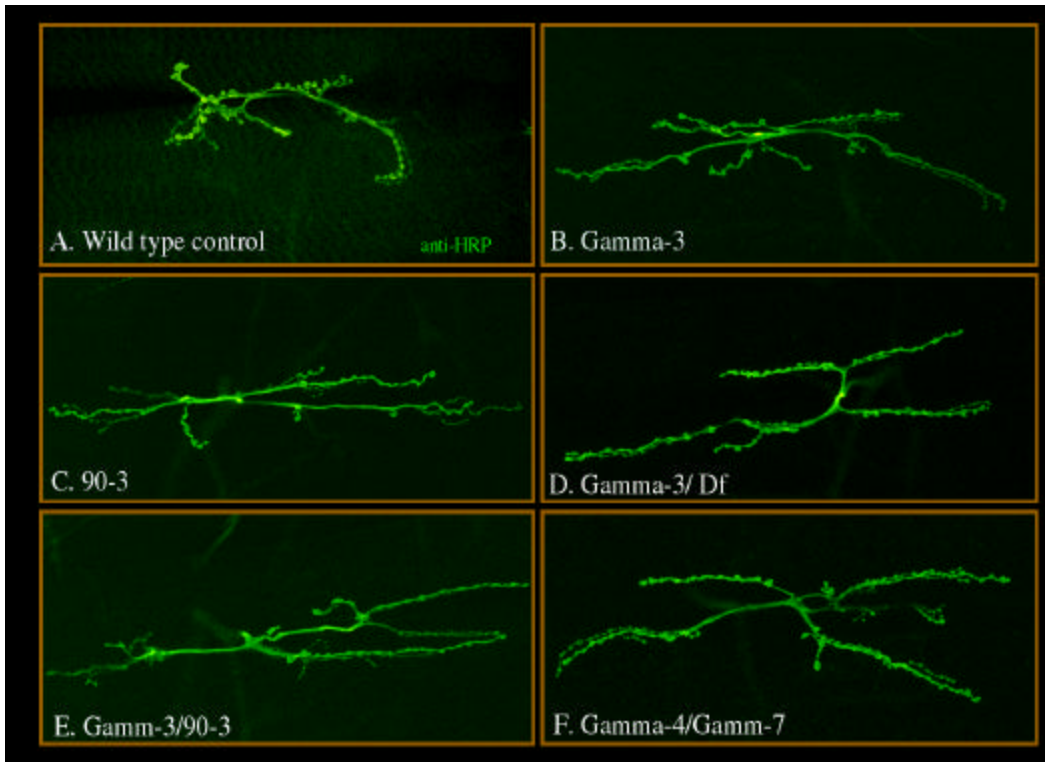


Figure 1: Confocal Images of Mutant and Wild Type Synaptic Terminals. The wild and mutant type alleles are shown. The green signals are extended anti-HRP that indicates synaptic branches and an increased number of synaptic boutons. Picture taken using a 16x magnification; actual size would be 300x160 μ m.⁸

Light and electron microscopy are used in these studies to observe and quantify the mutant phenotype*. Morphological preservation of the samples is important because the appearance of the synapse *in vivo* needs to be as clear as possible to properly see the mutant when observing it under a microscope.³

Dissection Technique

In order to dissect *Drosophila melanogaster*, it is necessary to use a dissection microscope and a dissection chamber. The fly is first pinned down with two pins and is then cut lengthways. (Figure 2) After all six pins are inserted and the fly is opened up, all of the organs are removed. The central nervous system and the muscle wall are all that remain. The fly must be continuously bathed in a calcium saline solution so that the normal functioning of the neural pathways can still be observed even though the specimen is not living.³

* For definitions see the glossary in Appendix B

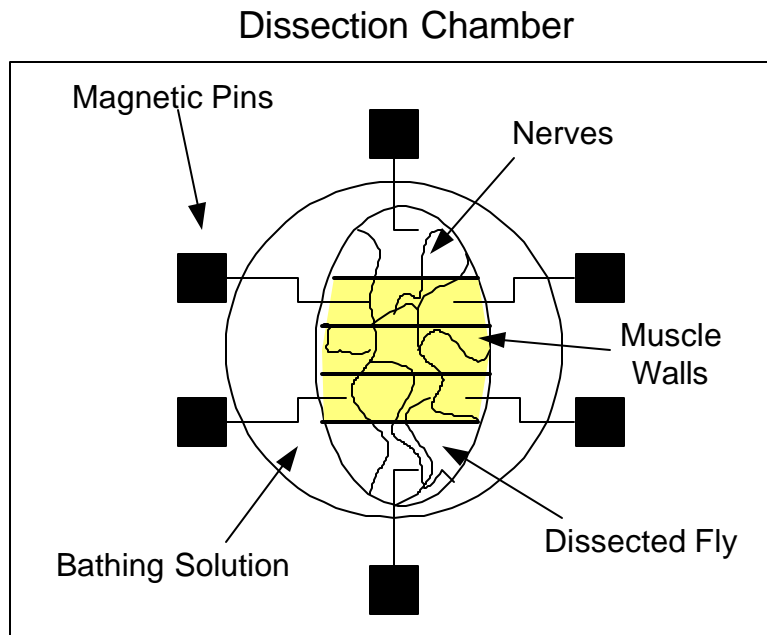


Figure 2: Dissection Chamber Used for *Drosophila melanogaster*. As part of the preparation process the *Drosophila melanogaster* are dissected, and their organs are removed so that their central nervous system and muscle walls are all that remains. After dissection the specimen is pinned out as in the above schematic, and at this point is ready to be fixed.⁸

Fixation Techniques

Chemical fixation of tissues, which is the most commonly used procedure, often introduces preparation artifacts that can affect analysis and interpretation. In chemical fixation, the specimen under consideration is stained with a dye to enhance the visibility of the neural pathways. Although this type of sample preparation has been used thus far to prepare specimens for use with the confocal microscope, other preparation techniques need to be implemented in order to use the electron microscope. The confocal microscope only requires chemical fixation of the sample, whereas in electron microscopy the sample must be cryofixed for further preparation. The preparation techniques used for electron microscopy allow for increased resolution and samples free of preparation artifacts. These preparation modifications are necessary to advance the research.⁸

The most common method of cryofixation uses a primary and a secondary cryogen*. The primary cryogen* (usually liquid nitrogen or liquid helium) is used to facilitate the cooling of the secondary cryogen, which comes in contact with the specimen. Secondary cryogens (typically ethane or propane) are condensed when passed through piping that is surrounded by the primary cryogen. The liquefied secondary cryogen is then used to vitrify the sample.¹⁰

Studies have shown that the quality of morphological preservation in a specimen is directly related to the rate at which it is frozen. Also, rapid solidification reduces the artifacts in a sample.³

Existing Commercial Systems

There are several commercial companies that offer cryofixation units, such as Leica and VG Microtech. The units that these companies provide are directed toward sample preparation for specific applications such as Scanning Electron Microscopy (SEM) or Tunneling Electron Microscopy (TEM). Having the units directed toward these specific applications limits the universality of the equipment. Preparing the samples for applications such as SEM includes applying a metal coating. These extra features such as the metal coating stage and others such as mounted microscopes, vacuum environment preparation and UV lamps significantly increase the cost of the commercial devices and will not benefit the client in his research. Also, the cryofixation mechanisms in the commercial devices did not include both the spraying and immersion options; a key requirement specified by the client.^{6,12}

Product Design Specifications

Commercially available units designed for rapid freezing are expensive.⁶ Therefore, the design and production of such a device in-house would provide an affordable alternative to achieve the desired end result. With the opportunity to design a device whose specifications are tailored for the client needs, the performance will be superior to commercial devices designed for more general uses.

The key requirements outlined by the client include:^{3,8}

- Rapid freezing of sample
 - Preservation of morphology
 - Minimal freezing artifacts
- Spray unit
- Soft budget of \$500
- Incorporation of immersion tank (tank size ~5cm diameter)

A complete product design specification is located in Appendix A.

Design Solutions and Evaluation

Phase I Alternatives

Phase I design solutions consisted of three alternatives: an immersion tank, a spraying mechanism and a spray/plunge unit. This phase of product development focused on determining the overall mechanism for bringing the specimen in contact with the secondary cryogen.

Immersion Device

This device freezes samples by plunging them into a cryogen. Using suction or water tension, the sample is mounted on the point of the plunger. (Figure 3) The plunger submerges the sample at a velocity around 3 m/s, via a spring-loaded mechanism. When the plunger is in the loaded position, the lower spring will exert a downward force, which along with gravity will immerse the sample in the cryogen tank when a release button is pushed. It may be useful to make the plunging arm detachable so that loading the plunger will be easier. The primary cryogen encompasses a pipe that the secondary cryogen flows through, thus cooling and liquefying the secondary cryogen. When the sample is immersed in the secondary cryogen, the sample quickly freezes and the morphology is preserved.

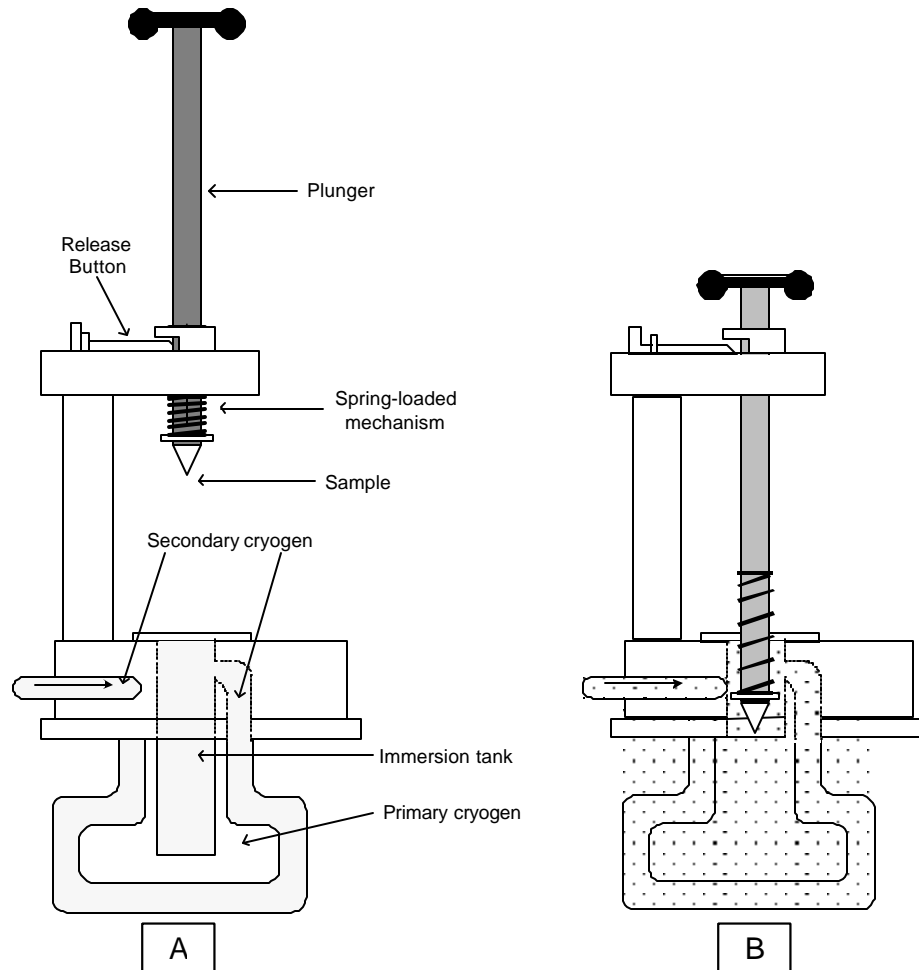


Figure 3: The Immersion Device. The immersion device involves plunging the specimen into a cryogen bath. In the above device, the specimen would be attached to the plunger head labeled sample. This head is spring loaded (A) and when released will drive the specimen into the immersion tank (B), which holds the cryogen bath, at a rate of 3 m/s. The base of the device is approximately 10 cm in diameter and the height of the device is roughly 50 cm.

The immersion device will effectively freeze small samples, such as the head of *Drosophila melanogaster*. This method will be less effective in rapidly freezing an entire body sample of *Drosophila melanogaster*.^{3,8}

Sprayer Device

The ultimate goal of the sprayer is the same as that of the immersion device; to vitrify the sample. Cooling of the secondary cryogen by the primary cryogen and the attachment of the sample work is done similar to what was done in the previous device. However, there is one significant difference. After the secondary cryogen is liquefied, it is sprayed out of a small nozzle onto the sample. The mounting device remains in a fixed position at all times, in contrast

to the immersion device. It may also be necessary to angle the nozzle due to the affect of gravity. This would allow the distance between sample and nozzle to remain close, while decreasing the initial velocity of the spray. A remote valve could control the spraying duration. (Figure 4)

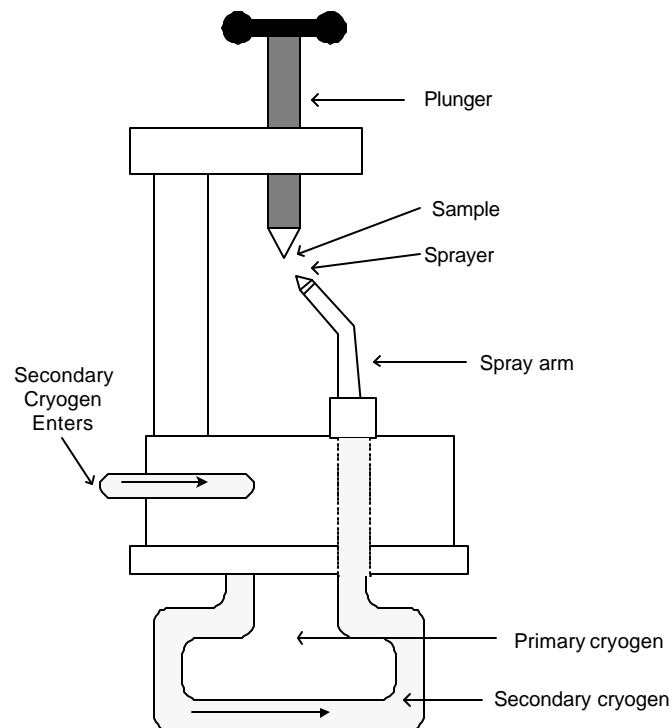


Figure 4: The Sprayer Device. The sprayer device aims to freeze the sample by spraying the secondary cryogen onto the specimen. The specimen would be attached to the device at the head labeled sample, and the cryogen would be sprayed onto the specimen by some spraying mechanism. The base of the device is approximately 10cm in diameter and the height of the device is roughly 50 cm.

The sprayer device will be more advantageous for whole-body samples of *Drosophila melanogaster*, but it will be less effective for smaller preparations.

Spray/Plunge Device

The plunging and spraying techniques have different advantages for different types of samples. Both of the previous designs have several similarities; therefore combining them into a singular multi-purpose device could be easily accomplished. (Figure 5) To do this, a rotating spray mechanism must be used so that it can be moved to allow for a clear the path for the plunging apparatus. It may also be convenient to spray a sample directly on a slide. The nozzle could be adapted for this by allowing rotation in a different plane. By implementing a

valve, the flow of the secondary cryogen could be shifted from the immersion tank to the spraying mechanism.

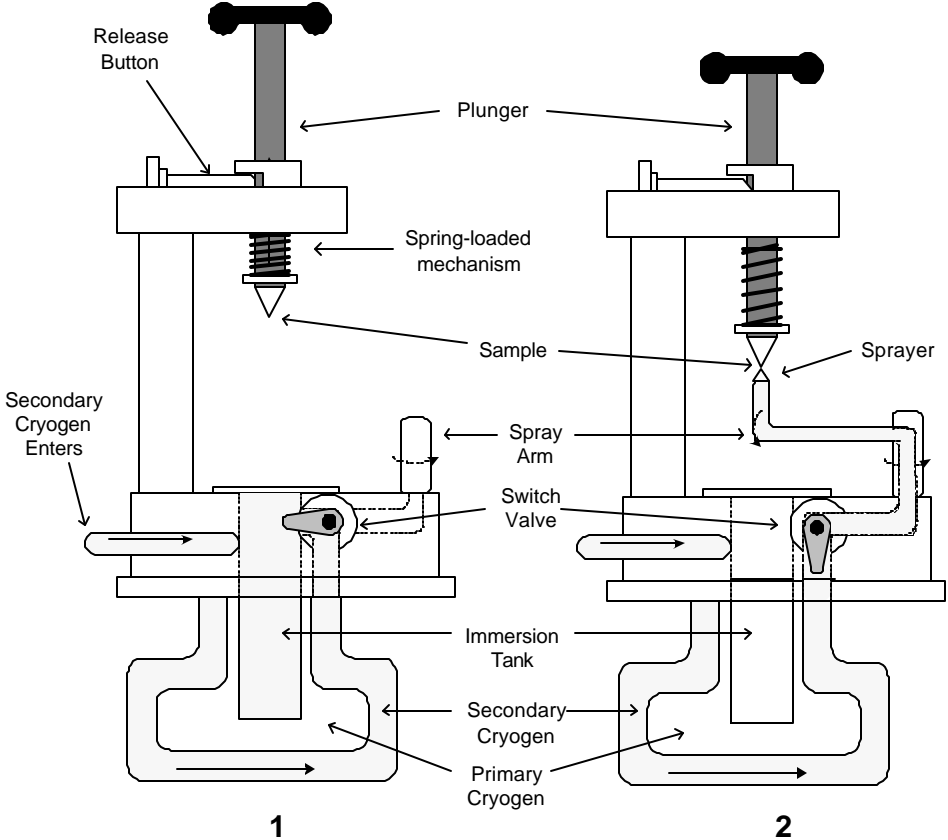


Figure 5: Spray/Plunge Device. This device combines the spray and immersion techniques and makes them available with the use of one device. The individual mechanisms would function the same as in the separate devices. The spraying mechanism would be implemented on a swinging arm so that it would not interfere with the immersion unit. A valve system would also be integrated so that the secondary cryogen would be directed to the appropriate fixation technique. The base of the device is approximately 10cm in diameter and the height of the device is roughly 50 cm.

Phase I Evaluation

Phase I of the product development focused on determination of the overall design components. It was evident that there would need to be a component that used a primary cryogen to liquefy a secondary cryogen. The delivery of the secondary cryogen to the specimen was the area in which alternative components were evaluated. There were two main alternatives: an immersion tank and a spraying mechanism. The third option was a combination of the two ideas. The three alternatives were evaluated using the criteria included in the product design specifications. For the overall Phase I design, the

specifications used for evaluation were: preservation of morphology, rate of freezing, versatility of the device, and overall cost. (Table 1) The advantage of the immersion tank is that it is useful when preparing samples of small body parts, such as *Drosophila melanogaster* heads. Due to the forces placed on the specimen when it is driven into the cryogen bath, preservation of the morphology of a dissected sample might be compromised using this method. In the spray unit, the force with which the secondary cryogen hits the specimen can be regulated, therefore favoring preservation of morphology. Thicker samples may not freeze at a rate rapid enough to maximize vitrification using this method, so the mechanism is somewhat limited in the types of samples it can prepare. The third option incorporated both components and implemented them in a single device. This design is optimal because it has multiple functions. Although it would cost more, it is less expensive than buying a separate device for each preparation technique.

Table 1: Decision Matrix for Phase I Alternatives

	Immersion Tank	Sprayer Device	Spray/Plunge Device
Preservation of Morphology	-	+	+
Rate of Freezing	+	+	+
Versatility	-	-	+
Cost	+	+	-

The important design specifications that the Phase I design must incorporate. A (+) indicates that the design meets that requirement and a (-) means that the design is deficient. The specifications are arranged according to priority, however, all of the specifications are important and should be met in the design solution.

Based on this preliminary evaluation, the spray/plunge unit was the design pursued for the remainder of the semester, moving the project in to Phase II of development.

Phase II Alternatives

Phase II development focuses on developing the individual components of the overall design. The spray/plunge unit was broken down into three components: the spray mechanism, the cryogen cooling mechanism, and the immersion set-up. Currently, resources have been focused on alternative development of the spraying mechanism, because this delivery component will provide the greatest short-term benefit to the client.

Spray Mechanism Alternatives

Several alternatives were developed for the spray mechanism. The first is just a single spray nozzle that sprays the secondary cryogen directly onto the sample. (Figure 6)

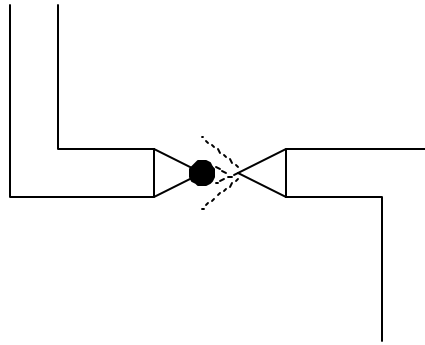


Figure 6: Single Spray Nozzle. The specimen is attached to the device at the sample head, which is bent at a 90°angle so that the cryogen is sprayed head on. The secondary cryogen is emitted from a single nozzle in a broad scatter pattern covering the specimen. Scale of the diagram is 1/4" (drawing) = 1/8" (real life)

The second alternative utilizes two spray nozzles so that the secondary cryogen can be sprayed onto the specimen from multiple directions. (Figure 7)

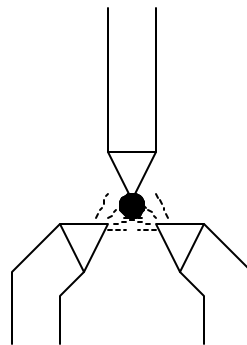


Figure 7: Double Headed Spray Unit. This spraying mechanism is similar to the single spray nozzle option, but the cryogen is emitted from two sources so that the cryogen is applied to the specimen from both sides. Scale of the diagram is 1/4" (drawing) = 1/8" (real life)

The angled spray alternative uses one nozzle to spray the secondary cryogen onto the specimen. By angling the release of the spray, the amount of interference created by falling cryogen is decreased. (Figure 8)

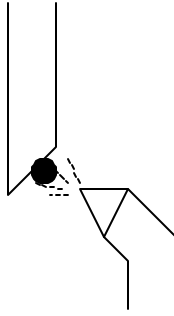


Figure 8: Angle Spray. In this alternative, the specimen and spray nozzle are both angled, so the cryogen is still directly being sprayed onto the specimen. The sprayed cryogen will not fall back onto the spray nozzle. Scale of the diagram is 1/4" (drawing) = 1/8" (real life)

The horseshoe alternative sprays the cryogen from multiple point sources in a semicircular ring that is placed around the specimen. (Figure 9)

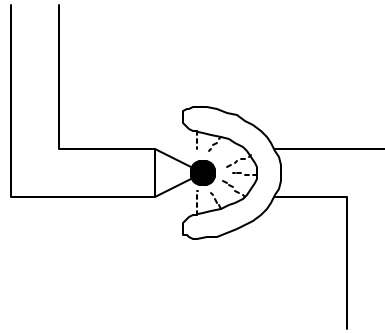


Figure 9: Horseshoe. In this design option, the spray mechanism is semi-circular with multiple holes for cryogen emission. The specimen is placed at what would be the center of the circle so that each point of spray emission is equidistant from the sample. Scale of the diagram is 1/4" (drawing) = 1/8" (real life)

In the showerhead alternative the specimen is placed at the center of a hollowed hemisphere that has multiple source locations for the release of cryogen. (Figure 10)

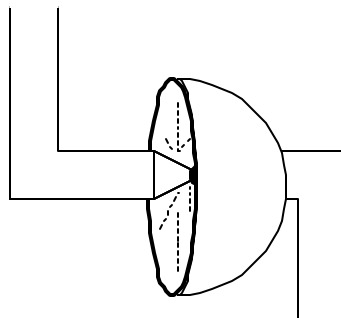


Figure 10: Showerhead. The spray mechanism in this option is in the shape of a hemisphere with multiple holes for cryogen emission. The sample is placed at what would be the center of the sphere so that the specimen is equidistant from all spray emissions. Scale of the diagram is 1/4" (drawing) = 1/8" (real life).

Spray Mechanism Evaluation

Each of the spray mechanism designs were evaluated based upon the following criteria: uniform coverage of specimen, simplicity of design, current availability, cost, and ability to adjust droplet size (Table 2). To accurately preserve morphology and minimize freezing artifacts, the sample needs to have a uniform distribution of secondary cryogen, thus making it the most important criteria. Also, the simplicity of the design is important to consider. The simpler the design is the less chance there is for the spray mechanism to malfunction. Another important aspect is the current availability of the spray mechanism. It will be less time consuming and much easier if the spray mechanism does not have to be manufactured. Cost was also taken into consideration, but it does not seem to be a major factor. Each criterion was then weighted based on its importance.

Table 2: Decision Matrix for Phase II Spray Mechanism Component

	Single Spray	Double Spray	Angle Spray	Horse Shoe	Showerhead
Uniform Coverage of Specimen (5x)	0	+	0	+	+
Simplicity of Design (4x)	+	+	+	0	-
Current Availability (3x)	+	+	+	-	-
Cost (2x)	+	+	+	0	0
Total +	+9	+14	+9	+5	+5
Total -	0	0	0	-3	-7
Total 0	5	0	5	6	2

The following table evaluates each of the spray mechanism designs based upon five criteria. Each specification is given a multiplication factor, with the most important criteria carrying the most weight. A (+) indicates the design meets the criteria, a (-) indicates that the design is deficient, and a (0) indicates the design is neutral.

Based upon the above specifications, the double spray mechanism was chosen. Most importantly, it will provide uniform coverage of the specimen. The spray nozzle is also currently available; a preliminary spray nozzle was found in a McMaster-Carr Catalog.⁷ Two of the nozzles will need to be purchased. The suggested nozzle is a full cone spray nozzle, as opposed to a hollow cone or flat fan spray (Figure 11).



Figure 11: Spray Types. The spray patterns for a flat fan, full cone, and hollow cone are displayed. The full cone spray nozzle will be used.

The full cone spray nozzle will allow the secondary cryogen to be distributed uniformly. The nozzle is available in brass, PVC, and stainless steel.⁷ For this design, the brass nozzle would be the best choice. Copper tubing will be utilized in the cooling of the cryogen, so brass fittings would be advantageous, since copper and brass have similar thermal expansion coefficients.² The material should be sufficient for the low temperatures it will be subjected to and it is available for 1/8" pipe size, which will be used for the copper tubing. One other option that must be decided is the spray angle, which can be either 60° or 120°. A spray angle of 60° should be adequate to cover the sample. Another component that will be necessary is a nozzle fitting, which allows the nozzle to be tilted and rotated 45°, thus ensuring a precise spray direction (Figure 12).

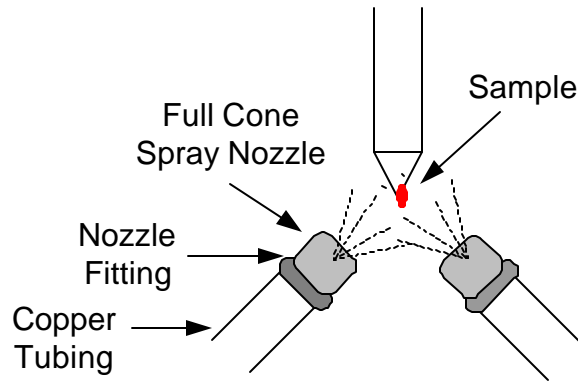


Figure 12: Double Spray Mechanism. The figure shows the double spray mechanism with a full cone spray nozzle and a nozzle fitting. The spray angle is 60°, providing full-coverage of the sample. Scale of the diagram is 1/4" (drawing) = 1/8" (real life)

A disadvantage of this design is that not all of the cryogen will hit the sample. This should not cause too much of a problem since the quantity being sprayed is so small.

The Cryogen Cooling Mechanism

The selection of the primary and secondary cryogen was central in the development of the cooling mechanism. Nitrogen and propane were selected as the primary and secondary cryogens respectively. These are the chemicals typically used in this type of system. Other options include liquid helium for the primary cryogen and ethane or halogenated hydrocarbons for the secondary cryogen. Liquid helium was not selected due to the high cost. Liquid nitrogen is at a sufficient cooling temperature, is available in most labs, and is far cheaper. Ethane and halogenated hydrocarbons both pose the same safety risks as propane. With proper precautions, the minimal amounts used should not pose a threat. (See the safety and ethical considerations section for a further discussion of safety issues) The propane, however, is available in most labs via a direct line. Therefore, propane was also chosen based on cost and availability.

In determining the cooling mechanism a basis of 1 mole of propane was set. Since the amount of propane needed to properly freeze the sample has not been calculated, the following series of calculations will need to be scaled down at a later time to account for this information.

In determining the ratio of nitrogen to propane required to run the process, the changes in enthalpy for the two chemicals was determined over the specified temperature range. In order to properly freeze the sample, the propane needs to be at or below -143°C .⁴ To ensure that the temperature requirement was met, an exiting temperature of -150°C for the propane was set. The calculations were performed under the following assumptions: 1) the propane enters the system at room temperature, 25°C and 2) the liquid nitrogen in the cooling tank is at -196°C , which is the boiling point for nitrogen. The amount of heat that needs to be removed from the propane in order to cool it from room temperature to the -150°C is -34.748 kJ/mol propane. The amount of heat required to vaporize the liquid nitrogen is $+5.577$ kJ/mol nitrogen. Therefore, seven moles of nitrogen are required to adequately cool one mole of propane.² (Specific calculations are in Appendix C)

The heat transfer from the propane to the liquid nitrogen occurs in the copper tubing which transports the propane through the liquid nitrogen cooling bath. Different copper alloys were considered in the selection of the tubing based on thermal conductivity. Alloy 122 copper tubing was selected since it has the highest thermal conductivity of any standard heat exchange material.⁷ Although the suggested operational temperature range for this material (-40 to 200°C)⁷ does not include the temperatures that are required for condensation and cooling of the propane; the extreme temperatures will not affect the system in this instance.⁹ The copper tubing will become brittle when exposed to such low temperatures, but it is only used to

transport the propane through the liquid nitrogen which causes little mechanical stress on the material. Thus, the slight weakening of the material due to extreme temperatures will not lead to failure. In order to maximize the surface area to volume ratio, 1/8" inner diameter tubing was selected.

Several valves are needed to regulate the flow of the propane through the cooling mechanism. The valves need to function at -200°C and adapt with 1/8" tubing. Three will be the minimum number of valves required for controlled regulation of the device: one for controlling the flow rate of the primary cryogen, one for controlling the flow rate of the secondary cryogen, and one for the controlled release of the cooled secondary cryogen. Pressure release valves on the dewar may also be necessary for safety reasons. A summary of the valve function and selection is presented in Table 3.

Table 3: Summary of function and specifications of selected valves.

	Function and Specifications	Selected Valve
Valve #1	<ul style="list-style-type: none"> • Control propane flow into system • Used under ambient conditions 	Solenoid
Valve #2	<ul style="list-style-type: none"> • Control nitrogen flow into system • Used under cryogenic temperatures • Regulated by thermocouple 	Cryogenic Solenoid
Valve #3	<ul style="list-style-type: none"> • On/off switch to control spraying of propane at -150°C • Regulated by thermocouple • Used under cryogenic temperatures • Directs flow of cryogen to spray or tank 	Cryogenic Solenoid
Pressure Release Valves	<ul style="list-style-type: none"> • Allow for release of built up pressure due to nitrogen vaporization • Safety consideration 	One-way valves will be implemented if needed, some of the dewar flasks already come with this modification

The function of the first valve is to control the flow of propane gas into the cooling system. This will most likely be controlled electronically in the final product, so a solenoid valve will be implemented here. The valve at this location is not subject to extreme operational conditions.

The second valve controls the flow of the liquid nitrogen into the cooling mechanism. This valve will be regulated by the negative feedback control loop associated with the

thermocouple at the propane exit site. If the temperature of the propane is not below a specified point, the second valve will open allowing more liquid nitrogen to flow into the system, thereby lowering the temperature. The temperatures under which this valve will need to operate under will be extremely cold. There are cryogenic solenoid valves commercially available that operate under these conditions. This valve will be electronically controlled by the feedback loop and manually controlled when the cooling chamber is initially filled.

The third valve functions as an on/off switch, which will be used in conjunction with a thermocouple placed at the outlet to the spray mechanism, to regulate the temperature of the exiting propane. This valve will only open if the temperature is below -150°C (the temperature required to freeze the sample); meaning this valve must operate under extreme temperature conditions. A solenoid valve will most likely be used here as well. There are cryogenic solenoid valves on the market, however they are rather expensive (around \$130 per valve)⁷ A cryogenic ball valve could be used. However, this type of valve could not be controlled electronically, meaning that the best choice would be the solenoid valve. This valve will be a three-way valve in order to direct the flow of the propane to either the immersion tank or the spray mechanism. The user will manually adjust this valve to direct the flow of the propane depending on the mechanism to be used.

A pressure release mechanism is necessary for safety. When the liquid cryogen vaporizes, there will be a significant increase of internal pressure in the system. If this pressure is not released there could be an explosion.⁹ Many of the thermoses contain seals or pressure release valves that protect against explosion. Depending on which thermos is selected, this feature may already be included separately. In addition, many of the cryogenic valves contain pressure release systems; therefore, there may not be a need for additional valves (Figure 13).

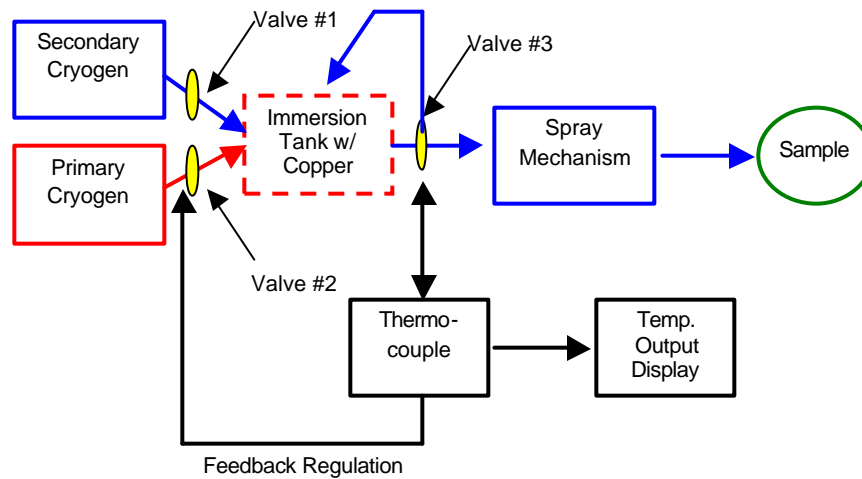


Figure 13: Valve Placement. There will be a minimum of three valves implemented into the design: one to control the flow of the primary cryogen (red arrows), one to control the flow of the secondary cryogen (blue arrows) and one to control the release of the secondary cryogen into the spray mechanism or the immersion tank.

The Immersion Set-Up

Most of the design thus far has focused on the spray mechanism and the cooling of the cryogen. The immersion tank will be incorporated into a liquid nitrogen dewar. The current option for the plunging mechanism is a spring-loaded plunger. The dimensions of the tank, recommended by the client as being 5 cm in diameter and 3 cm deep.³

Calculations/Engineering Analysis

In the development and implementation of the different components of the device, the following factors will need to be calculated and considered.

Spray Mechanism

- Scatter pattern
- Pressure
- Temperature
- Flow rate of secondary cryogen through primary cryogen
- Positioning of specimen in relation to spray flow: spray from side, below or above.

The Cryogen Cooling Mechanism

- Performance
- Safety
- Cost

Immersion Set-Up

- Spring constant
- Speed of plunger into the tank
- Distance between the sample starting point and the surface of the cryogen bath

Cost Analysis

A cost analysis was done for the current design. It includes estimated costs of the liquid nitrogen dewar flask, copper tubing, valves, and spray nozzles and accessories (Table 4).^{5,7}

Table 4: Estimated Manufacturing Costs

Component	Estimated Cost
Liquid nitrogen dewar flask	\$125.00
Copper tubing	\$3.18
Valves (3)	\$250.00
Spray nozzles (2)	\$14.60
Spray nozzle accessories (2)	\$22.80
Anticipated costs (plunging mechanism, thermocouple, temperature display)	\$150.00
Total manufacturing costs	\$565.58

The estimated costs of components obtained from catalogs.

Appendix D contains details and prices of the different component options. These costs do not take operational costs into account. Operational costs include the price of liquid nitrogen, propane gas, electricity, and disposal cost, which have not yet been determined.

Safety and Ethical Considerations¹⁰

The main ethical consideration is for the safety of the user. The device will be using liquid nitrogen and propane, which are potentially hazardous if proper guidelines are not followed when handling and disposing the chemicals.

The major concern with liquid nitrogen is inadequate ventilation in the room where the device will be used. One liter of liquid nitrogen expands to approximately seven liters of gaseous nitrogen, which can cause the oxygen content of air to fall. If the oxygen content in air falls below 18%, asphyxiation occurs. Most likely, this device will not be using large enough quantities of liquid nitrogen over a long enough period of time for this to be an issue, but using the device in a well-ventilated room can ensure this is not a problem.

Explosion of the gaseous propane is another safety concern that must be addressed. The device needs to be used in a room, which is not in a basement, therefore allowing the

propane gas to flow-off. Also, open flames should not be used in the room. Following these guidelines will prevent an explosion.

Cryogen splashing needs to be avoided to prevent injury to the operator. Liquid propane can cause burns if it contacts the skin, therefore some sort of protective facemask or shield around the device should be used. Splashing of the liquid nitrogen should not be an issue because it is contained within the unit. Precautions should be taken when filling the cooling tank as the low temperatures can lead to burns if excessive amounts of the liquid come in contact with the skin.

The last safety consideration is the potential bursting of cryogen containers. After the system is used, air must be allowed to flow freely through the device or there must be a pressure release valve. If it is sealed, leftover cryogen in the system will eventually evaporate and could explode due to the pressure build-up. In addition, the container used to transport the liquid nitrogen must never be completely shut, or it must contain a pressure release valve, to avoid an explosion.

A detailed safety and procedural manual needs to be written, providing guidelines which if followed should prevent any injuries to the operator of the system.

Current Status and Future Aspects

Background research has led to a specific problem statement that has provided the framework for the generation of alternative solutions. Phase I development of the design was focused on solidifying an overall conceptual design for the device; ultimately the decision was to incorporate the spray mechanism and immersion tank into one functional unit. This overall unit was split up into three components; the immersion set-up, the spray mechanism, and cryogen cooling component. (Figure 14)

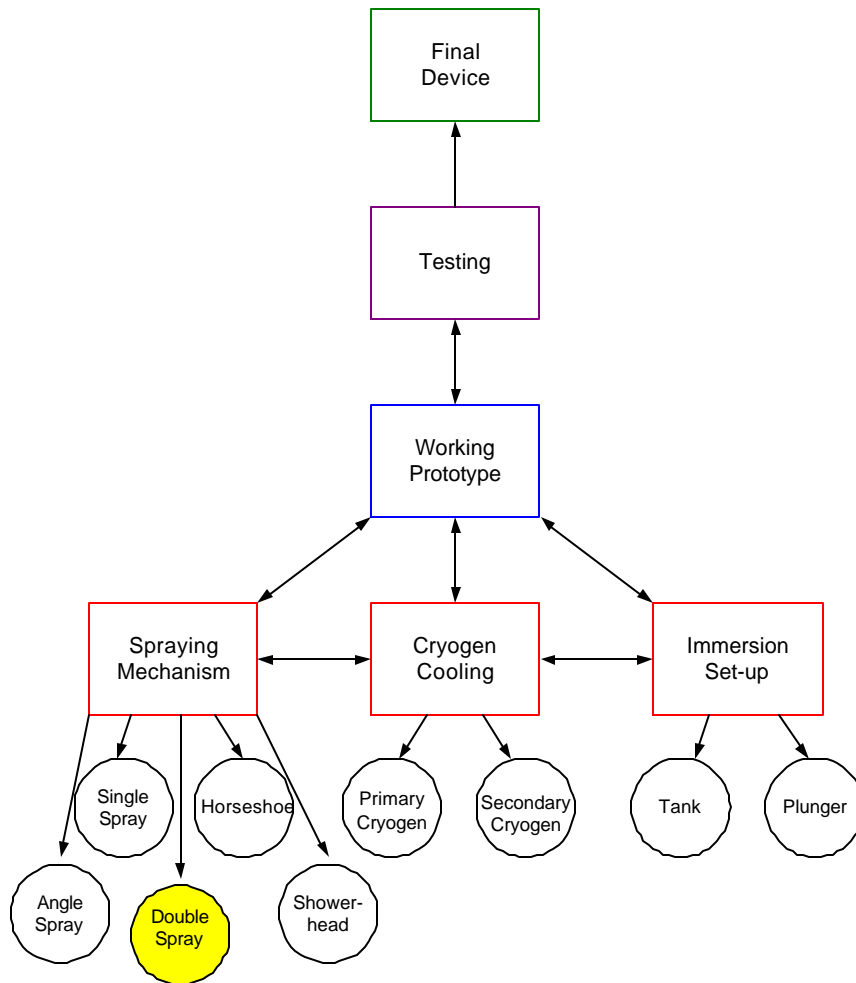


Figure 14: Overall Schematic of Design Development. The overall design was split up into three components: the spraying mechanism, the cooling component, and the immersion setup. Alternative design solutions for each of the different components were developed (Yellow indicates which selection was made). Evaluation of the components and selection of the optimal solution will lead to integration of components in a working prototype. This prototype will be tested and modifications based on performance will lead to the construction of a final device.

Phase II development was focused on generating solutions for each of the components. Several different designs were created and evaluated for the spray mechanism, which led to the recommendation of implementing an existing spray nozzle and fitting. Materials have been chosen for the construction of the cooling unit, being the nitrogen dewar flask, and the copper alloy tubing. Preliminary calculations related the amounts of primary and secondary cryogen needed. Further calculations correlating the heat transfer between the sample and the secondary cryogen are needed in order to obtain solid quantitative specifications. Once these numbers are known, determination of the size of the flask and length of the copper alloy tubing will be possible. Generation of alternatives for the immersion tank component is the next task

that will be focused on. An updated drawing was done on the current status of the design (Figure 15).

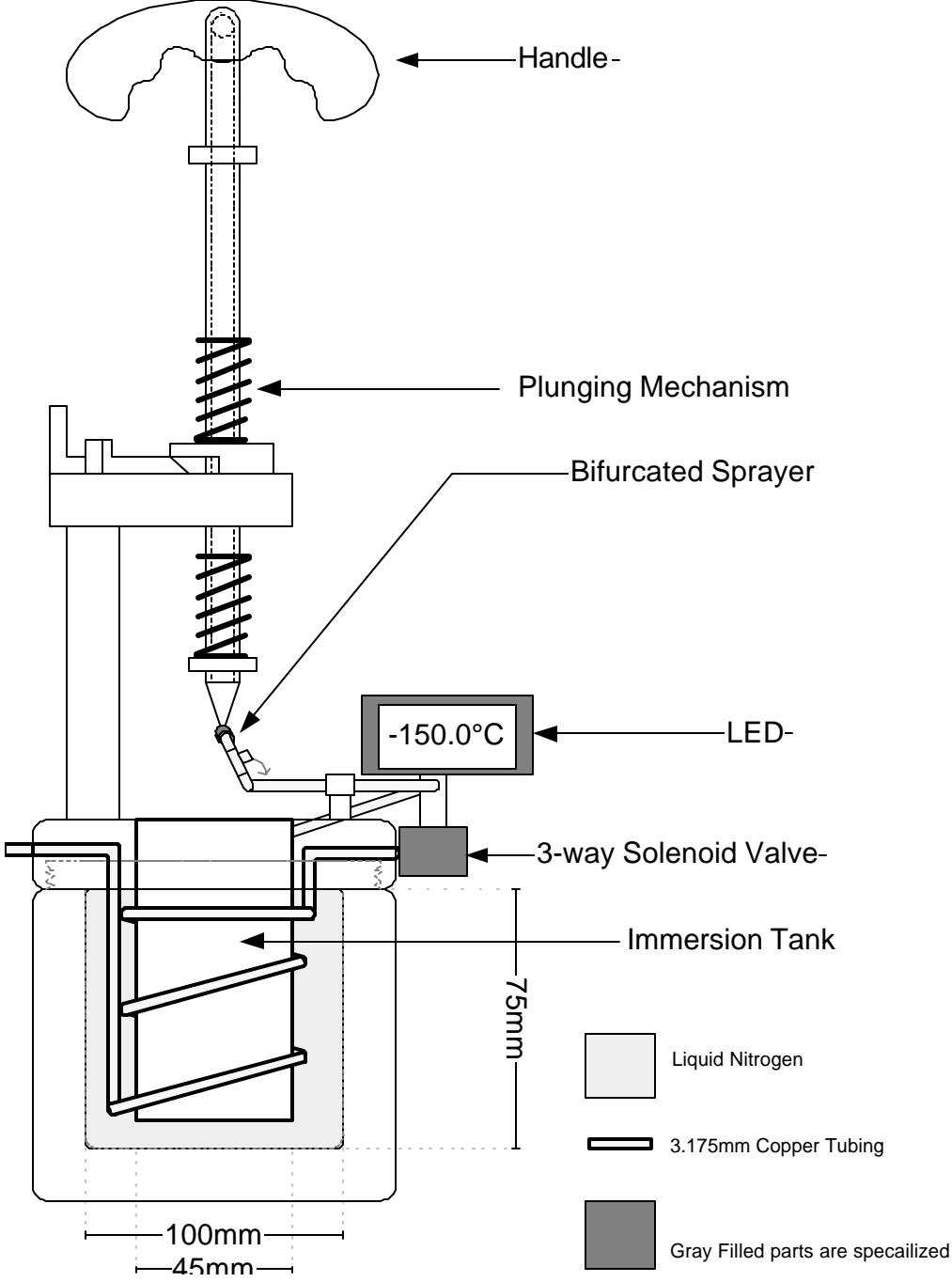


Figure 15: Current Design Drawing. Shows the double spray mechanism in conjunction with the plunging mechanism. The plunger is plunged into the immersion tank surrounded by copper tubing, which is insulated by a nitrogen dewar flask. A handle is provided to reset the plunger. A 3-way solenoid valve controls the flow of propane into the system. An LED display shows the temperature of the system, which should be -150°C.

Phase III development will be integration of the chosen design components and testing of the resulting prototype. This summer, preliminary calculations of the heat transfer of the total system will be completed. This will lead to finalized specifications for the cooling mechanism. Also, final determination of the immersion tank component will be completed allowing for the integration of all of the parts at the beginning of next semester. This summer a safety guidelines booklet will be compiled to account for the ethical issues involved with this project.

The beginning of the Fall 2001 semester will be focused on determining final dimensions for the overall integrated design leading to the construction of a prototype. By mid-semester, testing of the prototype should be underway allowing for modifications to be made in order to implement a final design by the end of the semester. (Figure 16)

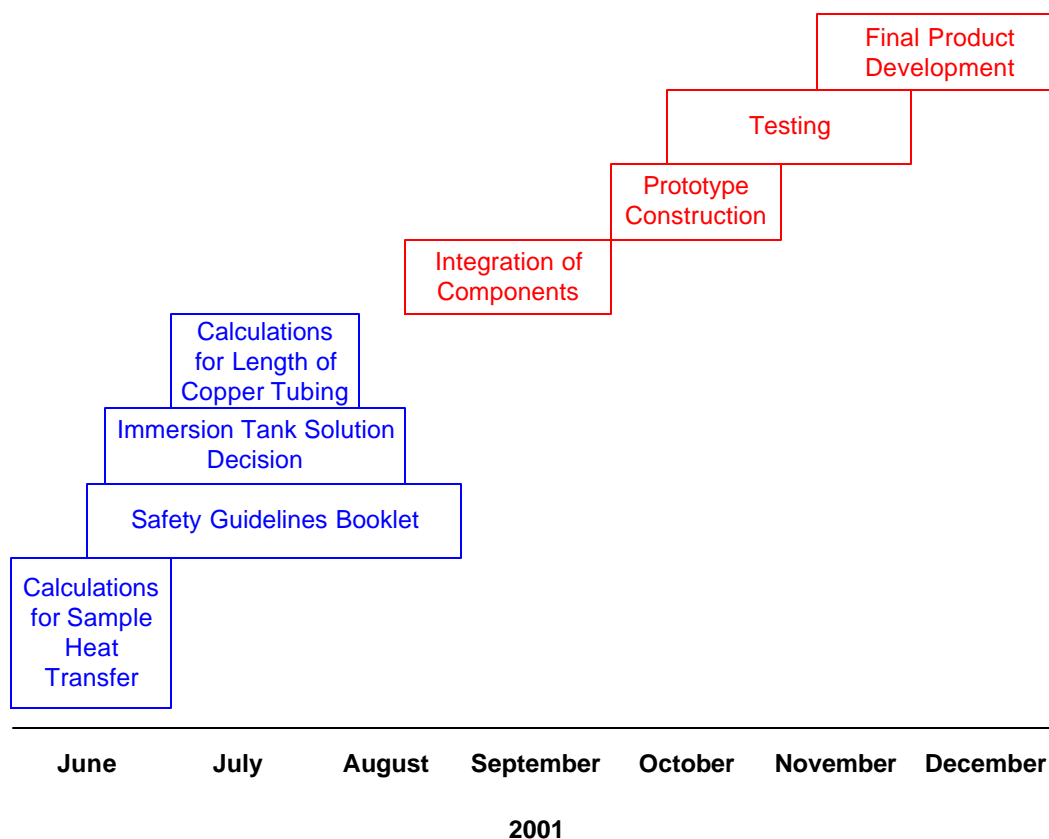


Figure 16: Timeline of Future Progress. This Gantt chart outlines the future goals and component deadlines in order to implement a final product by the end of the Fall 2001 semester.

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Appendix A

Product Design Specification

Cryogenic Freezing System

Version 4

April 24, 2001

Elan Bomstyzk, Gretchen Foltz, Marie Meyer, Kristy Wood

Problem Statement:

To design a device which is able to rapidly freeze biological specimens with maximum vitrification, while at the same time preserving the morphology of the mutant samples.

Currently, Professor Barry Ganetzky is involved in research focused on the synaptic connections in *Drosophila melanogaster*. Utilizing cryofixation techniques would allow the client to look at his samples using electron microscopy, thus improving the resolution from the confocal laser-scanning microscope he now uses.³ The commercial units available for this type of sample preparation are expensive, around \$10,000 and up per unit, due to the low demand and unnecessary features.⁶ The client believes modifications can be made to the conceptual designs of the commercial units and a personalized unit could be built in-house for a fraction of the cost. The personalized freezing unit is intended for preparing dissected *Drosophila melanogaster* samples. The device needs to include two different methods for freezing the flies; a spray mechanism and an immersion tank. The pressure and temperature of the delivered secondary cryogen needs to be regulated, so the sample is frozen quickly (~10ms) while still preserving its morphology. The selection of primary and secondary cryogens will be based on safety concerns and performance qualities.³

Client Requirements^{3,8}

- Rapid freezing of sample
 - Preservation of morphology
 - Minimal freezing artifacts
- Spray unit
- Soft budget of \$500
- Incorporation of immersion tank (tank size ~5cm diameter)
- Device should be able to be operated on a lab bench

Design Requirements

Physical and Operational Characteristics

A. Performance Requirements^{3,8}

1. Need to preserve the morphology in-vivo
2. Minimal freezing artifacts; vitrification (glass like solidification without the formation of crystals)
3. Extract the heat from the object as quickly as possible, because the quality of the result is determined by the speed of cooling.
4. Secondary cryogen needs to be cooled to -150°C, but not below – 187.678°C (freezing point of propane)
5. Plunging velocity should be 3m/s for optimal freezing of sample.

B. Safety¹⁰

1. Cryogen concerns

- a. Propane
 - 1. Flammable.
 - 2. Use less than 50ml of propane, because the dangers of combustion increase when this level is exceeded.
 - b. Liquid nitrogen
 - 1. Can freeze burn skin and eyes.
 - 2. Operational rooms should be ventilated, because high concentrations of nitrogen can be toxic when inhaled.
 - c. Explosions of copper tubing or dewar after usage.
 - 1. Leftover cryogen will evaporate if left in system.
 - 2. System must contain release valves to prevent pressure build-up and explosion.
 - 2. Transport and dispose of cryogens according to current handling guidelines: prevent explosions.
 - a. Never transport/store in a closed vessel.
 - b. Need a pressure release valve so transport vessel does not explode as cryogen vaporizes.
 - 3. Device user should wear goggles and gloves.
- C. Accuracy and Reliability
- 1. Minimal freezing artifacts.
 - 2. Rapid freezing time (less than 10 ms).
- D. Operating Environment
- 1. This device is intended to be used in a laboratory setting.
 - 2. The room will be well ventilated.
 - 3. Will be operating in a standard pressure and temperature setting.
 - 4. Will be used by trained laboratory techs and other researchers.
- E. Size³
- 1. Immersion tank size around 5cm in diameter to avoid need for cryogen stirrer (when a larger tank is used the cryogen temperature must be kept uniform with a mixing apparatus).
 - 2. Spray over 5mm square area to ensure entire specimen, which is approximately 1mm x 3mm, is completely covered with secondary cryogen.
 - 3. The thickness of the specimen is 150 μ m, but only the top 20 μ m needs to be frozen, because the nerve synapses lie above this level.
 - 4. Overall size should be minimized to increase ease of transport and decrease cost (i.e. materials and cryogen).
- F. Materials
- 1. Primary cryogens: liquid nitrogen
 - a. Used to cool secondary cryogen.
 - b. Immediately envelop the specimen or any warmer object with a thermally insulating layer of gas = Leidenfrost phenomenon; therefore cannot use directly on the specimen.
 - 2. Secondary cryogen (freeze specimen with direct contact): propane
 - 3. Liquid nitrogen dewar ~1L
 - 4. 2 spray nozzles
 - 5. Copper tubing for the secondary cryogen
 - 6. Flow valves
 - a. Control entering primary cryogen.
 - b. Control entering secondary cryogen.

- c. Make secondary cryogen go to either the immersion tank or the spray mechanism
 - d. Release valve at the end of the secondary cryogen tubing – most likely a solenoid
 - 7. Thermocouple – to measure the temperature of the secondary cryogen
 - 8. Spring-loaded plunger for the immersion tank
- G. Aesthetics, Appearance, and Finish

Production Characteristics

- H. Quantity
 - 1. One device is needed for professor Barry Ganetzky.³
 - 2. If the device can be built for a fraction of the cost of the commercial units, then it could be marketable for other laboratories.
- I. Target Product Cost; around \$500 (soft number; the initial amount that the client believes he can invest in the project; we want to stay away from using department money so that the client can have the finished product)³

Miscellaneous

- J. Standards and Specifications
- K. Ergonomics
 - 1. Specimen must be mounted and removed easily
 - 2. Spring loaded plunger must be easy to load
 - 3. Device must be light enough to carry
- L. Customer
 - 1. This product is being designed for Professor Barry Ganetzky
 - 2. Wants a variation of the commercially available freezing units, he believes if the device can be redesigned and built in house, so that the cost can be drastically reduced.
- M. Competition⁶
 - 1. The products on the market consist of the emersion tank component and run around \$10,000 per unit.
 - 2. Commercial companies advertising this type of product include: Leica Microsystems.

Appendix B: Glossary¹⁰

Allele: A member of a pair or series of genes that occupy a specific position on a specific chromosome.

Cryofixation: Solidification of a biological specimen by cooling with the aim of minimal displacement of its components.

***Drosophila melanogaster*:** The genus and species name for the fruit fly.

Freezing Artifact: Unwanted formation of ice crystals in a sample.

Morphology: The form and structure of an organism or one of its parts.

Neuromuscular Junction: A point that transmits an action potentials from the nerve to a muscle.

Phenotype: The observable physical or biochemical characteristics of an organism, as determined by both genetic make-up and environmental influences.

Primary Cryogen: The agent that cools the secondary cryogen.

Secondary Cryogen: The liquid that comes in direct contact with the specimen.

Synaptic Boutons: A knob-like enlargement at the end of an axon where it synapses with other neurons.

Synaptic Terminals: The end of the axon where release of the neurotransmitters takes place.

Vitrification: Glass-like solidification without the formation of crystals (i.e. amorphous structure).

Appendix C: Calculations^{1,2}

Energy released by cooling propane from room temperature (25°C) to spraying temperature (-150°C).

1. Cool propane from 25°C to -42.06°C.

$$\Delta H_1 = m \int_{25}^{-42.06} C_{p(\text{propane},g)} dT$$

$$C_p = 68.032 * 10^{-3} + 22.59 * 10^{-5} T - 13.11 * 10^{-8} T^2 + 31.71 * 10^{-12} T^3 \frac{kJ}{mol^\circ C}$$

$$\Delta H_1 = -4.429 \frac{kJ}{mol_{\text{propane}}}$$

2. Change propane phase from gas to liquid at -42.06°C.

$$\Delta H_2 = m(-\Delta H_{v(\text{propane})})$$

$$\Delta H_{v(\text{propane})} = 18.77 \frac{kJ}{mol}$$

$$\Delta H_2 = -18.77 \frac{kJ}{mol_{\text{propane}}}$$

3. Cool liquid propane from -42.06°C to -150°C.

$$\Delta H_3 = m \int_{-42.06}^{-150} C_{p(\text{propane},l)} dT$$

$$C_p = 0.58 \frac{Btu}{lb.^\circ F}$$

$$\Delta H_3 = -11.5487 \frac{kJ}{mol_{\text{propane}}}$$

Summation of the independent step enthalpies.

$$\Delta H_{total} = \Delta H_1 + \Delta H_2 + \Delta H_3$$

$$\Delta H_{total} = -34.748 \frac{kJ}{mol_{\text{propane}}}$$

Energy Balance based on 1 mol of propane

1. Change nitrogen phase from liquid to gas at -196°C

$$\Delta H_{\text{nitrogen}} = m(\Delta H_{v(\text{nitrogen})})$$

$$\Delta H_{v(\text{nitrogen})} = 5.577 \frac{kJ}{mol}$$

$$\Delta H_{\text{nitrogen}} = 5.577 \frac{kJ}{mol_{\text{nitrogen}}}$$

2. Amount of nitrogen needed per mole of propane

$$(\text{moles}_{\text{propane,g}})(\Delta H_{\text{total}}) + (\text{moles}_{\text{nitrogen,l}})(\Delta H_{\text{nitrogen}}) = 0$$

$$\text{moles}_{\text{nitrogen}} = 6.23 \frac{\text{mol}}{\text{mol}_{\text{propane}}}$$

Appendix D: Cost Analysis

Liquid Nitrogen Dewar Flasks⁵

Brand	Special Features	Inner Diam. (mm)	Inside Height (mm)	Vol. (L)	Price
Fisher-brand	Protective Aluminum casing, curved bottom to increase coolant capacity, vacuum tip-off tubulation minimizes space between bottom and magnetic stirrer	130	75	0.850	\$181.96
Nalgene	Unbreakable, chemical resistant, High-density polyethylene cover, double walls, heat leakage minimized by foam-filled annulus, polyethylene-coated steel handle, ribbed sides for safe handling	95	230	2.000	\$136.81
Nalgene	Unbreakable, chemical resistant, High-density polyethylene cover, double walls, heat leakage minimized by foam-filled annulus, polyethylene-coated steel handle, ribbed sides for safe handling	95	230	1.000	\$128.41
Fisher-brand	Protective Aluminum casing, curved bottom to increase coolant capacity, vacuum tip-off tubulation minimizes space between bottom and magnetic stirrer	80	75	0.350	\$125.37
Fisher-brand	Borosilicate glass with double silvered walls, space between walls evacuated and sealed, metal casing around glass to protect user from accidental breakage, vented polyethylene stopper minimizes evaporation	Not Listed	Not Listed	1.000	\$104.58
Fisher-brand	Borosilicate glass with double silvered walls, space between walls evacuated and sealed, metal casing around glass to protect user from accidental breakage, vented polyethylene stopper minimizes evaporation	Not Listed	Not Listed	0.600	\$104.58
Fisher-brand	Borosilicate glass with double silvered walls, space between walls evacuated and sealed, metal casing around glass to protect user from accidental breakage, vented polyethylene stopper minimizes evaporation	Not Listed	Not Listed	1.000	\$92.71

Copper Tubing⁷

Outer Diam. (in.)	Inner Diam. (in.)	Wall Thickness (in.)	Price/6-ft. Length
1/8	0.097	0.014	\$3.91
1/8	0.061	0.032	\$3.18

Valves⁷

Type	Pipe Size (in.)	Overall Length (in.)	Cv Factor	Min.-Max. Pressure (psi)	Temperature Range (°F)	Price
Three-way cryogenic solenoid	1/8	1 ¾	0.24	0-300	-320 to 356	\$112.07
Three-way cryogenic solenoid	1/8	1 ¾	0.35	0-225	-320 to 356	\$112.07

Spray Nozzles and Accessories⁷

Type	Pipe Size (in.)	Material	Min. – Max. Pressure (psi)	Price
Full cone spray nozzle	1/8	Brass	0 - 400	\$7.30
Square-pattern full cone spray nozzle	1/8	Brass	0 - 150	\$9.36
Right-angle full cone spray nozzle	1/4	Brass	0 - 100	\$31.37
Mini-mist nozzles (Full cone)	1/8	Brass	25 - 100	\$2.55
Nozzle fittings	1/8	Brass	0 - 300	\$11.40

**Highlighted products indicate the recommended purchase.