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Corresponding Author:	Elissa A. Hallem UCLA: University of California Los Angeles UNITED STATES
Corresponding Author's Institution:	UCLA: University of California Los Angeles
Corresponding Author E-Mail:	ehallem@ucla.edu
Order of Authors:	Michelle Castelletto
	Elissa A. Hallem
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TITLE:

Generating Transgenics and Knockouts in *Strongyloides* Species by Microinjection

AUTHORS AND AFFILIATIONS:

Michelle L. Castelletto[1], Elissa A. Hallem[1,2]

[1]Department of Microbiology, Immunology, and Molecular Genetics, University of California, Los Angeles, Los Angeles, CA 90095, USA

[2]Molecular Biology Institute, University of California, Los Angeles, Los Angeles, CA 90095, USA

Email address of co-author:

Michelle Castelletto (mcastelletto@ucla.edu)

Corresponding author:

Elissa Hallem (ehallem@ucla.edu)

SUMMARY:

The functional genomic toolkit for the parasitic nematodes *Strongyloides stercoralis* and *Strongyloides ratti* includes transgenesis, CRISPR/Cas9-mediated mutagenesis, and RNAi. This protocol will demonstrate how to use intragonadal microinjection to introduce transgenes and CRISPR components into *S. stercoralis* and *S. ratti*.

ABSTRACT:

The genus *Strongyloides* consists of multiple species of skin-penetrating nematodes with different host ranges, including *Strongyloides stercoralis* and *Strongyloides ratti*. *S. stercoralis* is a human-parasitic, skin-penetrating nematode that infects approximately 610 million people, while the rat parasite *S. ratti* is closely related to *S. stercoralis* and is often used as a laboratory model for *S. stercoralis*. Both *S. stercoralis* and *S. ratti* are easily amenable to the generation of transgenics and knockouts through the exogenous nucleic acid delivery technique of intragonadal microinjection, and as such, have emerged as model systems for other parasitic helminths that are not yet amenable to this technique. Parasitic *Strongyloides* adults inhabit the small intestine of their host and release progeny into the environment via the feces.

Once in the environment, the larvae develop into free-living adults, which live in feces and produce progeny that must find and invade a new host. This environmental generation is unique to the *Strongyloides* species and similar enough in morphology to the model free-living nematode *Caenorhabditis elegans* that techniques developed for *C. elegans* can be adapted for use with these parasitic nematodes, including intragonadal microinjection. Using intragonadal microinjection, a wide variety of transgenes can be introduced into *Strongyloides*. CRISPR/Cas9 components can also be microinjected to create mutant *Strongyloides* larvae. Here, the technique of intragonadal microinjection into *Strongyloides,* including the preparation of free-living adults, the injection procedure, and the selection of transgenic progeny, is described. Images of transgenic *Strongyloides* larvae created using CRISPR/Cas9 mutagenesis are included. The aim of this paper is to enable other researchers to use microinjection to create transgenic

and mutant *Strongyloides*.

INTRODUCTION:

Strongyloides stercoralis has long been overlooked as an important human pathogen compared to the more widely recognized hookworms and the roundworm *Ascaris lumbricoides*[1]. Previous studies of worm burden often severely underestimated the prevalence of *S. stercoralis* due to the low sensitivity of common diagnostic methods for *S. stercoralis*[2]. In recent years, epidemiological studies based on improved diagnostic tools have estimated that the true prevalence of *S. stercoralis* infections is much higher than previously reported, approximately 610 million people worldwide[2].

Both *S. stercoralis* and other *Strongyloides* species, including the closely related rat parasite and common lab model *S. ratti,* have an unusual life cycle that is advantageous for experimental genomic studies because it consists of both parasitic and free-living (environmental) generations[3] (**Figure 1**). Specifically, both *S. stercoralis* and *S. ratti* can cycle through a single free-living generation. The free-living generation consists of post-parasitic larvae that develop into free-living adult males and females; all progeny of the free-living adults develop into infective larvae, which must infect a host to continue the life cycle. Furthermore, this environmental or free-living generation can be experimentally manipulated in the laboratory. Because free-living *Strongyloides* adults and *C. elegans* adults share similar morphology, techniques such as intragonadal microinjection originally developed for *C. elegans* can be adapted for use with free-living adult *Strongyloides*[4,5]. While DNA is generally introduced into free-living adult females, both males and females of *Strongyloides* can be microinjected[6]. Thus, functional genomic tools are available to interrogate many aspects of the biology of *Strongyloides*. Other parasitic nematodes lack a free-living generation, and as a result, are not as readily amenable to functional genomic techniques[3].

[Place **Figure 1** here].

S. stercoralis shares many aspects of its biology with other gastrointestinal human-parasitic nematodes, including host invasion and host immune modulation. For example, human-parasitic hookworms in the genera *Necator* and *Ancylostoma* also infect by skin penetration, navigate similarly through the body, and ultimately reside as parasitic adults in the small intestine[7]. Thus, many gastrointestinal nematodes likely use common sensory behaviors and immune evasion techniques. As a result, the knowledge gleaned from *Strongyloides* will complement findings in other less genetically tractable nematodes and lead to a more complete understanding of these complex and important parasites.

This microinjection protocol outlines the method for introducing DNA into *Strongyloides* free-living adult females to make transgenic and mutant progeny. The strain maintenance requirements, including the developmental timing of adult worms for microinjections and the collection of transgenic progeny, are described. Protocols and a demonstration of the complete microinjection technique, along with protocols for culturing and screening transgenic progeny, are included, along with a list of all necessary equipment and consumables.

PROTOCOL:

NOTE: Gerbils were used to passage *S. stercoralis*, and rats were used to passage *S. ratti*. All procedures were approved by the UCLA Office of Animal Research Oversight (Protocol No. 2011-060-21A), which adheres to AAALAC standards and the Guide for the Care and Use of Laboratory Animals. The following tasks must be completed at least one day before microinjecting: worm culturing, preparing microinjection pads, creating constructs for the microinjection mix, and spreading bacteria (*E. coli* HB101) onto 6 cm Nematode Growth Media (NGM) plates[8]. The free-living females require a minimum of 24 h post-fecal collection at room temperature (20–23 °C) to develop into young adults before they can be microinjected. Microinjection pads must be completely dry. Bacterial plates must dry and establish a small lawn.

1. Preparation of microinjection slides: at least one day before injecting

NOTE: Worms are mounted on microinjection coverslips with dry agar pads for injection.

1.1. Set a heat block to 90 °C.

1.2. Add 5 mL of ddH_2O, then 100 mg of agarose to a borosilicate glass tube.

1.3. Heat the agarose mix in the tube over a flame until the agarose is dissolved.

1.4. Place the tube in a heat block set at 90 °C to maintain the agarose in the liquid state.

1.5. Drop ~180 µL of the agarose solution onto a coverslip using a glass Pasteur pipet or a pipet with a plastic tip. Immediately drop a second coverslip on top to flatten the agarose into a thin pad.

1.6. After 5–10 s, remove the top coverslip by sliding the two apart. Determine which slide the agar pad is on and lay it face up.

1.7. Select a tiny piece of glass shard from a broken coverslip and gently press it into the agar near the top edge of the pad using forceps (**Supplemental Figure S1**).

1.8. Continue making microinjection pads with the agarose solution.

1.9. Dry the agarose pads overnight on the bench or in an oven. Store in the coverslip box.

NOTE: The agarose pads can be used for up to 2 months but are only used for one injection run.

2. Culturing *Strongyloides* to obtain worms for microinjection: 1–2 days before injection

NOTE: A strain maintenance protocol can be found in the **Supplemental Material**, which includes a detailed description of how to infect gerbils and rats with nematodes and harvest nematodes from the feces of infected animals.

2.1. Two days before the injection day, place the infected animals[9,10] in collection cages overnight.

2.2. The next morning, collect infected feces and make fecal-charcoal plates[9,10].

2.3. Place a plate at 25 °C for 24 h to allow the free-living worms to develop into young adults.

2.4. The night before the injection day, place uninfected host animals in collection cages.

2.5. On the injection day, collect uninfected feces for post-injection cultivation.

3. Making the microinjection mix: prior to or on the day of injection

NOTE: The microinjection mix consists of the plasmids of interest diluted to the desired concentration in worm buffered saline (BU) (50 mM Na_2HPO_4, 22 mM KH_2PO_4, 70 mM NaCl)[11].

3.1. Determine the concentration of the plasmid stocks and the desired concentration in the microinjection mix (**Table 1**).

[Place **Table 1** here]

3.2. Dilute the plasmids in BU to a total volume of 10–20 µL.

3.3. Spin the mix through a filter column at 5,000 × *g* for 1–2 min.

3.4. Use the microinjection mix immediately or store it at -20 °C for future use.

4. Collect young adult *Strongyloides* for microinjection: morning of the injection day

4.1. Set up the Baermann apparatus with 1 fecal-charcoal plate of young adult *Strongyloides* (**Figure 2**).

NOTE: The fecal-charcoal plate may contain some infective larvae. Personal protective equipment consists of a lab coat, gloves, and eye protection. No skin should be exposed between the glove and the sleeve of the lab coat.

[Place **Figure 2** here]

4.2. Install a glass funnel with rubber collection tubing on a ring stand using an O-ring and secure it with a clamp. Close the collection tubing with 2 pinch clamps (**Figure 2A**).

4.3. Place a catch bucket under the funnel to catch drips.

4.4. Add warm (approximately 40 °C) water to the funnel to 5 cm below the rim. Verify that the system is not leaking.

4.5. Line the Baermann holder, a sieve made from 2 plastic rings with 2 layers of nylon tulle netting secured between them, with 3 overlapping pieces of lab tissue. Add the fecal-charcoal mixture to the Baermann holder (**Figure 2B,C**).

4.6. Place the Baermann holder with the fecal-charcoal mixture in the funnel. Fold the tissues around the fecal-charcoal mix and add enough water to submerge most of the fecal-charcoal. Do not fill above 2 cm from the rim of the funnel (**Figure 2D,E**).

4.7. Top the funnel with a 15 cm plastic Petri dish lid to contain the odor. Label the funnel as needed (**Figure 2F**).

4.8. Wait 30 min to 1 h to collect the worms from the Baermann apparatus.

4.9. Hold a 50 mL centrifuge tube under the rubber tubing at the bottom of the funnel. Carefully open the clamps at the bottom to dispense 30–40 mL of water containing worms into the 50 mL tube.

4.10. Transfer 15 mL of the Baermann water containing the worms to a 15 mL centrifuge tube. Spin the 15 mL centrifuge tube for 1 min at ~750 × *g* (slow). Alternatively, allow the worms to gravity settle for 10–15 min.

4.11. Remove the supernatant to ~2 mL and discard the supernatant into a waste liquid container with iodine to kill any worms.

4.12. Add more Baermann water to the 15 mL collection tube and repeat the spin. Remove the supernatant to ~2 mL and discard as in step 4.11.

4.13. Repeat steps 4.11 and 4.12 until all the worms are collected in the 15 mL centrifuge tube. After the final spin, remove as much water as possible.

4.14. Inspect the pellet of worms (40–100 µL) at the bottom of the tube. If no worms are visible, wait for another 1–2 h and try collecting more worms from the Baermann apparatus.

4.15. Transfer the worms in as little water as possible to a 6 cm 2% NGM plate with a lawn of *E. coli* HB101. Use this plate as the **source plate** for the microinjection.

4.16. Discard the fecal-charcoal mix by treating it with diluted iodine (a 50% dilution of Lugol's iodine in water), wrapping it in plastic film to catch drips, and placing it in a biohazard waste

container.

4.17. Add 10 mL of diluted iodine to the catch bucket and drain the excess water from the Baermann into it.

4.18. Wash the reusable components (the funnel, the catch bucket, the plastic holder with tulle, the plastic lid, and the clamps) with 10% bleach and rinse thoroughly.

5. Pulling and loading microinjection needles: just before injection

5.1. Prepare microinjection needles by pulling glass capillary tubes using a needle puller.

NOTE: Example settings for a commercial needle puller equipped with a 3 mm platinum/iridium filament are Heat = 810–820, Pull = 800–820, micrometer = 2.5.

5.2. View the tips under a dissecting microscope. If the needles have the desired shape (**Figure 3A–F**), pull 4–6 needles (2–3 capillary tubes). To achieve the proper needle shape, change the settings as needed: adjust the Heat or Pull settings by 10 and pull new needles until the shape of the taper and shaft are more appropriate.

[Place **Figure 3** here]

5.3. Store the pulled needles in a 15 cm plastic Petri dish with a piece of rolled tape to secure the needles and to avoid dust accumulation on the tips.

5.4. Place a 0.7 µL drop of the microinjection mix on the open end of the shaft. Hang the needle perpendicular to a shelf using a rolled piece of tape to fill the tapered shaft with the mix within 10 min. Prepare 2 needles at a time in case the first does not work.

6. Preparing the microscope and breaking the needle

NOTE: Microinjection uses an inverted microscope with 5x and 40x objectives equipped with a microinjector setup to control the movement of the needle. The inverted microscope should be placed on a heavy table or anti-vibration air table to reduce vibrational noise. The microinjector needle holder is connected to nitrogen gas that applies the pressure needed to deliver the microinjection mix. A smaller dissecting microscope nearby is used to transfer the worms.

6.1. Set the gas tank pressure to ~40–60 psi for breaking the needle and to ~30–50 psi for microinjecting, depending on liquid flow.

6.2. On the dissecting microscope, cover the shard of glass on the microinjection pad coverslip with halocarbon oil using a standard platinum worm pick.

6.3. Place the microinjection pad coverslip on the microinjection scope and locate the shard of

glass covered in oil. Align the glass shard such that an edge is perpendicular to the direction of the needle to serve as the surface used to break the needle.

6.4. Verify that the needle has no bubbles or debris in the tapered shaft using the dissecting microscope. Then, secure the needle 1–1.5 cm into the pressurized holder.

6.5. Position the tip of the needle in the center of the microscope field of view by eye. Then under low magnification, position the tip of the needle in the field of view, perpendicular to the side of the glass shard.

6.6. Switch to high power and align the tip of the needle with the edge of the glass, near but not touching it.

NOTE: When pulled, the needles are fused closed.

6.7. To break the tip of the needle to allow liquid flow, gently tap it on the side of the piece of glass while applying continuous pressure from the gas (**Supplemental Figure S1**). Once the liquid begins to flow, check the shape of the tip and ensure that it is sharp with easily flowing liquid.

NOTE: If the liquid is flowing too fast or the end is too blunt, the worms will be damaged during microinjection (**Video 1** and **Figure 3A–F**).

6.8. When the liquid is flowing well from the needle, move the microinjection slide to the dissecting scope and place drops of 1–2 µL halocarbon oil on the agar pad for placement of the worms.

6.9. Transfer 20–30 young adult *Strongyloides* to a 2% NGM plate without bacteria for at least 5 min to remove excess surface bacteria and select single worms for microinjection. Add more worms to the NGM plate as needed while injecting.

7. Microinjecting *Strongyloides*

7.1. Use a small amount of halocarbon oil on a worm pick to select a *Strongyloides* young adult female with 1–4 eggs in her gonad from the 2% NGM plate without bacteria.

7.2. Transfer the worm into a tiny drop of oil on the agar pad. Using the worm pick, gently position the worm so it is not coiled and the gonad is visible and easy to access. Note the direction of the gonad (**Figure 3G**).

7.3. Position the worm in the microinjection microscope field of view. Ensure the gonad is on the same side as the needle and positioned so that the needle will contact the gonad at a slight angle (**Figure 3H,I**).

7.4. Bring the tip of the needle to the side of the worm in the same focal plane. Aim for the gonad

arm near the middle of the worm. Use the microinjector to insert the needle gently into the gonad (**Video 2**).

7.5. Immediately apply pressure to the needle to gently fill the entire gonad arm with the DNA solution. Determine by eye when enough fluid has been injected (**Video 2**).

NOTE: It may take up to 2 s to fill the gonad.

7.6. Remove the needle and check to determine that the wound closes.

NOTE: The worm is too damaged to produce progeny if the gonad protrudes through the body wall (**Supplemental Video S1**).

7.7. Repeat with the other arm of the gonad if it is visible.

7.8. When finished injecting, quickly verify the needle is not clogged by applying pressure with the tip of the needle on the agar pad. Transfer the slide with the injected worm to the dissecting microscope.

7.9. To recover the injected worm, first place a few drops of BU on the worm to float it off the agar pad.

7.10. Collect a small amount of HB101 bacteria on a worm pick. Touch the worm with the adherent bacteria on the worm pick to remove it from the liquid.

7.11. Gently transfer the worm to the **recovery** plate, a 2% NGM plate containing an HB101 lawn.

NOTE: The worm should start crawling within minutes.

7.12. After a few females have been injected, add some uninjected males from the source plate.

NOTE: A minimum of one male for five females is a good baseline; an excess of males is preferred.

7.13. Repeat all steps until enough females have been injected for the experiment.

7.14. Leave the adults on the recovery plate for at least 1 h post-injection to allow the worms to recover and mate.

8. Recovery and culturing of injected *Strongyloides*

8.1. Collect feces overnight from uninfected host animals, using the same protocol as for infected animals.

8.2. Mix the uninfected feces with a small amount of charcoal (feces to charcoal ratio of

approximately 2 to 1 for these plates).

8.3. Pour a small amount of the fecal-charcoal mix into a 6 cm Petri dish lined with damp filter paper. Ensure that the mix does not touch the lid of the dish.

8.4. Flood the recovery plate with BU. Using a pipet set at 3 µL, transfer the worms to the feces in the fecal-charcoal plate. Place the worms directly on the feces, not on the charcoal.

8.5. Verify that the adults are on the fecal-charcoal plate using a dissecting scope.

8.6. To culture the worms, place the plate in a humidified chamber, *i.e.*, a plastic box with a tight-fitting lid lined with damp paper towels.

NOTE: After 2 days, there will be a mix of larval stages. After 5 days, most of the larvae will have developed into iL3s; a few younger larvae will remain. After 7 days, all the larvae should be iL3s.

9. Collecting and screening F_1 larvae to recover transgenics/knockouts

9.1. Using a Baermann setup, collect the larvae from the post-injection small-scale fecal-charcoal culturing plates. To get as many larvae as possible, wait for at least 2 h before recovering the worms from the Baermann apparatus.

9.2. Concentrate the larvae in a 15 mL centrifuge tube as in steps 4.10-4.14 and transfer the larvae to a small watch glass with BU.

9.3. If the larvae will be used for behavioral experiments, use 2% NGM plates with a thick lawn of HB101 for screening.

9.3.1. Transfer 20–30 larvae to the HB101 lawn.

NOTE: The bacteria will slow the movement of the larvae.

9.3.2. Under a fluorescence dissecting microscope, identify the larvae expressing the transgene of interest. Use a worm pick to select the transgenic larvae and move them to a small watch glass with BU.

9.3.3. Use a new HB101 plate to screen another small batch of larvae. When enough larvae have been collected for experimental use, treat the HB101 plates and the excess worms with diluted iodine (50% Lugol's iodine diluted in water) and discard them as biohazard waste. Alternatively, kill the excess worms using concentrated kennel cleaner containing alkyl benzyl ammonium chlorides.

9.3.4. Use the worms immediately or leave them in a shallow watch glass in a small amount of BU overnight.

NOTE: Worms may become hypoxic if the liquid is too deep. It is possible that leaving larvae in BU overnight may affect certain behaviors; therefore, use larvae for behavioral experiments within 6 h.

9.4. If the larvae will be used for microscopy and not behavioral assays, then immobilize the worms by nicotine paralysis reversibly for screening.

9.4.1. Using a razor blade, score a grid onto the plastic bottom of a 10 cm chemotaxis plate[12] to make it easier to keep track of the location of the worms on the plate.

9.4.2. Drop ~3 µL of larvae in BU into a square on the grid. Fill as many squares as needed. Do not use the ones near the edges of the plate, as the larvae may crawl to the sides of the plate.

9.4.3. Add 15–20 µL drops of 1% nicotine in water to the worm drops.

NOTE: After 4 min, the worms will be paralyzed.

9.4.4. Screen the worms using a fluorescence dissecting microscope.

9.4.5. Use a worm pick to transfer the transgenic larvae into a small watch glass with 1–2 mL of BU.

NOTE: The larvae will be paralyzed for several hours and can be easily mounted on microscope slides for microscopy. If left overnight in BU, the iL3s will recover and may be used for some assays or mammalian host infection. However, nicotine paralysis and the overnight incubation in BU may affect certain behaviors.

REPRESENTATIVE RESULTS:

If the experiment was successful, the F_1 larvae will express the transgene and/or mutant phenotype of interest (**Figure 4**). However, transformation rates are highly variable and depend on the constructs, the health of the worms, the post-injection culturing conditions, and the skill of the experimenter. In general, a successful experiment will yield >15 F_1 larvae per injected female and a transformation rate of >3% for fluorescent markers. If the total number of living progeny averages to fewer than 10 larvae/female, then it is possible that the construct is toxic, and the transformed larvae are not surviving. Finding large numbers of fluorescent eggs but not fluorescent larvae is another indication that the injection mix may be toxic. When first learning the technique, it is recommended to use a construct that expresses well, such as *act-2::mRFPmars,* which drives robust expression in body wall muscle[13] (**Figure 4**).

When generating mutants by CRISPR/Cas9-mediated targeted mutagenesis, the use of a repair template containing an *act-2::mRFPmars* or *act-2::GFP* transgene[13] is recommended so that potential mutants can be identified based on fluorescence[9,14,15]. It is important to note that because *Strongyloides* express transgenes from extrachromosomal arrays, fluorescent F_1

progeny may express mRFPmars or GFP from the array alone or express mRFPmars or GFP from both the array and an integrated transgene[3,9,16]. It is possible to identify larvae that are more likely to have integrated transgenes based on the pattern of fluorescent expression: "patchy" expression in the body wall muscle (**Figure 4A**) is more common when the transgene is not integrated into the genome, whereas consistent expression throughout the body wall muscle (**Figure 4B**) often, but not always, indicates that the transgene has integrated into the genome. However, expression patterns alone cannot be used to conclusively identify mutants—some worms with consistent expression throughout the body wall muscle may not have integration events. Moreover, expression patterns cannot distinguish mutants that are homozygous from those that are heterozygous or mosaic. Thus, each worm must be PCR-genotyped[9,14,15]. When disrupting genes that yield easily visible phenotypes, it may not be necessary to use a repair template. For example, disruption of the *Strongyloides unc-22* gene results in a dominant "twitcher" phenotype, with rates of heterozygous or homozygous disruptions above 10%[9].

FIGURE LEGENDS:

Figure 1: The *Strongyloides stercoralis* life cycle. The *S. stercoralis* parasitic females inhabit the small intestine of their mammalian hosts (humans, non-human primates, dogs). The parasitic females reproduce by parthenogenesis and lay eggs within the small intestine. The eggs hatch while still inside the host into post-parasitic larvae, which are then passed into the environment with feces. If the post-parasitic larvae are male, they develop into free-living adult males. If the post-parasitic larvae are female, they can either develop into free-living adult females (indirect development) or third-stage infective larvae (iL3s; direct development). The free-living males and females reproduce sexually to create progeny that are constrained to become iL3s. Under certain conditions, *S. stercoralis* can also undergo autoinfection, in which some of the post-parasitic larvae remain inside the host intestine rather than passing into the environment in feces. These larvae can develop into autoinfective larvae (L3a) inside the host, penetrate through the intestinal wall, migrate through the body, and eventually return to the intestine to become reproductive adults. The life cycle of *S. ratti* is similar, except that *S. ratti* infects rats and does not have an autoinfective cycle. The environmental generation is key to using *Strongyloides* species for genetic studies. The free-living adult females (P_0) can be microinjected; their progeny, which will all become iL3s, are the potential F_1 transgenics. This figure has been modified from Castelletto *et al.*[3].

Figure 2: The Baermann apparatus used to collect parasitic worms from cultures[10]. The contents of a fecal-charcoal plate are placed at the top of a column of warm water. The worms migrate into the water and collect at the bottom of the funnel. (**A**) To set up the Baermann apparatus, the stand for the Baermann funnel is clamped to the bench with a C-clamp. A rubber tube attached to the end of the funnel is closed with pinch clamps, and a catch bucket is placed underneath the tube for drips. Warm water is added to the glass funnel. (**B**) The plastic ring holder for the fecal-charcoal mix is then lined with 3 pieces of laboratory tissues (left). A wooden stick or tongue depressor (middle) is used to transfer the contents of a fecal-charcoal plate (right) into the plastic ring holder. (**C**) A close-up of the bottom of the plastic ring holder for the fecal-charcoal mix, showing the double layer of nylon tulle lining the bottom of the holder. (**D**) The fecal-charcoal holder is then placed on the top of the glass funnel. (**E**) The laboratory tissue is

dampened with water and closed over the fecal-charcoal mix. More warm water is added to mostly submerge the fecal-charcoal. (**F**) The complete Baermann setup, with the fecal-charcoal culture submerged under warm water.

Figure 3: Microinjection needles and a *Strongyloides stercoralis* adult female with optimal sites for microinjection identified. (**A–F**) Images of microinjection needles. The shaft taper (**A**) and the tip (**B**) of a needle that is correctly shaped for microinjection. The tip is sharp enough to pierce the cuticle and narrow enough not to cause excessive damage. The shaft taper (**C**) and the tip (**D**) of a microinjection needle that are incorrectly shaped for microinjecting. **D** is a higher magnification image of the tip of the needle in **C**. The tip is too blunt and wide and will cause excessive damage to the worm. The shaft taper (**E**) and the tip **(F)** of a needle that are likely to be too long and slender to work for microinjection. The tip in **F** is very similar to the tip in **D**. However, the shaft is narrower and too flexible to effectively pierce the cuticle. In addition, very slender needles clog easily. (**G**) An image of the whole worm correctly positioned for microinjection, assuming the needle is coming in from the right. Anterior is down and to the left; the vulva is indicated by the arrowhead. The gonad is visible along the right side of the female. This female has only one egg in her uterus (indicated by the asterisk). (**H, I**) Magnified views of the microinjection sites. The angle of the arrow approximates the angle of the injection needle. The vulva can be used as a landmark; it is on the opposite side of the worm from the arms of the gonad. The arms of the gonad curve around the intestine, and the ends with the dividing nuclei are opposite the vulva. (**H**) The posterior arm of the gonad; (**I**) the anterior arm. Either or both arms can be injected. For **H, I**, conventions are as in **G**. Scale bars = 50 µm (**B, D, F, H, I**); 100 µm (**A, C, E, G**).

Figure 4: Transgenic *Strongyloides stercoralis* larvae. (**A, B**) *S. stercoralis* larvae expressing an *act-2::mRFPmars* transgene, which expresses in the body wall muscle[13]. The transgene was incorporated into a repair template for CRISPR/Cas9-mediated disruption of the *Ss-unc-22* locus[9]. (**A**) An *S. stercoralis* larva with an incomplete, or "patchy," *act-2::mRFPmars* expression pattern that may indicate expression from an extrachromosomal array. (**B**) An *S. stercoralis* iL3 expressing the more complete *act-2::mRFPmars* expression pattern that may indicate gene disruption and integration of the repair template. For **A, B**, panels show differential interference contrast (left), fluorescent (middle), and merged (right) images. Scale bars = 50 mm.

Video 1: Demonstration of the needle-breaking process to make a useable needle. The tip of the needle is tapped against the edge of a glass shard (object on far left). When liquid emerges, the needle is pulled back and moved down onto the agar. The needle tip comes to a sharp point, and liquid flows moderately fast.

Video 2: Demonstration of a successful injection. The posterior arm of the gonad is visible as a light gray structure on the right. The tip of the needle and the gonad must be in the same focal plane. If the needle slides over or under the worm or along the body without catching, adjust the position. The tip of the needle will slightly indent the body wall. A quick tap on the needle holder attached to the micromanipulator will gently push the tip through the body wall and into the gonad. Once the needle is inside the gonad, apply pressure and inject the DNA solution. The liquid

should visibly flood the gonad. If the wound closes when the tip of the needle is removed, the worm is likely to survive.

Table 1: Examples of microinjection mixes. The plasmids and concentrations for three example microinjection mixes: one for a *gpa-3::GFP* reporter construct[10], one for CRISPR/Cas9-mediated disruption of the *Ss-tax-4* locus[14,15], and one for piggyBac-mediated integration of an *Ss-gpa-3::GFP* construct[13,17,18]. *strCas9* denotes the *Strongyloides* codon-optimized Cas9 gene. The final concentrations listed are commonly used in *Strongyloides* microinjection mixes.

Supplemental Material: *Strongyloides* strain maintenance. This protocol outlines the maintenance procedure for *S. stercoralis* in gerbils and *S. ratti* in rats. It includes the infective dose used for each nematode and host. Hosts are infected via subcutaneous injections under anesthesia. The progression of infections from patency to peak larval output to loss of patency is described for each nematode-host combination. The protocol used to collect infested feces and make the fecal-charcoal cultivation plates is also described.

Supplemental Figure S1: An image of a microinjection slide consisting of a dried agar pad on a coverslip with a small glass shard for breaking the microinjection needle. The agar outline and the shard outline are added here for clarity.

Supplemental Movie S1: Demonstration of an injection resulting in a damaged gonad. The anterior arm of the gonad is in focus. Applying slight pressure shows that the solution in the needle is still flowing out freely. The tip of the needle is in the same focal plane as the gonad and is aimed at a slight angle. Once the tip of the needle is inserted, the solution is injected into the worm and fills the gonad. However, when the needle is removed, a piece of the gonad protrudes through the wound in the cuticle. Material is visibly flowing out. This worm is unlikely to survive.

DISCUSSION:

This microinjection protocol details the methods for introducing constructs for transgenesis and CRISPR/Cas9-mediated mutagenesis into *S. stercoralis* and *S. ratti.* For both *S. stercoralis* and *S. ratti*, post-injection survival and the rate of transgenesis or mutagenesis are subject to several variables that can be fine-tuned.

The first critical consideration for successful transgenesis is how plasmid transgenes are constructed. Previous studies have found that expression of exogenous transgenes in *Strongyloides* requires the use of *Strongyloides* 5' promoters and 3' UTR elements[3,13,19]. Similar to *C. elegans* constructs, *Strongyloides* constructs generally use a gene-specific promoter and a common 3' UTR, such as the one from the *Ss-era-1* gene[13]. Codon-optimization of the coding region may also be important for expression in *Strongyloides*. Recently, the Wild Worm Codon Adaptor, a web-based app that codon-optimizes coding sequences for *Strongyloides* and other nematodes, was developed[20]. Finally, while not rigorously tested in *Strongyloides*, introns have been shown to increase expression of exogenous transgenes in both *C. elegans*[21] and the insect-associated nematode *Pristionchus pacificus*[22] and are presumed to increase expression in

Strongyloides as well. The Wild Worm Codon Adaptor has options for including up to three introns in the modified sequence[20].

The composition and delivery of the microinjection mix affect the transgenesis rate and the survival of the F_1 progeny. BU saline is routinely used as the diluent for mixes, although using ddH_2O is also an option. The concentrations and/or ratio of components in the mix can be adjusted to improve the transformation rate. Higher concentrations of the plasmids of interest can increase the rate of transgenesis but often result in fewer total progeny. If no transgene expression is observed or only dead transgenic eggs are found, it is possible that the transgenes are toxic, or that something in the plasmid stocks is causing the death of the transgenics. In the latter case, making new plasmid stocks using a different method (for example, using a different miniprep kit) may be sufficient for obtaining transgenics. Adding lipofectamine to the microinjection mix may also improve the rate of transgenesis[23].

The shape of the microinjection needle delivering the mix also affects the survival and transgenesis rates (**Figure 3A–F**, **Video 1**, **Video 2**, and **Supplemental Video S1**). The needle must be sharp enough to penetrate the cuticle and narrow enough to not result in excessive damage. It is recommended to pull needles just before use as needles stored for more than a day may accumulate debris and become clogged during microinjections. Recovering injected females from the microinjection pad without damaging them can be accomplished with a few different methods. One technique is to float the worms off the injection pad in a drop of BU, and then use HB101 on a worm pick to collect the worms. Other techniques for recovery include floating the worms in BU and collecting them using a pipette tip or a small paintbrush or simply using a worm pick alone to move the worms to a recovery plate.

If no progeny were obtained from the microinjected females, this suggests that either the injected females were damaged in the microinjection process or the post-injection culturing conditions were suboptimal. There are a number of different post-injection culturing conditions that can be tried. The small-scale fecal-charcoal cultures described above generally support better worm survival than NGM plates with HB101. However, it can be difficult to follow the survival of the injected worms and the development of the F_1 larvae on fecal-charcoal plates, and eggs are not visible on these plates. An advantage of culturing worms on NGM plates with HB101 instead of fecal-charcoal plates is to allow careful observation of egg-laying and larval development, which can be useful for troubleshooting. SV12 plates with HB101 can also be used to increase survival[24]. Finally, a chemotaxis plate[12] with a single rat fecal pellet can be used for *S. ratti* post-injection culturing. The males and injected females are transferred directly to the rat fecal pellet. In 5–7 days, worms are collected from the agar and feces using a Baermann apparatus, as described above.

To obtain *Strongyloides* adults for microinjection, freshly prepared fecal-charcoal plates can be incubated at 25 °C for 24 h or 20 °C for 48 h. *Strongyloides* adults reared at 25 °C for 24 h are young enough to produce a large number of progeny[25]. However, if the females are too young, they may not survive the microinjection process. Adults collected from fecal-charcoal plates that have been incubated at 20 °C for ~48 h are more likely to tolerate the microinjection process.

However, because these adults are older than adults obtained from a 24 h incubation at 25–26 °C, they are not as fecund and may have a lower transformation rate. Novices may prefer to start with older adults and then switch to slightly younger adults as skills improve.

Like *C. elegans*, *Strongyloides* species can express transgenes from extrachromosomal arrays and genome-integrated constructs in the F_1 generation. Unlike *C. elegans*, *Strongyloides* species will only express genome-integrated transgenes in the F_2 and subsequent generations even though the extrachromosomal arrays are still detectable by PCR[13]. The F_1 transgenic larvae expressing extrachromosomal arrays can be used for experiments that do not require genome integration or large numbers of transgenic worms. Genome integration may be required for experiments that require tagging endogenous genes or testing large numbers of worms in population-based assays. There are two methods for genome integration of transgenes in *Strongyloides*: piggyBac transposon-mediated integration[17] and CRISPR/Cas9-mediated integration[9]. piggyBac transposon-mediated integration uses the piggyBac transposase to target cargo to TTAA sites in the genome[26]. Because the TTAA motif is quite common in the AT-rich genome of *Strongyloides* species, integration is often at more than one site in the genome. In contrast, CRISPR/Cas9-mediated integration can be used to integrate transgenes at a specific target locus[9].

The CRISPR/Cas9 system can also be used to generate targeted gene knockouts[9,27]. Due to the AT richness of the *Strongyloides* genome, finding usable Cas9 target sites containing the optimal 5'-N_{18}GGNGG-3' sequence for nematodes[28] is a challenge. Frequently, there are only one or two sites in a gene for targeting. The preferred method involves the integration of a repair template containing a transgene with a fluorescent marker by homology-directed repair into the genomic locus, resulting in complete disruption of the gene. Potential knockouts can be identified by expression of the transgene[9,14,15,29]. However, transgene expression alone is not indicative of genotype as expression from arrays vs. integrated transgenes is often indistinguishable. Thus, the F_1 transgenic larvae require *post-hoc* genotyping to identify homozygous knockouts[9]. In the absence of a repair template, mutagenesis of the target locus occurs at high frequency but can result in large deletions[9].

Although generating transgenic or mutant F_1 larvae is relatively straightforward in *S. stercoralis*, generating stable lines is extremely difficult because of the need for host passage. In the laboratory, the Mongolian gerbil is a permissive host for *S. stercoralis* but requires a high dose of worms to establish an infection capable of producing enough F_2 larvae to establish the line[30]. Only approximately 6% of infective larvae become parasitic females[30]. Furthermore, if the transgenic larvae have array expression without genome integration, they will not produce the transgene-expressing progeny required to infect a second gerbil host. To increase the chances of sufficient numbers of genome-integrated larvae becoming reproductive parasitic adults, a minimum of 400–500 transgenic larvae in the initial inoculum is recommended. It may be possible to reduce the number of larvae required to establish a patent infection by treating the gerbils with prednisone[30]. Nevertheless, it is likely to be difficult to amass enough integrated transgenic or mutant worms to successfully establish a stable line of *S. stercoralis*. However, it is usually feasible to amass sufficient numbers of transgenic *S. stercoralis* F_1 larvae for single-worm assays[14,15].

Strongyloides ratti has the distinct advantage of the greater feasibility of stable transgenic or knockout lines[17,31]. *S. ratti* free-living adult females are less tolerant of the microinjection process than *S. stercoralis* free-living adult females; *S. ratti* females generally produce fewer overall larvae than *S. stercoralis* females, and the transformation rate is also lower[31]. However, only a few transgenic or knockout F_1 larvae are required to establish a stable line of *S. ratti*. As *S. ratti* is a natural parasite of rats, only a few *S. ratti* infective larvae are sufficient to establish a patent infection[32]. Thus, it is generally possible to amass sufficient numbers of transgenic or mutant larvae to establish a stable line. Because *Strongyloides* species will not express extrachromosomal arrays past the F_1 generation, only genome-integrated F_1 larvae can produce a stable transgenic line[13]. It is generally impossible to identify worms with integrated transgenes prior to genotyping, so the protocol is to collect all transgenic F_1 larvae and inject them into a rat. Some small percentage of these larvae will have the desired integration event; these larvae will form the basis for the stable line. As the piggyBac method often results in more than one integration event in any individual worm, almost 100% transmission of the transgene can be achieved after a few rounds of passaging transgenic larvae through a rat[17].

In summary, the technique described here can be used to generate transgenic or knockout *S. stercoralis* and *S. ratti*. This enables a wide range of potential experiments, including but not limited to the cell-specific expression of transgenes, the generation of mutants, and the endogenous tagging of proteins to determine spatial and temporal functions[14,15,29,33-35]. In the long run, knowledge gained from the use of transgenic *Strongyloides* can be used to develop new strategies to combat human infections with *S. stercoralis* and other intestinal parasitic nematodes.

ACKNOWLEDGMENTS:

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DISCLOSURES:

The authors declare no conflicts of interest.

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Figure 1

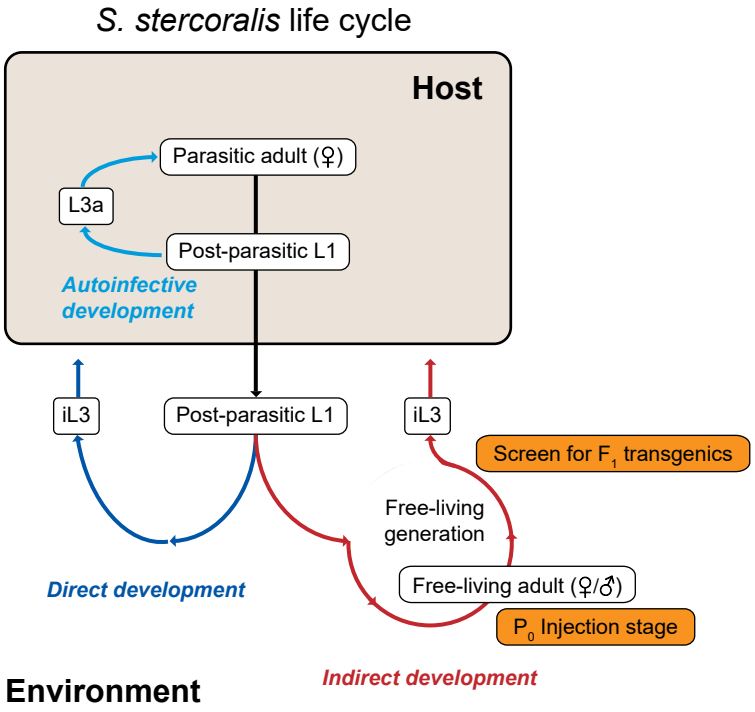

S. stercoralis life cycle
Host
Parasitic adult (♀)
L3a
Post-parasitic L1
Autoinfective development
iL3
Post-parasitic L1
iL3
Screen for F1 transgenics
Free-living generation
Free-living adult (♀/♂)
P0 Injection stage
Direct development
Indirect development
Environment

Figure 2

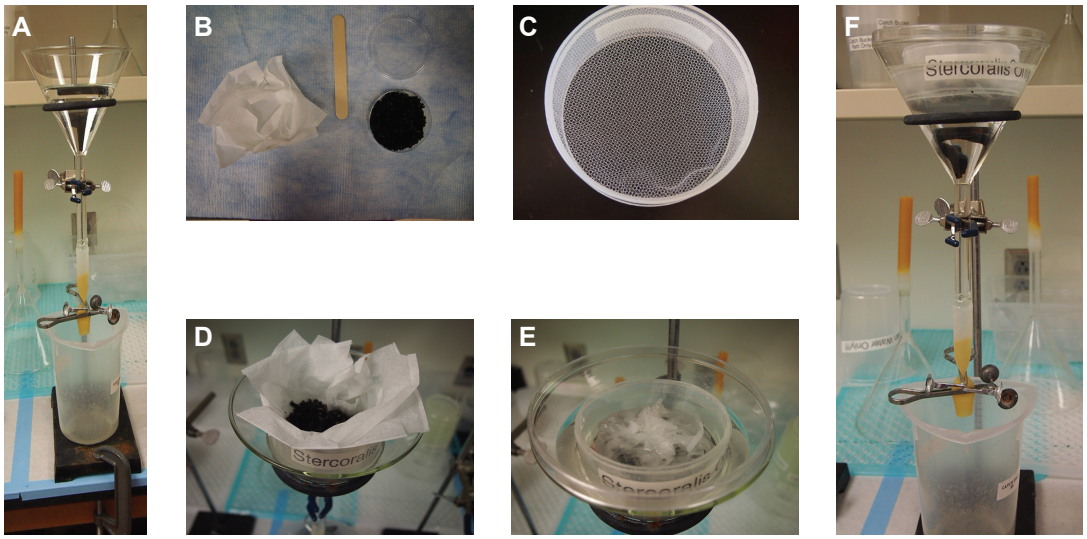


Figure 3

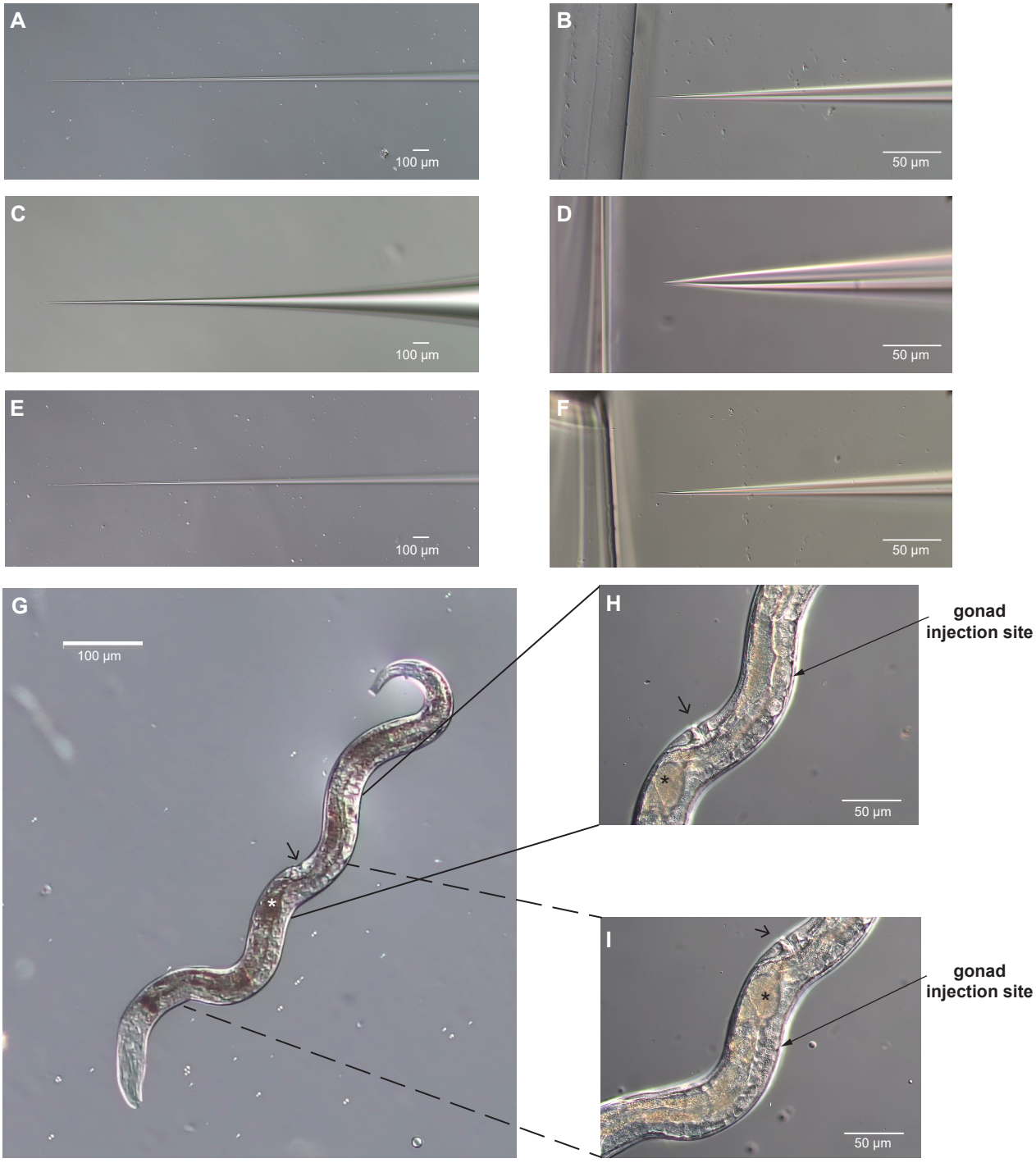
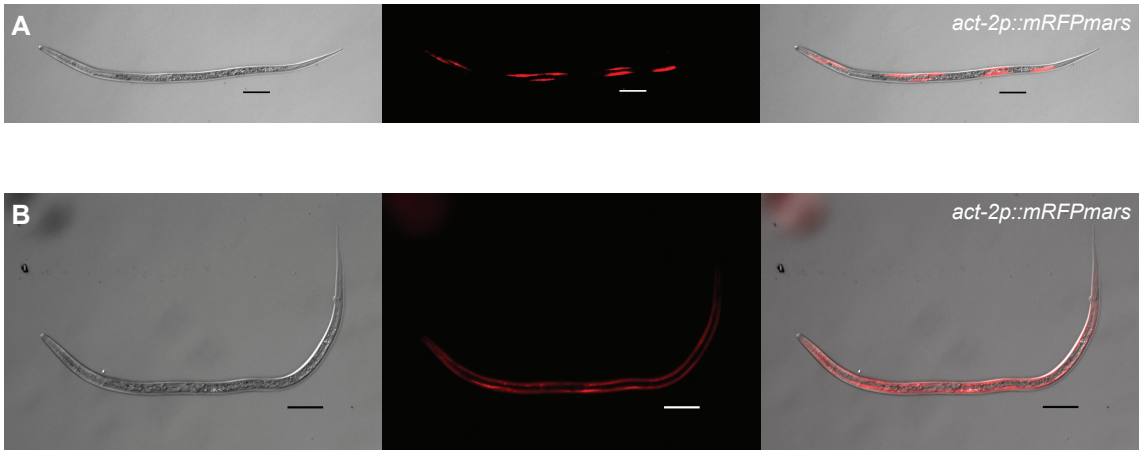


Figure 4



Movie 1

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Movie 1 2021-07-22.mov

Movie 2

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Movie 2.mov

Microinjection mix: reporter construct

Component	Stock Concentration	Amount	Final Concentration
pMLC30 *gpa-3::gfp*	300 ng/µL	1.6 µL	50 ng/µL
BU	na	8.4 µL	na
total		10 µL	50 ng/µL

Microinjection mix: CRISPR/Cas9 mutagenesis

Component	Stock Concentration	Amount	Final Concentration
pMLC47 *tax-4* sgRNA	300 ng/µL	2.6 µL	80 ng/µL
pEY11 *Ss-tax-4* HDR plasmid	400 ng/µL	2 µL	80 ng/µL
pPV540 *strCas9* plasmid	350 ng/µL	1.1 µL	40 ng/µL
BU	na	4.3 µL	na
total		10 µL	200 ng/µL

Microinjection mix: piggyBac integration

Component	Stock Concentration	Amount	Final Concentration
pMLC30 *gpa3::gfp*	300 ng/µL	2 µL	60 ng/µL
pPV402 transposase plasmid	450 ng/µL	0.9 µL	40 ng/µL
BU	na	7.1 µL	na
total		10 µL	100 ng/µL

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Table of Materials

Castelletto and Hallem 2021 materials list 2021-08-18.xlsx

Generating transgenics and knockouts in *Strongyloides* species by microinjection:
Response to reviewer comments

We thank the reviewers for their thoughtful and detailed comments on our manuscript. All of these comments have now been addressed in the revised version of our manuscript, as detailed below.

Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. Please define all abbreviations at first use.

All abbreviations have been defined.

2. Please provide an email address for each author.

We have now provided an email address for each author.

3. Please reduce the word count of your summary to be 10-50 words.

The summary has been rewritten to be less than 50 words.

4. Please remove citations from the abstract.

Citations have been removed from the abstract.

5. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. For example: QIAGEN miniprep kit, *etc.*

All commercial names, including “QIAGEN,” have been removed from the text. Please note that “Baermann apparatus”, “Pasteur pipet” and "Lugol's iodine" are the proper names, not commercial language.

6. Please revise the text, especially in the protocol, to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

The text has been revised to avoid the use of personal pronouns.

7. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., “Do this,” “Ensure that,” *etc.*). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.” However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, *etc.*

The protocol steps have been re-written in the imperative tense. Many of the “Notes” in our original manuscript have now been moved to the Discussion. All safety procedures are described.

8. The Protocol should contain only action items that direct the reader to do something. Please move the discussion about the protocol to the Discussion.

Most of the discussion points have been removed or moved to the Discussion. We strongly feel that the remaining discussion within the protocol is necessary to understand the protocol step. For example, Step 5.2 includes suggestions for how to optimize the shape of the pulled needles. This exposition is integral to the “View tips under a dissecting scope” action. If the shape is incorrect, immediate adjustments to the needle-pulling settings need to be made.

9. Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

We have ensured that our protocol contains sufficient detail.

10. Please format the manuscript as: paragraph Indentation: 0 for both left and right and special: none, Line spacings: single. Please include a single line space between each step, substep and note in the protocol section. Please use Calibri 12 points and one-inch margins on all the side. Please include a ONE LINE SPACE between each protocol step and then HIGHLIGHT up to 3 pages of protocol text for inclusion in the protocol section of the video.

Formatting has been changed to Calibri 12 point, one-inch margins, and specified paragraph indentation.

11. As we are a methods journal, please add any limitations of your technique to the Discussion.

We have moved the troubleshooting suggestions from the Representative Results section to the Discussion.

12. Please check and reupload movie 1 as it cannot be opened.

Movie 1 has been reformatted.

13. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source (ITALICS). Volume (BOLD) (Issue), FirstPage–LastPage (YEAR).] For 6 and more than 6 authors, list only the first author then et al. Please include volume and issue numbers for all references, and do not abbreviate the journal names. Make sure all references have page numbers or if early online publication, include

The references are in the specified format.

Reviewer #1:

Overall, this is a well-written and appropriately-detailed manuscript. Dissemination of the methods for generating transgenic *Strongyloides* spp. parasites with visual aids is important, as several labs have begun using *Strongyloides* spp. parasites for genetic studies.

Points:

-Line 61: *Strongyloides* spp. infect other vertebrate animals in addition to mammals.

We have now removed the phrase "Unlike other mammalian-parasitic nematodes" from the text.

-Line 63: Do you mean the post-parasitic generation consists of larvae, some of which develop into free-living males and females? As written, it sounds like there is more than one free-living generation.

We have reworded the text as follows: "Specifically, both *S. stercoralis* and *S. ratti* can cycle through a single free-living generation. The free-living generation consists of post-parasitic larvae that develop into free-living adult males and females; all progeny of the free-living adults develop into infective larvae, which must infect a host to continue the life cycle."

-Line 68 (or elsewhere in the introduction): It should be noted that both free-living females and males (at least for *S. stercoralis*) can be microinjected to generate transgenic progeny. PMID: 28577882.

This is now noted in the Introduction.

- Line 98: "room temperature" can be a wide range of temperatures, depending upon local climate. Can you be more specific (or provide ranges of temperatures and developmental timings)?

We have clarified that by “room temperature” we mean a temperature range of 20-23°C.

-Protocol 1.7: In humid climates, it can be helpful to dry the agarose pads in an oven overnight, and then allow them to cool to room temp, before use. It can help the worms "stick" to the pad better.

This point is now mentioned in the discussion.

-Protocol 4.2: Some people find it is possible to get enough worms in 15-30 min (depending on how many larvae were in the feces, length of the tubing on the Baermann, etc.), if they do not want to wait a full hour. Depends on how many worms are needed for injection.

The time frame for running the Baermann has been changed to 30 min - 1 h.

-Protocol 4.2.2 to 4.4.3: Something appears to be missing here: it is unclear how the worms get from the 30-40 ml of Baermann proceeds (in the 50 ml tube) to the 15 ml tube. Also the numbering skips.

We have now added more details to the protocol for collecting the worms from the Baermann apparatus. The numbering issue has also been fixed.

-Protocol 4.4.3.1: Some people prefer to sediment the worms at 1 x g to avoid pelleting the fecal debris along with the worms in a centrifuge.

Step 4.12 (formerly 4.4.3.1) has been changed to read, “Spin the 15 mL centrifuge tube for 1 min at about 750 x g (slow). Alternatively, worms can be allowed to gravity settle for 10-15 min.”

-Protocol 5.2: It would be really helpful to have a figure with images depicting the range of microinjection needle tips, including: too long and flimsy (where the needle bends, rather than pierces the worm cuticle), comes to a point too quickly and breaks open with too wide of an orifice, and the "Goldilocks" just right needle. This would help people visualize "proper needle shape."

We have added additional images of needles to Figure 3.

- Protocol 8: Do you have any data for the different post-injection culture conditions (e.g., injected female survival; number of progeny recovered)? A quantitative comparison of post-injection culture conditions might be useful (in addition to the qualitative comparisons in lines 449-462). This isn't necessary for this manuscript, but if you have the data, it would be helpful to include.

We do not have any quantitative data for the different post-injection culture conditions. In general, our impression is that at least for *S. stercoralis,* survival is often highest when the injected worms are cultured on small fecal-charcoal plates as opposed to plates with bacterial lawns. However, we do not generally count the total number of progeny recovered, so unfortunately we cannot provide quantitative comparative data.

-Protocol 9.3.3: One can also use concentrated kennel cleaner containing alkyl benzyl ammonium chlorides to kill excess worms (has a longer shelf-life than iodine solutions).

This is now noted in the protocol.

-Protocol 9.4.1: NGM agar or just agar?

We generally use chemotaxis plates. This is now specified in the protocol.

-Protocol 9.4.3: Does nicotine cause any morphological irregularities in the worms (as levamisole does)?

We have not noticed any morphological irregularities in nicotine-treated worms.

-Line 445: should also discuss the outcome of only getting GFP/RFP+ eggs and no GFP/RFP+ larvae.

We have now mentioned that getting fluorescent eggs but not fluorescent larvae is an indication the construct may be toxic.

- Line 469: have you tried using a repair template that does not have its own promoter driving expression of the fluorescent protein coding sequence, but instead relies on the endogenous promoter at the targeted locus? This might allow for the ability to distinguish between expression from extrachromosomal arrays vs. integrated transgenes in the F1.

We have not tried this yet, but it is something we have considered. We expect that whether this works will depend on where the CRISPR target site is in the gene. Since most genes in *Strongyloides* have very few good target sites, this approach may not work in many cases.

-Figure 1: the free-living life cycle appears continuous (i.e. multiple free-living generations) in the figure. This diagram should be altered to show that *S. stercoralis* only has a single free-living generation.

The figure has now been altered to make this clear.

- Line 492: there are some people in the parasitology community who would argue that invariant development of post-free-living larvae into iL3 is not a genetic, but rather chromosomal and gene dose-dependent. Maybe best to leave out the word "genetically."

We have removed the word “genetically.”

-Line 495: the autoinfective larvae are L3a and are not iL3. They are morphologically distinguishable (tail either comes to a point or is forked, respectively).

This has been corrected.

-Figure 2C: the image is a bit blurry. Could you use a better image to show how the mesh is attached to the bottom of the plastic ring?

We have now included a better image.

-Figure 3: it is a bit challenging to see the difference in the needle tips between panel D and panel E. Can you obtain images with greater magnification?

We cannot obtain images with greater magnification. However, we replaced the images in panel D and E and added some new images that should demonstrate the difference in needle tips.

- Figure 4: Could you also provide a set of images for what a heterozygous worm would look like, so that comparisons could be drawn for "more bright" vs. "less bright"?

The “more bright” vs. “less bright” comparison cannot be used to definitively distinguish heterozygotes vs. homozygotes. We have noticed that in a population of red worms, the brightest worms are more likely to be homozygotes than the dimmer worms; however, this is somewhat subjective and we have never quantified brightness. Thus, we have decided to remove this statement from our revised manuscript. The manuscript now reads: “It is possible to identify larvae that are more likely to have integrated transgenes based on the pattern of mRFPmars expression: “patchy” expression in the body wall muscle (Figure 4A) is more common when the transgene is not integrated into the genome, whereas consistent expression throughout the body wall muscle

(Figure 4B) often, but not always, indicates the transgene has integrated into the genome. However, expression pattern alone cannot be used to conclusively identify mutants – some worms with consistent expression throughout the body wall muscle may not have integration events. Moreover, expression patterns cannot be used to distinguish mutants that are homozygous from those that are heterozygous or mosaic. Thus, each worm must be PCR-genotyped."

-Line 534: Are you confident that the worm in panel A is an L2? It appears to have a more blunt-ended tail (similar to the iL3 in panel B), but it is difficult to tell on the PDF.

We have removed "L2" and simply labeled this worm as a "larva."

- Table 1: It might be helpful to include the plasmid numbers?

Plasmid numbers have now been included in the table.

-Line 581: maybe "*Strongyloides*" rather than "endogenous"? The promoter elements that are cloned out may not include all of the native/endogenous enhancers.

We have changed "endogenous" to "*Strongyloides*."

-Line 590: have these introns been tested in transgenesis studies? Do they splice out well?

These introns have not been tested in transgenesis studies in *Strongyloides*. This is stated in the text.

-Line 641: Roughly 6% of iL3 make it to the small intestine and become parasitic females in the gerbil (PMID: 8245532), so presumably it is possible to have an infective dose lower than 500 iL3, with sufficient numbers of post-parasitic L1. Additionally: By pre-treating a gerbil with prednisone/MPA/dexamethasone, can one reduce the number of iL3 necessary to establish a patent infection? Similarly, can treating a gerbil with prednisone/MPA/dexamethasone generate increased numbers of transgenic post-parasitic L1?

Line 641 has had the following text added: "Only approximately 6% of infective larvae become parasitic females[27]. Furthermore, if the transgenic larvae have array expression without genome integration, they will not produce the transgene-expressing progeny required to infect a second gerbil host. To increase the chances of sufficient numbers of genome-integrated larvae becoming reproductive parasitic adults, a minimum of 400 - 500 transgenic larvae in the initial inoculum is recommended. It may be possible to reduce the number of larvae required to establish a patent infection by treating the gerbils with prednisone[27]. Nevertheless, it is likely to be very difficult to amass enough integrated transgenic or mutant worms to successfully establish a stable line of *S. stercoralis*."

It is true that an initial inoculum of fewer than 500 iL3s will establish a patent infection in a gerbil. However, since only 6% of the inoculum will become patent adults, if 500 iL3s are injected into the gerbil, only ~30 worms are likely to survive. Because these worms cannot be definitively genotyped before injecting them into the gerbil, very few of them are likely to have the desired genotype. As a result, establishing a patent infection that yields sufficient numbers of F_2 progeny of the correct genotype to establish a stable line is very unlikely when fewer than 500 iL3s are injected into a gerbil.

-Line 654: It should be noted that extrachromosomal arrays can be maintained for multiple generations in *S. stercoralis*, even though the transgenes are not expressed.

We have added the following phrase to line 654: "even though the extrachromosomal arrays can be detected via PCR in the subsequent generations."

- For some of the consumables, it might be helpful to include catalog numbers with the items, rather than in a separate table?

JoVE style specifies that catalog numbers can only be included in the Materials List.

Supplemental protocol:

- In the supplemental protocol: Specify the strain and supplier of Mongolian gerbils as well as the rats?

We have added the animal suppliers to the supplemental protocol.

- section 1.1.2: it might be helpful to describe how to determine iL3 concentrations in mixes used for infecting animals.

Steps 1.3.1-1.3.5 describe the calculations for determining iL3 concentrations and an example for each step is given.

- section 1: it might be helpful to note that dogs can also be used as a laboratory host for *S. stercoralis*.

Step 1.7 has been added the Supplemental Protocol stating that dogs can be used as laboratory hosts for *S. stercoralis*.

- section 1: can the length of time in which a gerbil passes larvae in the feces be extended with low doses of prednisone?

Perhaps, but we do not routinely use prednisone on our gerbils.

Reviewer #2:

Manuscript Summary:
Michelle Castelletto and Elissa Hallem present a detailed and comprehensive protocol for intragonadal microinjection of DNA into *Strongyloides stercoralis* free-living females. This protocol exploits the unique lifecycle of *Strongyloides* species to introduce transgenes and CRISPR/Cas9 components into *Strongyloides* spp., a technique that is not yet possible in any other parasitic nematode species. As such, this manuscript is of significant interest to anyone involved in molecular parasitology research and will provide significant support to those attempting this type of microinjection experiment for the first time or, indeed, those that are troubleshooting issues with similar approaches. Hallem and Castelletto are absolutely best placed to author this protocol and accompanying media as they developed and optimised the CRISPR/Cas9 protocol in *S. stercoralis* and *S. ratti.* Overall, this manuscript represents a very valuable resource and represents an example of one of the most significant recent technical advances in nematode parasitology in addition, it provides insightful discussion on the application of this technique and its broader experimental uses. I have noted just a few very minor queries/comments.

Major Concerns:
None

Minor Concerns:
1. Line 105: should microinjection pads be stored at RT or +4?

We store the microinjection pads at room temperature. This has now been clarified in the protocol.

2. Line 124-126: an image of this might be useful, I realise that there is a video of tip breaking and a high power image of good vs bad tip, but a low power image of the shard on the agar pad might give some perspective?

We have added Figure S1, a photograph of the shard on the agar pad.

3. Line 141: add in 'free-living' for clarity

"Free-living" has been added to Line 141.

4. Line 187: nylon tulle, does this have a specific pore size?

The nylon tulle does not have a specific pore size. The lab tissue allows the worms through but holds most of the larger debris from the fecal-charcoal mix. The nylon tulle holds the lab tissues.

5. Is there some way to link the consumables in the protocol text to the table of information (or a specific section of the table) so that it is very clear what each component is referring to and linking the text and the consumables/equipment is easier? Also the table of information appears in five separate sections (on different pages) for me, I assume it is all one table?

We have followed the JoVE formatting guidelines, which unfortunately does not allow us to link the text to the table of materials. We have also followed JoVE formatting guidelines for the materials list.

6. STEP 4 -Collect young adult *S. stercoralis* for microinjection: morning of injection day. Can you add in a brief description of how you would dispose of faecal material/baermann waste given that it 'may' contain some infective larvae and that in step 9 you are collecting iL3's. Maybe this would be appropriate added into Supplementary protocol 1 as long as it is also cited in the main text.

We have now added steps 4.20 to 4.22 to the protocol, which outline the handling of the fecal-charcoal mix and the cleaning of the Baermann apparatus.

7. Line 296: should females be transferred to the NGM plate for a specific amount of time prior to microinjection to allow surface bacteria to be removed?

The phrase "for at least 5 min" has been added to the protocol step.

8. Line 524: conventions are (insert as) is in A

The legend for Figure 3 has been altered to say "conventions are as in A."

9. Line 412: Is there a specific reason why you score a grid onto the bottom of the plate as opposed to use a marker pen? I assume the grid is scored on the plastic of the base of the petri and not the agar itself.

The grid is etched onto the plastic bottom of the plate to make it easier to keep track of location of the worms on the plate. Even very fine-tip markers are larger than the worms one is trying to see. The thin scored line from the razor does not interfere with visualization of the worms.

10 Line 320: is the amount delivered judged just by eye as per the video? Can you add some clarification to this line.

Line 320 has been changed to read, "Determine by eye when enough fluid has been injected; it may take up to 2 seconds." The amount of DNA solution injected is variable and is determined by watching the fluid move through the gonad. More detail has also been added to Step 7.5 ("…gently fill the entire gonad arm with the DNA solution") in order to clarify how much fluid is considered enough.

Reviewer #3:

Manuscript Summary:
This is a really well organized description of transformation in *Strongyloides* to generate transgenic and CRISPR edited progeny. The videos are very clear and will help researchers, not only those studying *Strongyloides*, but also research studying other species of nematodes for which transgenesis is currently unavailable (I would include myself in the latter cohort and found the description very insightful). The protocol is very comprehensive and provides all necessary reagents and product codes that a researcher would need to duplicate the approach.

Major Concerns:
My only real concern with the protocol is that as it is currently organized it implies that a description of transgenesis will be outlined for more than one *Strongyloides* species but in actuality it only described the protocol using *S. stercoralis*. This should probably be re-framed to more precisely reflect the title *etc.*

We apologize for the confusion. We have now clarified that the same protocol can be used for *S. stercoralis* and *S. ratti*. We have also referred to the worms as "*Strongyloides*" throughout, except in the few cases where there are minor protocol differences between the two species.

Minor Concerns:
Some of the steps e.g. "pulling the needles" or "making the microinjection mix" need not be performed on the same day as the injection. This could be mentioned. This is very minor and I would be more than comfortable deferring to the author's judgement but it just caught my attention.

The label for Step 3 now includes "prior to or day of injection." We also added Step 3.5: "Use microinjection mix immediately or store at -20°C for future use." We recommend pulling needles the day of and only using them on that day. Otherwise, dust and particulate matter can collect on the glass and make the needles more likely to clog during injections.

Reviewer #4:

Manuscript Summary:
The authors describe the procedure for generating transgenic and knockout *Strongyloides* nematode species. This is a well described protocol, that could be useful not only for *Strongyloides* but also for other non-*Caenorhabditis* nematodes.

Major Concerns:
No major concerns

Minor Concerns:
I have only minor clarifications:

line 154: the authors mentioned that QIAGEN kit gave the best results when preparing plasmids. To which other kits/procedures was QIAGEN compared to?

We have removed the reference to the QIAGEN kit from the protocol, since commercial names are not permitted in the protocol. Instead, we have added a sentence to the discussion stating that some labs have found that some miniprep kits seem to work better for microinjection, in terms of adult and larval survival and transgenesis rate. QIAGEN miniprep kit will be in the parts list since we prefer this kit.

line 438: can the authors clarify what the 3% refers to? Is it the percentage of F1 that is fluorescent? I was a bit confused because in line 349 the transformation rate is stated as being 5%. I was curious about how many of those would have an integration, but I suppose it is difficult to tell because first a host has to be infected.

Line 349 listed a hypothetical transformation rate; this has now been removed. The transformation rate varies dramatically depending on constructs being injected, injector skill, age of worms, post-injection culturing system. Thus, it is not possible to give precise transformation rates. However, we do state in the manuscript that transformation rates for successful experiments are generally >3%. The integration rate can be determined via genotyping the transgenic larvae and depends on a number of factors, including the components of the mix and the activity score of the sgRNA target site in a CRISPR experiment.

In 2019, James "Sparky" Lok published a paper in which he claimed that lipofectamine increases the efficiency for CRISPR/Cas9 for *Strongyloides.* Have the authors tried adding lipofectamine to the injection mix?

We have tried using lipofectamine and have not seen an increase in transgenesis rate. However, we have now added a reference to this paper to the discussion section.

Reviewer #5:

In this manuscript Castelletto and Hallem provide a guide on generating transgenics and knock-outs in *Strongyloides* species. This protocol is highly detailed and thorough, providing a valuable resource for any researcher wishing to generate transgenic *Strongyloides* animals. One area to improve on is an expansion of the section on CRISPR knock-outs, given that it's a focus of the title and an area that other *Strongyloides* labs will likely be interested in.

We have changed the title to clarify that the focus of this manuscript is on the microinjection technique itself. The method we use for CRISPR/Cas9-mediated mutagenesis has already been published (Gang *et al., PNAS* 2017).

Suggestions are:

1. Clarify your preferred delivery approach of CRISPR reagents. From the author previous papers it looks like they use plasmid-based reagents. Have they tried Cas9 ribonucleoprotein complexes? If not, it could be worth adding a final "Future Perspectives" paragraph highlighting approaches that work well in *C. elegans* that may be worth importing into parasitic nematodes.

In our Gang *et al.,* 2017 paper, we detail the use of Cas9 both as plasmids and RNPs. Recent comprehensive reviews including Castelletto *et al.* 2020, Lok 2019, and Jaleta and Lok 2019 discuss functional genomics techniques, including CRISPR/Cas9 and transgenesis, being imported from *C. elegans* to parasitic nematodes. Our goal with this manuscript is to describe the process of microinjection, which is used to generate both knockouts and transgenics. This is now clarified in our revised manuscript.

In this protocol, we describe introducing the CRISPR components as DNA plasmids because this appears to work better than RNPs when making precise genome edits via homology -directed repair (Gang *et al.,* 2017).

2. How do the authors choose sgRNA sites? Do they use a program? Is there a cutoff for off-target? Is there any predicted activity that they can leverage?

We have now added a comment to the Discussion about the selection of sgRNA target sites. We use Geneious to select sgRNA sites. However, since JoVE does not allow product names in the text of the paper, the program we use is not listed.

For many *Strongyloides* genes, there are only one or two Cas9 targets sites available due to the AT-rich nature of the *Strongyloides* genome. We use the same guidelines that are used for selecting target sites in *C. elegans*; these guidelines generally work well for *Strongyloides* (Gang *et al.,* 2017). As long as the predicted activity score of a target site is above 0.3, it may be possible to use that target site. However, very few knockouts would be expected with an activity score that low.

Again, we apologize for the confusion over the focus of this manuscript. Our goal here is to outline the process of microinjection; the details of target site selection are already published in Gang *et al.,* 2017. This is clarified in our revised manuscript.

3. When they inactivate genes, do they insert an act-2::FP cassette at the start of the gene or use two sgRNAs to cut out the gene and replace it?

In the Discussion, we now explain that due to the limited number of sgRNA target sites in the *Strongyloides* genome, we use one target and insert a 2.5 kb cassette wherever the target cuts.

4. The authors make the point about the AT-rich genome of *Strongyloides* species. Do they ever run into issues finding an appropriate Cas9 sgRNA target? It could be worth noting in a "Future Perspectives" section that Cas enzymes like Cpf1/Cas12a with a more AT-rich PAM may be useful to circumvent this issues.

Yes, this can be an issue – see comments 2 and 3 above. Unfortunately, JoVE formatting guidelines do not seem to allow for a “Future Perspectives” section. However, we agree that Cas12 could be very useful for *Strongyloides* species. We have not yet tried Cas12 in *Strongyloides*, but plan to do so in the future.

5. The authors note that *unc-22* mutant produce a visible twitching phenotype. Given that this is a co-conversion marker in *C. elegans*, have they tried a co-conversion approach in *Strongyloides*. It's worth noting that Farboud and Meyer developed a *ben-1* co-conversion marker that results in resistance to benzimidazole which might be very useful for parasitic nematode genome editing. Again, this material is great fodder for a "Future Perspectives" section.

We have not used a co-conversion approach, in part because using an *act-2::mRFPmars* cassette has worked so well. Also, we are reluctant to add a drug resistance gene like *ben-1* to a human-parasitic nematode.

Figure S1

Movie S1

Click here to access/download

Supplemental Coding Files

Movie S1 2021-07-31v2.mp4