

Cryogenic Freezing System

Team Members:

Elan Bomsztyk, Gretchen Foltz, Marie Meyer, Kristy Wood

Biomedical Engineering Design 301
University of Wisconsin – Madison
February 17, 2001

Client:

Professor Barry Ganetzky
Department of Genetics
University of Wisconsin-Madison

Advisor:

Mitch Tyler
Department of Biomedical Engineering
Department of Rehabilitative Medicine
University of Wisconsin - Madison

Introduction

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

Problem Statement

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*. Cryofixation, would improve the resolution of the samples, allowing more information to be gained from the research. The commercial units available for this type of sample preparation are expensive, around \$10,000 and up per unit, due to the low demand. Some of these units are also multi-purpose and/or larger than needed, which increases the cost of the device. The client believes that modifications can be made to the conceptual designs of the commercial units and a personalized unit can be built for a fraction of the cost in house. The personalized freezing unit is intended for preparing dissected *Drosophila* samples. Delivering the cryogen via a spray mechanism would be optimal for preparing this type of sample. In commercially available units, the secondary cryogen is piped through a chamber containing a primary cryogen. The secondary cryogen is cooled and then sprayed directly onto the specimen, which may be mounted on a slide or a point. The pressure and temperature of the delivered secondary cryogen needs to be carefully regulated so that the sample is frozen as quickly as possible while still preserving the morphology. The selection of primary and secondary cryogens will be based on performance qualities and safety concerns.

Background Information

Current Research

Professor Barry Ganetzsky 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 the 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 of use exhibit particular characteristics. These mutants show a disturbance in development and morphology of synapses between an incoming motor axon and the neuromuscular junction. These 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.

The different alleles found in a gene, Gamma-3, 90-3, Gamma-3/Df, Gamma-3/90-3 and Gamma-4/Gamm-7, (Figure 1) are of interest because it is hypothesized that these wild types of synaptic terminals are involved in the development of the synapses. Comparing the mutations to 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.

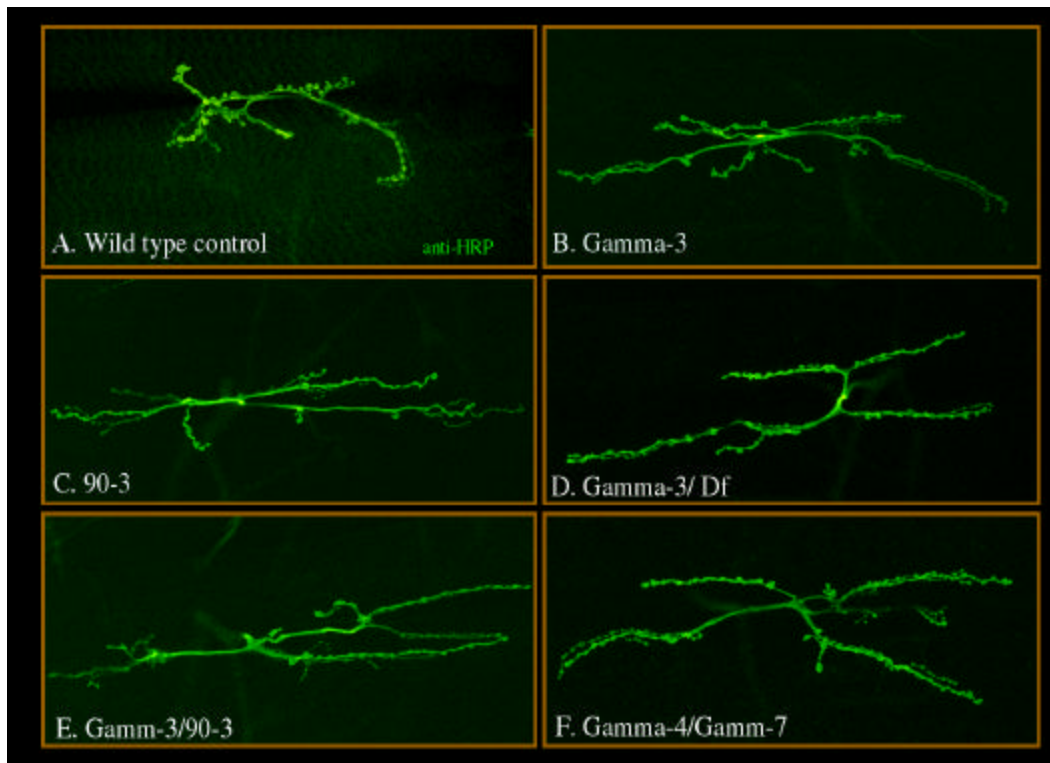


Figure 1: Confocal Images of Mutant and Wild Type Synaptic Terminals. The wild and mutant type alleles are shown. The green signals are extended anti-HRP that indicates synaptic branches and an increased number of synaptic butons.

A phenotypic analysis on the mutants should be done in order to culminate a definition that is as precise as possible to determine the affects of the mutant on synaptic morphology.

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 clearly see the mutant when observing it under a microscope.

Dissection Technique

In order to dissect *Drosophila*, 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. 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 is all that remains. The fly must be continuously bathed in a calcium saline solution.

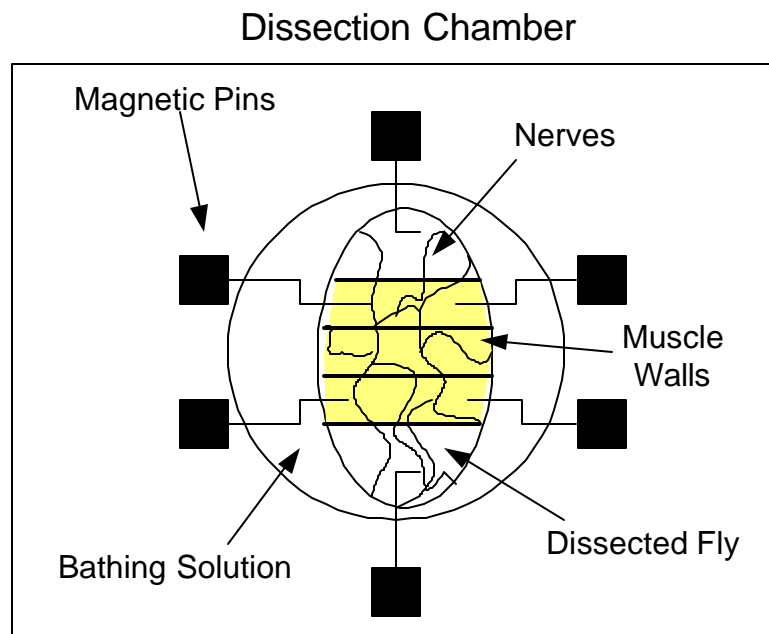


Figure 2: Dissection Chamber Used for *Drosophila*.

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 fixative measures need to be taken in order to use the electron microscope. The electron microscope is needed in order to increase the resolution of the synapse, thereby advancing the research.

Cryofixation is the solidification of a biological specimen by cooling with minimal displacement of its components. (REFERENCE) The most common method of cryofixation uses a primary and secondary cryogen. A cryogen is liquefied air used as a coolant. 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. (FIGURE) The liquefied secondary cryogen is then used to vitrify the sample.

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

Product Design Specifications

Commercially available units designed for rapid freezing are very expensive. 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 particular client's needs, the performance might be superior to commercial devices designed for more general uses.

The key requirements outlined by the client include:

- 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

The overall design was split into three components: the immersion tank, the spray arm and the secondary cryogen cooling mechanism. By varying these components, three designs were created which include an immersion device, a sprayer device, and a dual workstation which permits the use of both techniques.

Immersion Device

The device freezes samples by plunging them into a cryogen. Using suction, the sample is mounted on the point of the plunger (Figure 3). Which 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 easy. 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.

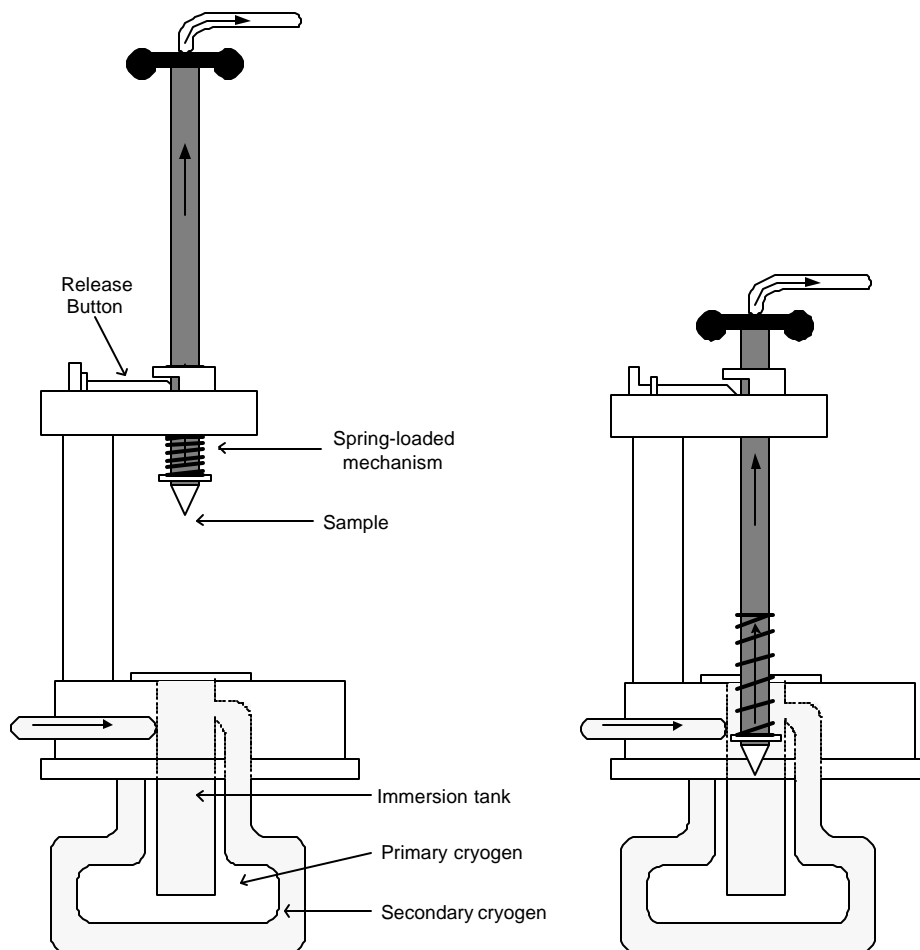


Figure 3: The Immersion Device in ready and activated position.

The immersion device will effectively freeze small samples, such as the head of *Drosophila*. According to the literature, this method will be less effective in rapidly freezing an entire body sample of *Drosophila* (REFERENCE CLIENT AND BOOK).

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 in exactly the same manner as 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 be useful to angle the nozzle because of gravity. This would allow the distance between sample and nozzle to remain close while decreasing the initial velocity of the spray. The nozzle of the device could have a single opening, or many. In addition, if one nozzle does not adequately encompass the entire sample in cryogen, multiple nozzles could be added to the design. A remote valve could be used to control the spraying duration.

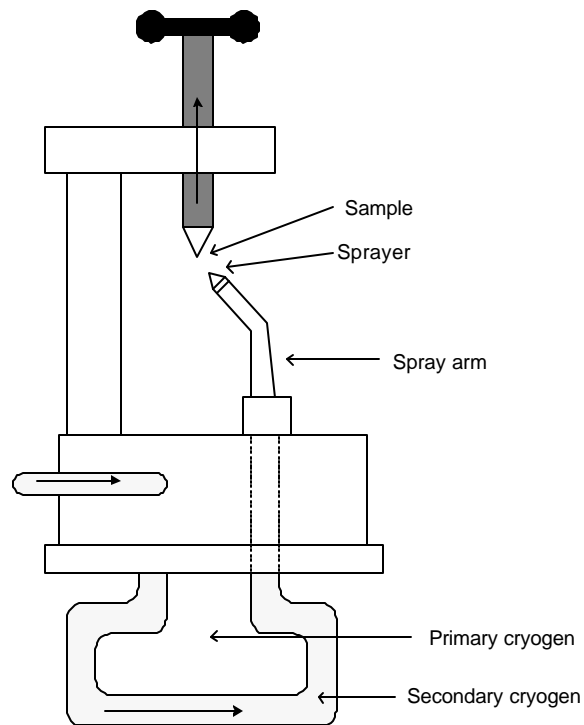


Figure 4: The Sprayer Device.

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

Combination Device

The plunging and spraying techniques have different advantages for different types of samples. Since both of the previous designs have so many similarities, combining them into a single multi-purpose device could be simple. To do this a rotating spray nozzle must be used to clear the path of 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. A valve could be used to shift the flow of the secondary cryogen from the plunger to the sprayer.

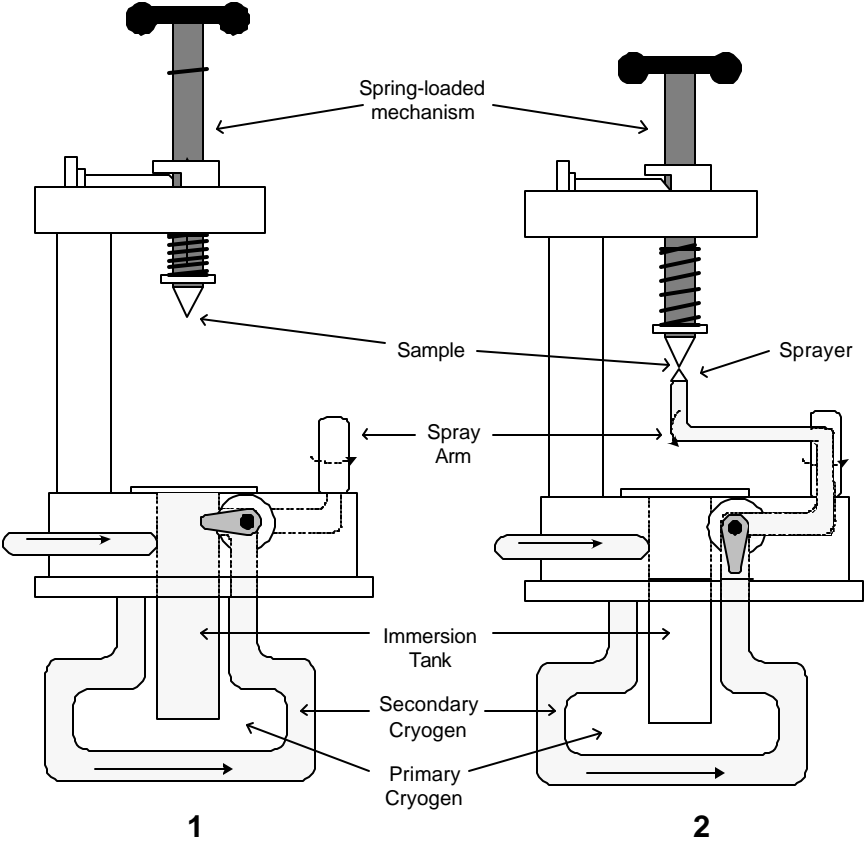


Figure 5: The Combination Device.

Calculations/Engineering Analysis Criteria

Immersion:

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

Spray:

- Droplet size
- Scatter pattern
- Pressure
- Temperature
- Flow rate of secondary cryogen through primary cryogen

Cryogen Selection:

- Performance
- Safety
- Cost

Conclusion

Currently, background research has led to a specific problem statement that has provided the framework for the generation of alternative solutions. The overall unit was split up into three components; the immersion tank, spray, and cooling components, which can be mixed and matched to form the different alternatives to create an overall design which fits the clients requirements.

Evaluation of alternative designs will potentially lead to the development of a modified cooling unit. Once a general design is selected, specific calculations and analysis can be applied so that the device is specifically tailored to the clients needs. Exact detailing of the design will lead to the building of a prototype, for testing, and eventually a functional unit.

References

Glossary

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

Cryofixation:

Morphology:

Neuromuscular junction: a point that transmits an action potential from the nerve to the muscle.

Phenotype:

Primary cryogen:

Secondary cryogen:

Synaptic boutons:

Synaptic terminals:

Vitrification: glass-like solidification without the formation of crystals.