

**PHYSIOLOGICAL EFFECTS OF RHYTHMIC MELATONIN ON
PERIPHERAL CELLS IN A SUPERFUSION SYSTEM**

A Senior Scholars Thesis

by

DIANA HONG TRAN

Submitted to the Office of Undergraduate Research
Texas A&M University
in partial fulfillment of the requirements of the designation as

UNDERGRADUATE RESEARCH SCHOLAR

April 2007

Major: Industrial Engineering

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Approved by:

Research Advisor:

Associate Dean for Undergraduate Research:

Vincent Cassone

Robert C. Webb

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ABSTRACT

Physiological Effects of Rhythmic Melatonin on Peripheral Cells in a Superfusion System (April 2007)

Diana H. Tran
Department of Industrial Engineering
Texas A&M University

Research Advisor: Dr. Vincent Cassone
Department of Biology

The immediate effects of melatonin from the pineal gland on peripheral cell types such as astrocytes, hepatocytes, and cardiac myocytes are relatively unknown. My research includes designing a superfusion or flow-through system that constantly pumps medium to light-entrained, chick pineal cells. The medium from the pinealocytes, enriched with pineal-specific secretions, will be directed to the above target cells, which are simultaneously housed in constant darkness. Radioimmunoassay for pineal melatonin and ELISA on the target cells' secretions examines the effects of melatonin ex-vivo. This study will shed light on the direct effects of pineal melatonin on the central nervous system and peripheral organ cell types.

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CHAPTER I

INTRODUCTION

The pineal gland in birds is the master circadian pacemaker, keeping an approximate 24-hour rhythm in biological functions. The pineal cells secrete melatonin, a process that is light and clock dependent. The dominant Zeitgeber, or ambient cue, for the avian pineal gland is a light:dark (LD) cycle since the pinealocytes are photoreceptive (Bell-Pedersen et al., 2005). Melatonin is a small, non-polar hormone that can diffuse through the plasma membrane. It is secreted rhythmically whether in vivo or in vitro, suggesting that the pineal gland contains endogenous circadian oscillator properties (Gwinner and Hau, 2000). Another structure that influences the circadian rhythm is the suprachiasmatic nucleus (SCN). The pineal gland produces melatonin at night, inhibiting the SCN output until the subjective day. In turn, the SCN is active during the day in birds, restricting melatonin biosynthesis to occur only at night.

Melatonin also affects peripheral tissues, though their effects have not been extensively investigated. To explore this question, the study is divided into two phases: (1) The Superfusion System and (2) The Effects on the Peripheral Cells. Phase I concentrates on designing and implementing a dispersed cell-perfusion system for two different cell types. Previous studies have used co-cultures of pinealocytes entrained to a LD cycle to confer rhythmicity with astrocytes in regards to metabolism and some clock genes (Adachi et al., 2002). Other previous data were acquired through exogenous melatonin

administered in a cyclic fashion to yield the same result. The limitation on these earlier culture systems is that melatonin levels never reach baseline since the media is stagnant cells (Earnest and Cassone, 2005). Moreover, these systems used whole tissues instead of. Dispersed cell flow-through systems have also been designed for a single cell type (Horvath et al., 2006). The design of this system is unique in the fact that two different cells will co-exist in the system with constant media flow. This attempts to reduce melatonin levels to baseline when light is present and mimic in-vivo secretions of the pineal gland.

The previous data relating melatonin to astrocytes rhythmicity identifies melatonin as a causative agent in cell regularity. Phase II of this study explores if the effects of melatonin on peripheral cells are the same. Melatonin's ability to quickly diffuse through cell membranes may allow it to act as a scavenger for free radicals, although most of its physiological effects have been attributed to three 7-transmembrane domain receptors, Mel_{1A}, Mel_{1B} and Mel_{1C} (Gwinner and Hau, 2000). Though many of the physiological effects of melatonin in vertebrates are known such as skin color changes, thermoregulation, aging, and puberty, these effects have not been established in birds (Gwinner and Hau, 2000).

The detection of the amount of melatonin secreted is tested using radioimmunoassay (RIA). A measured amount of antibodies to melatonin is added to media containing secreted melatonin, binding to the hormone. A radioactively labeled hormone is then

added to attach to the unbound antibodies. This radioactive signal can then be used to quantify the amount of melatonin in the sample; the higher the radiation, the lower the amount of melatonin.

The secretions from the peripheral cells will be examined using Enzyme Linked ImmunoSorbent Assays (ELISA). ELISA uses an antigen that binds to different cell products such as cytokines, antibodies, hormones, and different proteins. The product in question will be immobilized on a surface, allowing for unbound products to be removed. Antibodies linked with an enzyme will then attach to the antigen-antibody complex. A substrate is added that reacts with the enzyme to produce a detectable product, usually a color change or fluorescent light. This allows the product to be quantified.

Other effects that will be examined include glucose metabolism using C-14 labeled 2-deoxyglucose (2-DG) uptake. 2-DG is a synthetically manufactured glucose analog that competes with endogenous glucose for transport into the cell. It can be taken up by the cells, but not metabolized completely, allowing the uptake to be quantified. Exposure to rhythmic melatonin has been shown to affect astrocyte metabolism (Adachi et al., 2002).

This thesis follows the style of *The Journal of Biological Rhythms*.

CHAPTER II

PROBLEM

Phase I: Superfusion System

Phase I is the design and implementation of a dispersed cell flow-through system for two different cell types. This design is intended to be used to investigate Phase II. The goal is to maintain healthy cell growth for both the pinealocytes and the peripheral cells in the wells while collecting secretions for later analysis. Constraints for this design include maintaining a sterile environment in a controllable environment (37° C, 0.275 CO₂, 5.5 Air) with the ability to entrain chick pineal cells under LD cycles. The target cells must uniquely housed to minimize the potential effects from photosensitive cells as in the case for diencephalic astrocytes. Therefore, the peripheral cells are staged so that light has no effect. The entire system must deliver media compatible to all cell types at a constant rate without causing cells to lift off the surface.

Phase II: The Effects on Peripheral Cells

Evidence concluding that melatonin promotes cell rhythmicity from previous studies on astrocytes shows the relationship between melatonin and cell metabolism and some clock genes. Phase II explores if this relationship expands to peripheral cells such as cardiac myocytes, hepatocytes, etc. The superfusion system will be used to answer this question. The media collected from the flow-through system will be analyzed for

melatonin release and then cell secretions such as cytokinins, hormones, and other proteins. Non-treated target cells act as the control group.

CHAPTER III

METHODOLOGY

Generalized Design Features

The cell flow-through design phase incorporated many design alternatives. All systems consist of an initial pump to circulate media, a first stage consisting of the pinealocytes, a second stage housing the target cells, and a fraction collector to retain samples for future analysis (Fig. 1). The stages must culture the cells in six individual collection wells, which were the major design differences among the solutions.

The cell flow-through system major goals are the following:

1. Feed the pinealocytes and peripheral cells media at 250 uL/hour
2. Maintain the temperature at 37 °C
3. House the cells in a habitable environment contained in customized collection wells for feeding and collecting

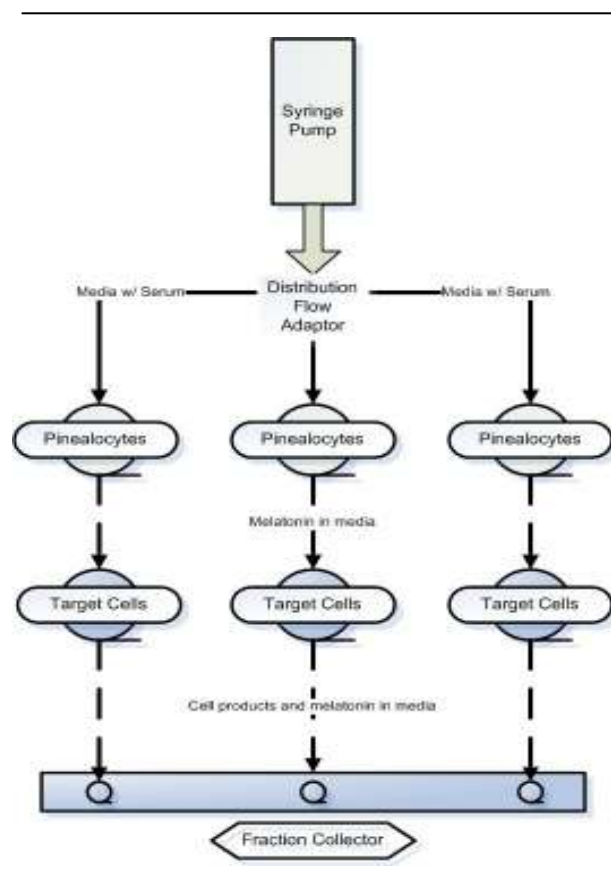


Figure 1. A generalized view of the flow through system that consist of Stage 1 for the pinealocytes and Stage 2, which houses the target cells.

4. Be able to remove cells after testing for RNA analysis
5. Be able to test six different samples simultaneously
6. Be able to collect 6 concurrent samples every hour
7. Maintain two different stages of cells at two different light environments
8. Eliminate biological adherence to all system materials
9. Preserve constant flow with no loss in volume

Implementation and Test

The implementation and testing of the different design alternatives included an initial run with only stage 1 cells, the pinealocytes (Fig 2). A successful run is characterized by the ability of the pinealocytes to entrain to a LD cycle. This is represented by a RIA analysis of the samples showing rhythmic melatonin release is maintained for 3 days. This initial run will allow the isolation of design problems before the stage 2 cells, the peripheral cells are integrated into the system. Six

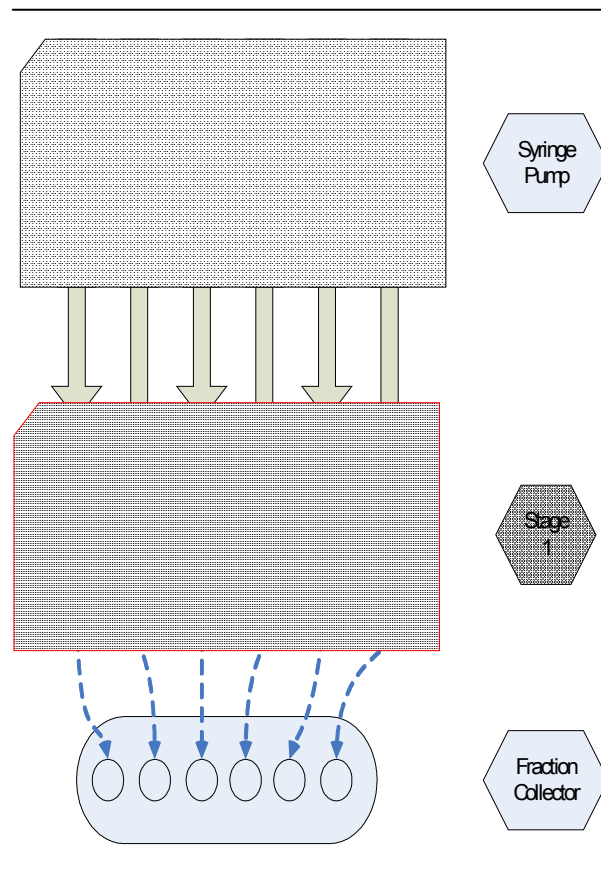


Figure 2. A generalized view of the initial run that show only Stage 1 (the pinealocytes) incorporated.

collection wells independent of the system with pinealocytes maintained in LD cycles are the control groups.

During setup, all design components are thoroughly sterilized for placement in a 37 °C incubator. Sterilization techniques for all non-autoclavable material included (1) clean with 10-70% bleach-ethanol (2) clean with 70% ethanol-water and (3) UV light for 20 to 30 minutes.

The Media Pump

In all the design solutions, there must be a force-generating component to maintain the flow of media throughout the system. The initial designs incorporated peristaltic pumps.

In the testing their accuracy and precision, several problems resulted including uneven flow among the tubes, tubing instability within the pump, and inaccurate flow at 250 uL per hour. Calibration tests were done with both water and media through three types of tubing material at different sizes. The major problem was that the peristaltic pumps could not maintain several channels of media at a low volumetric flow of 250 uL per hour. This led to the decision to use a different type of pump, the syringe pump.



Figure 3. The original Harvard Apparatus media pump could only handle three 60 mL syringes at once.

Originally, the cell culture system was designed to have three separate lines, dividing out to six plates. However, in the testing phase, the y-dividers for the tubes were failing. The y-dividers accurately divide the flow only when the pressures are equal for both sides. However, when air is caught in the system, they create bubbles that disrupt the flow. Different techniques were used to try to eliminate the air bubbles including long priming periods, different y-divider angles, and different tubing sizes; all of which did not eliminate the problem. So, the flow-through system was redesigned to flow 6 separate lines from the pump. However, the bottleneck became the current syringe pump, which could only handle up to only three 60 ml syringes (Fig 3).

After calling Harvard Apparatus, the manufacturer, it was determined that the pump could be modified to accommodate up to ten 60 ml syringes. Since the modifications were going to take up to 3-weeks if outsourced, the syringe pump was refitted at the Biology Dept. Instrument Shop (Phil Brand, personal communication). With the help of technical engineers at Harvard Apparatus, a new syringe holder, push bar, and stabilizer base were installed (Noah Strickland, personal communication).

The Tubing

The next step addressed the type of tubing to be used to carry the media to the different stages. The tubing needed to be gas-permeable, flexible, and designed for biological flows. Since the media would contain proteins and other secretions, the tubing material

should not adhere to these biological substances. Therefore, PharmMed© tubing (Tygon) was chosen for the system.

The tubing was also tested with the different collection wells and the fraction collector for even flow using gravitational and mechanical force. Two different conclusions were made. One addressed the tubing diameter. When the diameter was less than 1/8" ID, the capillary action pulled out the media from the wells without the need of external forces, but the capillary action also kept the media from draining out of the open end into the fraction collector. The other conclusion involved the tubing thickness. The thicker PharmMed tubing, which was 1/8" thick, interfered with the gravity flow. The inflexibility of the tubing due to thickness created an uneven flow to the fraction collector. Therefore, the best size for the tubing was 1/8" ID by 3/16" OD in systems where gravitational pull was necessary. When extraneous forces such as the syringe pump was used through the entire flow-through, the effects of ID and material thickness were insignificant.

The Fraction Collector

In all the different designs, the media and cell secretions are collected from the collection wells through the tubing into a modified fraction collector. The original fraction collector had the capacity to take only one sample at a time. The machine was taken apart to install a customized arm to collect six samples simultaneously. The first arm created was made out of plexi-glass with six holes to hold the straight-barb

connectors. These connectors act to consistently place the tubing directly over the test tubes, especially during movement. However, plexi-glass proved to be too fragile during runs, causing the arm to crack and become imprecise. Stainless steel replaced the plexi-glass in the final design.

To make room for the straight connectors and lessen the tension on the tubing, a cushion bar was attached to the arm. This both increase the distance of the arm from the fraction collector panel and to decrease the distance from the connector to the test tubes. Due to measuring difficulties, three different designs were created to ensure precision collection in the designated test tubes. The installation screws that were in the original fraction

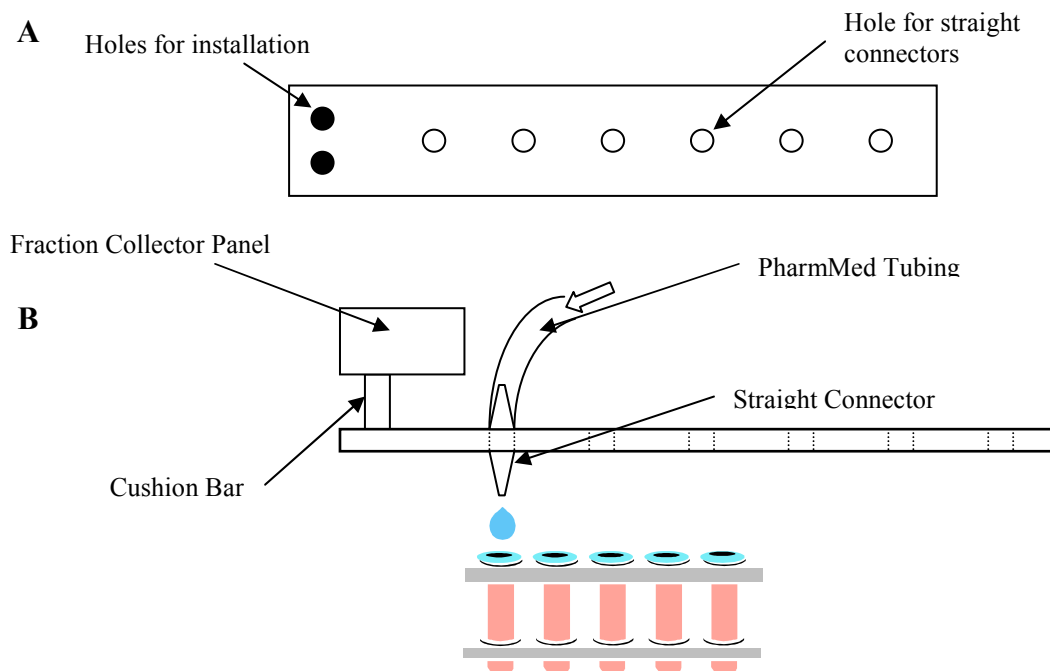


Figure 4: This stainless steel customized arm for the fraction collector consist of a cushion bar and the arm itself. The (A) top view shows how the holes were drilled and the (B) side view shows how the arm attaches to the fraction collector.

collector could not handle the weight of the customized arm. So larger holes were directly drilled into the panel to maintain the stability of the arm.

The key design differences among the solution alternatives are the collection wells and their incorporation into the system. Three different alternatives were developed through Phase I, and they are discussed in detail below.

Flow-through System A Design

The Collection Wells

This design consists of a customized BD Falcon Tissue Culture Plate with 6 wells (Fig. 5). To feed in the media from the syringe pump, six 21-gauge needles are heated via a Bunsen burner and inserted into the lid. The six feeding needles were cut to 0.5" so

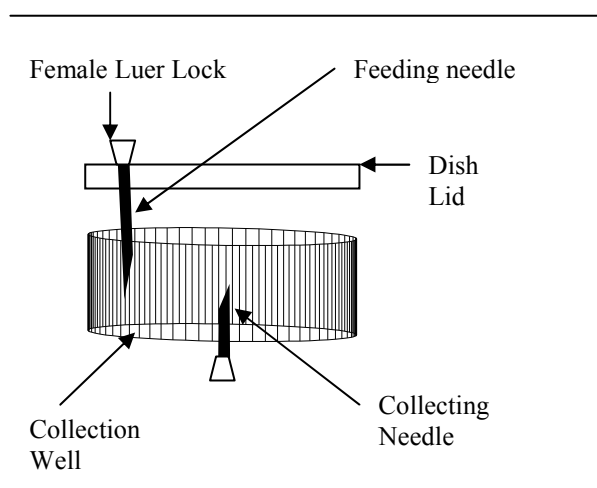


Figure 5. The collection well for Design A shows two 21-gauge needles maintaining the volume within the collection well.

the lid could fit over the plate. Six 21-gauge needles were also heated and inserted onto the bottom of the well to collect the media. They were set with superglue on the exterior side after insertion. The needles were cut at a height to only collect media when the volume was above 3 mL. Theoretically, if the media is fed at 250 uL per hour, the collection should also be maintained at 250 uL per hour. This system is

designed to feed with mechanical force from the syringe pump and collect with gravitational force. The needle length calculations are shown as follows:

Surface Area of Well Bottom: 9.6 cm^2

Volume of Cylinder: $r^2 \pi h$

where r = radius of circle, h =height of cylinder

Radius of cylinder = 1.748 cm

Volume needed of media= 3 mL = 3 cm^3

$= (1.748)^2 \pi H$ where H =height of needle

Height of Needle necessary = 0.3125 cm

The Connectors

In order to incorporate these collection plates into the system, the tubing discussed earlier was user in conjunction with different connectors. Since the syringes already contained female luer locks, different types of luer locks were used as connectors (Fig

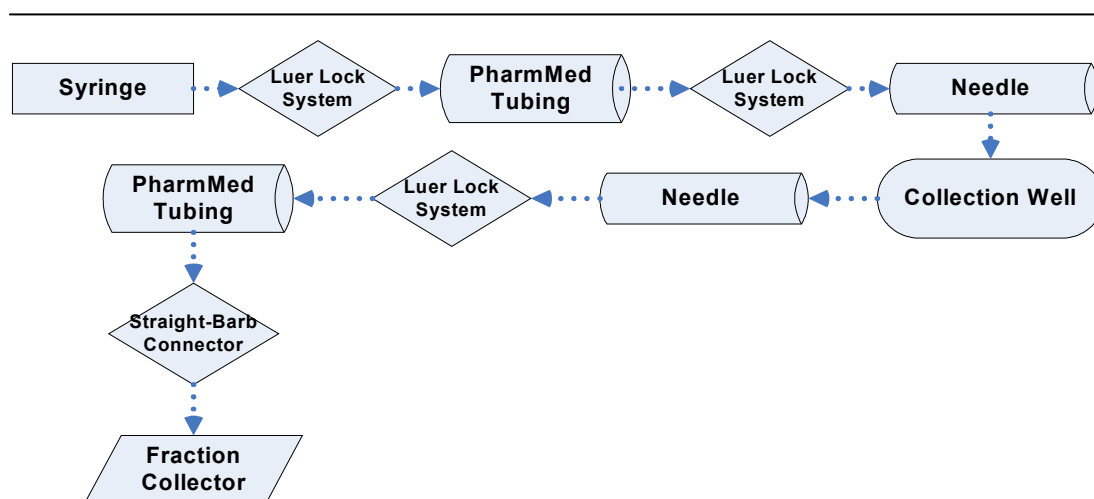


Figure 6. This flow chart shows the flow of the media throughout all the components of Design A.

6). The connectors were tested to make sure there was a tight fit with the tubing, and they did not interfere with the flow. In larger diameter tubing, the luer lock tip did create flow interference by creating back pressure (Fig 7). This was due to the space between the nozzle tip and the tubing walls. This was the reason why 1/8" ID tubing was chosen, creating a balance between unwanted capillary action and lowered backflow.

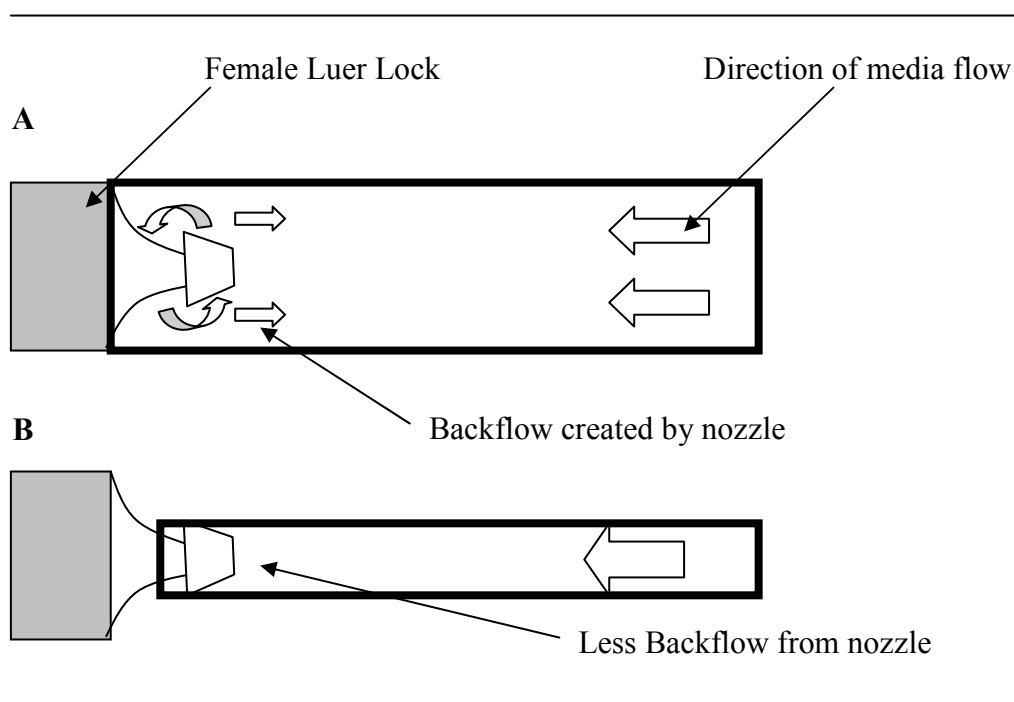


Figure 7. The backflow created by the luer lock system created great disturbance in gravitational pull designs. In larger tubing (> 3/16" ID), the nozzle interfered with the flow (A), unlike in smaller tubing (1/8" ID) where the backflow is reduced (B).

Discussion

After the initial run, several system problems were identified for redesign.

1. Uneven collection flow
2. Collection well contamination
3. Overheating of syringe pump and fraction collector
4. Collection well leakage
5. Volumetric problems within collection wells

The uneven collection flow was largely due to a setup error. During the routing of the tubes from stage 1 to the fraction collector, zip-ties were used to bind the tubing together. The effect was an upward bend of the tubing, forcing the media to flow against gravity. Without enough pressure, the melatonin-containing media settled in the tube for long periods of time until adequate pressure pushed the media to the fraction collector.

Upon completion of the system, the wells were removed, and contamination of the cells was apparent. Three different designed elements factored into this problem. One was the possibility of superglue contamination that interfered with cell growth. Second, the PharmMed tubing put great tension on the luer locks at the bottom of the well; this fractured the tight tolerance between the needle and the plate, causing leakage. Third, since the plate was created in an open environment, the sterilization technique was not adequate to eliminate contamination.

The overheating of the machines could not be helped. Due to the temperature of the incubator and long-term testing, the pump was too hot to be touched. However, syringe flows and collection movements were still accurate; therefore, this problem does not interfere with the major goals of the system.

During the run, there was the realization that the volume within the collection wells was too large. This steadily elevated the melatonin in the well since collection speed was not adequate to obtain all the melatonin released within that time period. This did not meet the requirement to maintain rhythmic release where melatonin levels reached baseline.

RIA analysis of the sample collection showed only 11.5% binding efficiency, well under the optimal 20%. This made the data unacceptable, but it did address a major point. The cells did survive for some time since melatonin was detected in the samples. Melatonin was being released, and system's flow material such as the tubing was not adhering to the different substances.

Design Modifications

The idea of flowing from the side has also been tested and addressed. The concern with inserting a needle at a right angle with side is the high volume required to initiate collecting flow. Another problem with this side-insertion design is the constant need to re-prime the system after adequate pressure has been reached. When a hole is punctured from the side of a liquid container, a certain amount of liquid above the hole is required

to create adequate pressure to push liquid out. The hypothesis was that needle diameter was controlling this required pressure. Larger needles such as 14-gauge needles failed to reduce the required volume. The reason is that the efflux speed is not dependent on the radius of the hole but dependent on the distance of the liquid level from the hole. This is based on Bernoulli's equation of conservation of total mechanical energy for ideal fluid flow. This equation and the efflux velocity calculations are highlighted below:

Bernoulli's Equation

$$P_1 + \frac{1}{2} \rho v_1^2 + \rho g y_1 = P_2 + \frac{1}{2} \rho v_2^2 + \rho g y_2$$

- P= pressure at two points along the flow
- ρ = density of the fluid
- v= flow speeds at the two pressure points
- g= gravity
- y= height of these two pressure points above a chosen reference level

Efflux Velocity Calculations

$$v_{\text{efflux}} = \text{sqrt}(2gD)$$

- D=distance from surface of liquid down to hole
- Assumptions:
 - $P_1 = P_2$ (both at atmospheric pressure)
 - $v_1 \cong 0$ since $\text{area}_{\text{well}} \gg \text{area}_{\text{hole}}$
 - $y_1 - y_2 = D$

Another design solution consisted of pushing the needle upward at an angle from the side of well. The same problem appears, though the volume necessary to create adequate pressure is much less. Accuracy is the major problem with this design concept. With the available resources, the needles cannot be accurately inserted a certain angle to a certain depth for each plate. This brings a new design model, the use of in-vitro fertilization (IVF) plates.

Flow-through System B Design

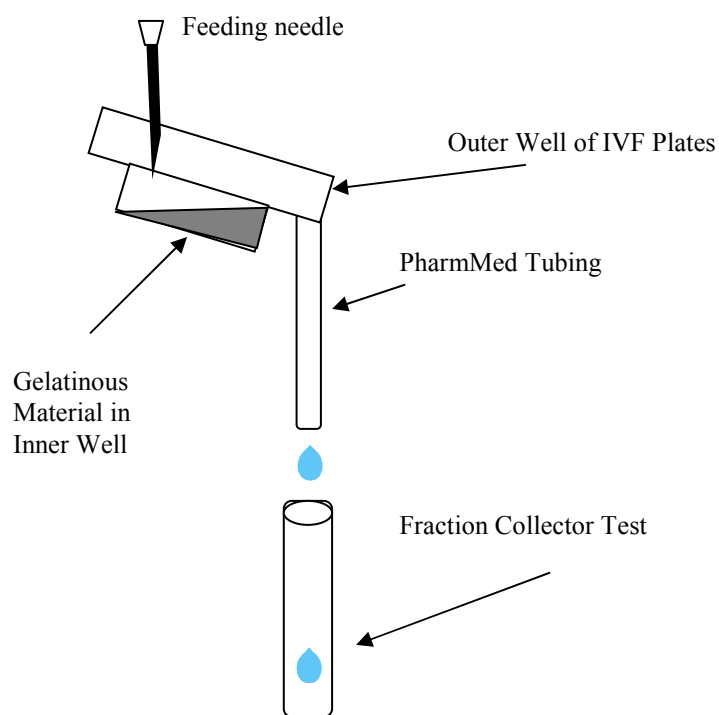
Collection Wells

Design B eliminates the use of the needles from the collection well to the fraction collector while still using the syringe pump to force media through the feeding needle.

This design proposal uses the inner well of IVF plates to house the cells and media.

When a certain volume is reached, the inner well overfills, spilling into the outer well (Fig 8). The media is collected through a hole at the bottom of the outer well, leading to the fraction collector via tubing. The (IVF) plates are slightly tilted ensure complete

draining of the outer well.



To maintain a 0.5 mL volume, the inner well is filled with a gelatinous material acting as the substratum for the cells. This gelatinous material must retain its semi-solid state at a 37 °C, be pH compatible to the cells and media, and act as a growth attachment site not as growth interference.

Figure 8. Design B uses IVF plates and a gelatinous coating to eliminate the need for needles for the collection flow.

Discussion

In the trial phase of this design, Type A gelatin (300 bloom), Type B gelatin (225 bloom), and 1% agarose was tested for compatibility with incubator temperature and cell pH. The gelatinous material is created by mixing the powdered gelatin to ddH₂O at certain percentages over heat. The mixture is autoclaved and then added to the inner well at 5mL per plate. During the design test phase, the plates were tested with Litmus paper and then placed into an incubator to determine melting temperatures. Both gelatin A and B had melting temperatures below 35° C when the % gelatin fell below 23% and were too acidic for cell growth (Table 1).

Type	% Gelatin (by weight)	Melting Temperature	pH	Description
A	10%	<37° C	5	Liquid
A	12%	<37° C	5	Liquid
A	23%	>37° C	5	slightly gel-form
Agarose	1%	>37° C	6	gel-form
B	10%	<37° C	5	Liquid
B	12%	<37° C	5	Liquid
B	23%	≅37° C	5	slightly liquid

Table 1. Initial tests showed that the best gelatin component able to withstand incubator temperature was either Type A or 1% agarose. The description describes the state of the coating after 24 hours at 37° C.

Type A, chosen since the bloom was higher, and 1% agarose were further tested with added sodium hydroxide during the mixing to increase the pH to 8; after coating the inner wells, 1 mL of media with serum (OK+SS) was added over the solidified gelatin.

After 24 hours, the test showed that 1% agarose with sodium hydroxide was the best coating solution (Table 2).

Sample Number	Gelatin Content	Media w/ Serum	NaOH	Description	pH
1	25% Gelatin A	1 mL OK+SS	0	Liquid	5
2	25% Gelatin A	1 mL OK+SS	1 drop	Liquid	7
3	1% Bacteriological Agarose	1 mL OK+SS	0	gel-form	6
4	1% Bacteriological Agarose	1 mL OK+SS	0	gel-form	6
5	1% Bacteriological Agarose	1 mL OK+SS	1 drop	gel-form	8
6	1% Bacteriological Agarose	1 mL OK+SS	1 drop	gel-form	8

Table 2. Further tests indicate that 1% bacteriological agarose is the best gelatin base to withstand the incubator temperatures and maintain cellular pH.

A test phase was created for 1% bacteriological agarose with the addition of pinealocytes. Different volumes of gelatin were added to the inner well to test the accuracy of maintaining a 0.5 mL volume of media over the coating. The exact volume of gelatin necessary at an 0° tilt was calculated and shown below. The theoretical calculations assume 1 cm³ equals 1 mL.

Large Well ID*: 31.5 mm

Larger Well Height*: 2.0 mm

Smaller Well ID*: 19.2 mm

Smaller Well Height*: 5.2 mm

Volume of Cylinder: $\pi r^2 h$

Where r=radius and h=height

Total Well Volume: 3064.174 mm³ or 3.06417 mL

Total Volume of Gelatin in Smaller Well=1.5586 mL

Total Height of Gelatin in Larger Well to maintain 0.5 mL of media:

$$3.06417 \text{ mL} - 0.5 \text{ mL} = 1.5586 \text{ mL} + \pi (3.15 \text{ cm}/2)^2 h$$

$$\mathbf{h=0.1290 \text{ cm}}$$

*Measurements were acquired from manufacturer (BD Biosciences, personal communication).

(Calculation ref. Mathworld)

The 1 % agarose solution proved difficult to coat the plates due to high viscosity.

Cooling at room temperature, the gelatin solidified too fast for efficient coating with a pipeter; therefore, accurate pipeting was not possible. The height of 0.1290 cm was also difficult to achieve since the gelatin condenses when cooling. Air bubbles, which are unsuitable for cell growth, were a constant problem. After one day of incubation, the inner wells were completely dissolved or there were no live cells. There was the possibility that the cells ate through the gelatin or pH imbalances throughout the coating were unsuitable for cell growth. Due to difficulty in achieving accurate and even coating as well as incompatibility with pinealocyte growth, Design B shifted to Design C.

Flow-through System C Design

Design C shifted the design phase from customized designs to look at professional cell culture systems. Many of the systems are infeasible to the previously discussed constraints. Many are created for tissue culture or are limited to one cell type; some designs were not created for gas permeability or due to size constraints, were too large to fit into the incubator. The major difficulty was finding one to adequately maintain the low volumetric flow accurately, which mostly eliminated many systems with a peristaltic pump.

Collection Wells

After extensive search, a cell culture component from IBIDI showed great potential. The IBIDI u-Slide VI has 6 channels on a slide fitted with 12 female Luer adapters to connect to a flow-through system (Fig 9). It can be coated for optimal cell growth and then incorporated into the system using the tubing kits, which included straight male Luer adapters and gas-permeable tubing. The only concern was the small channel volume at 30 uL. To adequately fill the channel with cells, the concentration of the

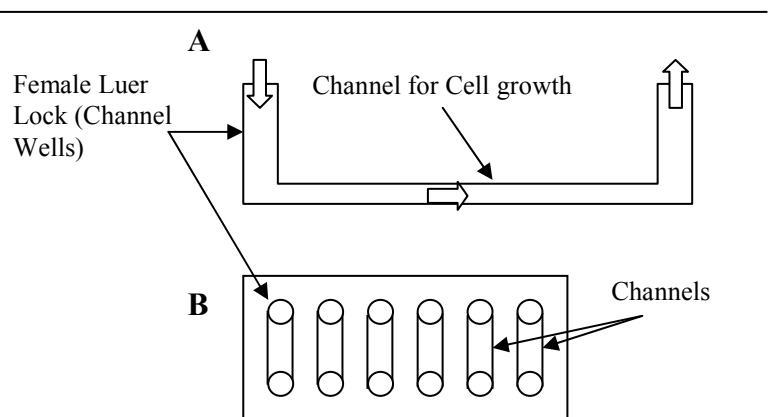


Figure 9. The IBIDI u-Slide VI contains 6 individual channels that can be connected to a flow-through system by Luer locks. Pumped media will flow over the cells grown in the coated channels as seen in the (A) side view and (B) top view.

cell culture solution was increased 10 fold during the seeding process.

In the initial trial with pinealocytes, three types of coatings were used: (1) collagen IV in acetic acid (2) laminin in PBS and (3) an equal mixture of both. The coating and feeding procedures are detailed in Appendix A and were developed based similar methods used in other cell-culture systems (Horn 2006). After two days of incubation, the coating

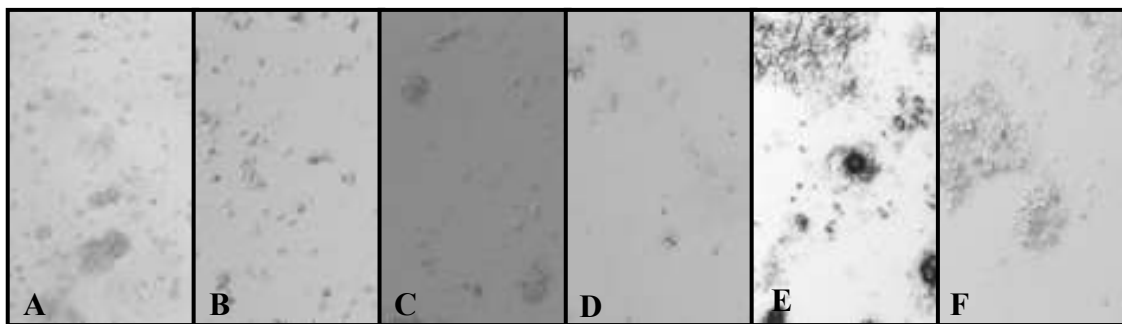


Figure 10. Photos of pinealocytes within the channels at 4X during initial testing with different coating procedures. Photos for Day 1 right after seeding on (A) collagen (B) laminin and (C) a mixture of both coating solutions showed living cells after seeding. Photos for Day 2 after feeding for the (D) collagen-coated channel (E) laminin-coated channel and (F) the mixture channel shows different results. The collagen-coated channel shows little cell adherence and growth (D), and the laminin-coated channel shows a great deal of cell debris and cell death (E). The mixture of laminin and collagen shows the greatest potential with cell adherence and growth.

mixture of collagen and laminin showed to be most compatible with cell growth (Fig 10).

Discussion

RIA analysis of the collagen series and mixed series showed melatonin release for over 2.5 days. The mixed series showed more rhythmic melatonin release with high CPM levels in light during ZT 0 through 12 with levels gradually decreasing (Fig 11). The

curve does not represent the true LD cycle since the system has a 5 hour delay. The 1/8" ID tubing from the channels to the fraction collection is 1 foot long, accounting for 2.413

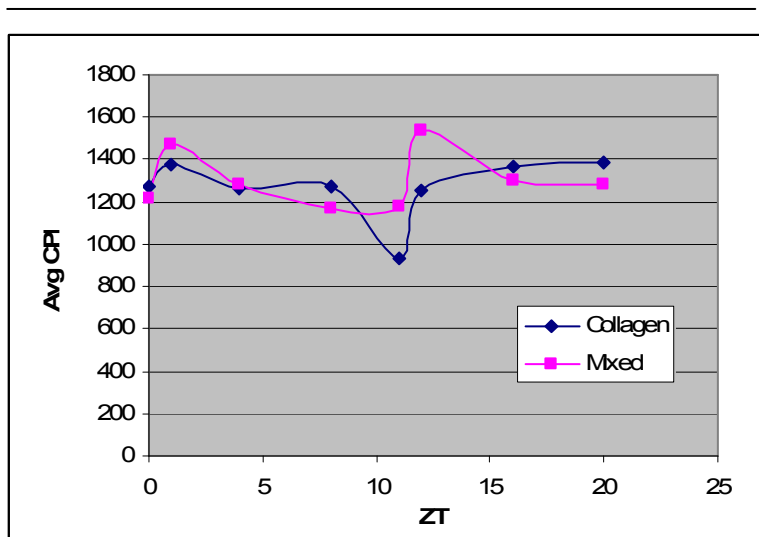


Figure 11. RIA analysis shows rhythmic melatonin release from pinealocytes for both coating series though the mixed series is more consistent.

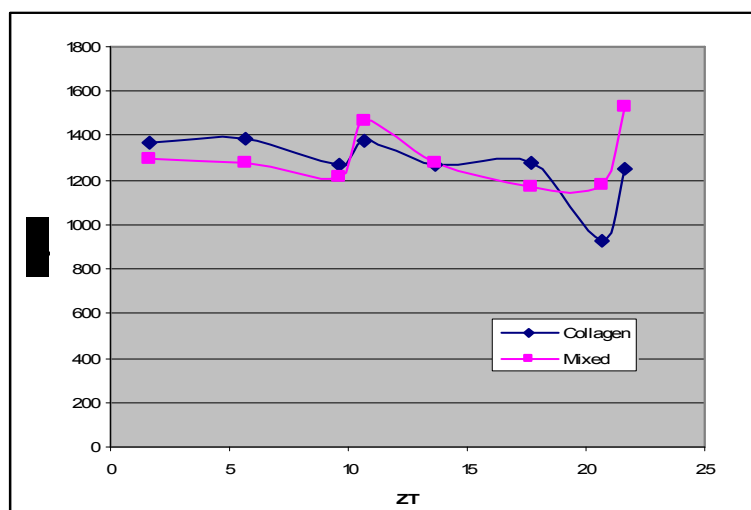


Figure 12. Due to the 9.653 hour delay inherent to the flow-through system, the graph is shifted to represent the true LD cycle.

mL of media with melatonin. So melatonin released from the pinealocytes is not collected in the test tube until 9.653 hours later if the syringe pump is set at 250 uL/hr (Fig 12).

In conclusion, Flow

Design C can meet all of the problem constraints with a few adjustments.

The use of the mixture of collagen and laminin is the best coating material for cell viability and adherence. The small channel volume is not an

adherence with adjustments to the seeding

formula, and the collection of media is consistent since the collection outlet depends on mechanical force from the syringe pump instead of gravitation force. The next step is to incorporate stage 2 into the system, the target cells. The same procedure in coating and seeding will be used for the target cells. However, the melatonin delay will be extended due to the extra system component.

CHAPTER IV

CONCLUSIONS

Phase I concluded in the decision to continue to Phase II with Flow-through System C Design. The use of the IBIDI channels within the generalized system resulted in many of the problem constraints. The cells survived in the small, 30 uL channel volume and did not lift off from the applied shear stress from the flowing media. The best coating procedure was determined to be the mixture of both laminin and collagen described in Appendix A. The pinealocytes were able to entrain for at least three days represented by the qualitative data. Qualitative data showed rhythmic melatonin release, even though quantitative data could not be acquired.

The next step is the addition of the second stage, starting with astrocytes in an attempt to replicate previous data to ensure the flow-through system is fully operational. We expect to see rhythmic release of melatonin from the entrained pinealocytes with baseline levels during light cycles. We also expect to see conferred rhythmicity in the astrocytes with regards to metabolism and some clock genes.

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- Personal Communication: Noah Strickland and Rick Toubeau, Harvard Apparatus
Technical Specialist, 1(800) 272-2775, www.harvardapparatus.com.

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Personal Communication: Phil Brand and Victor Johnson, Biology Instrument Shop,

(979) 845-9285, <http://www.bio.tamu.edu/INSTSHOP/INDEX.HTM>.

Cylindrical Segment Calculations: Mathworld

<http://mathworld.wolfram.com/CylindricalSegment.html>

APPENDIX A

Coating IBIDI u-Slides Procedures

Collagen IV (40 ug/mL) in Acetic Acid

Prep Work:

- 40 uL of Collagen (Sigma 1 mg/mL)
- 960 uL of 0.1 N acetic acid
- 300 uL of ddH₂O

Procedure:

1. Add 40 uL of Collagen (Sigma 1 mg/mL) to tube.
2. Add 960 uL of 0.1 N acetic acid to make 1 mL solution.
3. Fill channel with 30 uL of coating solution under the hood. Quick dispensing is better to avoid air bubbles. Lightly tapping while tilting the slide helps the channel fill.
4. Incubate at room temperature for 30 minutes.
5. Aspirate the channel completely.
6. Rinse the channel with pure water 10 times. Add water while removing water using 2 pipets simultaneously.
7. Let dry at room temperature.
8. Store under sterile conditions and use as soon as possible.

Laminin in PBS

Laminin is an extracellular matrix constituent used for the culture of neurons, epithelial cells, leukocytes, myoblasts and CHO cells.

Prep Work:

- Stock solution can be prepared by dissolving 1 mg/ml laminin in 1 mL of PBS. Filter, sterilize, and freeze in aliquots.
- Remove frozen stock solution from freezer and thaw at 4° C (approximately 30 minutes)

Procedure:

1. Fill channels with 30 uL of coating solution under the hood. Quick dispensing is better to avoid air bubbles. Lightly tapping while tilting the slide helps the channel fill.

2. Incubate several hours at room temperature.
3. Aspirate to remove laminin and rinse channels with media or PBS.
4. Immediately add cell suspension or growth media. Do not allow coating to dry.

Mixture of Laminin and Collagen

Prep Work:

- Prepare equal volumes of laminin in PBS and collagen in acetic acid as shown above.

Procedure:

5. Mix equal volumes of both solutions in an appropriately sized container.
6. Vortex for 1 minute.
7. Fill channels with 30 uL of coating solution under the hood. Quick dispensing is better to avoid air bubbles. Lightly tapping while tilting the slide helps the channel fill.
8. Incubate several hours at incubator temperature.
9. Aspirate to remove mixture solution and rinse channels with PBS.
10. Immediately add cell suspension or growth media. Do not allow coating to dry.

CONTACT INFORMATION

Name: Diana Tran

Address: 3258 TAMU
Biological Sciences Building West
Room 027A
College Station, TX 77843-3258

Email Address: diana2223@neo.tamu.edu

Education: B.S., Industrial Engineering, Texas A&M University, 2007