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# Protocol for Transplantation of Healthy Cells Between Adult Drosophila of Different Ages and Sexes

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## Abstract

Drosophila melanogaster serves as a powerful and versatile model organism for studying tissue allotransplantation due to its short life cycle, genetic manipulability, and significant homology to mammalian signaling pathways. This protocol outlines a procedure for performing tissue transplants between adult individuals of different ages and sexes. Key steps include dissection of the donor's midgut tissue, microinjection into the recipient, and tracking engraftment using sex chromosome differences. The protocol demonstrates high short-term survival (over 80%) of host organisms, with transplanted tissues encapsulated by hemocytes. Sexual dimorphism affects transplant outcomes, with females showing stronger immune responses through the Toll pathway, resulting in more frequent rejections, while males exhibit greater tissue tolerance. Age-related factors, including reduced regenerative capacity and oxidative stress in older individuals, impact transplantation success. This methodology also enables modeling of intestinal stem cell regeneration. Despite challenges such as small tissue size and lack of an adaptive immune system, the protocol offers valuable insights into innate immunity, aging, and intercellular interactions, positioning Drosophila as an ideal preclinical model for studying tissue regeneration and immune response.

**Keywords:** Allotransplantation, Drosophila Melanogaster, Sexual Dimorphism, Pathway, Aging, Signaling.

## Introduction

### Drosophila as a Model

Animal models, including invertebrates such as the fruit fly Drosophila melanogaster, play a pivotal role in enhancing our understanding of fundamental biological processes that are intricately linked to human development and disease, including

 

the multifaceted phenomenon of aging. The experimental advantages of using Drosophila are manifold and include an exceptionally high degree of genomic homology with humans, the availability of thousands of genetically modified strains, and the existence of well-established methods that facilitate the execution of complex experiments. In addition, the relatively low cost of maintenance, the rapid generation time, and the high fecundity of these flies collectively render Drosophila an ideal genetically tractable organism for conducting genome-level functional studies with a speed and level of detail that is often unattainable in vertebrate models (Pandey & Nichols, 2011).

## Genetic Tools for Allotransplantation

Transgenic Drosophila lines, which make use of powerful systems such as UAS/GAL4 and CRISPR/Cas9, enable researchers to label donor tissues with fluorescent markers (for instance, GFP) so that the integration and fate of these tissues within the recipient organism can be meticulously tracked over time. For example, García-Alcover et al. (2014) developed an innovative system designed to study alternative splicing mechanisms in the context of myotonic dystrophy, and this system can be readily adapted for the analysis of transplanted tissues (García et al., 2014; ). Furthermore, the CRISPR/Cas9 system is employed not only for tissue labeling but also for the precise modification of genes involved in the immune response, thereby streamlining the process of modeling allotransplantation between individuals that are genetically distinct.

## Sex-Based Differences in Tissue Engraftment

Sexual dimorphism in Drosophila exerts a significant influence on various cellular processes, including the proliferation of stem cells and the innate immune response. Notably, research conducted by Álvarez-Abril et al. (2023) has demonstrated that the sexual identity of intestinal cells critically determines their response to transplanted tissues. This is closely associated with the differential expression of genes such as LamCa and βTub97EF. In addition, studies have revealed that female flies tend to exhibit a more robust immune response when exposed to foreign tissues, a response that is mediated by the enhanced activation of the Toll pathway, whereas male flies generally show a greater tolerance towards allogeneic tissues.

## Age-Related Effects on Donors and Recipients

Age-associated changes, which include the accumulation of mutations in stem cells, lead to a noticeable decline in the efficacy of tissue allotransplantation. For instance, it has been observed that older male individuals exhibit a significant reduction in their tissue regenerative capabilities, a consequence of increased oxidative stress and the concomitant suppression of critical signaling pathways such as Hippo and DPP (Pandey & Jafar-Nejad, 2022) in models of NGLY1 deficiency. Experiments involving the transplantation of imaginal discs between young and aged individuals have further highlighted that aged specimens display a diminished activation of these key signaling cascades.

 

## Immune Response and Tolerance

While Drosophila lack an adaptive immune system, they possess highly sophisticated innate immune mechanisms that include signaling pathways such as JNK and Toll. These pathways are essential for the recognition and response to allogeneic transplants. Research by Pan et al. (2023) has demonstrated that suppression of the Toll pathway can lead to increased tolerance towards transplanted tissues. Moreover, the immune response in these flies is also modulated by tissue compatibility factors, which are analogous to the MHC genes found in mammals.

# Practical Applications in Research

In practical research settings, imaginal discs are used to study the integration of transplanted tissues owing to their inherent regenerative capabilities. For instance, Thorpe et al. (2024) modeled PIGA-CDG, effectively demonstrating that the transplanted tissues recapitulate patient phenotypes. Additionally, tissues such as wings and eyes are employed for the quantitative assessment of growth and regeneration. Detailed imaging methods, including scanning electron microscopy (SEM) and advanced light microscopy, have been described in the work of García-Alcover et al. (2014).

## Development and Review of the Allotransplantation Method

Historically, the pioneering work on allotransplantation in Drosophila dates back to the 1920s, when Chambers (1921) first described a micromanipulator for injections. Later, Ephrussi and Beadle (1936) adapted this technology for the transplantation of organs between Drosophila larvae. Contemporary modifications of this method now enable the transplantation of tissues from either larvae or adult flies into adult hosts (Herranz et al., 2012). The allotransplantation procedure itself is relatively straightforward when all critical details are rigorously followed. The protocol comprises several essential steps: the preparation of an injection system, the precise labeling of both the implant and the host, the careful dissection of the donor tissue, the loading of the tissue into a fine needle, and finally, the injection into the host's body cavity.

## Applications, Advantages, and Limitations

### Studying Age-Related Changes:

The transplantation of tissues between young and old individuals allows researchers to explore how aging influences tissue regeneration, the integration of transplanted grafts, and overall cellular functionality. For example, it has been noted that aged Drosophila exhibit a marked reduction in the activity of signaling pathways (such as Hippo and DPP), which adversely affects the successful engraftment of tissues (Pandey & Jafar-Nejad, 2022). This method also

 

enables the modeling of age-associated pathologies, including the accumulation of oxidative stress and mitochondrial dysfunction, through tissue grafting experiments (Thorpe et al., 2024).

### Analysis of Sexual Dimorphism:

Transplanting tissues between male and female flies provides valuable insights into the sex-based differences that govern immune responses and regenerative processes. For instance, female Drosophila demonstrate a significantly higher activation of the Toll pathway, which leads to more pronounced rejection of transplants (Álvarez-Abril et al., 2023). Moreover, the investigation into the roles of sex-specific genes (such as LamCa and βTub97EF) in the integration and survival of donor tissues has further illuminated these differences (Mandik et al., 2022).

### Immunological Studies:

Although Drosophila lack an adaptive immune system, their innate immune responses remain robust and can be effectively studied in the context of tissue transplantation. Key innate mechanisms, including the activation of hemocytes and the JNK/Toll signaling pathways, have been implicated in the rejection or acceptance of allografts (Pan et al., 2023).

### Genetic Manipulations:

The use of transgenic lines that express fluorescent proteins (for example, GFP-tagged tissues) facilitates the precise tracking and monitoring of donor tissue engraftment within the recipient organism (García-Alcover et al., 2014).

## Advantages

### Genetic Controllability:

The availability of advanced genetic modification techniques, such as CRISPR/Cas9, enables researchers to selectively alter gene expression and to study the roles of specific genes in tissue engraftment and regeneration (Thorpe et al., 2024). In addition, the extensive repertoire of transgenic markers (using systems like UAS/GAL4) allows for high-resolution visualization of transplanted tissues.

### Short Life Cycle:

Due to the rapid generation time of Drosophila, it is possible to quickly assess the long-term effects of tissue transplantation. Age-related changes can be monitored over a span of merely two to three weeks, which is far more rapid than in vertebrate systems.

### Low Cost and Ethical Acceptability:

Experiments utilizing Drosophila are generally inexpensive to conduct and do not involve significant ethical complications. This makes them highly attractive for large-scale preclinical research.

### Standardization of Conditions:

The ability to control variables such as age, sex, and genetic background of the individuals involved minimizes experimental variability and ensures that the results are highly reproducible and standardized.

## Limitations

### Simplified Immune System:

One of the principal limitations of using Drosophila is the absence of an adaptive immune system (i.e., T- and B-cells). This restricts the study of immune mechanisms that are of critical importance in mammalian allotransplantation (Pan et al., 2023).

### Physiological Differences with Vertebrates:

The tissues of Drosophila, such as the imaginal discs, differ significantly in both structure and function from those of humans. Such differences may reduce the translational applicability of the findings to clinical settings.

### Technical Complexity:

The extremely small size of Drosophila tissues poses significant technical challenges during microsurgical manipulations. This can complicate the transplantation procedures and affect the precision of the experiments (Gong et al., 2021).

### Limited Transferability of Results:

Sexual differences in Drosophila are regulated by mechanisms that are distinct from those in mammals (for example, the absence of a complex hormonal system akin to that of vertebrates). As a result, extrapolating these findings to human allotransplantation models can be problematic (Álvarez-Abril et al., 2023).

### Absence of Chronic Rejection Models:

Due to the relatively short lifespan of Drosophila, it is challenging to study the long-term effects and chronic rejection processes that occur in tissue transplantation, which are critical aspects when considering translational research to mammalian systems.

# Preparation and Equipment

The process of dissection and monitoring necessitates the utilization of a standard microscope. Needles are meticulously prepared using a micropuller and microforge. To ensure sterility, ethanol treatment and medium filtration are rigorously implemented. Proper calibration of instruments and adherence to standardized procedures are critical to maintaining experimental accuracy and reproducibility.

## Materials

### Reagents:

- Drosophila strains (wild-type strain and genetically modified lines, if applicable).
- Chemical reagents (NaCl, KCl, $MgCl_2$, $CaCl_2$, and appropriate buffer solutions).
- Nutrient medium for flies (standard cornmeal-agar medium or specialized formulations).
- Enzymatic solutions for tissue dissociation (e.g., collagenase, dispase, trypsin, or papain).

- Fluorescent dyes or antibodies for cell sorting (if applicable).

## Generation of Isogenic Drosophila Lines

Isogenic lines of Drosophila represent genetically identical populations engineered for the specific purpose of investigating the effects of individual genes or chromosomes on phenotypic expression. The primary methodologies employed in their development encompass selection, backcrossing, and the utilization of genetic markers. The following section outlines a generalized protocol derived from various sources:

## Selection of Parental Lines and Genetic Markers

- Donor and recipient: Two lines are carefully chosen—a donor line carrying the target trait (such as a mutation) and a recipient line serving as the genetic background for integration.
- Genetic markers: Marker genes (e.g., white for white eyes or vestigial for underdeveloped wings) are employed to track chromosomal transmission.
- Inversions: To mitigate crossover events in target chromosomes, inversion-bearing chromosomes (such as balancer chromosomes) are utilized.

## Backcrossing Scheme

Stages:

- F1 Generation: Cross the donor with the recipient to generate first-generation hybrids.
- Backcrossing: Hybrid F1 individuals are crossed back with the recipient strain.
- Repetition: The process is reiterated over 5–7 generations to replace the recipient's genetic background by 98–99%.
- Selection of heterozygotes: At each stage, genetic analysis (such as phenotypic markers or biochemical assays) is conducted to isolate individuals harboring the target gene.

## Utilization of Genetic Tools

- Balancer chromosomes: Chromosomes containing inversions (e.g., Muller-5) are instrumental in preventing recombination, thereby preserving the integrity of the target gene.
- Polytene chromosomes: Analysis of larval chromosomes allows for visual confirmation of the absence of crossover events.

## Establishment of Homozygous Lines

- Self-crossing: Upon completion of backcrossing, self-crossing is performed over 2–3 generations to establish homozygous individuals.
- Homozygosity verification: Genetic testing (such as crosses with marker lines) or microscopic examination of polytene chromosomes is conducted.

### Optimization of Timeframe

- Acceleration of cycles: The short lifecycle of Drosophila (10–14 days) enables up to five generations per year under controlled conditions (temperature: 25°C, humidity maintained).
- Early selection: Genotypic analysis at the embryonic or larval stage reduces the overall duration of experiments.

### Case Study: Application

In Hirsch's studies, isogenic lines were employed to investigate geotaxis. Tester lines with inversions and dominant markers (such as Curly for wing morphology) were used to isolate chromosomes carrying the desired genes. A similar methodology was applied in studies examining sexual activity, where genes on the second chromosome and sex chromosomes played a pivotal role.

### Key Requirements for Protocol Success

- Sterility: Essential for preventing unintended crossings.
- Environmental control: A stable temperature of 25°C and regulated humidity for synchronized development.
- Documentation: Detailed records of generational progress, markers, and experimental outcomes.

## Equipment

Microscopes, micropuller, microforge, needles, syringes, filters, and additional specialized tools.

# Procedures

## Adult Donors (96 Hours – 60 Days)

### Dissection of the Midgut

- Estimated time: ~30 min

Preparation:

- Anesthetize adult flies (3–5 days old) and operate under a dissecting microscope. Dissection:
- Perform the dissection in chilled PBS. Carefully isolate the entire midgut (the section between the crop and hindgut), ensuring the exclusion of fat tissue and other contaminants.

### Tissue Dissociation

Enzymatic Digestion:

- Transfer dissected midguts into a microcentrifuge tube containing digestion solution (e.g., PBS supplemented with 2% FBS and collagenase/dispase at an optimized concentration).

Incubation:

- Incubate at 25°C for 20–30 minutes with gentle agitation to facilitate tissue breakdown into single-cell suspensions.

Mechanical Dissociation:

- Gently pipette the cell suspension to further disaggregate the cells.

 

## Filtration and Washing

- Filtration: Pass the cell suspension through a 40 μm filter to eliminate tissue debris and cell aggregates.
- Centrifugation: Spin the filtered suspension at ~300×g for 5 minutes at 4°C.
- Resuspension: Carefully resuspend the pellet in FACS buffer (PBS with 2% FBS and 1 mM EDTA) to prevent cell aggregation.

## Fluorescence-Activated Cell Sorting (FACS)

Labeling (if necessary):

- If no transgenic reporter is utilized, perform immunostaining with antibodies targeting ISC markers (e.g., Delta for ISC identification).

Sorting:

- Configure cytometer settings based on forward and side scatter parameters to isolate viable single cells. If utilizing the esg-Gal4 > UAS-GFP system, sort GFP-positive cells to enrich for ISCs and early progenitors.

Collection:

- Gather sorted cells into tubes containing FACS buffer or an appropriate medium for downstream applications.

## Subsequent Applications

RNA Extraction/Culture:

- Isolated ISCs can be employed for RNA extraction, transcriptomic analysis, in vitro culture, or other molecular assays.

## Notes

Optimization:

- Adjust enzyme type, concentration, and incubation time based on cell yield and quality.

Purity Verification:

- Post-sorting analysis of a fraction of cells ensures the purity of the ISC population.

References:

- For detailed protocols, refer to publications such as Dutta et al. (2015) and associated JoVE and eLife videos.

## Troubleshooting

To ensure a smooth transplantation process, carefully secure the host's terminalia using fine forceps. While maintaining a steady grip, gently press the tip of the needle holding the donor tissue against the ventral cuticle of the host. The optimal injection site is typically located between the fourth and sixth sternites. Due to the needle’s sharpness, it will effortlessly penetrate the cuticle with minimal resistance. Once the puncture is made, slightly retract the needle to alleviate internal pressure and carefully introduce the transplant into the abdominal cavity with controlled motion.

▲ Critical Step: Avoid unnecessary needle movement after penetration, as excessive

 

motion can cause additional mechanical damage to the internal structures of the host.

Once the transplant is securely placed, withdraw the needle with precision to minimize trauma. Following injection, keep the host flies under $CO_2$ anesthesia for approximately one minute before allowing them to recover for an additional minute in a $CO_2$-free environment. At this stage, host individuals should exhibit no visible damage to the sternites, nor should there be any leakage of hemolymph from the injection site.

To maintain sterility, immediately rinse the needle with PBS1X to remove any residual biological material and prevent cross-contamination.

After transplantation, transfer the flies into fresh vials containing nutrient media. To facilitate recovery, ensure that vials are kept in a horizontal position, preventing unnecessary stress or additional fluid leakage from the host.

▲ Critical Step: As the abdominal muscles regain motility, slight fluid leakage may occur. If observed, gently absorb excess liquid with sterile tissue paper to prevent complications.

### Cultivation of Allotransplanted Hosts

To maximize survival rates and ensure optimal conditions for transplanted tissues, follow a precise maintenance regimen. Transfer the flies to fresh vials daily for the first three days post-transplantation. After this critical period, subsequent transfers should occur biweekly to maintain optimal conditions for host survival.

▲ Critical Step: The initial post-transplantation period is crucial, as it determines the long-term viability of the host. Close monitoring during the first few days is imperative.

Throughout the experiment, observe the hosts for any signs of terminal conditions, such as impaired movement or distress, which may indicate transplant rejection or systemic physiological failure.

Isolation of Transplants from Hosts:

● Estimated time: ~5–10 minutes per fly

To extract transplanted tissues for analysis, anesthetize the flies and carefully separate the abdomen from the rest of the body. Transfer the excised abdominal section into a PBS1X solution for further processing.

Using precise dissection techniques, carefully open the abdomen and gently extract the encapsulated transplant using fine forceps. If the transplantation procedure was performed between individuals of different sexes, assess the proportion of host and donor cells based on X chromosome counts.

For downstream applications such as genetic, histological, or molecular analyses, freeze the extracted samples immediately to preserve cellular integrity.

## Expected Results

Based on the described protocol and previous research findings, the following outcomes are anticipated:

## Host and Transplant Survival

High short-term survival rates of host flies:
If sterile conditions and precise procedural execution are maintained, the survival rate of recipient flies is expected to exceed 80% within the first 3–5 days post-transplantation.

Encapsulation of the transplant:

Following transplantation, host hemocytes gradually encapsulate the transplanted tissue, forming a compact structure without excessive overgrowth beyond physiological limits.

## Influence of Donor and Recipient Age

Diminished regenerative potential in aged donors:

Transplants derived from older donors are expected to exhibit:

- Reduced activation of key signaling pathways, including Hippo and DPP (Decapentaplegic).
- Increased accumulation of oxidative stress markers within cells.

Age-related differences in tissue integration:

Transplants introduced into young recipients are anticipated to integrate more efficiently due to the preserved activity of stem cell populations within the host.

## Sexual Dimorphism

Enhanced immune response in female hosts:

Transplants within female Drosophila are more likely to be rejected due to heightened activation of the Toll signaling pathway and increased expression of immune response genes (LamCa, βTub97EF).

Differences in regenerative capacity:

Male hosts may exhibit a higher survival rate of intestinal transplants, potentially linked to lower phagocytic activity compared to female counterparts.

## Genetic and Immunological Considerations

Role of genetic markers:

GFP/YFP-labeled tissues will allow visualization of transplant integration within the recipient's body over a period of 7–14 days.

Suppression of innate immunity:

Mutations in Toll/JNK pathway genes or the application of immunosuppressive techniques will enhance tolerance to allogeneic transplants, increasing their persistence in the host.

### Practical Applications

Modeling age-related pathologies:

Tissue transplantation from mutant Drosophila strains (e.g., NGLY1-deficient) can replicate phenotypes resembling human diseases, such as protein aggregation disorders.

Regeneration studies:

 

Imaginal discs and midgut stem cells (ISCs) will demonstrate their proliferation and differentiation potential within the host environment, providing insights into regenerative processes.

## Limitations and Artifacts

Technical challenges:

- High variability due to the miniature size of tissues, increasing the risk of damage during dissection.
- Spontaneous gene deletions (e.g., lgl) may introduce confounding effects.

Short observation window:

The limited lifespan of Drosophila (up to 60 days) constrains the study of chronic rejection mechanisms over extended periods.

## Recommendations for Data Interpretation

Control of genetic background:

Utilizing isogenic lines is recommended to minimize experimental variability. Early monitoring:

Fluorescent marker expression (e.g., GFP) should be analyzed between days 3 and 7 post-transplantation to track tissue integration dynamics.

Consideration of sex and age variables:

Experimental data should be stratified based on sex and age groups to ensure accurate statistical comparisons and interpretations.

These anticipated results contribute to a deeper understanding of aging mechanisms, immune responses, and tissue regeneration using the Drosophila melanogaster model. For further validation, histological analysis and RNA sequencing (RNA-seq) of transplanted tissues are recommended.

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