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Methods Article

An efficient injection protocol for *Drosophila* larvae

Sattar Soltani [1], Nhan Huynh[1] and Kirst King-Jones[1,*]

[1]Department of Biological Sciences, University of Alberta, G-504 Biological Sciences Bldg, Edmonton, Alberta, Canada, T6G 2E9

*Corresponding author. Department of Biological Sciences, University of Alberta, G-504 Biological Sciences Bldg, Edmonton, Alberta, Canada, T6G 2E9.
E-mail: Kirst.King-Jones@ualberta.ca

Abstract

Intravenous injection provides a direct, rapid, and efficient route for delivering drugs or other substances, particularly for compounds with poor intestinal absorption or molecules (e.g. proteins) that are prone to structural changes and degradation within the digestive system. While *Drosophila* larvae represent a well-established genetic model for studying developmental and physiological pathways, as well as human diseases, their use in analyzing the molecular effects of substance exposure remains limited. In this study, we present a highly efficient injection method for *Drosophila* first- and second-instar larvae. Despite causing a slight developmental delay, this method achieves a high survival rate and offers a quick, easily adjustable protocol. The process requires 3–5 h to inject 150–300 larvae, depending on the microcapillary needle, microinjection system, and the compound being administered. As proof of concept, we compared the effects of injecting ferritin protein into *Fer1HCH*^{00451} mutant first instar larvae with those of dietary ferritin administration. Our results show that ferritin injection rescues *Fer1HCH* mutants, a result that cannot be achieved through dietary delivery. This approach is particularly valuable for the delivery of complex compounds in cases where oral administration is impaired or limited by the digestive system.

Keywords: drug administration; injection; larval injection; ferritin; ferritin injection; *Drosophila* larval drug delivery

Introduction

The study of disease mechanisms and the evaluation of novel active pharmaceutical compounds in model organisms are critical steps in drug development. *Drosophila melanogaster* has been widely used in pathophysiological and biomedical research due to its evolutionarily conserved biological processes and the availability of advanced genetic and molecular tools [1–3].

Oral administration is the most straightforward method for testing substances in *Drosophila* adults or larvae. However, ensuring consistent absorption through the digestive system presents challenges when studying biological processes and assessing compound efficacy [4]. The digestive system's chemical properties—such as pH, digestive enzymes, intestinal permeability, transit time, presence of gut microbes, and interactions between compounds and the fly food—can potentially alter the physical properties of the drug or limit its absorption [4–6]. Cell culture and *ex vivo* tissue culture have been used to evaluate cellular responses to compounds [7, 8]. However, these methods often struggle to replicate *in vivo* conditions, and issues such as compound solubility, contamination risks, and limited cell and tissue longevity can complicate studies [9, 10].

Injecting drugs offers several advantages over oral administration. It ensures efficient and targeted delivery with precise dosage control, especially for compounds with poor oral absorption or in cases requiring rapid systemic effects [11, 12]. Injection also bypasses the digestive system, avoiding compound degradation or precipitation [11, 12]. In *Drosophila*, embryo injection is a commonly used strategy for genome editing and introducing new genetic material, facilitating the study of gene function and chemical effects on development [13]. Injection into the adult thorax has been developed to study nutritional sensing mechanisms controlled by insulin signaling [12, 14–16].

Larval *Drosophila* injection is often preferable to embryo and adult injection when investigating the molecular underpinnings of physiological processes, as larvae continuously consume nutrients, and undergo substantial growth and development over a period of ∼4 days [1, 17, 18]. As such, *Drosophila* larvae are particularly suitable for studying the impact of new compounds on larval physiology and development. Here, we describe an improved injection method for first and second instar *Drosophila* larvae, overcoming many of the limitations of previously described techniques. This method presented here efficiently delivers compounds to internal target organs and allows for the study of molecular responses throughout development from larval to adult stages. Our protocol, adapted and modified from a larval injection protocol developed for *Tribolium* (*Coleoptera*) [19], allows for high survival rates and rapid, large-scale compound screening with reduced workload.

As a proof of concept, we injected human and horse spleen holo-ferritin into *Fer1HCH*^{00451} mutant larvae, which display impaired ferritin assembly due to a mutation in the ferritin heavy chain (*Fer1HCH*) gene. Ferritin, a protein complex comprising 24 subunits, and generally composed of 12 Heavy (H) and 12 Light (L) chains, can store over a thousand iron atoms [20]. Mutations in the *Drosophila* ferritin genes cause embryonic or early larval lethality [21]. The *Fer1HCH*^{00451} allele causes lethality in mid-first instar larvae [22, 23]. While dietary supplementation with holo-ferritin failed to rescue *Fer1HCH*^{00451} mutants, injection of

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holo-ferritin into first instar larvae allowed their development to adulthood.

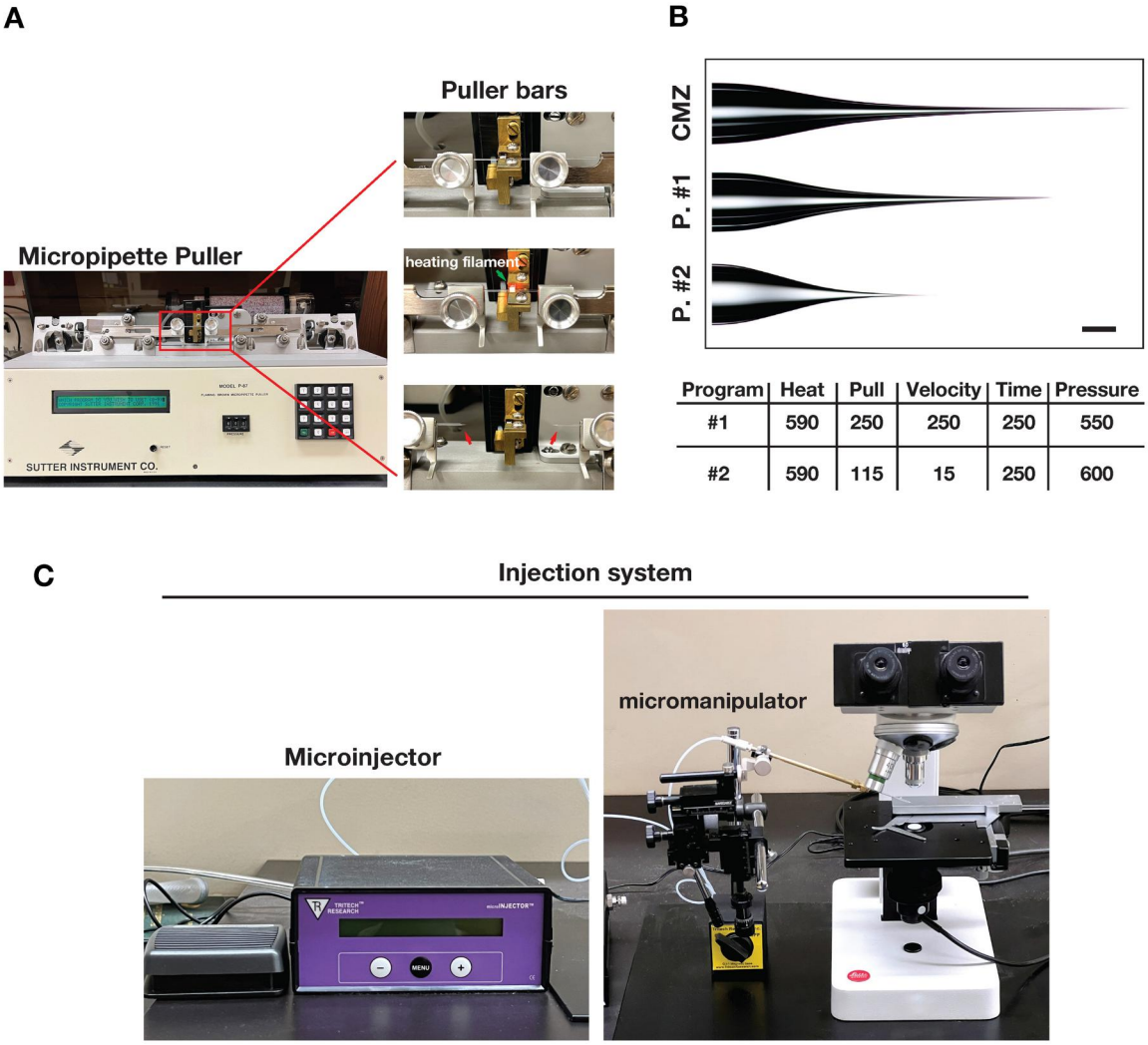
Materials and methods

Injection

Needle preparation and opening

a) A well-prepared needle is crucial for injection efficiency and success. We used a microcapillary needle puller (Fig. 1A, Sutter Instrument Company. Model P-87) with two distinct needle programs to create needles with unique tip lengths and shapes (named P. #1 and P. #2, Fig. 1B). Needle program parameters are provided in Fig. 1B. We used unpulled microcapillary needles with an outer diameter of 1.2 mm and an inner diameter of 0.9 mm (SUTTER Instruments, No. B120-90-10). Commercially available pre-pulled microcapillary needles (10/pack, Cat. No. MINI-PP, named CMZ, Fig. 1B) were purchased from Tritech Research. The needles were loaded with 5–10 µl of the 1X PBS (Sigma No. P3813-10PAK, pH 7.4), human liver holo-ferritin (Ferritin^{Hu}, Sigma, Cat. No. F6754, Concentration: 10 µg/ml, MW = 440 kDa, Stock solution: 22.7 nM), horse spleen holo-ferritin (Ferritin^{Ho}, Sigma, Cat. No. F4503, Concentration: 25 mg/0.5 ml, MW = 440 kDa, Stock solution: 113.6 µM), and Brilliant Blue FCF (BBF) dye (Sigma, Cat. No. 80717-100MG, Fig. 3A) using Eppendorf microloader tips (Sigma, Cat. No. EP5242956003). Ferritin solutions were injected at the final concentration of 5 pM (~10–20 ng of ferritin, for details, see the Injection procedure) into late first instar larvae, while the final concentration of injected BBF was 100 nM. *Note*: All three needles were capable of penetrating larvae efficiently; however, needles created with program #2 performed better than those made with program #1 or the commercial needles (Fig. 1B). Needles can be prepared in advance and stored at 4°C for up to 24 h.

b) We attached the loaded needle to the micromanipulator and opened the tip (Fig. 1C). The needle tip plays a crucial role in injection efficiency and larval survival. The needle was opened under a microscope by gently tapping the tip



Program	Heat	Pull	Velocity	Time	Pressure
#1	590	250	250	250	550
#2	590	115	15	250	600

Figure 1. Larval injection setup. (A) Micropipette puller. Red box highlights the puller bars (a part of the pulley adjustment setup) and a glass microcapillary needle. The needle is secured in the V-groove of the pull bars and positioned through the heating filament (arrow). The vertical pull produces two needles (arrows). (B) Needle comparison. Tips of standard commercial needles (CMZ) compared to needles created using Program 1 (P.1) and Program 2 (P.2) on the micropipette puller. Table shows parameters for both programs. Scale bar: 100 µm. (C) Injection setup. Left: Digital Multi-pressure Microinjector system. Right: Three-axis coarse/fine micromanipulator attached to the microscope, both of which are mounted on top of an anti-vibration base plate.

with a sharp surface. We used a broken slide piece to create a sharp edge for this purpose.

Larval collection

a) Animals were reared on the desired medium until the required stage.
   Note: We used w^{1118} (control animals, ID: 3605) and $Fer1HCH^{00451}$ mutant flies [21, 22] from the Bloomington *Drosophila* Stock Center (BDRC, Indiana University, Bloomington, IN, USA). All flies were reared on standard fly medium (NUTRI-fly, Cat. No. 66-113), which was prepared following the company's instructions. To prepare Brilliant Blue-supplemented food (BBF), we added Brilliant Blue dye (Sigma, Cat. No. 80717-100MG) to a final concentration of 100 μM.
b) We collected 10- to 15-h-old first-instar or ~5-h-old second-instar larvae (L2) with forceps (#5) or a fine paintbrush. Larvae were rinsed in ice-cold distilled water or 1× PBS buffer and gently tapped on dry tissue paper to remove excess liquid (avoid over-drying as it impacts survival rates). For this study, we used first- and second-instar larvae, but third-instar larvae (16–20 h old) may also be used.

Slide preparation

a) We adhered double-stick tape (5 mm × 15–20 cm, Scotch™ Brand No. 665) to a glass slide (25 × 75 × 1 mm) in order to immobilize larvae for injection (Fig. 2A and B). *Note*: An alternative method using extracted glue is also an option (place double-sided tape in a small glass bottle with 500 μl to 1 ml heptane, shake overnight, to extract the smooth glue).
b) We individually transferred 10–15 semi-dried larvae onto the tape. Consistent alignment of larvae ensures easier injections, which can be carried out by positioning the mouth hook (anterior) toward the slide's outer edge. Enough space should be left between larvae to allow for injections into the T3 and A1 segments (Fig. 2B and C)
c) Prepared injection slides were stored in a slide holder or Petri dish lined with moistened filter paper to prevent the larvae from drying out. Up to 10 slides can be prepared in advance, preparing more than 10 at once is not recommended, as it increases larval lethality due to prolonged time on tape without food or proper humidity, leading to dehydration and stress.

Larval immobilization on slides

a) To increase injection efficiency, second-instar larvae were immobilized by anesthetizing them with CO_2 gas for ~2 min or by placing the slide at 4°C for 3–5 min. Larvae were monitored frequently to avoid over-immobilization, which can reduce recovery rates.
b) Immobilized larvae were covered with 200S halocarbon oil (Sigma #H8898-100ML). *Note*: Halocarbon oil can be applied before or after the injection, depending on room humidity.

Injection procedure

We used a Digital Multi-pressure Microinjector (Fig. 1C, Tritech Research, Cat. No. MINJ-D) connected to an inverted microscope. The following parameters were used: 5 PSI pressure, 0.2-s intervals, and 5–10 s repeats. This allowed for injection of ~0.5–1 μl per larva into the desired larval segment. Given that the human and horse spleen holo-ferritin compounds were injected at 5 pM, injecting ~0.5–1 μl corresponded to ~10–20 ng of ferritin protein per larva. For ring gland and CNS analysis, injections were performed in the T3 and A1 segments (Fig. 2C).

Post-injection recovery

a) After injection, larvae were covered with halocarbon oil (if not applied earlier) and allowed to recover on the slide for 1–2 h at room temperature. This prevents hemolymph leakage from the injection site.
b) Injected larvae were gently rinsed with distilled water to remove the oil and carefully release them from the tape adhesive. A piece of lint-free paper can be used to remove excess oil as well.
c) Larvae were then transferred to fresh medium using forceps (#5) or a fine paintbrush.

Media preparation and survival analysis

All flies were reared on standard fly medium (NUTRI-fly, Cat. No. 66-113) and maintained at 25°C with 60% to 70% humidity. For media supplemented with holo-ferritin, we used 50 μM final concentration of human liver holo-ferritin ($Ferritin^{Hu}$, Sigma, Cat. No. F6754) and horse spleen holo-ferritin ($Ferritin^{Ho}$, Sigma, Cat. No. F4503), and 100 μM BBF (Sigma, Cat. No. 80717-100MG). For the larval survival analysis, 50 injected first-instar larvae (L1) were transferred into the tested media and the number of second-instar larvae (L2), third-instar larvae (L3), pupae (P), and adults (Ad) were quantified. For the feeding analysis, 50 embryos of w^{1118} (control) and $Fer1HCH^{00451}$ mutants were transferred to the appropriate media, and the number of L1, L2, L3, P, and Ad were counted. All experiments were performed in triplicate.

Larval hemolymph collection and BBF absorbance analysis

Hemolymph was extracted from larvae following a protocol by Hiroyasu *et al.* [24]. Samples were collected from 50 third-instar larvae (24 and 44 h) in batches of 25 individuals. Larvae were washed in 1× PBS and gently pierced near the mouth-hooks using #5 forceps. Hemolymph was collected immediately and snap-frozen in liquid nitrogen to prevent melanization. A total of 50 μl (per well) of hemolymph was added to a 96-well flat-bottom UV-star plate (Sigma-Aldrich, No. M3812-40EA). Absorbance was measured at 595 nm using a VICTOR Nivo Multimode Microplate Reader.

Results

Drosophila digestive tract restricts compound release into hemolymph

The *Drosophila* larval gut consists of several structures, including the peritrophic membrane, epithelial cells, the basement membrane, circular and longitudinal muscles, and the serosal barrier. These structures create a defense barrier against pathogens and limit the uptake and release of unsuitable compounds into the hemolymph (larval blood) [25].

To test whether the larval digestive tract limits the release of dietary compounds into the hemolymph, we compared larvae fed BBF food dye with larvae injected with BBF (Fig. 3A and B). We reared w^{1118} animals on a standard diet (no-supplement) until 20 h after the L1/L2 molt (~65 h after egg deposition) ensuring comparable conditions between the injected and fed groups. One group was transferred to a diet supplemented with 100 μM of BBF, whereas another group was injected with 100 nM BBF (~1 μl per larva). Injected larvae were allowed to recover on standard

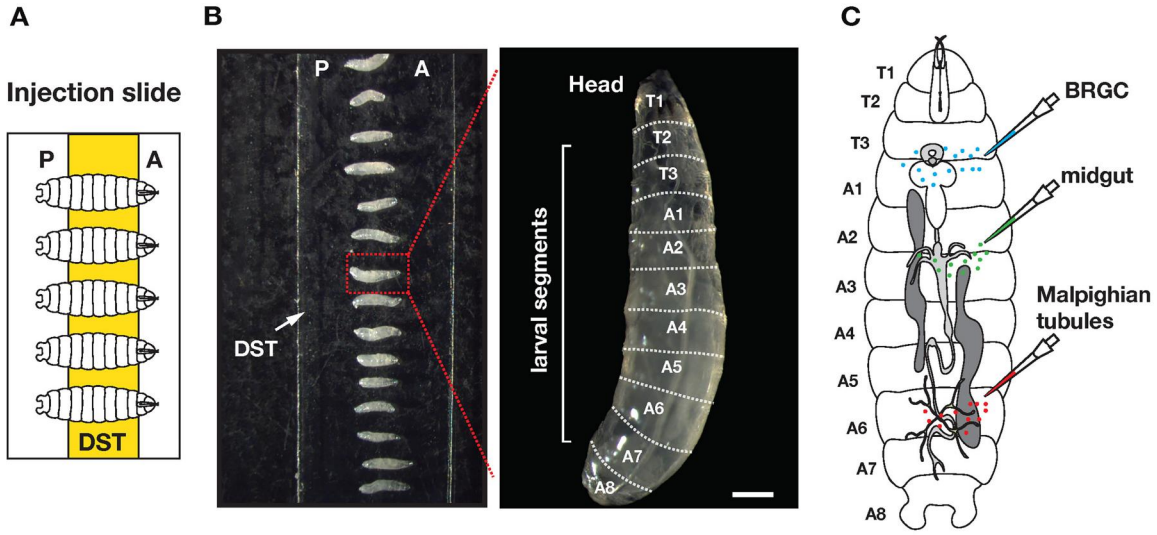


Figure 2. Larval alignment and injection. (A) Schematic illustration of larval alignment on injection slide. First (L1) or second (L2) instar larvae were placed on double-stick tape (DST) and oriented from posterior (P) to anterior (A). (B) Left: an injection slide of L2 larvae aligned on double-stick tape (DST), oriented from posterior (P) to anterior (A). Right: Larval segments highlighted, showing thoracic (T1–T3) and abdominal (A1–A8) segments, indicating needle entry sites. (C) Schematic illustration showing larval segments used for injecting near the Brain-Ring Gland Complex (BRGC, T3/A1 segments), the anterior midgut (A2–A3 segments) and the Malpighian tubules (A6–A7 segments).

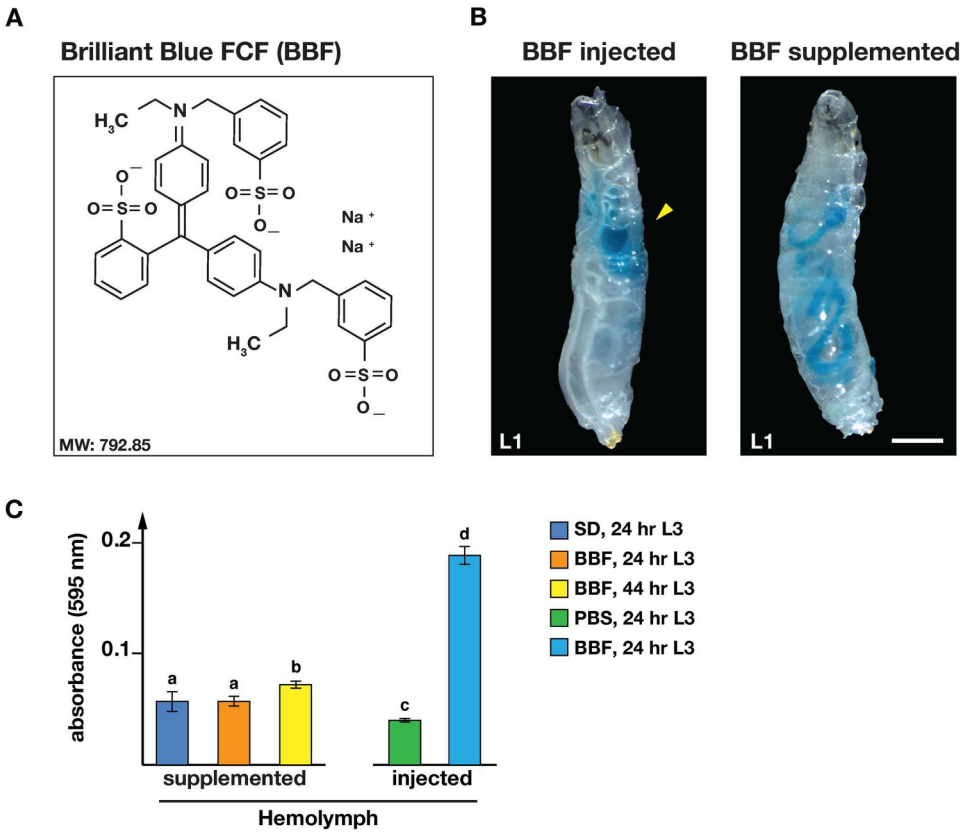


Figure 3. Comparison of BBF absorption in *Drosophila* L1 larvae via injection and feeding. (A) BBF structure and molecular weight (MW). (B) BBF Injection Visualization. Comparison of an L1 larva injected with 10 μM BBF into the third thoracic segment (T3) and an L1 larva fed with 150 μM BBF. w^{1118} first instar larvae (L1) were used for both injection and feeding analysis. Scale bar: 500 μm. (C) BBF absorbance at 595 nm of hemolymph samples isolated from fed and injected w^{1118} L1 larvae. Animals were reared on a standard diet (SD), or on diets supplemented with 100 μM BBF. Larvae were injected with 100 nM BBF and 1× PBS buffer. Significance letters correspond to *P*-values obtained via a two-tailed, paired Student's *t*-test (***$P < .001$). Error bars represent standard deviations, with the center denoting the average of three biological samples.

food (no BBF) for 24 h before hemolymph collection. Hemolymph from the fed group was collected at 24 and 44 h during the L3 larval stage, and BBF absorbance at 595 nm was measured (Fig. 3C).

As expected, the absorbance of BBF in the hemolymph of injected L3 larvae was notably higher (~3.5-fold) than in corresponding hemolymph samples isolated from animals reared for

24 h on BBF-supplemented medium (0.189 versus 0.055). Extending the feeding period to 44 h only slightly increased BBF absorbance from 0.055 to 0.072 in the fed group.

Taken together, these results suggest that the *Drosophila* larval digestive system limits the absorption of dietary compounds into the hemolymph, whereas injection into the larval cavity bypasses this limitation, providing a more efficient method for compound delivery.

Injection of human and horse ferritin rescued *Drosophila Fer1HCH* mutants

To assess the efficacy of larval injection versus larval feeding, we used the *Fer1HCH*^{00451} mutant allele, a null mutant that causes lethality at the first-instar larval stage (Fig. 4A). First, we attempted to rescue *Fer1HCH*^{00451} mutants by feeding larvae with holo-ferritin (i.e. iron-bound protein) isolated from human liver and horse spleen. Both ferritin supplements failed to rescue the arrested L1 larvae, with approximately 60–75% of embryos hatching but failing to develop beyond the L1 stage (Fig. 4A). The arrested larvae died after 7 days, consistent with previous attempts to rescue *Fer1HCH* mutants using dietary iron supplementation.

Next, we reasoned that injecting ferritin directly into the larval cavity would increase the concentration of ferritin in the hemolymph, which in turn should allow for cellular ferritin uptake and thus compensate for the ferritin deficiency. To test this, we injected human liver (Ferritin^{Hu}) and horse (Ferritin^{Ho}) holo-ferritin, into arrested *Fer1HCH*^{00451} first-instar larvae. Remarkably, injecting Ferritin^{Hu} and Ferritin^{Ho} allowed the mutants to progress through the L2 and L3 stages, pupation and even reach adulthood, with survival rates of 27% and 43%, respectively (Fig. 4B). In contrast, injecting PBS buffer showed no noticeable rescue, suggesting that the ferritin-deficient cells were able to import ferritin from the hemolymph when directly injected. These data indicate that microinjection of ferritin bypasses the digestive system's limitations and provides a rapid, efficient method for rescuing *Fer1HCH* mutant larvae.

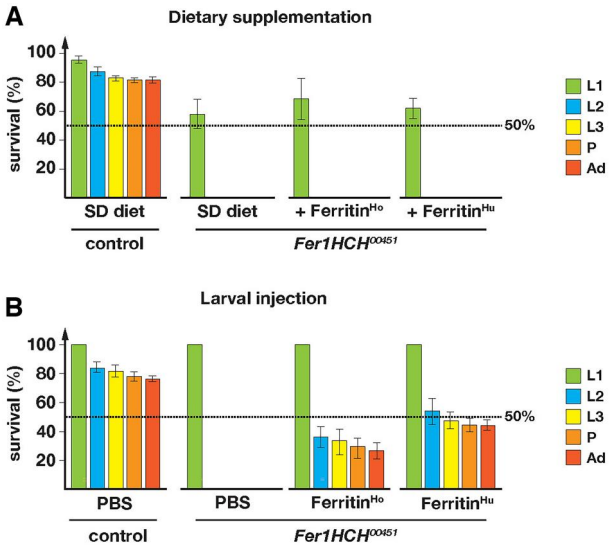


Figure 4. Survival analysis of *Fer1HCH*^{00451} mutants on ferritin-supplemented diets and following larval ferritin-injection. (A) Survival analysis of *Fer1HCH*^{00451} and control (w^{1118}) animals on a standard diet (SD) and media supplemented with holo-ferritin obtained from horse (Ferritin^{Ho}) and human (Ferritin^{Hu}). (B) Survival analysis of *Fer1HCH*^{00451} larvae injected with holo-ferritin solutions. First instar larvae of *Fer1HCH*^{00451} and control (w^{1118}) animals were injected with PBS buffer, horse holo-ferritin (Ferritin^{Ho}) and human holo-ferritin (Ferritin^{Hu}). First-instar larvae (L1) were scored for survival at the larval, pupal, and adult stages. Error bars represent the standard error of three biological replicates. PBS buffer was used as the control compound in injections. L1: first instar, L2: second instar, L3: third instar, P: pupae, Ad: adults.

Discussion

In this study, we used a *Drosophila* larval injection strategy to deliver compounds directly to internal tissues, bypassing the limitations of dietary supplementation. The previous reports describing injection procedures for *Drosophila* larvae have significant shortcomings (Table 1). The described methods lack precise reporting on critical parameters such as needle tip size, injection volume, injection site, and the number of larvae injected per round, making reproducibility and scalability difficult. The published methods were designed for third-instar larvae, limiting their adaptability to earlier developmental stages, which produce much smaller larvae. For smaller larvae, the exact parameters for needle production are more critical, as these small larvae are more vulnerable and less likely to tolerate inappropriate needles. In addition, critical injection tools, in particular glass capillary tubes, were either not specified or unsuitable for handling larger injection volumes. Furthermore, the prior injection strategies were not intended for higher throughput approaches, as they focused on injecting only a few larvae. The previous studies did not provide a detailed step-by-step protocol, further complicating attempts to replicate or refine their methods. Unlike the technique presented here, the earlier reports make controlled survival studies difficult to achieve, given that this requires a reasonably high throughput strategy that does not cause significant lethality. The absence of defined injection sites for tissue-targeted studies limits the precision by which compounds can be delivered, thus limiting the scope of possible experimental approaches. These limitations, along with the absence of survival data across larval, pupal, and adult stages, prevent thorough evaluation of previous methods [19, 26, 27].

Overall, our injection approach proved very effective, allowing for the controlled dosing and administration of substances into the body cavity by injecting into specific larval segments. This approach was efficient, and allowed for rapid and controlled delivery of substances with minimal impact on larval survival and development. Notably, injected larvae exhibited full recovery within 5–8 h, with no observable lethality or long-term developmental defects, underscoring the practicality and robustness of this technique [28].

One of the key insights gained from this work was the limited ability of the *Drosophila* gut to absorb certain compounds. The gut's complex architecture—including the peritrophic membrane, epithelial cells, and other barriers—creates a defense mechanism that restricts the release of dietary compounds into the hemolymph. Our results with BBF confirmed the gut acts as a significant barrier, as feeding larvae BBF did not translate into significant BBF levels in the hemolymph, even after extended feeding periods (Fig. 3B). This is consistent with the well-known "first-pass effect" where compounds are metabolized, chemically altered, or prevented from absorption during their initial passage through gut or liver tissues, thereby limiting their therapeutic potential [29, 30]. In contrast, direct injection of BBF into the hemolymph resulted in significantly higher levels of the dye, demonstrating that bypassing the gut is critical for exposing target tissues to certain substances.

Finally, we injected human or horse ferritin into *Fer1HCH*^{00451} mutant larvae to test whether this could rescue the developmental arrest of these animals. We previously demonstrated that

Table 1. Comparison of larval injection protocols.

Method / Parameter[a]	Method 1 [19]	Method 2 [25]	Method 3 [26]	This publication
Injected animal	*Tribolium castaneum*	*Drosophila melanogaster*	*Drosophila melanogaster*	*Drosophila melanogaster*
Experimental purpose	Evaluating RNAi efficacy via dsRNA injection	Evaluating RNAi efficacy via dsRNA injection	Evaluating innate immune response to bacterial infection	Assessing the survival of controls and mutants after sub-stance injection
Needle tip size and shape	Not reported	Not reported	Program reported	Program details with supporting images
Capillary tube[b]	10-µl glass capillary tube	Not reported	3.5″ glass capillary tubes	Microcapillary (1.2 mm OD, 0.9 mm ID) and commercial needles
Injection volume[c]	∼0.4 to 0.5 µg dsRNA	∼0.25 to 0.5 µg dsRNA	Not reported	0.5–1 µl per larvae
Larval stage for injection	Last instar larvae	Third-instar larvae	Wandering third instar larvae	first-, second- and third-instar larvae
Adaptability for different larval stages	Unknown	Unknown	Unknown	Can be applied to all larval stages
Larval size	Large	Large	Large	Small to large
Larval injection site	First or second abdomi-nal segment	Larval dorsal side	Uncontrolled, on the dorsal side of the posterior	Injection performed at user-selected sites
Larval mortality	Not reported	Not reported	Only 72 h after injection reported	Survival was evaluated for all larval instars, pupae, and adults
Injection variability	dsRNA	dsRNA	Bacteria	Soluble compounds
Number of injected larvae (per round)	Not reported	Not reported	Larvae injected individually	100–150 larvae

[a] All parameters are based on reported methods [19, 25, 26].
[b] OD: outer diameter and ID: inner diameter.
[c] Injection volume can affect larval viability significantly.

injecting exogenous horse spleen holo-ferritin into *Drosophila* second-instar larvae with prothoracic gland (PG)-specific ferritin depletion (via PG-specific expression of *Gal4* driving a *Fer1HCH*-RNAi transgene, aka *PG>Fer1HCH*-RNAi) successfully rescued the lethality typically observed in these animals [28]. In contrast, the injection of horse spleen apo-ferritin, which lacks iron, failed to rescue the larval lethality, indicating the essential role of iron in the ferritin-mediated rescue process [28]. In *Drosophila*, the PG requires substantial amounts of iron to produce the steroid hormone ecdysone [28, 31], and PG-specific RNAi targeting *Fer1HCH* causes a developmental arrest in second-instar larvae, leading to 100% lethality [28]. Injection of equine holo-ferritin allowed *PG>Fer1HCH*-RNAi larvae to reach adulthood, indicating that internal larval tissues can endocytose mammalian holo-ferritin to acquire iron or utilize ferritin's residual capacity to store or detoxify excess iron [28]. However, we did not compare the survival rates of injected *PG>Fer1HCH*-RNAi larvae to those reared on a diet supplemented with equine spleen holo-ferritin, nor did we test a Fer1HCH mutant. Consistent with our previous findings, injection of human liver and horse spleen holo-ferritin rescued the arrested $Fer1HCH^{00451}$ first-instar larvae, whereas dietary supplementation with these ferritins failed to do so (Fig. 4A and B). These results further support the idea that gut cells metabolize dietary ferritin proteins, likely digesting them in the gut and preventing their intact absorption.

Finally, we did not systematically monitor BBF diffusion in larval hemolymph following injection; however, preliminary observations indicated that BBF remained locally enriched for at least 24 h post-injection. In contrast, obtaining comparable diffusion data for ferritin compounds was not possible due to their transparent solutions. This localized enrichment may be influenced by the relatively high viscosity of larval hemolymph [32], which likely plays a role in restricting diffusion. We recognize that diffusion patterns may vary depending on the physical and chemical properties of the injected compounds, as well as the specific injection sites. Therefore, we suggest that injecting compounds into larval segments near the target tissues may enhance their effectiveness on the tissues of interest.

In conclusion, despite challenges such as the requirement for specialized equipment, technical expertise, and potential risks of larval lethality, larval injection offers several distinct advantages over other administration methods. These include bypassing first-pass metabolism, allowing for controlled site-specific delivery, and enabling a rapid, efficient response to target substances.

Author contributions

Sattar Soltani (Investigation [equal], Methodology [equal], Validation [equal], Writing—original draft [equal]), Nhan Huynh (Investigation [equal], Methodology [equal]), and Kirst King-Jones (Conceptualization [equal], Funding acquisition [equal], Methodology [equal], Project administration [equal], Supervision [equal], Writing—review & editing [equal])

Conflict of interest statement. The authors declare there are no competing interests.

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Data availability

All the data presented in this manuscript are available upon request.

References

1. Yamaguchi M. *Drosophila Models for Human Diseases*. Singapore: Springer Nature Singapore, 2018.
2. Ugur B, Chen K, Bellen HJ. Drosophila tools and assays for the study of human diseases. *Dis Model Mech* 2016;**9**:235–44.
3. Millburn GH, Crosby MA, Gramates LS. *et al.*; FlyBase Consortium. FlyBase portals to human disease research using Drosophila models. *Dis Model Mech* 2016;**9**:245–52.
4. Homayun B, Lin X, Choi HJ. Challenges and recent progress in oral drug delivery systems for biopharmaceuticals. *Pharmaceutics* 2019;**11**:129.
5. Lou J, Duan H, Qin Q. *et al.* Advances in oral drug delivery systems: challenges and opportunities. *Pharmaceutics* 2023;**15**:484.
6. Domínguez-Avila JA, Wall-Medrano A, Velderrain-Rodríguez GR. *et al.* Gastrointestinal interactions, absorption, splanchnic metabolism and pharmacokinetics of orally ingested phenolic compounds. *Food Funct* 2017;**8**:15–38.
7. Wise C. *Epithelial Cell Culture Protocols*. Berlin, Germany: Springer Science & Business Media, 2008.
8. Centenera MM, Raj GV, Knudsen KE. *et al.* Ex vivo culture of human prostate tissue and drug development. *Nat Rev Urol* 2013;**10**:483–7.
9. Buckley ST, Fischer SM, Fricker G. *et al.* In vitro models to evaluate the permeability of poorly soluble drug entities: challenges and perspectives. *Eur J Pharm Sci* 2012;**45**:235–50.
10. Gumbleton M, Audus KL. Progress and limitations in the use of in vitro cell cultures to serve as a permeability screen for the blood-brain barrier. *J Pharm Sci* 2001;**90**:1681–98.
11. Jain KK. *Drug Delivery Systems*. Berlin, Germany: Springer Science & Business Media, 2008.
12. Jin JF, Zhu LL, Chen M. *et al.* The optimal choice of medication administration route regarding intravenous, intramuscular, and subcutaneous injection. *Patient Prefer Adherence* 2015;**9**:923–42.
13. O'Connor MJ, Chia W. Gene transfer in Drosophila. *Methods Mol Biol* 2002;**180**:27–36.
14. Webster CS, Merry AF, Gander PH. *et al.* A prospective, randomised clinical evaluation of a new safety-orientated injectable drug administration system in comparison with conventional methods. *Anaesthesia* 2004;**59**:80–7.
15. Anselmo AC, Mitragotri S. An overview of clinical and commercial impact of drug delivery systems. *J Control Release* 2014;**190**:15–28.
16. Xu Y, Shrestha N, Préat V. *et al.* Overcoming the intestinal barrier: a look into targeting approaches for improved oral drug delivery systems. *J Control Release* 2020;**322**:486–508.
17. Mirzoyan Z, Sollazzo M, Allocca M *et al. Drosophila melanogaster*: a model organism to study cancer. *Front Genet* 2019;**10**:51.
18. Rubin GM. Drosophila melanogaster as an experimental organism. *Science* 1988;**240**:1453–9.
19. Tomoyasu Y, Denell RE. Larval RNAi in Tribolium (Coleoptera) for analyzing adult development. *Dev Genes Evol* 2004;**214**:575–8.
20. Rosas-Arellano A, Vásquez-Procopio J, Gambis A. *et al.* Ferritin assembly in enterocytes of Drosophila melanogaster. *Int J Mol Sci* 2016;**17**:27.
21. Missirlis F, Kosmidis S, Brody T. *et al.* Homeostatic mechanisms for iron storage revealed by genetic manipulations and live imaging of Drosophila ferritin. *Genetics* 2007;**177**:89–100.
22. González-Morales N, Mendoza-Ortíz MÁ, Blowes LM. *et al.* Ferritin is required in multiple tissues during Drosophila melanogaster development. *PLoS One* 2015;**10**:e0133499.
23. Li S. Identification of iron-loaded ferritin as an essential mitogen for cell proliferation and postembryonic development in Drosophila. *Cell Res* 2010;**20**:1148–57.
24. Hiroyasu A, DeWitt DC, Goodman AG. Extraction of hemocytes from Drosophila melanogaster larvae for microbial infection and analysis. *J Vis Exp* 2018;**135**:57077.
25. Shanbhag S, Tripathi S. Epithelial ultrastructure and cellular mechanisms of acid and base transport in the Drosophila midgut. *J Exp Biol* 2009;**212**:1731–44.
26. Miller SC, Brown SJ, Tomoyasu Y. Larval RNAi in Drosophila. *Dev Genes Evol* 2008;**218**:505–10.
27. Tafesh-Edwards G, Kenney E, Eleftherianos I. Drosophila melanogaster Larva Injection Protocol. *J Vis Exp* 2021;**176**:e63144.
28. Soltani S, Webb SM, Kroll T. *et al.* Drosophila Evi5 is a critical regulator of intracellular iron transport via transferrin and ferritin interactions. *Nat Commun* 2024;**15**:4045.
29. Kwan KC. Oral bioavailability and first-pass effects. *Drug Metab Dispos* 1997;**25**:1329–36.
30. Doherty MM, Pang KS. First-pass effect: significance of the intestine for absorption and metabolism. *Drug Chem Toxicol* 1997;**20**:329–44.
31. Huynh N, Ou Q, Cox P. *et al.* Glycogen branching enzyme controls cellular iron homeostasis via Iron Regulatory Protein 1 and mitoNEET. *Nat Commun* 2019;**10**:5463.
32. Zabihihesari A, Parand S, Rezai P. PDMS-based microfluidic capillary pressure-driven viscometry and application to Drosophila melanogaster's hemolymph. *Microfluid Nanofluid* 2023;**27**:8.

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