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Tumor Allotransplantation in Drosophila melanogaster with a Programmable Auto-Nanoliter Injector

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Corresponding Author:	Wu-Min Deng Tulane University School of Medicine New Orleans, LA UNITED STATES
Corresponding Author's Institution:	Tulane University School of Medicine
Corresponding Author E-Mail:	wdeng7@tulane.edu
Order of Authors:	Shangyu Gong
	Yichi Zhang
	Hongcun Bao
	Xianfeng Wang
	Chih-Hsuan Chang
	Yi-Chun Huang
	Wu-Min Deng
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TITLE:

Tumor Allotransplantation in *Drosophila melanogaster* with a Programmable Auto-Nanoliter Injector

AUTHORS AND AFFILIATIONS:

Shangyu Gong[1#], Yichi Zhang[1#], Hongcun Bao[1], Xianfeng Wang[1], Chih-Hsuan Chang[1], Yi-Chun Huang[1], Wu-Min Deng[1]

[1]Department of Biochemistry and Molecular Biology, Tulane University School of Medicine, New Orleans, Louisiana, USA

Email Addresses for All Authors:

Shangyu Gong (sgong1@tulane.edu)
Yichi Zhang (yzhang52@tulane.edu)
Hongcun Bao (hbao2@tulane.edu)
Xianfeng Wang (xwang52@tulane.edu)
Chih-Hsuan Chang (cchang18@tulane.edu)
Yi-Chun Huang (yhuang30@tulane.edu)
Wu-Min Deng (wdeng7@tulane.edu)

Correspondence:

Wu-Min Deng (Wdeng7@tulane.edu)

#These authors contributed equally to this work.

KEYWORDS:

Drosophila, salivary gland, NICD, tumor allograft, transplant, allotransplantation, autoinjector, live imaging

SUMMARY:

This protocol provides detailed guidance for the initial and continued generational allotransplantation of *Drosophila* tumors into the abdomen of adult hosts for studying various aspects of neoplasia. Using an autoinjector apparatus, researchers can achieve improved efficiency and tumor yields compared to those achieved by traditional, manual methods.

ABSTRACT:

This protocol describes the allotransplantation of tumors in *Drosophila melanogaster* using an auto nanoliter injection apparatus. With the use of an autoinjector apparatus, trained operators can achieve more efficient and consistent transplantation results compared to those obtained using a manual injector. Here, we cover topics in a chronological fashion: from the crossing of *Drosophila* lines, to the induction and dissection of the primary tumor, transplantation of the primary tumor into a new adult host and continued generational transplantation of the tumor for extended studies. As a demonstration, here we use Notch intracellular domain (NICD)

overexpression induced salivary gland imaginal ring tumors for generational transplantation. These tumors can first be reliably induced in a transition-zone microenvironment within larval salivary gland imaginal rings, then allografted and cultured in vivo to study continued tumor growth, evolution, and metastasis. This allotransplantation method can be useful in potential drug screening programs, as well as for studying tumor-host interactions.

INTRODUCTION:

This protocol provides a step-by-step guidance for allotransplantation of *Drosophila* larval salivary gland (SG) imaginal ring tumors into abdomens of adult hosts using an auto nanoliter injection apparatus (e.g., Nanoject). This protocol also provides directions for the subsequent re-allografting of tumors into new generations of adult hosts, which provides opportunities for continued longitudinal study of tumor characteristics, such as tumor evolution and tumor-host interactions. The protocol can also be applied toward drug screening experiments.

This method was developed to improve upon the efficacy of performing tumor allotransplantation in *Drosophila* using manual injectors[1], which are often inconsistent in their suction and injection forces, leading to suboptimal results for tumor allotransplantation. An autoinjector apparatus provides better control and can result in lower rates of fly mortality post-allograft. A trained operator could achieve a host-survival rate of over 90% with the autoinjector, compared to around 80% when the manual injector was used[1]. The overall tumor acquisition rate is 60%–80% at day 8–12 post-allograft. The average injection time has also been improved from 30–40 s per fly using a manual injector to 20–25 s per fly using the autoinjector.

This protocol is among the first few protocols to use the autoinjector apparatus in *Drosophila* tumor allotransplantation. A recent study also used the autoinjector for allotransplantation of tumorous neural stem cells[2]. Previously, the autoinjector apparatus was used in *Drosophila* to study bacterial virulence[3], parasitic infections and host defense[4], as well as screening for bioactivity of different compounds[5]. Our protocol adapts the autoinjector apparatus for tumor injection use and seeks to provide *Drosophila* researchers with higher quality and more consistent results while saving them considerable time. This protocol can not only be used for the allotransplantation of tumors, but can also be tailored to the allotransplantation of wildtype and mutant tissues of similar caliber[6].

The *Drosophila* NICD tumor used in this protocol was first introduced by Yang et al.[7] in the SG imaginal ring transitional zone, a “tumor hotspot” that exhibits high levels of endogenous Janus Kinase/Signal Transducer and Activators of Transcription (JAK-STAT), and c-Jun N-terminal Kinase (JNK) activity. Additionally, the transition zone has high levels of matrix metalloproteinase-1 (MMP1)[7], which makes this region particularly conducive to tumorigenesis. Notch pathway activation through NICD overexpression alone is sufficient to consistently initiate tumor formation. These tumors can be subsequently allotransplanted to allow investigation of a broad range of topics, including tumor cell division, invasion, and tumor-host interactions.

PROTOCOL:

1. Preparation of SG imaginal ring tumor

1.1. Cross adult flies with genotypes of *UAS-NICD* (Male: 10–15 flies) and *Act-Gal4, UAS-GFP/CyO; tub-Gal80^{ts}* (Virgin female: 10–15 flies) and allow them to breed for 1 day at 18 ˚C. The selected adult flies should be 5–9 days old to ensure high fertility.

1.2. Allow the adult flies to lay eggs in the fly food contained in vials for 24 h at 18 ˚C, then remove the adult flies.

NOTE: Fly food is prepared using the standard cornmeal food recipe from *Drosophila* Stock Center[8]. Each vial should contain around 10mL of fly food.

1.3. Allow the eggs to incubate for 6 days at 18 ˚C. During this period, the larvae will hatch.

1.4. Transfer the vials containing larvae to a 29 ˚C incubator and incubate for another 7 days.

NOTE: This incubation step is optional depending on the specific experimental design.

2. Preparation of adult wild type *Drosophila* for allotransplantation

2.1. Anesthetize wild type or appropriate mutant adult flies with 100% CO_2 and sort flies based on sexes. Both male and female flies can be used as tumor hosts.

2.2. Secure a 5 cm long piece of fly tape to a microscope slide with the sticky side up by securing it with two smaller pieces of tape at each side.

2.3. Immobilize the flies by adhering their wings to the tape. Use forceps while maneuvering the flies.

2.3.1. Repeat the above step for 60–80 adult flies used as allograft acceptors.

NOTE: It is best to organize flies into neat rows, with their body axis aligned parallel to each other for a more time-efficient injection process later. **Figure 1** shows rows of host flies taped down in this manner.

3. Assembly of the autoinjector apparatus

3.1. Connect both the autoinjector apparatus and power cord to the controller box.

3.1.1. Set the injection volume to 59.8 nL. This will help maintain the appropriate amount of suction and injection forces during allotransplantation.

3.2. Place the controller box and the autoinjector on opposite sides of the light microscope.

NOTE: For right-handed operators, the control box should be placed on the left side of the microscope with the injector on the right side. Vice versa for left-handed operators.

3.3. Prepare the 3.5'' glass capillary for use by clipping off the closed end with forceps.

3.3.1. Process one end of the glass capillary using a four-step micropipette puller and heat to a narrow, closed end using the following specifications in the instrument: Heat = 650, Force = 200, and Distance = 8. Neatly place the capillaries into the puller apparatus and run the program after inputting the above settings.

3.3.2. Use forceps to clip the capillary at an approximately 60˚ angle to make a sharper end for easy entry into the adult fly abdomen[1]. See **Figure 2** for an example of a well-clipped capillary.

3.4. Lightly unscrew the cap of the injector. Hold down the **Empty** button to advance the injector needle until 70%–80% of its total length is showing. To accelerate capillary advancement, press the **Fill** button once while simultaneously holding down the **Empty** button.

3.5. Use a syringe to fill the glass capillary with mineral oil. Then, carefully insert the glass capillary onto the injector needle until the former is firmly attached to the rubber stopper of the injector. Now screw the injector cap tight.

3.5.1. Wipe off the mineral oil residue on the external surface of the glass capillary cover to avoid contaminating the medium during allotransplantation.

4. Dissection of the SG imaginal ring tumor

4.1. Select one of the larvae and transfer it to a dissection plate filled with 100 µL of Schneider's Medium to prepare for SG imaginal ring tumor dissection.

NOTE: Only the specimens harboring the tumor will remain as larvae. This is because tumor growth delays larval development and progression[9]. Two thirds of the specimens will not harbor the tumor and will thus have progressed to pupae/adults.

4.1.1. For dissection and allotransplantation purposes, use a stereomicroscope with a 10x–20x magnification range.

4.2. Using one pair of forceps to hold the mid-section of the larval body, pinch the larval head using another pair of forceps and apply a stretching force lengthwise.

4.3. Locate the Y-shaped SG of the larvae and isolate it from the rest of the larval tissue[10].

4.4. Dissect and isolate the SG imaginal ring tumor by removing the adjacent tissue. See **Figure 3** for a depiction of this dissection process.

4.5. Repeat steps 4.1–4.4 for an additional 10 to 20 SG imaginal ring tumors based on research needs.

5. Allograft of primary SG imaginal ring tumor

5.1. Submerge the capillary into the Schneider's Medium containing the primary SG imaginal ring tumors. Hold the **Fill** button to fill the glass capillary with Schneider's Medium all the way to the top 0.5 cm segment. This top segment should remain filled with mineral oil.

NOTE: Accelerate this process by pressing the **Empty** button once while simultaneously holding down the **Fill** button.

5.2. Locate a primary tumor and press the **Fill** button until the tumor is suctioned into the capillary.

5.2.1. Ensure that the tumor sits at the tip of the capillary or several millimeters away from the tip of the capillary. This helps avoid the tumor drifting and becoming lost in the solution contained within the capillary. See **Figure 4A** for a demonstration of the appropriate tumor location as it sits in the capillary.

5.3. Locate an adult fly immobilized to the tape on the microscope slide. Using forceps, gently hold down the lower abdomen. Then, pierce the lower lateral cuticle of the abdomen with the capillary. Press the **Empty** button until the tumor enters the new host abdomen. See **Figure 4B** for a demonstration of this technique.

5.4. Using forceps, gently pinch the wings of the host up to remove it from the tape. Place the host into a new vial with fresh food. It is best to place the vial sideways for the initial 24 h after injection. Each vial should only contain up to a maximum of 20 flies.

NOTE: Some host flies will have missing wings and other wounds on their bodies after injection and may stick to the fly food if the vial is placed upright.

5.5. Repeat steps 5.2 through 5.4 to transplant the remaining primary tumors into their new adult hosts.

5.6. Dispose capillaries into sharps' container and clean the mineral oil residue from the exterior of the autoinjector before replacing the apparatus back into its box.

5.7. Store the vial of hosts at room temperature for 1 day, then transfer the vial to an incubation chamber at 29 ˚C. Transfer fly hosts to new vials every 2–3 days.

5.8. Monitor the fly hosts daily and calculate survival rates. After a week, tumors should be visible under a stereo microscope with fluorescence adapter and can be continually monitored for their size and progression.

6. Re-allograft of transplanted tumors

6.1. Around 10–14 days post-allograft, screen for tumors that have grown in the host abdomens using a fluorescence microscope.

6.2. Anesthetize a host with CO_2 and place it in a dissection plate filled with 100 µL Schneider's Medium. Dissect the grown allografted tumor out of the host using two pairs of forceps.

6.2.1. Use one pair of forceps to hold down the abdomen and the other pair to incise open the abdominal cuticle, exposing the allografted tumor[1].

6.2.2. Carefully isolate the tumor from the attached host tissues as much as possible using fluorescence markers as guidance.

6.3. Repeat step 6.2 to prepare for two to three additional allografted tumors.

6.4. Transfer the dissection plate containing the harvested tumors onto the stage of a light microscope.

6.5. Use sterile needles and dissect the tumors into smaller pieces that are appropriate for the capillary size.

6.6. Repeat steps 4.1–4.5 to prepare the new generation of adult hosts and repeat steps 5.1–5.7 to complete the allotransplantation of the tumors.

NOTE: Success rates are generally higher for non-primary tumors compared with those of primary tumors.

6.7. Repeat steps 6.1–6.6 for every subsequent generation of flies used in the study.

NOTE: Choose *Drosophila* host lines appropriate for the experimental needs.

REPRESENTATIVE RESULTS:

Here, we carried out generational allotransplantation of SG imaginal ring tumors using the nanoliter injection autoinjector apparatus and conducted subsequent tumor live-imaging with a confocal laser scanning microscope, which allowed for a deeper dive into topics of tumor growth, tumor cell migration, and tumor-host interactions. When mounting flies, glue them to a microscope slide and restrain them via a polydimethylsiloxane (PDMS) block[11].

Figure 5A features a live imaging capture of a 1st generation (G1) SG imaginal ring tumor growing in an adult host abdomen on day 10 post-allotransplantation. This level of imaging can be used to track the process of tumor division. **Figure 5B** depicts a 6th generation (G6) SG imaginal ring tumor occupying a large portion of the host abdomen on day 10 post-allotransplantation.

Imaging at this stage may help reveal tumor growth patterns, as well as its migration and invasion behaviors. It is important to note that even though this image was captured using a confocal laser scanning microscope, a stereomicroscope with a GFP fluorescence adapter could also be used at 2x to 5x magnification, depending on the tumor size.

FIGURE AND TABLE LEGENDS:

Figure 1: Host flies taped and secured for allotransplantation. The host flies are taped down by their wings and oriented neatly to prepare for the subsequent transplantation procedure.

Figure 2: A well-clipped injection capillary. The red arrow points to a sharp edge needed to effectively pierce the abdominal cuticle of adult *Drosophila* hosts.

Figure 3: Dissection of the primary salivary gland ImR tumor. The process of dissecting and isolating two primary salivary gland ImR tumors is demonstrated chronologically from panels A to B, using two separate incisions Panel A shows the salivary gland before tumor dissection. The red arrowheads indicate the first incision points. The blue arrowheads indicate the second incision points. The tumor lies between the red and blue arrowheads. Panel B shows the isolated ImR tumor after the two incisions are made to separate it from normal salivary gland tissue.

Figure 4: Appropriate tumor location within the capillary and injection of tumor into *Drosophila* host abdomen. (**A**) shows the most appropriate tumor location within the capillary. The tumor expresses eGFP (488 nm). Panel (**B**) shows the injection process. The red arrow indicates the tumor injection site. The blue arrow shows the placement of forceps to help hold down the terminalia of the fly for easier injection.

Figure 5: A G1 and G6 ImR tumor seen in WT *Drosophila* host abdomen on day 10 post-allotransplantation. These are ventral views of the fly abdomen with the transplanted tumors in green. Panel A shows a G1 tumor on day 10 post-allotransplantation expressing eGFP (488 nm). Panel A is captured using a confocal microscope using a 20x lens with 0.8 NA, and 3x zoom. Panel B shows a G6 tumor on day 10 post-allotransplantation expressing eGFP (488 nm). Panel B is captured using a confocal microscope using a 5x lens with 0.25 NA, and 1x scan zoom.

DISCUSSION:

Tumor allotransplantation can help researchers address certain problems that arise during *Drosophila* tumor growth and progression. One such challenge is the circumvention of premature deaths of tumor-bearing larvae or adults during primary tumor culture[12]. In this context, continued tumor allotransplantation allows tumors to grow indefinitely, which facilitates longitudinal studies of tumor growth, metastasis, and evolution. Tumor allotransplantation is also useful for assessing various aspects of host-tumor interactions[7,13]. Host genotypes can be manipulated prior to tumor allograft to allow for evaluation of the host effect on tumor growth and migration, and on tumor-induced cachexia[14,15]. Fly hosts with different genotypes may exhibit distinct manifestations of cachexia-like wasting in response to the same tumor. Post-

allotransplantation, the tumor hosts can be mounted in preparation for in vivo imaging using a protocol adapted from Koyama et al. and Ji et al.[11,16].

The application of the autoinjector apparatus toward *Drosophila* tumor allotransplantation provides a convenient and straightforward protocol that possesses enhanced efficiency. As compared to the manual injector[1], this method allows for reproducible and large-scale allotransplantations, which can expedite and standardize tumor behavior studies and drug screening procedures. This improved method produces impressive host survival and tumor yield rates. A trained researcher can achieve post-allograft host survival rates of >90%. Tumor yield rates can differ depending on whether the tumor is primary or re-allografted. Researchers can expect to achieve tumor yield rates of >50% for primary tumors and >70% for re-allografted tumors. In addition, this method reduces injection time per host fly by nearly 50% compared to the manual injector method.

This procedure has its limitations, however, mainly due to inconsistency of tumor incision, injection location and wound size. If the primary tumors are not cut into uniformly sized fragments prior to allotransplantation, certain fly hosts can receive larger fragments than others. This is a confounding factor affecting studies that aim to track the rate of tumor growth. This can potentially be mitigated by measuring the differential rate of tumor growth at two-day intervals post-allograft. In addition, during the injection, the operator should choose a consistent site in the abdominal cuticle across all host flies. This helps mitigate another confounding variable that may affect fly host survival and the final location of tumor attachment.

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DISCLOSURES:

There are no conflicts of interest to declare among the authors.

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Figure 1

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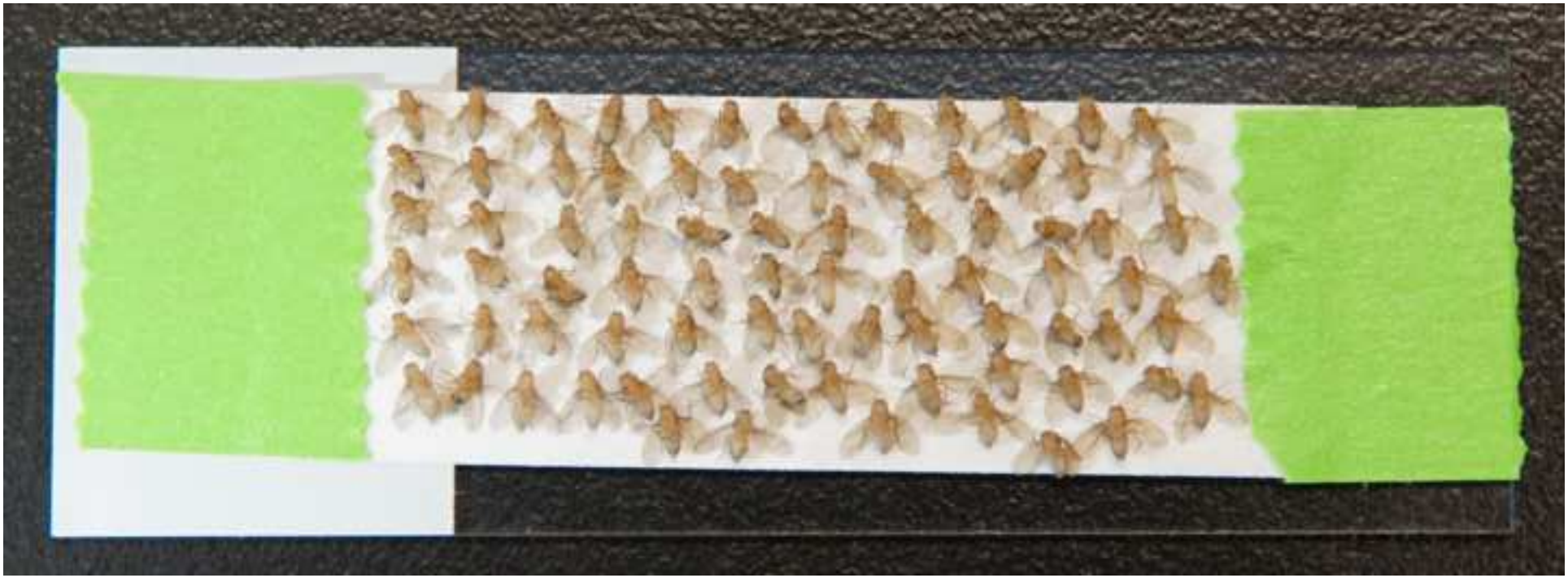

Figure 2

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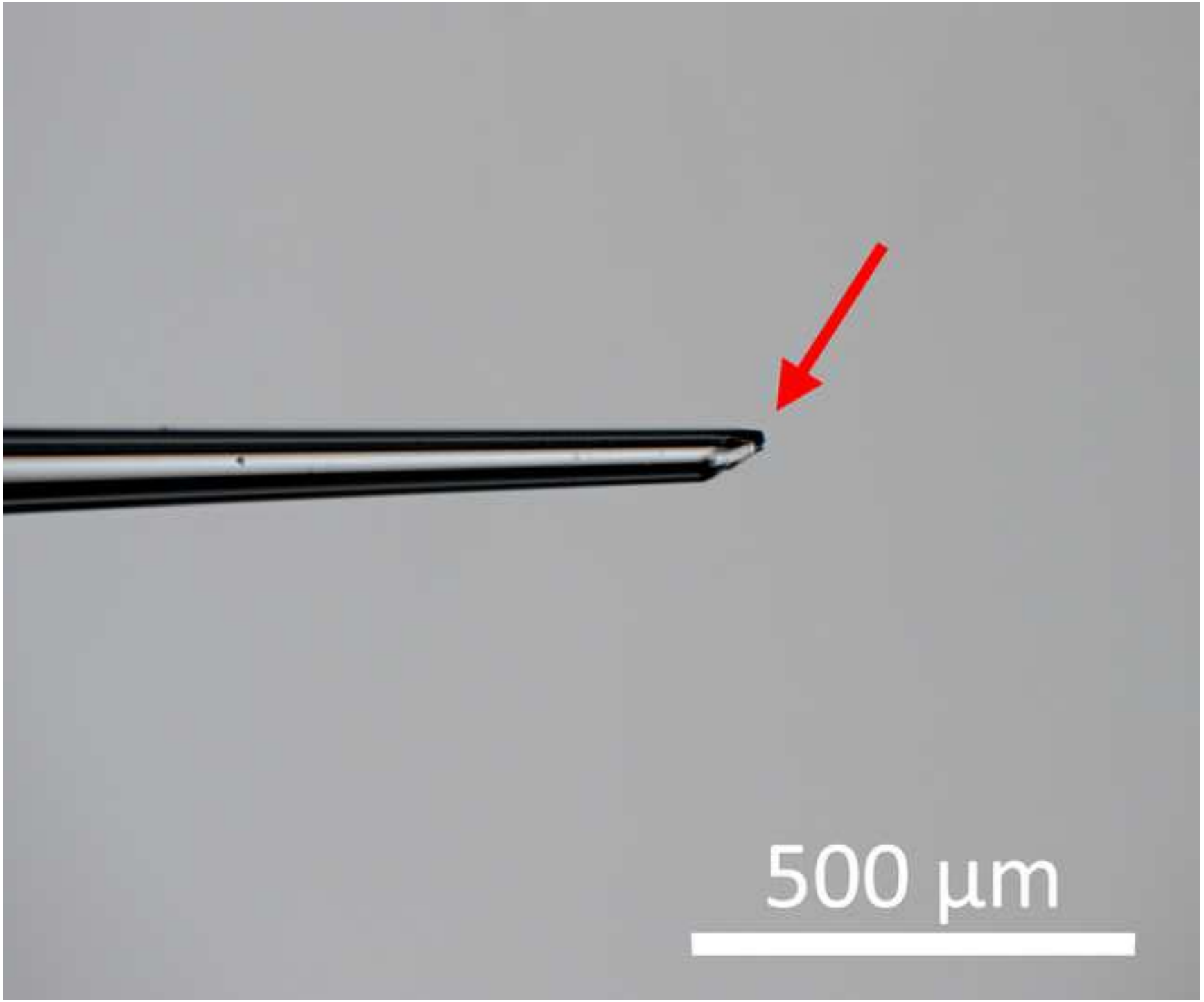

Figure 3

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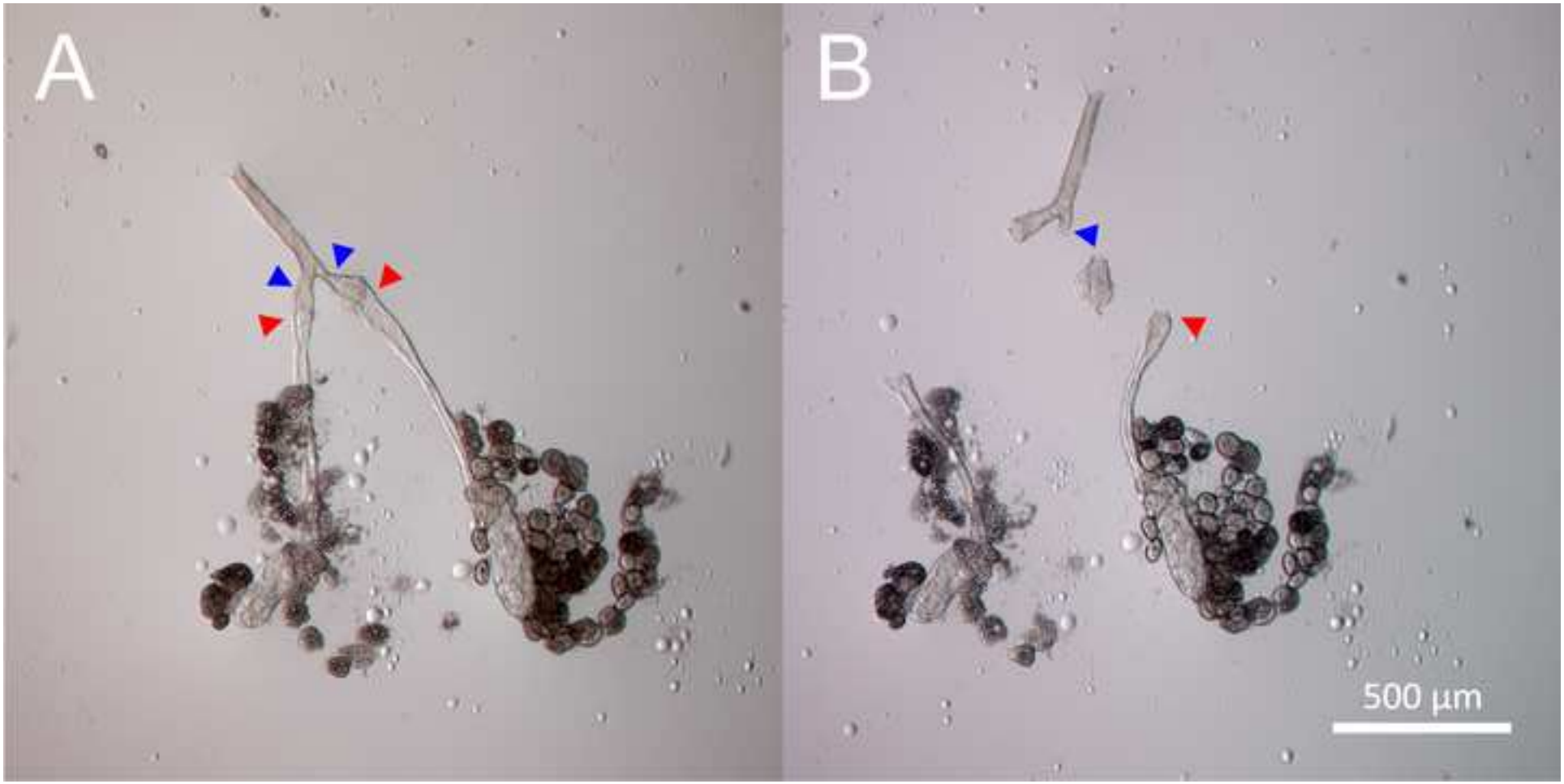

Figure 4

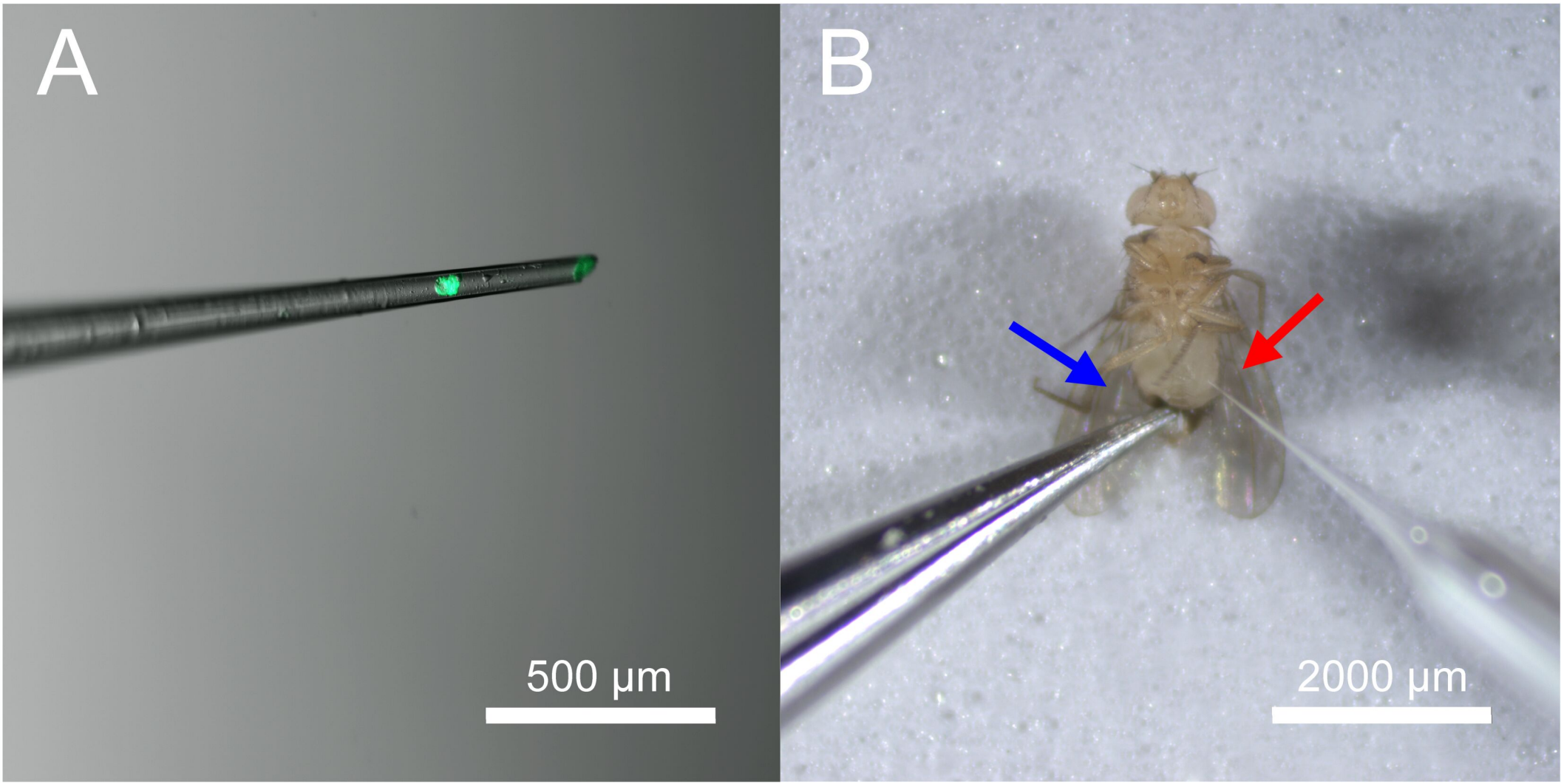

Figure 5

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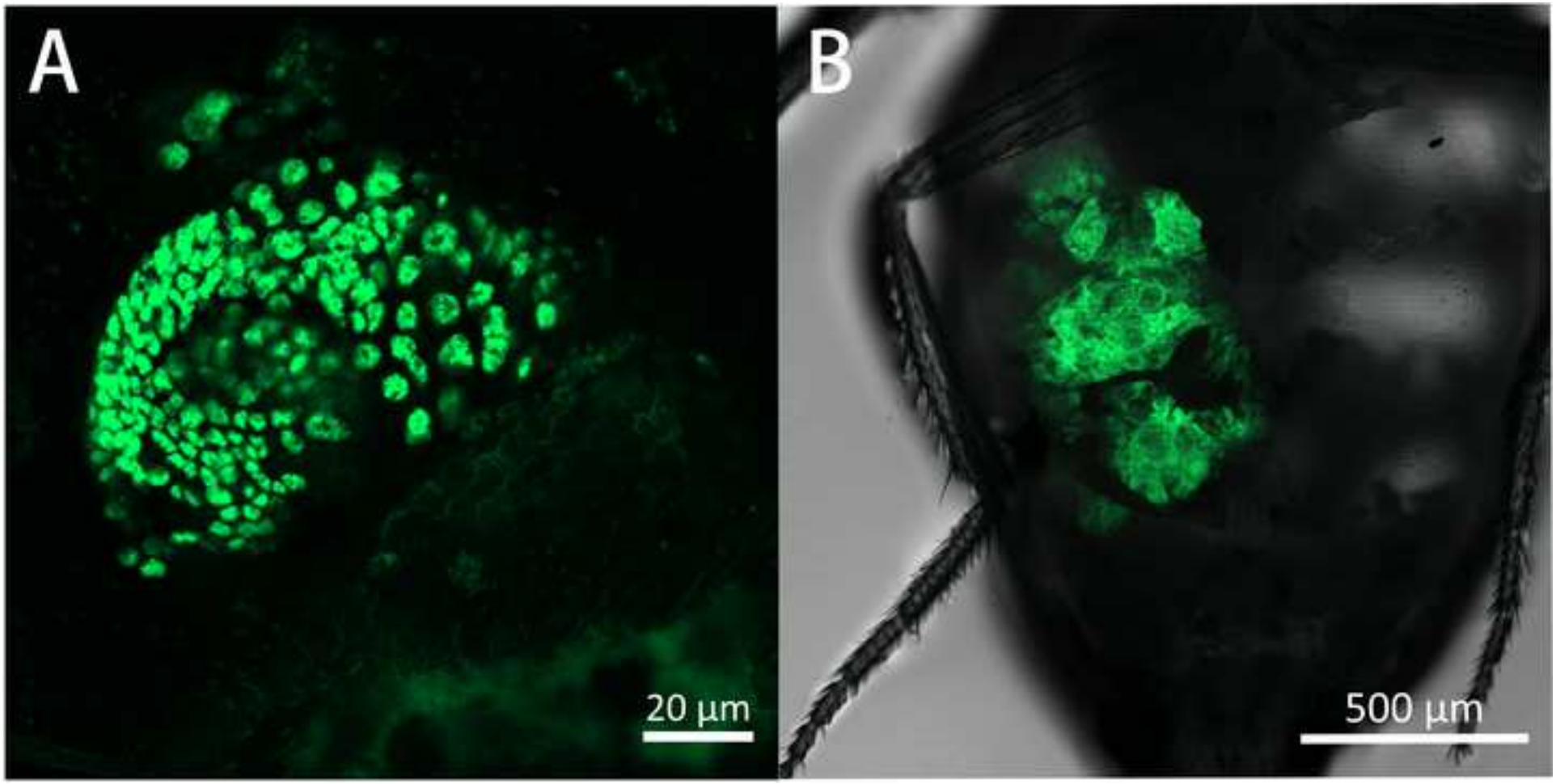

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Confocal Laser Scanning Microscope	Zeiss	LSM 980	Also known as "Zeiss LSM 980"
Cornmeal Fly Food	Bloomington *Drosophila* Stock Center	N/A	Also known as "BDSC Standard Cornmeal Food"
Dissection Needle (30Gx1/2)	BD PrecisionGlide	305106	
Dissection Plate	Fisher Scientific	12-565B	
Fly Tape	Fisherbrand	159015A	
Fluoresence Adapter for Stero Microscope	Electron Microscopy Sciences	SFA-UV	Also known as "NightSea Fluorescence Adapter"
Fluoresence Microscope	Zeiss	495015-0001-000	Also known as "Zeiss Stereo Discovery.V8"
Forceps	Fine Science Tools	11251-10	Also known as "Dumont #5 Forceps"
Glass Capillary (3.5'')	Drummond	3-000-203-G/X	
Glue	Elmer	E305	Also known as "Elmer Washabale Clear Glue"
Light Microscope	Zeiss	435063-9010-100	Also known as "Zeiss Stemi 305"
Micropipette Puller	World Precision Instruments	PUL-1000	Also known as "Four Step Micropipette Puller"
Nanoject Apparatus	Drummond	3-000-204	Also known as "Nanoject II Auto-Nanoliter Injector"
Schneider's Medium	ThermoFisher	21720001	
Syringe (27G x1/2)	BD PrecisionGlide	305109	
Vial	Fisherbrand	AS507	

Dear Editors and Reviewers:

Thank you for reviewing our work. We appreciate your sincere comments, suggestions and corrections and have made corresponding point-to-point responses below, in bold. Please kindly let us know any further concerns you may have.

Best wishes,
The Deng Lab

***A Brief Clarification on Authorship:**

Dear editors, please note that Shangyu Gong and Yichi Zhang are co-first authors of this manuscript. We have denoted this using the "#" sign on the title page. Thank you.

Editorial Changes

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.
   - **Response: We have proofread and edited the manuscript again.**

2. Please remove the embedded figure(s) from the manuscript. All figures should be uploaded separately to your Editorial Manager account.
   - **Response: Figures removed from manuscript.**

3. Figure 3: Is this figure necessary as there is a lot of commercialization present in it.
   - **Response: Figure removed.**

4. Figure 2/4/5/6: Please include and define scale bars in the lower right corner of the panels.
   - **Response: Scale bars added.**

5. Figure 7: Is this figure necessary? Please note that if accepted, we will film the protocol and the information conveyed by Figure 7 can be shown in the video.
   - **Response: Figure removed.**

6. Please consolidate some of your figures into a figure with multiple panels: Figure 5 and 6 can be combined. Figures 8 and 9 can be combined.
   - **Response: Figures combined.**

7. Please revise the title to remove the commercial product (Nanoject). Please use generic language whenever possible and specific commercial products can be named in the Table of Materials.
   - **Response: Changed the title.**

8. Please reconsider the usage of the term “Improved” in the title as the term may not age well as time passes.

- **Response: Changed the title.**

9. JoVE policy states that the video narrative is objective and not biased towards a particular product featured in the video. The goal of this policy is to focus on the science rather than to present a technique as an advertisement for a specific item. To this end, we ask that you please reduce the number of instances of “Nanoject” within your text. The term may be introduced but please use it infrequently and when directly relevant. Otherwise, please refer to the term using generic language.

- **Response: Changed accordingly.**

10. Why are the notes in quotations? If the quotations are not required, please remove them.

- **Response: Unnecessary quotations removed.**

11. Please add more details to your protocol steps. Please ensure you answer the “how” question, i.e., how is the step performed?

- **Response: Added more details where necessary**.

12. 1.2: What vials are used? How much fly food is included in the vial?

- **Response: Specified in manuscript as well as table of materials**.

13. 1.2.1: Can the recipe be provided? Alternatively, please provide a citation.

- **Response: Provided citation.**

14. 2.1/6.2: What percentage of CO2 is used for anesthesia?

- **Response: 100% CO2 is used, and we have specified this in manuscript.**

15. Please ensure that the protocol numbering is sequential.

- **Response: Checked and corrected the numbering sequence.**

16. 4.1: Please provide the composition of Schneider’s Medium.

- **Response: It was purchased from Thermofisher, and the catalog number is listed in table of materials.**

17. Please include microscope parameters in the protocol.

- **Response: Parameters added.**

18. Please include a one line space between all protocol steps and substeps. Once this is done, please highlight up to 3 pages of protocol text that identifies the essential steps of

the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

- **Response: Made the requested changes.**

Reviewer #1:

Manuscript Summary:

The manuscript of Gong et al describes a simple procedure to produce allografts in Drosophila using a relatively simple apparatus/strategy.

Major Concerns:

The wording and grammar of the manuscript requires intensive revision, since the current form of the text is ambiguous and incorrect. Of particular relevance is the use of the central term allograft, which refers to the heterologous, transplanted tumor rather than the process of transmission.

- **Response: Fixed relevant grammar and wording to improve clarity.**

Minor Concerns:

Giant larvae is not the suitable description.

- **Response: Changed to "Remaining Larvae", because only the specimens that harbor the tumor will remain as larvae at this stage. The ones that do not have the tumor would have progressed along to pupa/adult stages. We will also note this in the manuscript itself.**

More details about the puller and the glass capillaries are required.

- **Response: More details added. The video protocol will also help with clarification.**

Reviewer #2:

Manuscript Summary:

In this article Gong et al describe a method to improve the allograft of tumor samples in adult drosophila using a Nanoject apparatus. The method of allograft transplantation has been described very nicely before (Rossi and Gonzalez 2015) but being a technique that heavily depends on the hands of the experimenter, the method described in this article may be very helpful in terms of increasing the consistency of the process. The article is well written, the protocol is clear and it is easy to follow. I therefore fully support its publication after addressing minor concerns.

Major Concerns:

I have no major concerns

Minor Concerns:

I have few minor issues that should be addressed before publication:

1) It's not clear form the text (maybe the video will show it) whether they use a pipette holder or whether the injection is performed by freehand.

- **Response: The injection is performed by hand because it allows for convenient adjustment of the angle in injection. The video will help clarify this as well.**

2) In point 3.3 they describe the preparation of the 3.5" glass capillary. Would it be possible to add a picture of the Four step micropipette puller and point to the capillary to be used after heating?

- **Response: This will be shown in the video.**

3) The point 3.4 is a little bit confusing, the capillary refers to the glass capillary? If so, shouldn't it to be put into the injection needle after filling it with mineral oil (point 3.7)? I would suggest the authors to rewrite this point to make it more clear.

- **Response: Rewritten for clarity.**

4) In point 5.1 they mention to fill the capillary with Schneider's medium until 0.5 cm remains unfilled. Are this 0.5 cm filled with mineral oil?

- **Response: That is correct. Changed wording for clarity.**

5) It is not mentioned whether host flies are anesthetized at the moment of the injection.

- **Response: They are not anesthetized at time of injection.**

6) Finally, there are some minor inconstancies in numbering the points. For example, point 1.3 (line 98) should be 1.4, or point 3.7 (line 131) should be point 3.5

- **Response: Corrected, thank you!**

Reviewer #3:
Shangyu Gong et al describe a protocol to transplant small tissue pieces to adult Drosophila flies using the Nanoject apparatus. The manuscript is clearly written and easy to follow. To substantiate their claims for high efficiency, the authors could provide a table with numbers from a couple of representative experiments (eg one with primary tumours and one with re-allografts), reporting the number of injected hosts, survival after the procedure and number/percent of tumour-bearing flies at a given time post allograft. They should also provide a troubleshooting section: micromanipulation apparati often present with problems (e.g. needle does not fill, etc), so some tricks and tips on how to circumvent obstacles would be useful. Finally, for the sake of completeness and reproducibility I recommend the inclusion of some more experimental details, as follows:

Line 102: How old are the host flies? Do you recommend conditioning them with special food or any other treatment before the procedure? Do you take any measures to avoid microbial infection?

- **Response: For keeping the tumor lines, we use 2-3 days old wildtype female hosts (W^{1118}) flies. Different types of foods and flies of various ages can be used for the procedure, depending on specific experimental purpose. Before the injection, we use 75% EtOH to sterile the needle as much as possible to reduce chances of microbial infection.**

Line 121: What type of forceps do you use to clip off the end of the microcapillary needle? Dumont #5 or something else?

- **Response: That is correct, it is now specified in table of materials.**

Line 128 "until 70%-80% of its total length is showing" You clearly do not mean the capillary, but the plunger that goes into the capillary. Please clarify.

- **Response: It is the injector needle that is advancing. Corrected in manuscript.**

Line 162: Are the glued hosts under CO2 anesthesia for the entire duration of the procedure or not?

- **Response: They are not under anesthesia at moment of injection.**

Line 163: If you use the "Empty" button, not the "Inject" button, to transfer the tissue fragment, what is the point of setting the injection volume earlier (line 116)?

- **Response: This injection volume setting helps us control and limit the flow rate during both suction and injection. And this particular setting was the one that we found that led to the best results.**

Line 166: How do you remove the hosts from the tape without leaving their wings behind?

- **Response: Unfortunately, the wings would be left behind. This, however, should not affect fly survival, and we are careful not to damage their legs.**

Line 173: The Nanoject is a delicate piece of equipment. How do you clean it at the end of the procedure?

- **Response: At the end of the procedure, we will remove the glass needle, dispose of it in a sharps' container, clean the mineral oil residue on the exterior and then put the apparatus back into the original box for storage.**

Line 175: It should be mentioned that incubation at 29oC is optional, depending on experimental design.

- **Response: Added to manuscript as requested.**

Line 193: Please specify the type of sterile needles used for the microdissection.

- **Response: Listed in tables of materials.**

Line 244: Figure 3 should contain an inset with a closeup of the capillary, to show the level to which it is filled with mineral oil and how far down the plunger is.

- **Response: This will be shown in the video.**

Lines 269/274: The legends should include a brief mention of the mounting method and they type of optics used for imaging (stereoscope? confocal? type of lens?)

- **Response: The mounting method is described in the representative results section of the manuscript and the microscope parameters are added to the figure legend.**

And a minor remark:
Line 65: Recently at least one paper reported the use of the Nanoject apparatus for tumor allografting (Magadi et al, doi: 10.1242/dev.191544)
- **Response: This paper was published on November 23rd, 2020, which dates after our original manuscript submission to JoVe. We will take note and cite this paper, but we do believe that our paper is more methods-oriented and provides more details into the technique itself.**

Reviewer #4:

Manuscript Summary:

The method reported here by Gong and colleagues is a slightly modified version of the ancient tissue transplantation technique that was first reported decades ago. Compared to the several most recent descriptions of this technique, the innovation reported here is the use of a Nanoject that affords for the amount of injected material to be better controlled and more reproducible than what is possible with fully manual methods. Incremental as it is, I believe that this improvement makes the submitted manuscript acceptable for publication once the following standing issues have been addressed.

Concerns:

1— As said before, it is obvious that the use of Nanojet makes the method more reproducible, but I do not see any improvement with regards to efficiency. After all, aside from the automated quantification of the injected volume, the method remains manual. The authors themselves acknowledge that consistency ultimately depends on the operator's skills. The repeated claims in abstract, results, and discussion on improved efficiency seem therefore as unjustified as they are unnecessary.

- **Response: Our method requires a shorter training period and time-to-mastery for operators. In addition, it can help operators achieve higher efficiency because it reduces the time needed to inject one fly by almost 50% compared to that of previous protocol. And given that each injection cohort may consist of hundreds of flies, our method can reduce operator workload signifcantly. Thank you for pointing this out and we have clarified and substantiated our claims in the manuscript.**

2— Lines 59-62. Checking on the several published versions of the method I did not find any that is limited to a maximum host survival rate of 80%. The >90% reported here is also achievable with any of the other published protocoles.

- **Response: In our lab, we have had several experienced operators compare the use of the older protocol to this new one that we present today. They have found that the latter does indeed help increase post-injection survival rates from the original 80% to over 90%, which is substantial when considering the context of injecting several hundred flies in any given experimental cohort.**

3 — Figure 5. The authors may wish to chose a better example of a well-clipped capillary - this one does not look perfect- and take a higher magnification and better quality photograph to document it.

- **Response: Updated the figure to improve clarity.**

4 — Lines 162-4. The manipulations described in this paragraph sound cumbersome. It is hard to imagine how one single person can manage to press the Nanojet button while manipulating the forceps with one hand and the needle with the other.

- **Response: This process will be very clear with the associated video protocol.**

5 — Figs. 5 and 6. The copies of Figures 5 and 6 that I had access to were far too dark and lacked detail.

- **Response: We adjusted the brightness of the figures to better accentuate the tumor tissue.**

6 — Line 305-6. I do not see the arguments that justify the conclusions regarding time saving and scaling up.

- **Response: Our method helps reduce injection time per fly by almost 50% compared to previous methods and helps achieve a consistent tumor acquisition rate. Thus, when applied to large-scale drug screening platforms, considerable time can be saved when the experiment involves hundreds or even thousands of flies receiving allografts.**

7 — Lines 309-20. There is not much new in this paragraph.

- **Response: Removed the redundant parts of the paragraph.**

8 — Lines 336-344. Once more, consistency is much more dependent upon the operator than on the method itself

- **Response: This is a good point, but our methods require a shorter training time for operators to become proficient and consistent. In addition, we have mentioned several times in the manuscript that more consistent results can only be achieved by an experienced operator. Furthermore, this improved method can also help experienced operators achieve even better results than before.**