

## Video Article

# Embryo Microinjection and Transplantation Technique for *Nasonia vitripennis* Genome Manipulation

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## Abstract

The jewel wasp *Nasonia vitripennis* has emerged as an effective model system for the study of processes including sex determination, haplo-diploid sex determination, venom synthesis, and host-symbiont interactions, among others. A major limitation of working with this organism is the lack of effective protocols to perform directed genome modifications. An important part of genome modification is delivery of editing reagents, including CRISPR/Cas9 molecules, into embryos through microinjection. While microinjection is well established in many model organisms, this technique is particularly challenging to perform in *N. vitripennis* primarily due to its small embryo size, and the fact that embryonic development occurs entirely within a parasitized blowfly pupa. The following procedure overcomes these significant challenges while demonstrating a streamlined, visual procedure for effectively removing wasp embryos from parasitized host pupae, microinjecting them, and carefully transplanting them back into the host for continuation and completion of development. This protocol will strongly enhance the capability of research groups to perform advanced genome modifications in this organism.

## Video Link

The video component of this article can be found at <https://www.jove.com/video/56990/>

## Introduction

The jewel wasp, *N. vitripennis*, is one of the four species within the genus *Nasonia* that are ectoparasitoids of flesh eating flies such as *Sarcophaga bullata*<sup>1</sup>. Due to their fast-generational periods, ease of rearing in the laboratory, and a range of unique and important biological attributes, *N. vitripennis* has been a focus for the development of multiple experimental tools found in traditional model organisms. For example, some unique biological attributes include a haplo-diploid reproduction system<sup>2</sup>, a relationship with microbial and genetic parasites<sup>3,4</sup>, and a supernumary (B) chromosome<sup>5,6,7</sup>. Taken together, these make *N. vitripennis* an important experimental system for experiments aimed at elucidating the molecular and cellular aspects of these processes, in addition to others including venom production<sup>8,9</sup>, sex determination<sup>10,11</sup>, and evolution and development of axis pattern formation<sup>12,13,14</sup>. Moreover, the genetic toolkit to study the biology of *N. vitripennis* has dramatically increased over the last decade or so, with the sequencing of a high-resolution genome<sup>15</sup>, several gene expression studies<sup>16,17,18</sup>, and the ability to functionally disrupt gene expression relying on RNA interference (RNAi)<sup>19,20</sup>, which together have improved the tractability and capabilities of performing reverse genetics in this organism.

Despite the many important scientific advancements and expanded toolkits in this organism, as of present knowledge only one group has successfully performed embryonic microinjections to generate heritable genome modifications<sup>21</sup>. This is primarily due to the difficulties of working with embryos of *N. vitripennis* as they are quite fragile and small, being ~2/3 the size of *Drosophila melanogaster* embryos, making them generally difficult to manipulate. Additionally, *N. vitripennis* females deposit their eggs into pre-stung blowfly pupae, within which the entirety of embryogenesis, larval, and pupal development occurs. Therefore, for successful microinjections, pre-blastoderm stage, embryos must be efficiently collected from host pupae, quickly microinjected, and immediately transplanted back into their hosts for development. These steps require precision and dexterity to avoid damaging the microinjected embryos, or the pupal hosts, making the technique exceptionally challenging. Notwithstanding, there is one short protocol published over a decade ago that describes *N. vitripennis* embryo microinjection<sup>22</sup>. However, this procedure requires that the freshly laid embryos be desiccated, it uses sticky tape to anchor the eggs for microinjection, and does not include a visual demonstration of the technique. Therefore, described here is an updated and revised protocol, including a visual procedure, detailing an improved step-by-step protocol for *N. vitripennis* embryo microinjections that can be followed by any basic lab to generate heritable genome modifications in this important model insect.

## Protocol

### 1. *N. Vitripennis* Colony Rearing

- Set up several colonies (~ 3-5) by placing 200-500 *N. vitripennis* adults (with a 3:1 ratio of female:male) in bug dorm cages.  
NOTE: Alternative to using bug dorms, wasps can also be propagated in multiple, small glass test tubes plugged with cotton with approximately 15-20 wasps/vial.
  - Maintain the wasps at  $25 \pm 1$  °C with 30% relative humidity and a 12:12 light dark cycle, feeding them daily with small droplets of 1:10 (v/v) sucrose/water solution.  
NOTE: Propagation of wasp colonies and collections can also be conducted at room temperature without controlled humidity; however, temperatures lower than 25 °C will slightly lower the developmental timing of the embryos.
- Allow wasps to mate for at least 4 days prior to injections.
  - For the first two days, allow mated females to feed on fresh *S. bullata* pupae, as well as small droplets of a 1:10 (v/v) sucrose/water solution.
  - For the following two days, remove the blowfly pupae to deprive the female wasps of an oviposition site, making them very gravid.

### 2. Collection and Alignment of *N. Vitripennis* Pre-blastoderm Stage Embryos

- Allow female wasps to parasitize (oviposit embryos) into young hosts (*S. bullata* pupae). To keep hosts fresh, store hosts in 4 °C immediately after obtaining them and only remove when needed.  
NOTE: Young hosts can be determined by having a red colored puparium whereas older hosts have a darker colored puparium (**Figure 1A**).
  - Place individual fresh *S. bullata* pupae into a foam stopper with a pupae-sized hole cut into the center. Expose only ~ 0.2 cm of the anterior end (preferred oviposition site) of the host for maximum concentration of parasitization and easier embryo collection.  
NOTE: The anterior end is rounded, while the posterior end is thicker and contains a 'crater-like' opening (**Figure 1B**).
  - Place host pupae (roughly 5 host pupae per 100 wasps) in this arrangement into the cage and wait roughly 45 min to allow wasps to parasitize them (**Figure 2- ii**).  
NOTE: It is very important that the embryos are as young as possible, ideally within the first hour of being oviposited, to ensure that they are in the pre-blastoderm stage. Old embryos (> 1.5 h) should not be injected.
  - Remove parasitized hosts by retrieving them by hand and gently brushing/blowing off any residing wasps. Replace them with fresh hosts after every ~ 15 min to continue collecting early staged eggs during injections.
- Remove the freshly parasitized hosts from cage by hand. Under a dissecting microscope, using forceps, carefully peel away the posterior end (0.4 cm) of the exterior puparium (pupal shell) to expose the *N. vitripennis* eggs (**Figure 2- iii**).  
NOTE: Care must be taken to avoid puncturing the pupal skin; if this happens, the hemolymph will moisten the wasp embryos, which will be very difficult to remove for microinjection.
- Use liquid adhesive to glue a coverslip onto a clean glass slide to prepare an embryo alignment slide (**Figure 3**).  
NOTE: A high performance instant adhesive is used to increase bond strength and drying speed, however any glue that can keep the coverslip from moving around on the slide is acceptable.
- Brush-off the embryos from the host carefully with a wet fine-tip paintbrush, making sure not to damage the soft pupal skin of the host.  
NOTE: An 'embryo pick,' which is essentially a half of a pair of ultrafine forceps, can be used instead of a paintbrush.
- Transfer ~ 20 embryos one-by-one onto the slide, immediately adjacent to the side of the coverslip with a wet paintbrush. Orient the anterior end of the embryo against the cover slide's edge so that the posterior end of the embryos is pointed in the same direction (**Figure 2- iv**). This will allow for higher precision of injecting into the same position among all embryos.  
NOTE: This improved technique does not require double-sided sticky tape to fix embryos to the slide as described previously<sup>21,22</sup>. Additionally, do not desiccate embryos, or cover the eggs with halocarbon oil during injection as described previously for *N. vitripennis* embryo microinjection<sup>22</sup>.

### 3. Needle Preparation for Microinjections

- Place an aluminosilicate capillary glass into a needle puller.  
NOTE: A good needle is critical for successful *N. vitripennis* embryos injections. The injection needles should have a sharp and fine-tip to allow easy penetration through the chorion. Quartz and borosilicate capillary needles also can be used (**Figure 4**).
- Set the **Heat** to 605, the **Velocity** to 130, the **Delay** to 80, the **Pull** to 70, and the **Pressure** to 500 on a needle puller.  
NOTE: Additional needle puller settings for pulling quartz and Borosilicate needles can be seen in **Table 1**; the parameters may vary for different pullers and filaments.
- Activate the needle puller to create the correct needle. Repeat as needed for production of multiple needles.  
NOTE: Pulled needles can be stored by placing them into a Petri dish containing several parallel rolls of commercial modeler's clay.
- Bevel the needle tip by slightly touching the tip to the diamond abrasive plate around 10 s at 25 °C (**Figure 2- iii**).  
NOTE: The injection needles benefit from beveling by promoting entry into the embryos with minimal damage while simultaneously enhancing the flow of regents through the needle.

## 4. Embryo Microinjection

1. Prepare the injection mixture consisting of genome modification reagents (e.g., transposon + helper transposase, or CRISPR reagents, etc.), and keep it on ice.
2. Load the injection needle with 2  $\mu$ L of injection mixture by using microloader tips.  
NOTE: The injection mixture demonstrated in the protocol consists of single-guide RNA (sgRNA)(s) and recombinant Cas9 protein mixed in H<sub>2</sub>O.
3. Place the glass slide with the lined embryos onto the stage of a compound microscope.
4. Carefully insert the needle into the posterior end of the embryo with a vertical angle of 25-35 °. Inject 1-5  $\mu$ L of injection mixture (around 2-10% of the embryo's volume) and ensure that the embryo appears to swell slightly as the mixture is injected into it (**Figure 2- iv**).  
NOTE: If too much injection mixture is injected into the embryo it may burst dispelling the cytoplasm from the embryo. Additionally, improperly beveled or broken needles with too large of an opening may also cause embryo rupture.
  1. Try re-beveling needles if clogging occurs. The cytoplasm of *N. vitripennis* embryos is unusually viscous and sticky, which leads to frequent needle clogging (1/25 injections).  
NOTE: It is important to keep the embryos moist during the injection period by regularly adding de-ionized water using the paintbrush. The amount of water on the brush is key to move embryos around and align with ease. Too much water results in embryos floating and too little water makes them difficult to move around.
  2. Inject ~ 20-40 eggs at a time (should take roughly 10 min), then stop. Transfer the injected eggs with a wet paintbrush by lightly touching the injected eggs and place them into a host. Then continue injecting again using a fresh newly laid batch of eggs (> 1 h old).  
NOTE: Ideally this protocol is most effective if one person is continuously collecting and lining up eggs, while another person is injecting the genome modification components and transplanting injected embryos to host pupae.

## 5. Transplanting Injected G<sub>0</sub> *N. vitripennis* Embryos onto Pre-stung Host

1. Carefully place the injected G<sub>0</sub> embryos onto a previously stung *S. bullata* pupae (**Figure 2- vi**) with a wet paintbrush after microinjection.  
NOTE: The host pupae used to collect embryos for microinjection will work for this purpose. Also, despite significant effort, there is currently no available artificial diet that can be used<sup>22</sup>.
  1. Place up to 40 injected embryos one at a time onto a single pre-stung host to avoid overcrowding and larval competition.  
NOTE: Injection will cause damage to the embryos; careless transplantation of injected embryos to the host pupae may squeeze the injected component or the yolk out, and lead to the death of the embryos.
2. Place the host pupae into a Petri dish with damp filter paper and cotton balls, and incubate them at 25 °C with roughly 70% humidity until injected embryos hatch (roughly 1-2 days) (**Figure 2- vii**).  
NOTE: Importantly, hosts can be left with a peeled off puparium and the *N. vitripennis* eggs will develop normally so long as they are incubated in a humidified chamber (Petri dish with damp filter paper and cotton balls) to prevent desiccation. Incubating at room temperature and with humidity from the dampened cotton balls is sufficient in case humidity cannot be controlled.
3. Transfer the host pupae into a new Petri dish at 70% humidity and 25 °C after G<sub>0</sub> embryos hatch.
  1. Monitor the host pupae and injected G<sub>0</sub> larvae daily. If the host pupae have a foul smell, transfer the injected larvae to new healthy host pupae.

## 6. Screening for Genome Modifications

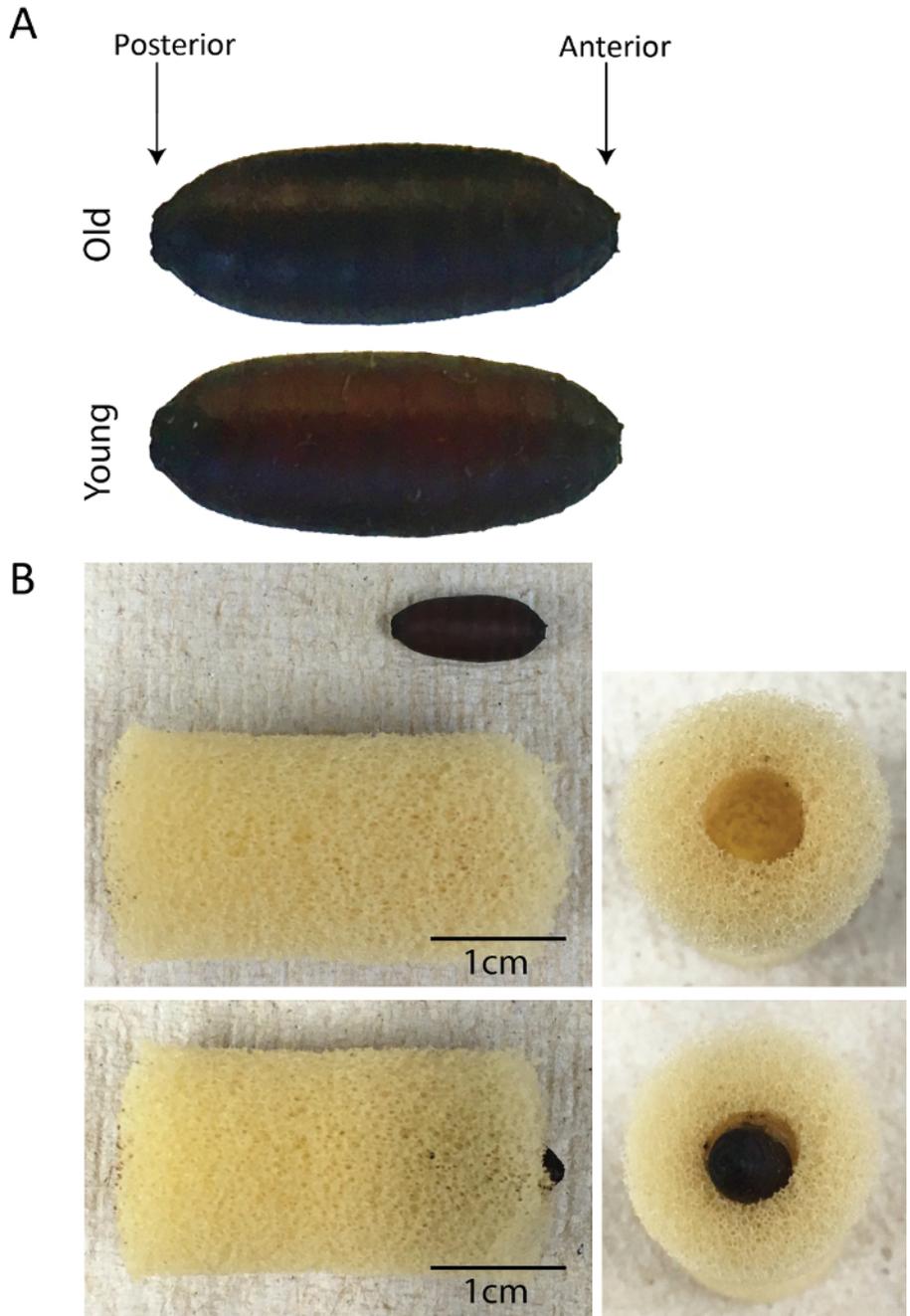
1. Remove each *N. vitripennis* pupae from the host using either a fine paintbrush or embryo pick. Injected G<sub>0</sub> larvae should pupate around 8 days post injection.
2. Place each removed pupa into an isolated glass vial plugged with cotton and allow them to emerge as G<sub>0</sub> adults, ensuring that the hatched females will be virgin which will help with downstream genetic crosses.  
NOTE: Females can be sorted from males by wing length: females have longer wings which extend past the body profile when turned on the side. All microinjected female pupae can be placed together into a plugged vial, and the same applies to male pupae.
3. Screen G<sub>0</sub> individuals, after eclosion, by either PCR or visually (if disrupting a phenotypic gene). For example, if CRISPR is used to create deletions in a given gene and the disrupted gene confers a visible phenotype, then sort and keep individuals with a mutant version of the affected phenotype (**Figure 2- viii**)<sup>21</sup>. If, however, the affected gene encodes for a non-visible phenotype, such as a mutant of an essential gene, perform a crossing scheme (cross G<sub>0</sub> male with wild type female, or cross G<sub>0</sub> female with wild type male) to recover and screen for heritable mutants through assaying lethality or sterility.  
NOTE: Similar to *D. melanogaster*, *N. vitripennis* adults can be immobilized by exposure to CO<sub>2</sub> allowing for straightforward manipulation. Moreover, unmated females will give rise to 100% haploid male broods, so therefore a mutant unmated female can give rise to large number of mutant males that can be used for subsequent analysis.  
NOTE: Mutations in essential genes will need to be kept by mating surviving G<sub>0</sub> injected females (presumably heterozygous for a mutation) to wild type males; in this particular case, half of the G<sub>1</sub> male progeny should die due to inheritance of the lethal mutation, while half of the G<sub>1</sub> female progeny will be carriers of the lethal.
4. Outcross G<sub>0</sub> adults and screen G<sub>1</sub> progeny for transgene insertions using transgenesis markers if the goal is transgenesis. Currently transgenesis remains to be demonstrated in *N. vitripennis*.

## Representative Results

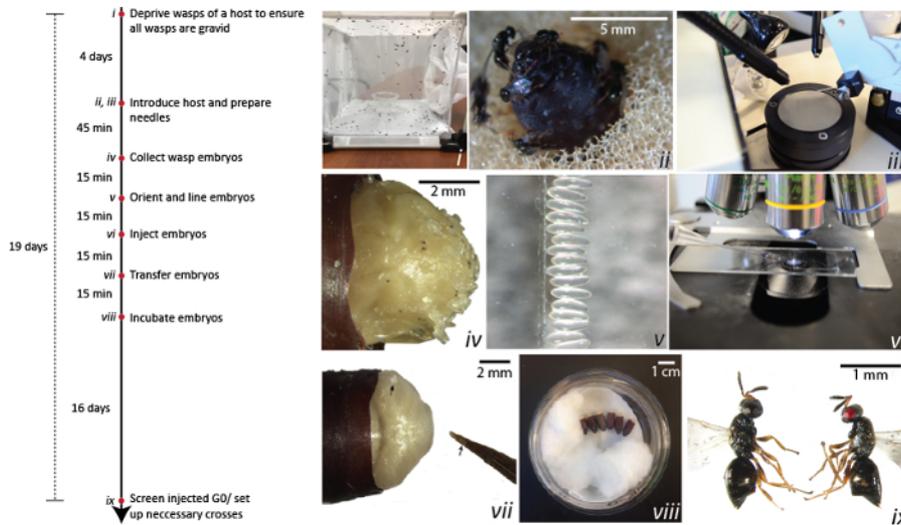
This protocol provides detailed guidelines for colony rearing, pre-blastoderm embryo collection, alignment, microinjection, and subsequent transplantation after injection and can be used for efficient genome engineering in *N. vitripennis*. As shown in **Figure 2**, the general sequence of steps for a successful microinjection into *N. vitripennis* include: (i) permitting male and female adults to mate (~ 4 days), (ii) supplying fresh host fly pupae (*S. bullata*) placed in a modified foam plug to mated females and allowing for oviposition (~ 45 min), (iii) carefully peeling away the parasitized host pupae cuticle to expose and collect pre-blastoderm stage wasp embryos (~ 15 min), (iv) aligning collected embryos (~ 15 min), (v) microinjecting genome modification components into embryos (~ 15 min), (vi) carefully placing injected embryos back into pre-stung hosts to allow for proper development (~ 15 min), and (vii) preventing dehydration of the injected embryo/host by transferring them into a humidified chamber with roughly 70% relative humidity (~ 15 min). Parasitized hosts are then incubated for roughly 14 days to allow for complete development of *N. vitripennis* embryos. Once injected, adults emerge from the host (viii), isolate, mate, and screen them individually for the presence of expected mutations.

For effective needle penetration and microinjection into *N. vitripennis* embryos, several types of capillary glass needles with filament including quartz, aluminosilicate, and borosilicate types are tested. It is found that the quality of needle is critical for avoiding breakage/clogging during injection, and for achieving high rates of both embryo survival and transformation efficiency. For each glass type, an effective protocol was developed to pull needles in order to have a desired hypodermic-like long tip effective for *N. vitripennis* embryo microinjection using different micropipette pullers (P-1000, and P-2000) (**Table 1**, **Figure 4**).

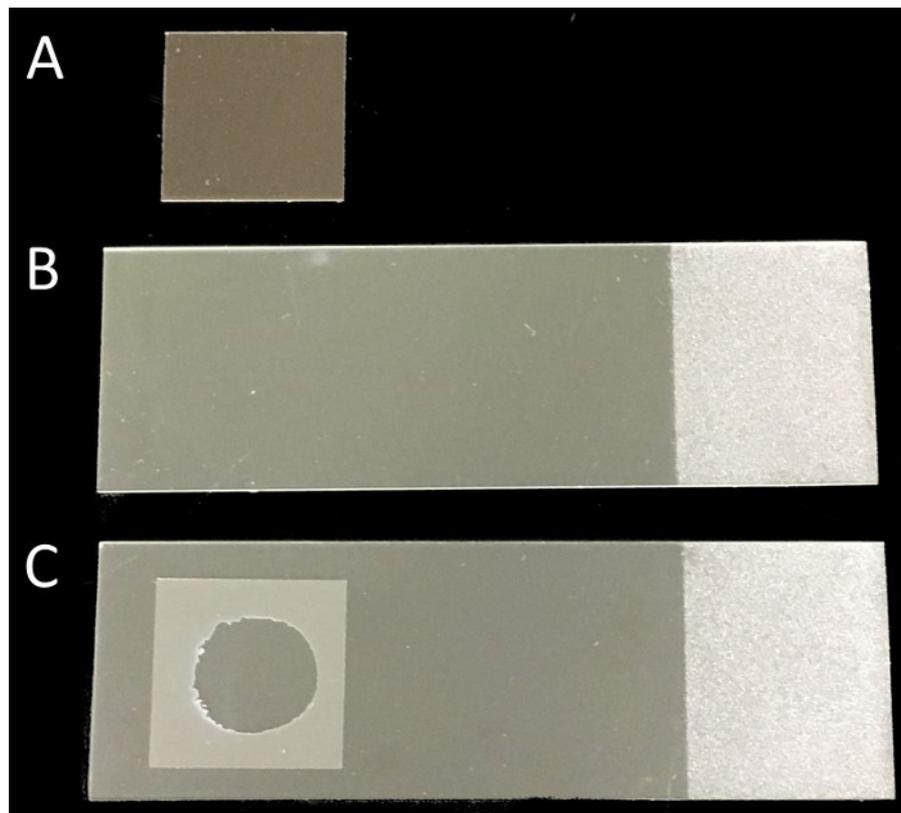
To optimize the procedure, survival rates are measured following injection of varying amounts of genome modification components. The genome modification components used here were mixed guide RNAs and Cas9 protein for CRISPR-mediated genome editing, which were demonstrated previously to work well in *N. vitripennis*<sup>21</sup>. Similar to what was previously reported, here a sgRNA targeting the *cinnabar* gene is designed and synthesized. By targeting and disrupting this gene, an easily identifiable phenotypic change is seen in the eye color of the organism<sup>19,21</sup>. An injection mixture combining a variety of concentrations of sgRNA (0, 20, 40, 80, 160, and 320 ng/μL) with Cas9 protein (0, 20, 40, 80, 160, and 320 ng/μL) is created and injected into embryos of wild type *N. vitripennis*. Survival rate of injected embryos is found to be dose-dependent (**Table 2**)<sup>21</sup>. The increased concentration of sgRNA and Cas9 protein lead to decreased survival rates (**Table 2**), perhaps due to additive off-target effects. High humidity (~ 70%) is also found to be important for embryo survival after transplantation to hosts, as low humidity (~ 10%) resulted in 100% death to all injected embryos.



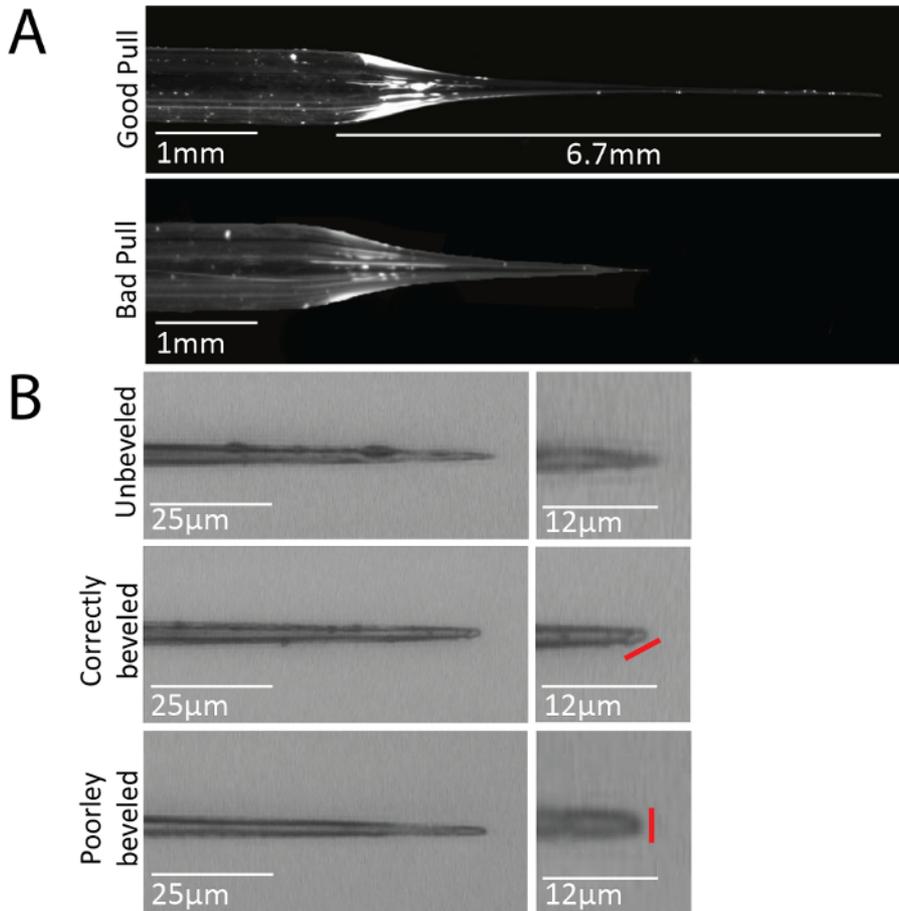
**Figure 1. Preparation of host pupae (*S. bullata*) for *N. vitripennis* embryo oviposition. (A)** Young and old *S. bullata* pupae. Older pupae have a darker cuticle whereas younger pupae have a more reddish tint to their cuticle. Younger pupae are preferred for maximizing oviposition. Posterior and anterior ends of the pupae can also be distinguished by a "crater-like opening on the posterior end, whereas the anterior end comes to a rounded point. (B) Host (*S. bullata*) pupa preparation for *N. vitripennis* embryo oviposition. Inserting the host pupae into a foam plug that has had a pupae sized hole carved out. Have the posterior side of the host pupae face inside the plug while 0.2 cm of the anterior end exposed to allow for maximum concentration of oviposition into the anterior area. [Please click here to view a larger version of this figure.](#)



**Figure 2. Timeline for creating *N. vitripennis* mutants by microinjection.** Timeline of *N. vitripennis* embryo collection, CRISPR/Cas9 microinjection, and post-injection procedures. *N. vitripennis* adults were allowed to mate in absence of an oviposition site for 4 days (i). Following, a fresh, flesh fly host pupae, *S. bullata*, placed inside a foam stopper as to only expose 0.5 cm of the posterior end, was introduced to the gravid females for 45 min to allow for parasitization (ii). Concurrently injection materials including microinjection needles and CRISPR/Cas9 components were prepared (iii). Embryos were collected from the host (iv), aligned (v), and injected with CRISPR/Cas9 components (vi). The injected embryos were carefully transferred back to a pre-stung host (vii) and incubated till fully developed (14 days) (viii). When the adults emerged, mutants were screened for phenotypes of expected CRISPR/Cas9 induced mutations in the target gene (ix). The entire procedure generally takes 19 days to complete. [Please click here to view a larger version of this figure.](#)



**Figure 3. Modified microscope slide for lining embryos.** (A) A coverslip. (B) A microscope slide. (C) An embryo alignment device. A coverslip can be glued onto a microscope slide to be used to line embryos for injection. The purpose of the coverslip is to act as an edge to allow for easy manipulation and lining of embryos. [Please click here to view a larger version of this figure.](#)



**Figure 4. Injection needle preparation.** (A) Examples of good and bad aluminosilicate glass needles. (B) The tips of an unbeveled, correctly beveled, and poorly beveled needle. Aluminosilicate glass capillary tubes were pulled by using a micropipette puller. The produced needle tips were then gently opened and refined using a beveler. The good bevelled needle has a very sharp tip, and the bad bevelled needle has a blunt tip. [Please click here to view a larger version of this figure.](#)

Capillary Glass Type	Sutter Needle Puller Model	Heat	Filament	Velocity	Delay	Pull	Pressure
Quartz	P-2000	750	4	40	150	165	-
Aluminosilicate	P-1000	605	-	130	80	70	500
Borosilicate	P-1000	450	-	130	80	70	500

**Table 1. Settings for needle puller.**

Note: Needle puller setting vary from machine to machine so each lab will need to optimize their own needle puller settings. This table has been modified from Li *et al.*<sup>21</sup>

sgRNA-1	Cas9	Total embryos	Transplantation (10% humidity)	Transplantation (70% humidity)			
			Larvae Survivors	Larvae Survivors	Adult survivors		Total (%)
			Total (%)	Total (%)	♂	♀	
No injection	No injection	100	94 (94)	96 (96)	66	26	92 (92)
Water	Water	100	0 (0)	78 (78)	44	32	76 (76)
20 ng/μL	20 ng/μL	100	0 (0)	74 (74)	34	34	68 (68)
40 ng/μL	40 ng/μL	100	0 (0)	67 (67)	30	32	62 (62)
80 ng/μL	80 ng/μL	100	0 (0)	53 (53)	24	22	46 (46)
160 ng/μL	160 ng/μL	100	0 (0)	41 (41)	16	22	38 (38)
320 ng/μL	320 ng/μL	100	0 (0)	25 (25)	10	10	20 (20)

**Table 2. Injection and transplantation survivorship and mutagenesis rates based on injections of different concentrations of sgRNA and Cas9.** This table has been modified from Li *et al.*<sup>21</sup>

## Discussion

The recent sequencing of the *N. vitripennis* genome has unleashed an important need for molecular tools to functionally characterize unknown genes within this species<sup>23</sup>. The CRISPR-Cas9 system, and many other gene editing tools, have proven to be valuable in investigating gene functions for a number of organisms<sup>24</sup>. However, to generate heritable mutations, these tools require performing embryo microinjections. Therefore, demonstrated here is a detailed visual technique that includes a number of innovations that allow for efficient *N. vitripennis* embryo microinjections.

Overall, this detailed technique offers a number of significant innovations, with respect to existing methods<sup>23</sup>, that allow for efficient *N. vitripennis* embryo microinjections. For example, to facilitate the rapid collection of embryos, an oviposition tool (foam stopper) is created and used to restrict egg laying entirely to the posterior end of the host (**Figure 1**), which greatly facilitates collection of numerous embryos within a short period of time. Techniques for rearing wasp colonies with large numbers to collect greater numbers of eggs are also improved and defined. Additionally, to accelerate embryo alignment, an embryo alignment device which allows for embryos to be efficiently aligned and injected without having to use double-sided sticky tape to secure the embryos in place (**Figure 3**) is developed.

Furthermore, it is found that by keeping the embryos moist with water during injection and not desiccating the embryos or covering them with oil improved survival rates. Additionally, several capillary glass types are tested and parameters to construct the perfect needles for *N. vitripennis* microinjection (**Table 1, Figure 4**) are determined. Moreover, following microinjection, embryo survival rates are able to significantly increase due to incubating injected eggs in pre-stung hosts placed in high-humidity (70%) chambers. These innovations allow for a more streamlined and successful microinjection procedure for *N. vitripennis*.

Minor modifications in terms of injection apparatus, and rearing procedures can be made to this protocol, depending on user preferences. However, a number of critical steps will be essential for successfully generating mutants in *N. vitripennis*. For example, working quickly so that injected embryos are > 1 h old, and ensuring that injection needles are sharp enough to minimize damage to the embryo will both be essential. While this protocol should be effective for generating mutations in many (if not all) genes, one major limitation is the CRISPR/Cas9 requirement to target a PAM (NGG) sequence which will dictate the target sequence.

In conclusion, this improved technique can be used to generate many kinds of genome modifications, such as mutations, deletions, and possibly even insertions using CRISPR/Cas9 technologies<sup>21</sup>, or even transgene insertions to generate transgenic *N. vitripennis*, which should greatly accelerate functional research in this organism.

## Disclosures

The authors have nothing to disclose.

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