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Literature Replication: Insect Gut Cell Isolation Protocols

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Abstract

A large problem in science is the lack of literature replication, especially for subjects in which there is little published research. Literature replication is an integral part of the scientific method and is important for validating methods and results of published studies. A study from *Nature* found that out of 1,500 scientists interviewed, over 70% have been unable to reproduce literature studies and over half of them have been unable to reproduce even their own studies (Baker, 2016). This problem is even more pronounced in the field of biology, with 80% of scientists reporting that they are unable to replicate published experiments and 60% are unable to replicate their own. Literature that reports methods of cell isolation from insect tissue, specifically that of Lepidopteran species, is not robust and has few published protocols demonstrating novel cell isolation methods. This project examines the reproducibility of two methods from published studies, the cell migration and forced cell separation protocols, by attempting to replicate their cell isolation and culture methods. While the two studies both isolate midgut cells from Lepidopteran species, they utilize different cell isolation methods and have different applications of the isolated cells.

In this study, midgut cells were isolated from the Lepidopteran species *Helicoverpa zea* in order to create and maintain primary cell cultures. The success of this project was assessed based on a comparison of replication results to the literature results. Mixed results were obtained through the replication process. The forced cell separation protocol achieved more successful results, while changes had to be made to the cell migration methods to combat consistent contamination issues and irreproducible methods. Even after these changes were made, the methods and results were only partially replicated successfully. Steps can be taken to combat the widespread issue of literature irreproducibility, starting from addressing the issue on a broader

scale in the scientific community and including encouraging replication studies and external validation and increasing the acceptance of publishing these replication studies.

Introduction

Literature replication and the reproducibility of research are integral parts of the scientific method that are important for validating study findings and confirming the accuracy and applicability of pieces of research. Literature replication efforts can show where changes need to be made in a protocol or can potentially produce conflicting results. In the last decade, there has been an increasing focus around a reproducibility crisis in science, as several large-scale reproducibility projects produced disappointing results (Fidler & Wilcox, 2021). A 2016 study by Nature found that out of over 1500 scientists interviewed, around 70% said they were unable to replicate published studies and 50% said they were unable to replicate even their own (Baker, 2016). This issue becomes even more prevalent in the field of biology, the scientific discipline with the second highest numbers behind chemistry, where close to 80% of scientists were unable to replicate published literature and 60% were unable to replicate their own (Baker, 2016).

Many factors contribute to the irreproducibility of research, with the scientists interviewed in the Nature study citing selective reporting, pressure to publish, lack of internal validation, and poor experimental design as some of the top factors (Baker, 2016). Another influential factor is the lack of published replication studies in literature. The absence of published replication studies often results in a lack of external validation for literature studies. This stems from historical policies held by many science funding bodies, where importance is placed on novel research and replication studies are discouraged or not published at all (Fidler & Wilcox, 2021).

The aim of this project was to approach the issue of literature replication through the recreation of two protocols from published studies. The two protocols chosen are both methods for isolation of Lepidopteran gut cells, but they differ in their cell isolation method as well as the application of the isolated cells. Focus was placed on assessing the reproducibility of each of the two protocols and then comparing the reproducibility of the methods and results to each other. These protocols were chosen in part because they have different factors that may affect the reproducibility of their methods or results.

The Lepidopteran gut contains three distinct sections, the hindgut, located posteriorly, the midgut, and the foregut, located anteriorly (Figure 1). For both literature protocols as well as this project, the midgut was the region that was isolated. The Lepidopteran species used in this study was the fourth instar *Helicoverpa zea* larva, which is also known as the corn earworm. In the midgut, three main epithelial cell types are found, stem cells, columnar cells, and goblet cells (Caccia et al., 2019) (Figure 2). The columnar cells are most abundant, with there being three to six columnar cells for every one goblet cell (Sadrud-Din et al., 1996).

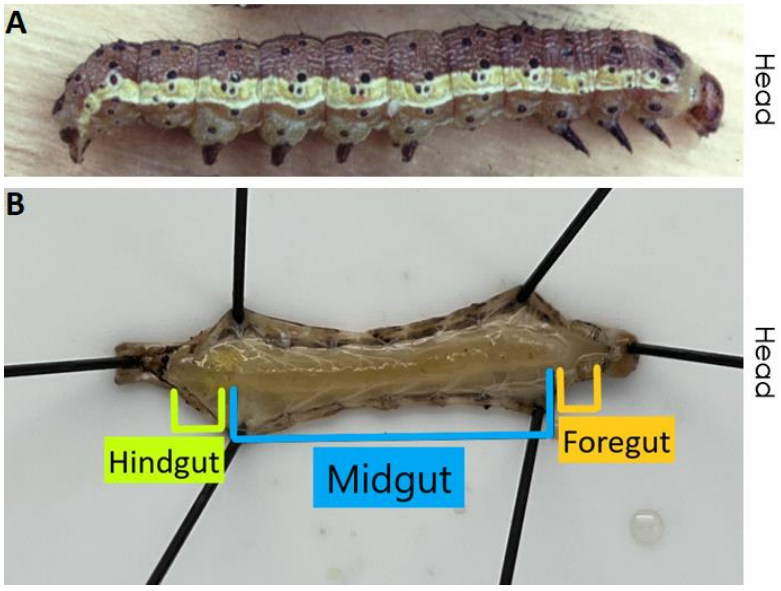

Figure 1. (A) Exterior of *H. zea* larva lined up with (B) gut anatomy, distinguished between hindgut, midgut, and foregut.

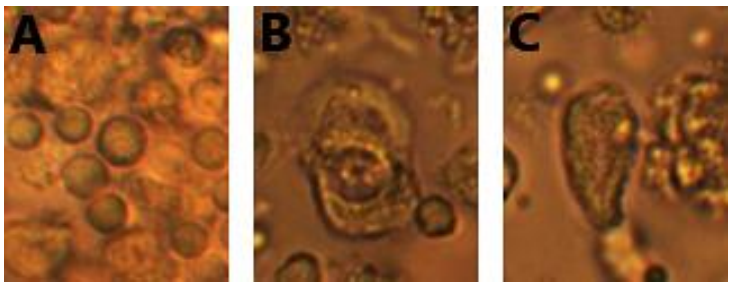

Figure 2. Microscope images of (A) stem cells, (B) a columnar cell, and (C) a goblet cell from *H. zea* larvae midguts taken at 400X magnification.

The first protocol came from multiple studies written by the Sadrud-Din, Loeb, and Hakim research group published between 1994 and 2009. Their protocol was developed over time throughout these studies, and the methods were not always clearly written or sometimes referenced steps developed and described in a previous publication. As a result, the complete protocol to be used in this project had to be pieced together from their many studies. This protocol will be referenced by the name cell migration (CM) because the isolation method in this protocol involved inducing the migration of cells out of the midguts through gentle shaking over time. The goal of this method was to create primary gut cell cultures from *Manduca sexta*, or tobacco hornworm, and induce proliferation and differentiation of stem cells through the addition of fat bodies from pupating insects, 20-hydoxyecdysone (20-HE), and fetal bovine serum (FBS). While there is significant internal validation for this protocol, as it has been described in at least five papers, obtaining a complete protocol was difficult and steps of the protocol were often not described well. The aforementioned factors could result in issues with protocol replication due to possible poor experimental design that could have resulted in missed or poorly described methods.

The second protocol chosen for this project came from a Darif et al., 2023 paper. This study isolates gut cells from the Lepidopteran species *Spodoptera frugiperda*, or fall armyworm,

for testing of cell penetrating peptides. This protocol will be referenced by the name forced cell separation (FCS) because the isolation method in this protocol involved pushing the guts through a filter basket and isolation of the cells for immediate use. The protocol used for isolation of cells is novel and did not reference other papers for its methods. The study itself is very new, being published in 2023, and likely has not had any replication studies done on it or published. As a result, the lack of external validation for the methods would be the greatest factor that could play a role in the reproducibility of this protocol.

This project assessed the question: Are these protocols replicable? This was tested by attempting to replicate the two protocols with an assessment of success based on the comparison of replicated results to the results published in the literature. Successful isolation of cells, cell proliferation, viability, and longevity were the variables used to measure the success of the CM protocol. The criteria for replication of isolation of cells should have seen successful isolation of cells with no contamination and the creation of primary cell cultures. For proliferation of cells, stem cells numbers should have quadrupled in 14 days when cultured with fat body from pupating insects. Viability and longevity were assessed by taking initial cell counts and subsequent cell counts throughout a period of 31 days and comparing the trends to cell count data from the various studies.

Results for the FCS protocol were assessed based on isolation of cells and viability and longevity of cultures. Literature results were focused only on the successful isolation of cells and the study did not include any assessment of culture creation or contamination due to the immediate use of the cells. The initial cell counts should have been at least 3.2×10^6 cells for 10 guts and 1.6×10^6 cells for 5 guts. There were no literature results beyond initial cell counts but longevity was measured in this study for comparison against the CM protocol.

Methods

Insect pre-dissection starvation procedure

H. zea larvae were individually added to souffle cups with Whatman grade 4, 42.5 mm diameter filter paper. Each filter paper was wetted with 0.5 mL of 1X sterile phosphate buffered saline (PBS; 11.83 g/L KH_2PO_4 and 2.299 g/L K_2HPO_4) + 10X antibiotic-antimycotic solution (AAS; Sigma Aldrich) + 5 mg/mL geneticin (g418). The *H. zea* larvae were left in a hatching incubator set at 28-31° C for 16-72 hours.

Sterilization and dissection of *H. zea* larvae

H. zea larvae were surface-sterilized with one, 2-minute wash in 70% ethanol (EtOH), one, two-minute wash in 0.1% Bleach, and two, two-minute washes in sterile water. They were left in the final water wash until dissected, for up to 1 hour.

All tools and dissection plates were sterilized with 70% EtOH before use and in between each dissection. A surface-sterilized *H. zea* larva was pinned to the dissection plate at its anterior end and the body was pulled taut with a second pin in the posterior and then fixed to the plate. A dorsal incision was made with scissors and the epidermis and fat body layers were pulled back and pinned on each side with pins. The peritrophic membrane and all gut contents were removed with forceps through a small incision in the gut. The attached nerves and trachea were removed, and the midgut was severed from the fore and hind gut regions and removed. The midgut was then flooded with Lepidopteran physiological solution (LPS; 47 mM KCl, 20.5 mM $MgCl_2$, 20 mM $MgSO_4$, 4.3 mM K_2HPO_4, 1.1 mM KH_2PO_4, 1 mM $CaCl_2$, 88 mM Sucrose), and any remaining gut contents or trachea were removed with forceps. The dissected midgut was then placed in a solution of LPS/0.001% bleach and the dissection steps repeated for all insects.

Midgut washing and sterilization

A 6-well plate (Thermo Scientific, Cat. No. 140675) was set up with five wells of LPS/0.001% bleach and one well of LPS + 1X AAS + 10 mg/mL g418. All dissected midguts were transferred to the first LPS/0.001% bleach well, stirred, and let sit for 2-10 minutes. This was followed by 2–3-minute washes in the remaining four LPS/0.001% bleach wells and the final LPS + 1X AAS + 10 mg/mL g418 well.

The previous sterilization and dissection steps were used for both of the following isolation protocols.

Cell migration method

A 70 µm-pore-size filter basket (VWR, Cat. No. 76327-100) was placed in a well of a 6-well plate filled with 6 mL of LPS + 1X AAS + 10 mg/mL g418. Fifteen midguts were added to the filter basket and the plate was placed on a VWR rocking platform at 17 rocks/min and left overnight.

The next day the filter basket was removed from the well and the media was transferred to 1.5 mL sterile conical tubes. The tubes were centrifuged at 400xg for 5-10 minutes. If no visible pellet was seen after 10 minutes of centrifugation, the speed was increased to 500xg, and the media was centrifuged for another 5-10 minutes. The supernatant was decanted, and two of the cell pellets were resuspended in 1 mL LPS. During this resuspension step, half of the tubes were resuspended and combined into 1 mL and the other half resuspended and combined into another 1 mL of LPS. The cells were centrifuged again at 400xg for 5-10 minutes. The supernatant was decanted, and all the cells were resuspended and combined in 1 mL Modified Grace's Insect Media (Supplemented Grace's media) (Sigma Aldrich), 10 µg/mL g418, 1X AAS,

1 mg/L 20-hydroxyecdysone, 0.35 g/L $NaHCO_3$, 10% fetal bovine serum (FBS). The cells were added to 3 mL of Modified Grace's Insect Media in a single well of a sterile 6-well plate.

Forced cell separation method

Due to the immediate use of the isolated cells, the published protocol did not include any sterilization or culture maintenance methods. For this study, the insects and the guts were prepared and sterilized like the Cell Migration method, see previous.

Once the gut sterilization was complete, they were transferred to a 70 µm-pore-size filter basket and placed in a well of a 6 well plate. For this method, the guts were split into groups of 10 and 5 and each group was placed in its own sieve. The guts were pushed carefully through the sieve using the pistil of a 1 mL syringe. Cells were collected by rinsing the sieve with 2 mL of Modified Grace's Insect Media. The medium and collected cells were then passed through a new 70 µm-pore-size filter basket. The media that passed through the filter basket was transferred into two 1.5 mL sterile conical tubes and centrifuged at 300xg for 5 min. The supernatant was discarded, and the pellets resuspended and combined in 1 mL Modified Grace's Insect Media. The cells were transferred to a sterile 6-well plate and brought up to 4 mL with Modified Grace's Insect Media.

Maintenance of primary insect gut cell culture

The following steps were used for culture maintenance and determination of cell viability for both the cell migration and forced cell separation protocols.

Cells were incubated in a 6-well culture plate at 27°C. They were checked daily for contamination for the first few days. Starting on day 6, and then repeating every 3-4 days, media was replaced, and cells were fed. This was done by removing 1 mL of media, approximately 1/3 of the media volume, while taking care not to draw up any cells by tilting the plate and drawing

up media away from the cells. The removed media was then replaced with 2 mL of Modified Grace's Insect Media.

Determining cell viability

Cells were counted every 3-4 days starting at day 6, with dissections occurring on day 1. The contents of a well were shaken gently and 15 µL of media was taken from the middle of the well. 15 µL of 0.4% (w/v) Trypan blue (Gibco, Ref. No. 15250-061) was then mixed with the aliquot of cells. The Trypan blue stains dead cells and debris while viable cells will exclude the dye. Cells were then visualized and counted using white light on a Hausser Levy hemacytometer (Hausser Scientific, Cat. No. 3500) with a Zeiss Axiovert 40 CFL microscope at 400X magnification. A cover slip was placed over the chambers of the hemacytometer and 5 µL of the cell mixture was pipetted into the slit underneath the cover slip and sucked up onto the slide, resulting in an even distribution. Under the microscope, live cells were counted within five of the 4x4 squares in the middle of the chamber. Total cell counts were obtained using the following formula, where x_n represents the cell count for each square and V is the total volume of the culture in milliliters.

$$\frac{x_1 + x_2 + x_3 + x_4 + x_5}{5} \times 25 \times 1000 \times V = Total\ Cells$$

The cell counts were averaged to and then multiplied by 25 to get the count for the inner grid of the hemocytometer, which is 1 mm^3 and equivalent to 1 µL. This is then multiplied by 1000 to get the number of cells per 1 mL and then multiplied again by the volume of the culture in milliliters to get the total number of cells in the culture. The total volume was estimated to be 4 mL throughout the study, so 4 mL was used as the final multiplication factor.

All graphs were made in GraphPad Prism.

Results

Results were measured by taking initial cell counts and continual cell counts thereafter, as well as assessing cell density through microscope images. The initial cell counts were taken on day 6 after dissection. The 10-gut forced cell separation replicates had an average cell count of 3.7×10^6 cells while the 5-gut replicates had an average initial cell count of 2.4×10^6 cells. The initial cell counts for each replicate of 10 guts were 4.4×10^6 cells, 3.6×10^6 cells, and 3.2×10^6 cells. For 5 guts the initial cell counts were 1.8×10^6 cells, 2.8×10^6 cells, and 2.6×10^6 cells. The average initial cell count for the three replicates of the cell migration method using 15 guts was 9.0×10^5 cells. The third replicate had a much higher cell count than the first two replicates, with an initial cell count of 2.0×10^6 cells compared to 1.0×10^6 cells and 6.0×10^5 cells. The initial cell count data is summarized in Figure 3 with a literature comparison for the FCS replicates. There is no cell count for the CM literature because these counts were not well described in the literature and there was no clear number for their initial cell counts.

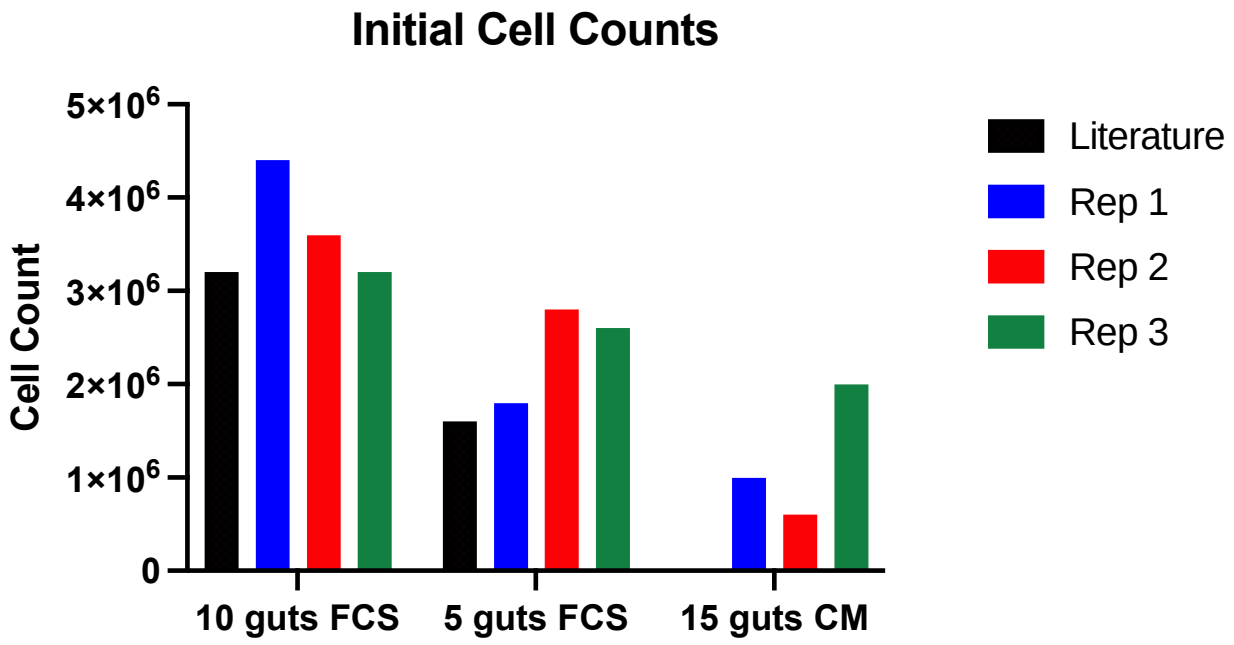

Figure 3. Initial cell counts of three replicates of FCS and CM protocols compared to literature results.

Cell counts were measured every 3-4 days for the duration of 31 days, starting with the initial cell counts on day 6. The 10 gut FCS replicates maintained the highest cell counts throughout the 31-day observation period. The two CM replicates with lower cell counts reached their lowest possible counts, between 0 and 2.0 x 10^6 cells around day 17. For these counts, some cells were seen when looking at the whole well under the microscope, but there were either zero or one cell within the counting area of the hemocytometer. Neither of the FCS groups reached cell counts this low during the 31 days. Figure 4 shows all the cell counts for all three groups with the trends being indicated by the solid lines. When co-cultured with fat bodies, the stem cells in this study were able to proliferate and quadrupled in number within 15 days (Figure 5). The open circles with the solid line show the growth trend of stem cells cultured in media alone, which are the conditions achieved in this replication study. The published literature indicates that stem cells do not proliferate in this environment and instead retain a constant number of cells.

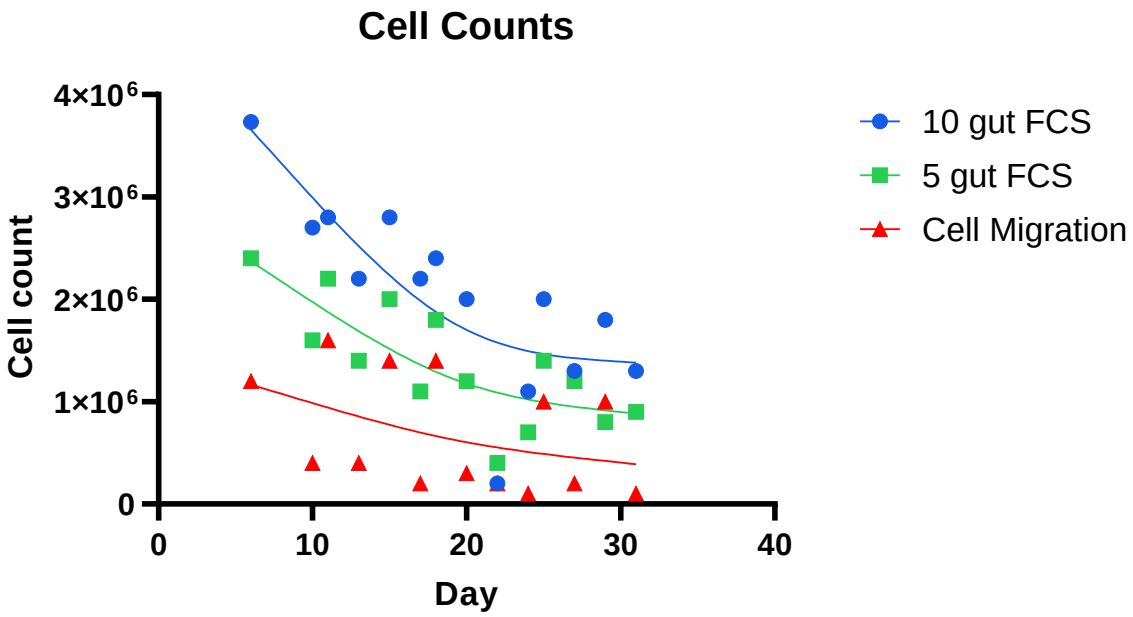

Figure 4. Average cell counts of FCS and CM protocols through 31 days. Data points represent averages of cell counts from three replicates of each protocol.

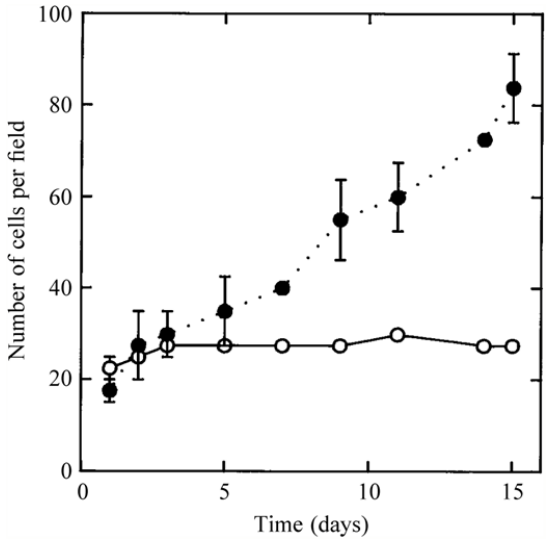

Figure 5. Cell counts showing the proliferation of isolated stem cells only when in the presence of co-cultured fat body. The open circles with the solid line are stem cells incubated in medium alone. The filled circles with the dotted line are stem cells co-cultured with fat body. (Sadrud-Din et al., 1996)

By visual inspection, the density of cells from a 10 gut FCS culture was significantly higher than the density from a CM culture (Figure 6). While the 10 gut FCS group maintained high cell counts throughout the 31 days, the CM group had a more significant decrease in cell density from days 1 to 17, and through day 31. The microscope images of the cell migration culture also confirm that cell counts were very low around day 17 and had very few cells remaining by day 31. The lack of proliferation of cells is also seen in these images, as there is no visible increase in cell density and only a decrease in the number of cells is seen.

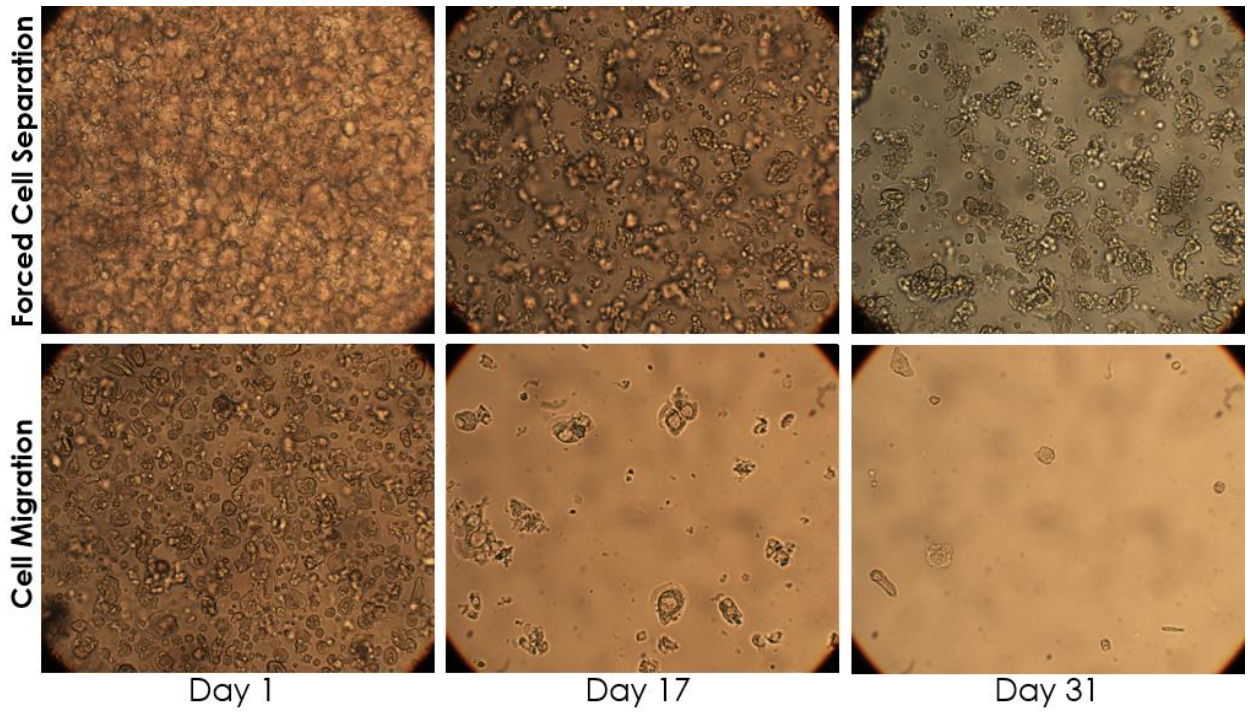

Figure 6. Cell density comparison of 10 gut forced cell separation protocol vs cell migration protocol at days 1, 17, and 31. Microscope images were taken from the cell culture wells at 400X magnification.

Discussion

Replication success was measured by comparison of results to those outlined in the literature. The variables measured to gauge success of the cell migration protocol were the isolation of cells, proliferation of cells, and longevity. Successful isolation of cells was eventually achieved, but not without changes to the protocol to eliminate contamination issues. Sterilization methods for the pupae fat bodies were not clearly described in any of the literature referenced, so attempts to add them into the cell cultures were unsuccessful as they led to contamination and therefore there was no cell proliferation. The cell counting with trypan blue staining provided reliable numbers for total live cell counts but did not allow for accurate counting of cell types. More accurate counting methods, such as counting cells directly from the

culture in a set field of view, are often more difficult or complicated to do by hand or are less described in the literature. Changing to a more accurate counting method would also require changes to the protocol.

Early isolation trials were unsuccessful in replicating the literature as there were consistent contamination issues. Additional antibiotic steps were added throughout the protocol in order to try and combat the various bacterial, yeast, and other fungal contaminations encountered. The concentration of AAS in the feeding solution was increased from 1X to 10X to reduce contamination that may have come from gut contents. The final midgut wash with LPS + 1X AAS + 10 µg/mL g418 and the use of antibiotics (1X AAS + 10 µg/mL g418) in the media for the shaking step of the cell migration protocol were also added. These steps, in addition to the replacement of old dissection tools and dissection plates, were essential for eliminating the contamination issues. Care was also taken to ensure that all reagents were sterile, including re-sterile filtering them, as well as having dedicated sterile tips and tubes. Another change essential for successful recreation was in the shaking step of the cell migration method. In the literature, this step was only for 1-2 hours, and yielded enough cells to start a culture. When this protocol was attempted with the shaking step being 1-2 hours there were not enough cells isolated to generate a pellet in the subsequent washing process. When shaken overnight, this step was able to produce enough isolated cells to generate a pellet in the washing process. A contributing factor to the need for this change may have come from the species of Lepidopteran used in the literature versus in this study. Several of the Sadrud-Din, Loeb, and Hakim studies used fourth instar *Manduca sexta* larvae, which can grow up to lengths of 49 mm in its fourth larval stage (Capinera, 2008b). Since *H. zea* are much smaller, averaging at lengths of about 11.4 mm in their fourth larval stage, the number of cells isolated overall would be less than for *M. sexta*, and more

time may be required to reach a high enough cell count to be able to get a pellet (Capinera, 2008a).

Since the forced cell separation protocol isolated cells that were to be used immediately in testing, the variables measured to assess this method were the successful isolation of cells and initial cell count. Successful isolation of cells was achieved, and due to the additional use of the sterilization and washing processes, there was no contamination, and the cells were able to be maintained in a culture for 31 days. The initial cell count described in the literature was at least 2.4×10^5 cells per 3 guts. This equates to 3.2×10^6 for 10 guts and 1.6×10^6 for 5 guts. These numbers were met, and surpassed, with the average initial cell count for 10 guts being 3.7×10^6 cells and the average initial cell count for 5 guts being 2.4×10^6 cells. The cultures for all three replicates of the FCS protocol were kept alive with high cell counts through the 31 days of the experiment, with the last cell counts taken on day 31 having an average of 1.4×10^6 cells for 10 guts and 8.6×10^5 cells for 5 guts. Overall, the replication results for the FCS protocol met the outlined literature results successfully and went above and beyond in the additional areas measured.

Conclusion

Based on the results of each of the two protocols, their reproducibility can be assessed. The forced cell separation protocol involved methods that were easy to pull from the literature and well described. These factors contributed to the ability to replicate this protocol, and therefore replicate the results, much more easily than the cell migration protocol. Results for this method were successfully met and even surpassed the literature results. While in the literature they did not create cultures from this protocol and only used the cells for immediate testing, this

study was able to combine the protocol with the sterilization and culture methods from the cell migration protocol and create cultures that were superior to the CM cultures in their cell counts, viability, and longevity of cells.

Replication of methods for the cell migration protocol was partially successful and was not without many failed trials and changes to the methods. Results were also not completely replicated. Isolation of cells was successful, but proliferation of cells was not met due to methods issues and the cell counts did not have clear literature numbers to compare to. The protocol itself was difficult to put together due to the incomplete nature of steps described in each of the papers. This piecing together of methods may have played a role in the difficulty of replicating the protocol and the results.

As seen through this project, literature replication is not always a straightforward process. Attempted replication of a piece of literature may involve changes that are necessary to achieve successful recreation of the protocols or results. It is also possible that conflicting results could be obtained. Improving reproducibility of literature as a whole is an issue that should take precedence in science if having validated data is considered important. This could be improved by increasing external-lab validation for studies, as well as increasing the number of validating replication studies in literature. Encouraging replication studies and increasing acceptance of publishing these studies is an important step in this process that relies on the greater scientific community, especially major science funding bodies and influential journals. An important part of validating literature is ensuring that the methods are reproducible because reproducibility of methods is essential for having reproducibility of results. In the case of the cell migration protocol in this study, partially successful replication of the results was impossible until changes were made to the protocol because the original methods were not reproducible and therefore led

to irreproducible results. This method had clearly been replicated internally many times, as it was used in 5 studies from this group, but due to the way it was written in the literature, external replication was very difficult. Through addressing replication issues on a broader scale and encouraging replication studies and external validation the reproducibility crisis in science can hopefully lessen, leading to better publishing practices and data that is more credible.

Resources

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