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Experimental use of Drosophila to Model Proliferation of SARS-CoV-2 Infected Cells

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Experimental use of *Drosophila* to Model Proliferation of SARS-CoV-2 Infected Cells.

A Research Proposal.

An Honors College Thesis

by

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Table of Contents

Abstract

Drosophila have been vectors for scientific research since the beginning of the 20th century. Their successful use within experiments that investigated mendelian genetics inspired their continuous application to biological research. This includes topics that detailed what is now known as innate immunity. The innate immune system of *Drosophila* is a first line of defense against pathogens. Innate immunity has been extensively researched and has reserved responses between *Drosophila* and mammals. Specifically, *Drosophila* have been experimentally targeted to draw conclusions on human infecting viruses such as Human Immunodeficiency Virus (HIV) and Severe Acute Respiratory Syndrome Coronavirus (SARS-Cov-1). Such experiments modeled viral protein function resulting from viral gene expression. *Drosophila* can additionally be applied to research pertaining to the novel Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-Cov-2). Using transgenic *Drosophila* that contain a modified SARS-CoV-2 gene would ultimately force expression of the chosen gene within ocular tissue. This ocular tissue can be isolated and properly stained to observe cell proliferation. Evaluating proliferation can help develop standard knowledge of

SARS-CoV-2 infected cells.

1. Introduction

The use of model organisms within scientific research has been essential to understanding a wide array of biological phenomena. Their use is continuously relevant and can be applied to new uprising questions and concerns. The novel Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) can potentially be investigated through the application of a model organism. When considering the historical use of *Drosophila* to evaluate topics such as modern

genetics, bacterial susceptibly, and viral infection, a successful application to Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) can be assumed. This concept is strengthened due to the homologies between *Drosophila* and mammalian innate immunity. Tailoring transgenic *Drosophila* to express a portion of the SARS-CoV-2 genome can provide answers to questions on proliferation of contaminated cells.

2. *Drosophila* as a Model Organism

A model organism refers an organism that is extensively used within research as a replacement for human beings, allowing scientists to develop a template for biological phenomena without inflicting harm to human subjects. (Urry, et al., 2017) Favored qualities for a model organism include quick generation, high progeny production, and easy maintenance. *Drosophila melanogaster*, a specific species of fruit flies, is one of the earliest developed model organisms and has been used to evaluate an array of phenomena including the behavior of genetics, inheritance factors and modeling a wide range of human diseases. *Drosophila* have a rapid generation time of approximately two weeks with the potential of producing hundreds of progenies. They are also fairly simple genetically with a genome composed of only four chromosomes. Three being autosomes while the remaining one is a sex chromosome. Their abilities and characteristics were deemed ideal and suitable by the famous embryologist, Thomas Hunt Morgan for investigating mendelian genetics. Morgan's experimental use of *Drosophila melanogaster* remains one of, if not the, textbook example of the use of model organisms in biological research. This experiment was deemed scientifically successful due to its observation of red-eyed wildtype and mutant, white-eyed male *Drosophila*. This proved that inherited factors, specifically eye color and sex are somehow linked to chromosomes. An exact link was not clear however, Morgan suspected that the factors were located on the same chromosome.

(Gleason, 2017) Morgan outlined these observations in immense detail in his publication, "Sex limited inheritance in *Drosophila*." These observations were the stimulus for "Modern Genetics" and for the understanding that inherited factors known as genes (that can have variants known as alleles) are located on chromosomes. (Urry, et al., 2017) (Vilmos and Kurucz, 1998) Overall, this experiment solidified the chromosome theory of inheritance and allowed it to become a steppingstone for future knowledge. Examples of future knowledge include the examination of linked genes (genes located closely to each other on the same chromosome) and crossing over (genetic material exchange) during meiosis. (Vilmos and Kurucz, 1998) It also proved that *Drosophila melanogaster* can successful be used as a simplified model of genetics and can be applied to a more in depth and complex discussion of genetics. Overall this solidified Drosophila's status as a model organism. Since this experiment, which was performed in 1910, research using and manipulating *Drosophila* has become overwhelmingly popular. Fortunately, *Drosophila melanogaster*-based research has proved to extremely applicable to human biology. This category of research covers a diverse and expansive list of topics and processes. Examples include extensions of genetics, metabolic procedures, implementations of medications and questions on pathogen susceptibility.

3. Inspiration for *Drosophila* use in Immunity Research

A majority of research using *Drosophila melanogaster* is aimed at understanding their methods of survival. This includes proper environment, successful mating, and first-line immune defenses. An original experimental question was "Can *Drosophila* become ill?" This question was originally investigated by Hans G. Boman along with contribution from his colleagues in 1970. His inspiration being the major health concerns of that time. One example was Malaria, a disease caused by parasitic *Plasmodium spp* which is commonly spread by female *Anopheles*

mosquitos. (Faye and Lindberg, 2016) To properly understand questions like these, which relate to human survival, we must start on a smaller and broader scale. Discussion and observations on how *Drosophila* conduct first-line immune defenses offered a beginning to this understanding. Boman was able to experimentally determine that fruit flies certainly possess a mechanism for recognizing and defending themselves against pathogenic material through a humoral immune response. This was seen through trials of inoculation of test flies with pathogenic gram-negative bacteria and observing the host for survival. Three specific bacteria, *Pseudomonas aeruginosa*, *Aerobacter cloacae,* and *Escherichia coli* were isolated in the log phase of replication and tested at varied quantities. Although, all three bacteria exhibited colonies, only *Pseudomonas aeruginosa* caused host fatality. (Faye and Lindberg, 2016) This inspired Boman to experiment with non-pathogenic bacteria. *Aerobacter cloacae* was modified with an additional antibiotic marker to avoid similar responses to primary infection. The modified sample was then used to cause secondary infection within the same test flies. No growth was observed. Additionally, secondary *Pseudomonas aeruginosa* and *Escherichia coli* infections were alleviated as a result, which is said to model immunization by vaccination. Boman's findings conclude that upon primary infection, *Drosophila* initiate a robust immune response that can differentiate self and non-self-material which was independent of cellular activity. (Faye and Lindberg, 2016) The mechanism observed can be put under the blanket term of innate immunity which has since been extensively investigated for more detail.

4. Immunity

Immunity is a collection of responses within an organism that resist infection by a pathogen. This includes identification of pathogenic and unviable material while attempting to combat damage done by it. There are several ways that responses can prevent damage including

physically blocking and destroying pathogens as well as interfering with their replication and growth. Responses are carried out through many circulating cell types and can be categorized into two subsets, Innate and Adaptive. Innate immunity is an immediate and non-memory-based defense against infection that is present in all organisms. It is carried out by innate cells that can broadly identify foreign material and pathogen-associated molecular patterns (PAMPs). This is done through use of Toll-like receptors (TLRs.) These cells, however, cannot identify specific strains of pathogens. For example, bacterial infection will be recognized but innate cells cannot differentiate *E. coli* from *S. aureus* infections. Within humans there are many types of innate cells. Examples include some white blood cells (eosinophils, monocytes, neutrophils, basophils), mast cells, and dendritic cells. (Features of an immune response) A substantial process executed by these cells is phagocytosis. Phagocytosis is the physical engulfing of pathogenic or apoptotic material. (Govind, 2008) Innate immunity is essential to stimulate the adaptive immune system. Adaptive immunity exhibits more specificity and is composed of three cell types, B lymphocytes, T lymphocytes and Helper T cells. These cells will be rapidly produced if the innate immune system senses danger. B lymphocytes and T lymphocytes have specialized receptors, B-cells receptors (BCRs) and T-cell receptors (TCRs) that can identify foreign material when bound to their corresponding antigens. (Features of an immune response) Antigen presentation results from innate responses. For example, dendritic cells are antigen-presenting cells. (Théry and Amigorena, 2001) Upon antigen detection, B-cell receptors synthesize antibodies, proteins that bind to pathogens and destroy them and T-cell receptors focus on recruiting other immunity cells and destroying already infected cells. Additionally, Helper T cells will encourage the production of B lymphocytes and T lymphocytes through the release of cytokines. As pathogenic threat lessens, B lymphocytes and T lymphocytes will stop replicating

and be maintained within the host as memory cells. If the host were to be presented with the same pathogen again, memory cells will carry out processes to eliminate the pathogen before infection. (Features of an immune response)

5. The Innate Immunity of *Drosophila*

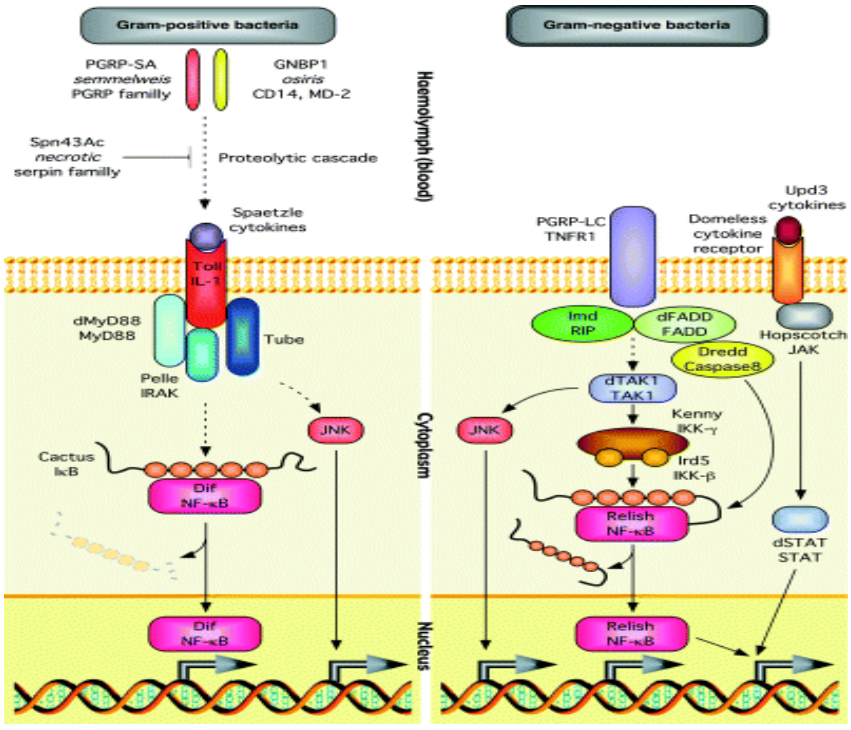
Drosophila are an excellent model for studies of innate immunity due to their lack of adaptive immune responses. The species allows for conclusions based purely on innate interaction without contamination of adaptive processes. A variety of pathogen types such as bacteria, fungus, viruses, and parasites can pose a threat to *Drosophila*. (Govind, 2008) There are three main anatomical components that contribute to innate immunity within *Drosophila*. The fat body, which produces antimicrobial peptides, the lymph gland that produces hemocytes and hemocytes themselves. Hemocytes circulate within hemolymph, fluid within *Drosophila* that is equivalent to vertebrate blood and develop through a process called hematopoiesis. (Vilmos and Kurucz, 1998) They are diverse in morphology and can be classified into three subsets, crystal cells, lamellocytes and plasmatocytes. Plasmatocytes utilize the process of phagocytosis, where foreign pathogens and debris are engulfed and discarded. Crystal cells are responsible for secreting the necessary factors for melanisation, a process that encourages encapsulation of pathogens. Lamellocytes are also involved in encapsulation, however, are less likely to circulate within a healthy host. (Williams, 2007) In general, hemocytes are multi-functional and provide both chemical and mechanical barriers to pathogens. There are three categories of innate responses that *Drosophila* initiate including physical barriers, humoral responses, and cellular responses. Physical barriers include epithelial cells of the respiratory, intestinal and reproductive tract. If pierced by a pathogen a clotting cascade is initated to enclose it. (Govind, 2008) Humoral responses are generally carried out by the antimicrobial agents with cellular response

interjections. Upon foreign material recognition, rapid antimicrobial peptide (AMPs) production is initiated. Antimicrobial peptides are a diverse group of factors which can be distinguished specifically by morphology. Every peptide contains 60 amino acids and has a positive charge. However, the specific amino acid sequences and types of bonds between them can vary between peptides. These are the factors that determine the class of AMPs a given peptide falls into. AMPs are expressed in many immune cell types such as phagocytotic cells and physical barriers cells of the digestive tract. (Diamond et al., 2009) One humoral response includes melanisation, a process where hemocytes in the presence of a pathogenic microbe synthesize the proenzyme, prophenoloxidease, which initiates encapsulation, a cellular process. The synthesized enzyme, prophenoloxidease is transported to sites of infection such as wounds where hemocytes will then employ encapsulation. Through the catalyzation of melanin, the invading pathogen can be encapsulated and enclosed on. (Faye and Lindberg, 2016) An additional example of a humoral response is the coagulation of hemolymph. This process includes a serine protease reaction that striggered by lipopolysaccharide detection. Lipopolysaccharide can be found in the exotoxins of some pathogens such as bacteria. This mediates a clotting cascade that encloses on an invading microbe. In addition, intermediates of this process are secreted that produce toxins inferring that hemolymph clotting also has a role in denaturing pathogens. (Muta, and Iwanaga, 1996) During these procedures other cellular responses have freedom to interject however, they are not well modeled through research. (Parsons and Foley, 2016)

Each humoral or cellular response requires stimulation by the recognition of foreign material. Stimulation is regulated and carried out mainly through two signaling pathways within *Drosophila*, the Toll pathway and the Immune deficiency (Imd) pathway. The Toll pathway is initiated by fungal and gram-positive microbe recognition and signals AMP production in

response. This pathway has also shown to interact with phagocytosis, encapsulation, melanisation and hemocyte production. The Toll pathway employs circulating pattern recognition proteins that contain a Toll receptor. Example proteins would include peptidoglycan recognition proteins (PGRPs) and Gram-negative binding protein (GNBP1) which are both encoded for by different genes. (DeVeale and Brummel and Seroude, 2004) There are currently nine known genes that encode for Toll receptors. Spatzle, a ligand necessary for Toll efficiency, is activated and then binds to the Toll receptor. Additional proteins are then recruited to create a MyD88-Tube-Pelle complex which marks activation of the Toll pathway. Once activated, cactus, another protein that is bound to NF-κB transcription factor Dif is phosphorylated and then degraded. Dif will then isolate and move to the cytoplasm where it will initiate gene activation. Mutations that hinder any of these steps, the toll receptor, Spatzle or recognition proteins encourage host fatality upon infection of gram-negative or fungal microbes. (Valanne, Wang, and Rämet, 2011)

Figure 1: Toll and Imd Signaling Pathways of *Drosophila*



https://onlinelibrary.wiley.com/doi/full/10.1111/j.1474-9728.2004.00106.x

In contrast, the Imd pathway detects gram-negative bacteria using peptidoglycan-recognition protein (PGRP-LC). PGRP-LC binds to PGN, a component of the cell wall of gram-negative bacteria. Upon activation, an IKK-β/IKK-γ kinase complex initiates the phosphorylation of the NF-κB transcription factor Relish. Relish then cleaves and migrates to the nucleus where it activates several genes. (DeVeale and Brummel and Seroude, 2004) The Imd pathway is responsible for most of the AMP production in *Drosophila. Drosophila* that are mutant for Imd necessary factors are more susceptible to gram-negative microbes. (Myllymäki and Valanne and Rämet, 2014)

6. Parallels between *Drosophila* and Vertebrate Innate Immunity

When reviewing *Drosophila* innate immunity, there are several parallels that can be seen with vertebrate innate immunity. These connections can be seen in multiple instances. For example, in anatomic structures. The fat body within fruit flies, is comparable to the liver within a mammal. The mammalian liver is a detoxifying organ that is responsible for the production of clotting factors. The procedure of clotting is also a homologous factor between the two. Hemolymph clotting initiated within fruit flies is comparable to the blood clotting mechanism of a vertebrate and contains regions comparable to the von Willebrand factor. The von Willebrand factor is a protein that's essential to mammalian coagulation. (Vilmos and Kurucz, 1998) In tandem with this conclusion, hemocytes, the foundation for insect innate immunity, can be successfully compared to the blood cells of vertebrates. More specifically, macrophages, phagocytic blood cells of vertebrates share a common function with plasmatocytes. Both engulf

smaller pathogenic or apoptotic cells and dispose of them. (Parsons and Foley, 2016) In addition, another humoral response can also draw parallels. During the process of melanisation, the resulting enzyme, prophenoloxidease, is comparable to proteins C3 and C4 within vertebrates since they contain a like sequence. Specifically, the thiol-ester section of C3 and C4 is almost identical to the prophenoloxidease sequence. Additionally, the Toll and Imd signaling pathways show similarities as well. (Imler, 2014) The employment of NF-κB transcription factors that result in gene expression remains a reserved process. Within vertebrates that have an adaptive immune system, using NF-κB transcription factors allows stimulation of adaptive immunity. This is seen in the expression of cytokines that cause inflammation. (DeVeale and Brummel and Seroude, 2004) Based on these examples, it can be concluded that there is a definite link between vertebrate and *Drosophila* innate immunity. The most viable suggestion implies that there is an evolutionary link (either a common ancestor or survival of favorable traits) between the two. (Vilmos and Kurucz, 1998) This link allows for *Drosophila melanogaster* to be an appropriate vessel within research where conclusions on vertebrate immune responses are sought.

7. The Innate Viral Response of *Drosophila*

Within research, *Drosophila* are commonly chosen to model infection by a variety of pathogens. Bacterial and fungal infection are well studied and understood. Knowledge of viral pathogen infection would benefit from additional research. Viruses are infectious genetic material that are encased in a protein enclosure. They can be separately identified by protein capsid shape and infectious nucleic acid type. Viruses are completely dependent on a host and take advantage of natural cellular processes for survival. Viral infection occurs when a virus introduces their genome into a host cell which will consequently replicate the viral genome. The replicated genome is then encapsuled into new viral microbes which can travel across the tissue

of a host. (Urry, et al., 2017) Injected genetic material can be in the form of RNA or DNA. RNA viruses contain either single stranded RNA (ssRNA) or double stranded RNA (dsRNA). ssRNA can be further categorized into either positive ssRNA (ssRNA(+)) or negative ssRNA (ssRNA(-)). ssRNA(+) can be quickly transcribed by a host cell due to its similarity to mRNA. ssRNA(-) must be converted into ssRNA(+) to be translated. RNA viruses tend to have small genomes therefore encode for few proteins. In contrast, the genome of a DNA virus is large and complex. DNA viruses can contain double stranded DNA (dsDNA) and sometimes single stranded DNA (ssDNA). Both require DNA polymerase to efficiently replicate. (Durmuş, and Ülgen, 2017) *Drosophila* recognize invaders broadly using pattern recognition receptors (PRRs) that interact with pathogen-associated molecular patterns (PAMP.) PAMP interaction stimulates effector cells and gene activation of anti-microbial peptides which is done through several pathways. This was seen previously within humoral innate responses that were initiated by the Toll and Imd pathways. *Drosophila* express specific processes such as transcriptional pausing, autophagy, and RNA interference/silencing in effort to combat viral pathogens. Transcriptional pausing takes place early in the innate response and primes genes with a pause sequence. This will consequently interfere with RNA biogenesis. (Xu and Cherry, 2014) A pausing sequence prevents the process of transcribing DNA sequences into RNA sequences which is done by RNA polymerase. (Saba, et al., 2019) Autophagy is a natural cellular regulatory process where components of cytoplasm, specially lysosomes of targeted cells are broken down. Although there are three types of autophagy, macroautophagy, microautophagy and chaperone-mediated autophagy, the term “autophagy” commonly refers to solely macroautophagy. Macroautophagy includes the formation of an autophagosome which will attach to and deliver degradation agents to lysosomes. (Mizushima, 2007) RNA silencing is a step of RNA interference. RNA

interference negatively impacts gene expression of foreign genetic material. Upon recognition of a viral pathogen, small interfering/silencing RNA (siRNAs) are produced in effort to degrade corresponding mRNA. (Xu and Cherry, 2014) Nonviable mRNA will interfere with efficient gene expression. (Meng and Lu 2017) Experimentally, mutant *Drosophila* for RNA silencing are seen to be highly susceptible to RNA type viruses. (Bronkhorst, and P van Rij, 2014) In addition to these responses, there is evidence that the Toll, Imd, and JAK/STAT pathways are involved in viral defense. The Toll pathway is commonly observed in antibacterial and fungal mechanisms however, in terms of antiviral defense, it is said to be carried out untraditionally. In fact, understanding the exact mechanism in which the Toll pathway stimulates antiviral reactions requires more experimental investigation. Currently, Toll-7, one of the nine known Toll receptors, is known to initiate autophagy. This can be observed through G protein of Vesicular Stomatitis Virus (VSV) that attaches to the Toll-7 receptor. In result, the protein is recognized as a PAMP and autophagy is initiated. This hinders viral replication and survival within the *Drosophila* host. Vesicular Stomatitis Virus currently remains the only virus where this phenomenon is observed leaving room for future investigation. Additionally, through research, the Imd pathway is noted to halt the replication process of some viruses in *Drosophila* as well. For example, the antimicrobial peptide DiptB which is produced downstream in the Imd pathway, shows some support against Sindbis Virus (SINV.) The JAK/STAT pathway is also stimulated in viral innate immunity. This pathway is not initiated by direct virion contact but, does stimulate gene expression for antiviral components. *Drosophila* that exhibit weak or dulled JAK/STAT pathway responses, are more likely to succumb to infections of *Drosophila* C Virus (DCV) and Cricket Paralysis Virus (CrPV.) (Xu and Cherry, 2014). Further research would be

needed to detail the steps and factors specifically involved within these pathways that cause their antiviral responses.

8. Parallels between *Drosophila* and Mammalian Viral Immunity

The innate viral immunity of *Drosophila* is quite comparable to that of a mammal. For example, the Toll pathway stimulation within fruit flies is conserved. Within mammals the homologous pathway is called the Toll-like signaling pathway which is regulated with Toll-like receptors, a type of protein recognition receptor. This was concluded through the observation of that both pathways are dependent on NF-κB transcription factors. Exact transcription factors however, differ. Additionally, the Imd pathway of *Drosophila* parallels the Tumor necrosis pathway of mammals. Similarities can also be seen in specific responses. For example, autophagy, a vital process for elimination of pathogens, is evolutionarily preserved (Xu and Cherry, 2014) Due to these similarities *Drosophila melanogaster* can be used as a suitable vector to model antiviral responses that are consequently applicable to mammals. Research collectively has moved toward using experimental fruit flies to observe viruses that are infectious to humans.

9. Human Infecting Viruses Modeled through *Drosophila*

Human infecting viruses such as Human Immunodeficiency Virus (HIV) and Severe Acute Respiratory Syndrome Coronavirus (SARS-Cov-1) have been used within research that utilize *Drosophila* in hope of expanding knowledge of their infection motives. Specifically, research manipulating Human Immunodeficiency Virus and Severe Acute Respiratory Syndrome Coronavirus has been beneficial to understanding how each virus' proteins overtake a host's environment. Human Immunodeficiency Virus is a broad term that umbrellas infections of HIV-1 and HIV-2, two types of the virus. HIV-1 has a higher infection rate and accounts for 95% of

Human Immunodeficiency Virus infections worldwide. Due to this, HIV-1 has been prioritized in scientific research. HIV-1 is a retrovirus that injects its genomic RNA into a host cell. Its RNA is then transcribed into complementary DNA using the enzyme reverse transcriptase. (Hiv strains and types) (Coffin, et al., 1997) As the infected host cell replicates, the viral genome is expressed. Through research that has used *Drosophila,* three proteins encoded by HIV-1 have been modeled. Within cultures of Schneider 2 (S2) cells of *Drosophila*, HIV-Nef protein and human CD4 protein were both observed. (Hughes, et al., 2012) Schneider 2 cells are embryonic cells of *Drosophila* that are excessively used for research due to their ability to take in foreign RNA. They can also be easily maintained within a laboratory setting. (Lee and Peng and Guo, 2013). Human CD4 protein commonly collaborates with helper T cells during human immune responses. (Sekaly and Rooke, 1998) Additionally, HIV-Nef was observed to suppress human CD4 through the process of endocytosis. (Hughes, et al., 2012) Future investigation utilizing RNA silencing, showed that endocytosis was initiated by HIV- 1 Nef and AP2 protein complex interaction. This Nef-AP2 interaction was also validated to be a conserved procedure within humans through experiments that utilized HeLa cells. (Hughes, et al., 2012) HeLa cells are one of the most commonly used type of human cell within research. In a laboratory setting, these cells continuously replicate and divide making them "immortal." (Butanis, 2017) HIV-1 Rev protein has also been evaluated through Schneider 2 cell culture. Rev was observed initiating the movement of viral mRNA from the nucleus of a host cell to the cytoplasm. This facilitation has been previously identified within mammalian cells concluding that Rev utilizes conserved factors to cause this movement. In contrast, transgenic *Drosophila* have been used experimentally with HIV-1 Tat protein. Transgenic *Drosophila* contain foreign genetic material that is introduced for experimental purposes. HIV-1 Tat is necessary for viral survival,

expression, and replication. Transgenic *Drosophila* that expressed Tat, consequently experienced inefficient microtubule formation. Microtubules are a structural component within cells that are comprised of tubulin. (Sept, 2007) HIV-1 Tat directly disrupts congregation of tubulin. Human cells also mimic this process. (Hughes, et al., 2012)

Drosophila have also been useful within studies of Severe Acute Respiratory Syndrome Coronavirus (SARS-CoV-1.) In 2003, an outbreak of SARS-CoV-1 became a global concern. The virus is infectious to lung and intestinal tissue of humans and is transmitted through respiration particles. SARS-CoV-1 is a pathogenic, enveloped single strand of RNA. The experimentation of transgenic fruit flies has helped to understanding the functionality of the SARS-CoV-1 3a protein. 3a protein was observed to cause death of ocular tissue within experimental flies. This was determined to be carried out through a mitochondrial pathway. Within mammalian cells, the mitochondria initiate apoptosis, a process of calculated cell death. Through a release of proteins and caspase proteases, cell destruction is activated. Phagocytosis is then stimulated to clean up and engulf the dead cells. (Wang and Youle, 2009) This mechanism was also seen to take place within mammalian lung and intestinal tissue. Additionally, the suppression of the 3a protein by medications was tested. Medications were used to block the ion pathway of 3a protein within transgenic flies and human cells. Apoptosis was not observed in either samples, solidifying that the SARS-CoV-1 3a protein and induced apoptosis have a direct correlation. (Hughes, et al., 2012) Utilization of *Drosophila* within viral research of Human Immunodeficiency Virus (HIV) and Severe Acute Respiratory Syndrome Coronavirus (SARS-CoV-1) has aided in creating an improved understanding of these viruses as well justifying the use of *Drosophila* as a model organism. When considering the global pandemic caused by Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV- 2,) experiments pertaining to

Severe Acute Respiratory Syndrome Coronavirus (SARS-CoV-1) could be extremely useful. Both are considered to be epidemic Coronaviruses and are estimated to share 79.5% genetic homology. Coronaviruses are single stranded RNA viruses. Although, coronaviruses target several animal hosts ranging from bats to felines, there are currently seven identified human infecting coronaviruses (hCoVs) including both SARS-CoV-1 and SARS-CoV-2. (Zhu, 2020) Due to their genetic and host similarities, proposing an experiment that utilizes *Drosophila* to investigate SARS-CoV-2 infection, inspired by previous use for SARS-CoV-1 infection could elaborate knowledge on the subject.

10. Proposal Introduction

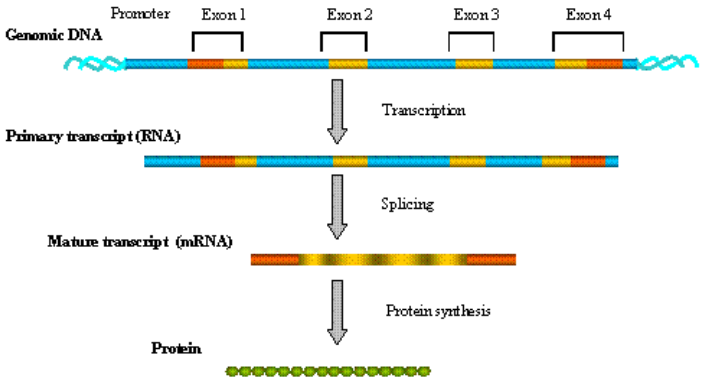
In March, 2020, Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV- 2) was declared to be the cause of a worldwide pandemic by the World Health Organization. As of May, 2021, this coronavirus remains a threat to global populations despite aggressive controlling efforts. As of May, 4th, 2021 the Centers for Disease Control and Prevention (CDC) has reported a total of 32,228,003 positive cases and 574,220 fatalities due to SARS-CoV- 2 within the United States since January 21st, 2020. (CDC COVID Data Tracker) Due to the novelty of this virus, complete understanding of its methods of transmission and infection are still pending. Currently, most knowledge is based on assumptions made from observation of human infections. (Petersen, 2020) Trends include the infection of respiratory and gastrointestinal tissue. Transmission occurs through contact with infected respiratory particles. This is referred to as airborne transmission. The virus is highly infectious and can spread easily between humans. (How coronavirus spreads) There is currently a desperate cry for concrete data as fatalities are still exponentially raising. Designing an experiment which employs transgenic *Drosophila* to evaluate specific SARS-CoV-2 protein expression could create and influence a concrete set of data pertaining to the virus. If

the nonstructural protein 15 (NSP15) encoding gene of SARS-Cov-2 is isolated and modified to create transgenic *Drosophila*, ocular tissue could then be harvested and examined to model the proliferation of SARS-CoV-2 infected cells.

11. Gene Expression

To observe an accurate example of proliferation of a cell infected with SARS-CoV-2, the targeted gene which encodes for NSP15 must be efficiently expressed throughout the tested flies. Gene expression is the process where genes are transcribed and translated to create a functional protein. A gene is a portion of DNA which is comprised of nucleic acids. During gene expression, a gene's specific nucleic acid sequence is copied and transcribed to create RNA. Then by translocation, the RNA will synthesize a protein. (Gene expression) Proteins are functional factors that carry out cellular processes.

Figure 2: Overview of Gene Expression



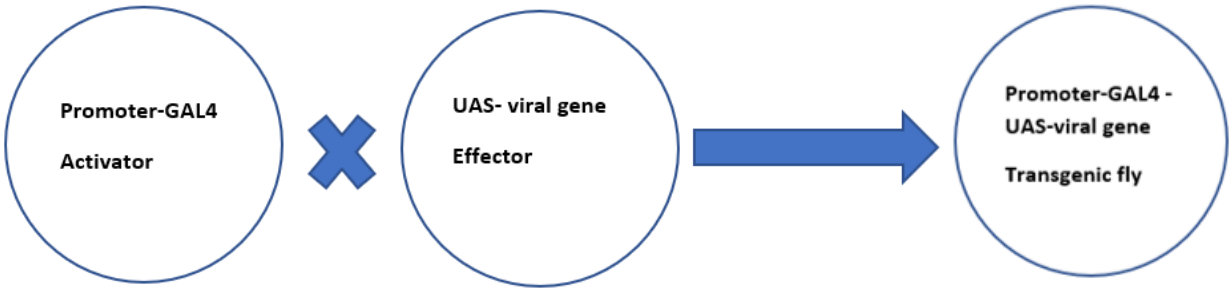
https://www.ncbi.nlm.nih.gov/probe/docs/applexpression/

12. GAL4/UAS (upstream activating sequence) System.

Strains of *Drosophila* can be manipulated to express foreign genetic material. One of the most common ways this is done within research is by using the GAL4/UAS (upstream activating sequence) system. This expression strategy has been used to tailor experimental *Drosophila* to express viral genes *in vivo.* The system is highly effective due to its ability to overcome obstacles from genetic differences and temperature. It requires the creation of two lines of fruit flies, the activating (GAL4) and effector (UAS) line. (Scheer and Campos-Ortega, 1999) GAL4 is a transcriptional factor that binds to UAS when crossed. As a result, transcription of a select gene is stimulated. (Busson and Pret, 2007) GAL4 lines can be purchased through Bloomington *Drosophila* Stock Center in Bloomington, Indiana. This facility has thousands of GAL4 lines available and is easily accessible due their location. These lines are already modified to contain regulatory and promoter sequences for GAL4 which are necessary for gene expression. (Gal4 lines) Additionally, the UAS line of *Drosophila* must be created. The genome of this line must include the upstream activating sequence and the nonstructural protein 15 (NSP15) encoding gene of SARS-CoV-2. This gene would have to be harvested from the SARS-CoV-2 genome in the form of mRNA and modified before being implemented. (Scheer and Campos-Ortega, 1999) After modification, the gene will be introduced into the genome of the flies that will make up the UAS line. Once introduced into a new genome, the gene will be a transgene. Transgenes are altered genes that are purposely placed within a foreign genome. (Nishu, et al.,2020) The transgene should be placed directly next to the upstream activating sequence within the genome. This placement allows for fusion and activation of the transgene when crossed with the GAL4 line. When the GAL4 and UAS lines mate, transgenic progeny that has a mutant phenotype and a complete GAL4/UAS system will be produced. Ideally, the progeny will express the SARS-CoV-2 transgene in every tissue that GAL4 is expressed. Within this system, viral transgene and

GAL4 expression are directly correlated. This also means that the SARS-CoV-2 transgene will not be expressed where GAL4 is not.

Figure 3: Overview of GAL4/UAS system



the probability of host fatality. For example, if the transgene were to encode for NSP15 and NSP15 were to stimulate apoptosis, apoptosis would be reserved to nonessential tissue. Apoptosis would not be expected to be observed throughout other tissue. Within *Drosophila*, ocular tissue, specifically, eye-antennal disks are nonessential. (Hughes, et al., 2012)

13. Modifications of the GAL4/UAS System for Transgene Expression

The GAL4/UAS expression system favors higher temperatures. Higher temperatures have been seen to increase efficient transgene expression. Through evolution viruses have shifted to survive within human body temperature. Viruses that could replicate and withstand the temperature within humans had a survival advantage over ones that could not. The physiological temperature of a human is higher than the favored temperature of a fruit fly. The normal body temperature of a human is 37°C. (Protsiv, 2020) The body temperature of *Drosophila* is directly influenced by their environment. Their body will take on the temperature around them and favors ~25°C. (Goda and Hamada, 2019) Therefore, creating an environmental temperature of over 25°C for both the GAL4 and SARS-CoV-2 UAS lines of *Drosophila*, will encourage transgene expression within progeny. This temperature, however, should not be increased by more than

5°C, as *Drosophila* cannot internally regulate their body temperature. (Hughes, et al., 2012) Environmental temperatures of over 30°C decrease the probability of survival. (Goda and Hamada, 2019)

14. SARS-CoV-2 Nonstructural Protein 15

Through elementary research of SARS- CoV-2, its known that 28 SARS-CoV-2 proteins interact with 417 human proteins during infection. There are 326 proteins within *Drosophila* that show homology with these human proteins. Therefore, choosing to isolate a gene that encodes for at least one of these 28 proteins would be ideal to ensuring its expression within *Drosophila*. When observing the genome of SARS-CoV-2 the nonstructural protein 15 (NSP15) interacts with four human proteins, NUTF2, RNF41, ARF6, and PPIA. All of these proteins have a corresponding protein within *Drosophila* of either Ntf-2r, elgi, Arf51F, or Cyp. The remaining 27 viral proteins interact with groups of human proteins that do not all have a homologous link to *Drosophila*. By choosing the gene that encodes for NSP15, this link is ensured and lessens error. Once this gene is modified and implanted within the genome of the UAS line of *Drosophila*, the corresponding protein (NSP15) should be expressed. (Hussain, et al., 2020)

15. Modification of SARS-CoV-2 Nonstructural Protein 15

An isolated gene should ideally be modified to increase the probability of producing a phenotype when expressed within a transgenic host. Injecting one altered gene into a host's genome may not be efficient to produce a phenotype. Therefore, more than one should be adjusted and implanted to ensure the desired level of expression. (Hughes, et al., 2012) This would require the isolation of several NSP15 encoding genes and the introduction of them into

the same UAS genome. By using this technique in combination with considerations to the GAL4/UAS system's temperature preference, the ideal presentation can be ensured.

16. Culturing of Eye-antennal Discs

As transgenic progeny is produced, eye-antennal discs of third instar larval (L3) *Drosophila* should be isolated for culture. L3 is a pre-pupal developmental stage of *Drosophila.* During L3 ocular tissue has reached maturity and can replicate. Within developmental stages prior to L3 such as L1 and L2, eye-antennal discs are still morphing into the correct shape and size. (Spratford and Kumar, 2014) Therefore, L3 ocular tissue is the most suitable to harvest for culture. Harvard University's method for extraction of eye-antennal discs would be appropriate to use. 30 grams of transgenic flies should be gathered and frozen at -80°C for 60 minutes. After 60 minutes, the frozen contents should be centrifuged until room temperature is reached (20°C.) The flies should be blended on high speed in a blender with Shields and Sang M3 medium. (Cell culture (fly) Shields and Sang M3 medium is commonly used within insect cell cultures and is available for purchase through https://www.sigmaaldrich.com. (Shields and Sang M3 Insect MEDIUM S8398) Once a reddish colored liquid is reached, blending can be stopped. The entire liquid contents should be transferred to 250mL centrifuge tubes and spun at 2600 rpm for 20 minutes. Following centrifugation remaining fly carcasses and oil should be removed. The supernatant (remaining liquid) will be transferred to new 250mL centrifuge tubes and then spun at 3000 rpm for 30 minutes. Additional oil achieved through this step should be removed as well. The remaining liquid should be transferred to a new set of 250mL centrifuge tubes and placed into a 60°C water bath. After a precipitate forms, the tubes can be removed. This usually take about 40 minutes. The tubes should be centrifuged at 3200 rpm for 45 minutes and then transferred to a tissue culture hood. Using a 0.22µm filtering unit the supernatant should be

filtered. Several filtering units might be needed, as this supernatant can clog units. The final extraction liquid should be approximately 200mL and represent a large sample of eye-antennal discs. This sample should be frozen in liquid nitrogen and stored at -20°C (Cell culture (fly)) This sample can be used to create cultures of *Drosophila* eye-antennal discs using a POC-R Chamber system. POC- R chamber systems can be easily sterilized and allows for flexibility of cultures. This system can be used for both open or closed culture and perfusion. In addition, the system can be used for either short or long term culturing projects. (Cell culture + microscopy poc-r - ueb.cas.cz.) A small amount of the sample of ocular tissue should be placed directly onto a coverslip that is mounted onto a POC-R Chamber system. (Tsao, C.,) Agarose gelling agent should be added to fixate the sample onto the coverslip. This should be followed by 1 mL of Schneider's *Drosophila* culture medium added into the plate of the chamber system. Schneider's *Drosophila* culture medium is available for purchase through https://www.thermofisher.com and was originally developed as a way of growing and observing S2 cells of *Drosophila*. This medium has also proved to successfully grow eye-antennal discs within culture, therefore, is an appropriate medium to use. (Schneider's *Drosophila* Medium) The completed system should then be incubated.

17. BrdU Staining Technique

BrdU (Bromodeoxyuridine / 5-bromo-2'-deoxyuridine) solution staining techniques can be used to evaluate eye-antennal discs *in vitro* for cell proliferation. *In vitro* refers to the observation of cells outside of their host. For example, cells within a culturing medium would be *in vitro*. Cellular proliferation is the physical increase in cell number resulting from cell replication and division. Observations of cellular proliferation look to evaluate the homeostasis between cellular gains (increasing cell number) and losses (cell death.) (The cell proliferation

guide) Use of a BrdU assay followed by immunofluorescence staining, allows cell proliferation to be visually observed under a Florescent microscope. BrdU stain, when introduced to a cell sample will naturally incorporate itself into the sample cell's replication process. Specifically, BrdU stain targets DNA polymerase. DNA polymerase is an enzyme that facilitates DNA replication during cellular replication. (Maga, 2019) BrdU solution can be used to stain the transgenic *Drosophila* eye-antennal disc culture so it can be observed for proliferation. Staining kits can be purchased from different providers however, most follow a standard protocol. A 10mM stock solution of BrdU should be made containing 3mg per 1mL of water. Various bottle quantities ranging from 250mg to 5g of BrdU can be purchased through https://www.abcam.com. (Brdu (5-bromo-2'-deoxyuridine), thymidine analog (ab142567)) The 10mM stock should be diluted to a 10 µM solution using Schneider's *Drosophila* Medium. The diluted solution should be then filtered using a 0.2 µM filter. The filtered solution is now considered to be a BrdU labeling solution. The Schneider's *Drosophila* Medium from the POC-R chamber system should be removed and replaced with 1mL of BrdU labeling solution. The entire system should then be put into a CO_2 incubator for up to 24 hours. The actual incubation time will depend on the rate of cell proliferation. Rapidly dividing cells require a shorter incubation time than slower cells. Finding the optimal incubation time for this culture would require several trials. Once removed from incubation the cells should be washed twice with phosphate buffering solution (PBS.) Each wash should be for five seconds. These cells should be rinsed three additional times with phosphate buffering solution. These washes should be two minutes each. Following, PBS washes, the sample should be soaked in 1M HCl for approximately for 10 minutes. After, three phosphate buffering solution rinses should be repeated. (Brdu staining and

brdu assay protocol) This staining procedure ensures that BrdU will interact within the replication of the targeted eye-antennal discs.

18. Immunostaining Technique

To visualize BrdU implication, an additional staining procedure of Immunostaining must be completed. Immunostaining utilizes BrdU antibodies that target areas of BrdU and sample cell interaction. Antibodies will bind to fluorophores resulting in the bright visualization of sample cell proliferation. Immunostaining is one of the most accurate techniques to visualize cellular changes due to encoded protein activity. This is extremely helpful when evaluating transgene expression which will consequently encode for a protein. This technique can be categorized into either direct staining, when a single primary antibody binds with fluorophores or indirect staining, when a secondary antibody binds to fluorophores. An indirect staining technique that utilizes BrdU antibody 6326 (ab6326) as a primary and AlexaFlour555 as a secondary antibody would be suitable. Ab6326 has been extensively used as a primary antibody within research that includes a BrdU and immunostaining process. (Goding, 1996) AlexaFluor555 is commonly paired as a secondary antibody to ab6326. AlexaFlour555 produces exceptional coloring, has pH stability and is water soluble. (Alexa fluor® conjugated SECONDARY ANTIBODIES.) To prepare for staining of the sample of eye-antennal discs, microscope slides and coverslips should be sterilized using either 70% ethanol or heating techniques. Using a smear technique, a sample of the BrdU stained eye-antennal discs should be placed onto a microscope slide. The slide can be set onto a slide warmer until completely dry. (smear preparation) The slide should be incubated at -20 °C in 100% Methanol for five minutes. This step will fix the sample to the slide. Once fixed the slide should be rinsed with chilled phosphate buffering solution three times. By using a methanol-based method of fixation, the slide does not have to be permeabilized.

Permeabilization would require an additional step and materials. Ab6326 requires an antibody retrieval process to enhance its performance. This antibody can only recognize single stranded DNA, making it is essential to denature its DNA strands before use. This process would include mixing ab6326 with 2M HCl in effort separate its DNA strands. Acid denaturing methods are more efficient for antibody retrieval rather than using a DNA enzyme. (Anti-BrdU antibody) Once ab6326 is modified, it should be diluted with 1% Bovine Serum Albumin solution in phosphate buffering solution. This should be done in a ratio that creates a 1/250 solution. The fixed slide should then be incubated in 1% Bovine Serum Albumin solution for 30 minutes. Then the slide should be incubated in the diluted ab6326 solution overnight at 4 °C. Following incubation, the slide should be washed with phosphate buffering solution three times. AlexaFluor555, the secondary antibody, should be mixed with 1% Bovine Serum Albumin solution and then used to incubate the slide for one hour. This should be done in a dimly lit setting. After one hour the remaining solution should be removed, and the slide should be washed again with phosphate buffering while remaining in a dimly lit setting. An additional counterstaining procedure would be required to ensure the proper fluorophore-antibody binding. This can be done using Hoechst stain (1mg/mL concentration) incubation for one minute. Hoechst stain is known to be the least toxic choice of possible counterstaining agents and more suitable for cell survival. Following, counterstaining, the side should be rinsed three times with Phosphate buffering solution. A coverslip can then be mounted over the sample area making the slide suitable for observation under a florescent microscope.

19. Evaluation of Proliferation

Under a florescent microscope, stained eye-antennal discs that encode for SARS-CoV-2 NSP15 can be observed for proliferation. The fluorescent coloring achieved through fluorophore

binding allows for the observer to actively view cellular replication. This includes viewing and quantifying the number of cells participating in each stage of the cell cycle (G1, S, G2, M.) Each stage of the cell cycle includes specific procedures within the cellular replication process. (The cell proliferation guide) Observation can additionally be used to establish a ratio of cells that are actively replicating to cells that are not. Replicating cells can be followed through the cell cycle to their production of daughter cells. Nonreplicating cells could be within cell stage G0 or dead. Cells that are in the G0 phase are at a resting state. (Pizzorno, 2016) These cells, however, can be initiated to rejoin the cell cycle. Dead cells can potentially result from apoptosis. Apoptosis can be initiated as a regulatory cellular process which induces death of unviable cells. However, some viruses have the ability to encode for proteins, like SARS-CoV 3a that stimulate apoptosis without reason. Possible ratios that can be observed could include an excess number of cells experiencing death in comparison to cells being replenished. Excessive/uncontrolled cellular replication with unbalanced cell death could be noted as well. Additionally, proliferation can be evaluated to estimate replication time of given cells. Feasible observations could include hasty or prolonged replication. In addition to quantifications, comments could be made pertaining to the quality of replication. If quick replication were to take place, noting whether the produced daughter cells are fully developed and viable would be important. Overall, conclusions can be made to help develop a numerical and characteristic standard for SARS-Cov-2 infected cells.

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