Video Article

Delivery of Nucleic Acids through Embryo Microinjection in the Worldwide Agricultural Pest Insect, *Ceratitis capitata*

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Abstract

The Mediterranean fruit fly (medfly) *Ceratitis capitata* (Wiedemann) (Diptera: Tephritidae) is a pest species with extremely high agricultural relevance. This is due to its reproductive behavior: females damage the external surface of fruits and vegetables when they lay eggs and the hatched larvae feed on their pulp. Wild *C. capitata* populations are traditionally controlled through insecticide spraying and/or eco-friendly approaches, the most successful being the Sterile Insect Technique (SIT). The SIT relies on mass-rearing, radiation-based sterilization and field release of males that retain their capacity to mate but are not able to generate fertile progeny. The advent and the subsequent rapid development of biotechnological tools, together with the availability of the medfly genome sequence, has greatly boosted our understanding of the biology of this species. This favored the proliferation of new strategies for genome manipulation, which can be applied to population control.

In this context, embryo microinjection plays a dual role in expanding the toolbox for medfly control. The ability to interfere with the function of genes that regulate key biological processes, indeed, expands our understanding of the molecular machinery underlying medfly invasiveness. Furthermore, the ability to achieve germ-line transformation facilitates the production of multiple transgenic strains that can be tested for future field applications in novel SIT settings. Indeed, genetic manipulation can be used to confer desirable traits that can, for example, be used to monitor sterile male performance in the field, or that can result in early life-stage lethality. Here we describe a method to microinject nucleic acids into medfly embryos to achieve these two main goals.

Video Link

The video component of this article can be found at https://www.jove.com/video/54528/

Introduction

The Mediterranean fruit fly (medfly) *Ceratitis capitata* is a cosmopolitan species that extensively damages fruits and cultivated crops. It belongs to the Tephritidae family, which includes several pest species, such as those belonging to the genera *Bactrocera* and *Anastrepha*. The medfly is the most studied species of this family, and it has become a model not only for the study of insect invasions[1], but also for optimizing pest management strategies[2].

The medfly is a multivoltine species that can attack more than 300 species of wild and cultivated plants[3,4]. The damage is caused by both the adults and the larval stages: mated females pierce the surface of the fruit for oviposition, allowing microorganisms to affect their commercial quality, whereas the larvae feed on the fruit pulp. After three larval stages, larvae emerge from the host and pupate into the soil. *Ceratitis capitata* displays an almost worldwide distribution, including Africa, the Middle-East, Western Australia, Central and South America, Europe, and areas of the United States[5].

The most common strategies to limit medfly infestations involve the use of insecticides (*e.g.*, Malathion, Spinosad) and the environmentally-friendly Sterile Insect Technique (SIT)[6]. The latter approach involves the release into the wild of hundreds of thousands of males rendered sterile by exposure to ionizing irradiation. The mating of such sterilized males to wild females results in no progeny, causing a reduction in population size, eventually leading to eradication. Although SIT has proven effective in multiple campaigns worldwide, its major drawbacks include the high costs of rearing and sterilizing millions of insects to be released. Marking of released individuals is necessary to distinguish sterile from wild insects captured in the field during monitoring activities and it is currently achieved using fluorescent powders. These procedures are costly and have undesirable side-effects[7].

In order to optimize and/or to develop more effective approaches for the control of this pest, medfly biology and genetics have been widely explored by numerous researchers worldwide. The availability of the medfly genome sequence[8,9], will facilitate novel investigations on gene functions. RNA interference is a powerful tool for such studies and it can be achieved through the microinjection of dsRNA (double-stranded RNA) or siRNA (small interfering RNA). This technique has been used, for example, to demonstrate that the sex determination molecular cascade in *C. capitata* is only partially conserved with respect to that of *Drosophila*[10].

The development of protocols to microinject medfly embryos allowed *C. capitata* to be the first non-Drosophilid fly species to be genetically modified. As its eggs are similar to those of *Drosophila*, both in terms of morphology and resistance to desiccation[11], the protocol to deliver plasmid DNA into pre-blastoderm embryos first developed for *D. melanogaster*[12,13] was initially adapted for use in *C. capitata*. These first experiments allowed medfly germ-line transformation based on the transposable element Minos[11]. Subsequently, the original system was modified[14] using other transposon-based approaches. This is the case of *piggyBac* from the Lepidoptera *Trichoplusia ni*[15]. The protocol has since been further optimized and this has permitted the transformation of other tephritid species[16-21] and also of many other Diptera[22-31]. All these systems rely on the use of a typical binary vector/helper plasmid transformation system: artificial, defective transposons containing desired genes are assembled into plasmid DNA and integrated into the genome of the insect by supplying the transposase enzyme[32]. A number of transgenic medfly lines have been generated, with multiple features including strains carrying a conditional dominant lethal gene that induces lethality, strains producing male-only progeny and thus not requiring additional sexing strategies, and strains with fluorescent sperm, which may enhance the accuracy of the SIT monitoring phase[33-37]. Although the release in the wild of transgenic organisms has occurred in pilot tests against mosquitoes only[38,39], at least one company is evaluating a number of transgenic medfly strains for their use in the field[40].

Embryo microinjection can also favor the development of new genome-editing tools, such as transcription activator-like effector nucleases (TALENs), clustered regularly interspaced short palindromic repeats (CRISPR) / CRISPR associated protein 9 nuclease (Cas9) and homing endonucleases genes (HEGs), which will enable novel evolutionary and developmental studies, as well as expanding the available biotechnological toolbox. Genome-editing approaches already allowed the generation of gene-drive systems in mosquitoes[41], and their transfer to the medfly is imminent. Here we describe a universal protocol for microinjecting nucleic acids in medfly embryos that can be useful for all the above mentioned applications.

Protocol

1. Experimental Set-up

1. **Insectary requirements**
    1. Maintain all *C. capitata* life stages at 25 °C, 65% humidity and 12/12 hr light/dark photoperiod.
    2. Place about 1,500-2,000 medfly pupae in a 6 L cage. Use a cage with a brass mesh on one side with holes small enough to stimulate oviposition[42]. Insert a sponge strip through a small opening in the cage's base to provide flies with water by means of capillary action. Use a mixture of yeast and sugar (1:10) as food source for the adults (**Figure 1A**). Increase the amount of yeast (up to 1:3, yeast:sugar) should the females fail to lay eggs.
    3. Place a plate filled with water below the cage to collect the eggs deposited through the mesh. As virgin females can oviposit, wait until the flies are at least 6-7 days old before collecting eggs for the microinjection experiments. This will ensure that the females will have mated and that the eggs will have been fertilized.
    4. Collect the eggs by filtering the water with a fine net strainer. Transfer the eggs from the strainer in a plastic box containing standard larval food (1.5 L H_2O, 100 ml HCl 1%, 5 g broad-spectrum antimicrobial agent dissolved in 50 ml ethanol, 400 g sugar, 175 g demineralized brewer's yeast, 1 kg soft wheat bran) using a Pasteur pipette.
    5. Place each box in a transparent container closed with a net lid to allow air circulation and containing a layer of bran to favor pupation (**Figure 1B**).
    6. After about 10 days, check for 3rd instar larvae that emerge from the larval food and jump down into the bran layer for pupation.
    7. 24 hr later, collect the pupae in a small cup using a soft brush and transfer them to a new adult cage. Adults normally emerge 10 days after pupation. The emerging fly uses the *ptilinum*, an inflatable pocket located on its head above the base of the antennae, to force off the end of the puparium. After emergence, the *ptilinum* permanently collapses back inside the head.

2. **Preparation of 1 L food for microinjected larvae**
    1. Dissolve 2.5 g agar in 300 ml H_2O.
    2. Warm 500 ml H_2O on a hot stirring plate and dissolve 4 g Sodium Benzoate, 4.5 ml 37% HCl, 42 g yeast extract and 115 g dehydrated carrot powder.
    3. Add to the mixture a solution of 2.86 g broad-spectrum antimicrobial agent dissolved in 10 ml absolute ethanol.
    4. Distribute the medium in 15 cm round Petri dishes and allow to cool. If necessary, store at 4 °C.

3. **Slide preparation**
    1. Place a 2 cm strip of double-sided tape on a microscope slide (75 x 26 mm^2) and mark the edges with a china marker, to prevent oil spread and the consequent desiccation of the injected embryos.

4. **Nucleic Acid Preparation**
    1. Isolate plasmid DNA using commercially available kits[34]. Precipitate the plasmid using ethanol and re-suspend in injection buffer (0.1 mM phosphate buffer pH 7.4, 5 mM KCl) at the desired concentration.
    2. Purify *in vitro* synthetized long dsRNA using phenol-chloroform, precipitate using isopropanol and re-suspend in injection buffer[43].

2. Embryo Preparation

1. Place a tray filled with water under a cage containing 6-7 day old adults.
2. Allow the females to oviposit for 30 min and then collect the embryos by filtering the water using a fine strainer. Always keep the strainer in water to avoid desiccation of the embryos.
3. Dechorionate the embryos by immerging the strainer for 5 sec in a commercial 50% bleach solution.
4. Wash the embryos carefully by repeatedly immerging the strainer in clean ultrapure water (at least 4-5 times). Finally, place the strainer in clean ultrapure water. Use the embryos within maximum 2 hr.

NOTE: The timing of microinjection is related to the necessity to inject into pre-blastoderm embryos, during a phase of nuclear divisions prior to cellularization. This allows the injected DNA to be taken-up into the nuclei, specifically into the primordial germ cell nuclei that will originate the gametes. In *Drosophila*, cellularization at the pole cells begins at about 90 min after fertilization (at 25 °C), with blastoderm formation occurring about 30 min later[44], whereas in the medfly cellularization takes place between 9 and 12 hr[45,46].

5. **Embryo orientation in rows**
    1. Using a fine paintbrush, collect about 50 embryos and place them on a disk of black filter paper soaked with water.
    2. Under a dissecting stereomicroscope, arrange the embryos in a row on the upper surface of the double-sided tape on the microscope slide using a fine paintbrush (000) (**Figure 1C**).
    NOTE: Align the embryos all in the same orientation, as injection is performed in the posterior pole, where germ-line will originate; the posterior pole is opposite to the micropyle region.
    3. Cover the embryos with a layer of chlorotrifluoroethylene oil making sure that the oil does not spill over china marker borders (see 1.3).
    NOTE: Before covering the embryos with oil, a further step can be performed: the embryos can be desiccated for a few minutes to reduce their liquid content and thus facilitate the entry of the injected DNA solution. This may help avoid embryo bursting during injection or excessive leakage of the injected solution, as a consequence of the high liquid pressure.

3. Embryo Microinjection

1. Fill a needle (1-2 µl) with plasmid DNA or dsRNA solution (1-2 µg/µl) using a micropipette.
2. Connect the needle to the microinjection apparatus, which controls the injection time (0.5 sec), the pressure (800 hPa) and the backpressure (150 hPa).
3. Perform microinjection under a stereomicroscope, using a micromanipulator to control the movements of the needle (**Figure 1D**). Use the microscope stage to move the slide.
    1. Place the slide on the stage of the scope.
    2. Insert the tip of the needle into the posterior pole of the embryo (**Figure 1E**).
    3. Inject the solution by activating the microinjection system with the pedal. The injected liquid induces an increase in internal pressure, resulting in a slight movement of the embryo.
    4. Release the pedal and immediately, but gently, remove the needle from the embryo.
    5. Observe a little cytoplasmic leakage at the injection site.
    NOTE: To discriminate between potential lethal effects of DNA/dsRNA/siRNA and mortality due to the microinjection procedure itself, control experiments must be performed. These comprise the injection of buffer only, or, in the case of reverse genetics experiments, of a green fluorescent protein (GFP)-derived double-stranded RNA (dsRNA-GFP), or siRNA.
4. Following injection incubate the embryos at 25 °C.

4. Post-injection Procedures

1. Two days after injection, check the slides for hatched larvae (**Figure 1F**) under a stereomicroscope. Larvae can move in the oil but need to be transferred to larval food as soon as possible. For this reason, check the slides several times a day.
2. Using a fine paintbrush (000), gently transfer the hatched larvae to the carrot-based larval food. Transfer up to a maximum of 200 larvae per each Petri dish.
3. Incubate the larvae at 65% humidity (25 °C). Ensure that the lid does not stick to the dish, impairing air exchange.
4. Keep the Petri dishes with the lid mostly closed so that there is still air flow to the larvae. To avoid excessive drying, check the dishes daily and, if needed, add water to compensate for evaporation.
5. Place the Petri dishes in a clear container closed by a net-lid and containing a layer of bran to favor pupation. As for routine insectary rearing, 3rd instar larvae jump out of the larval food and pupate in the bran.
6. Collect the pupae and place them in a small cage for adult rearing and screening (this is Generation 0, G0). Rear the progeny on standard larval food.
7. In the case of germ-line transformation experiments, check for possible transformed individuals in the next generation (G1), as injected individuals usually are chimeras. Transposition events, indeed, might have occurred in any of the nuclei of the embryonic syncytium, which will form either the germ-line or the soma.
    1. Soon after emergence, anesthetize G1 individuals using CO_2 and check for transformation events under an epifluorescence stereomicroscope. To screen for the presence of red fluorescence, use a DsRedwide filter (Ext. 546/12; Emm. 605/75), while to screen for green fluorescence use the EYFP filter (Ext. 500/20; Emm. 535/30).
    2. Place transformed individuals in a small cage with wild-type adults of the opposite sex and originate a new, initially heterozygous, strain.

Representative Results

Here we report two applications of embryo microinjection directed at the functional characterization of a gene of interest (Case 1), and at the generation of transgenic strains (Case 2), respectively.

Delivery of dsRNA into embryos to unravel gene function.

The *innexin-5* gene encodes a gap-junction that, in insects, is expressed specifically in the male and female gonads[43,47,48]. Based on the information available for closely related species, the dsRNA-induced gene silencing was expected to result in the ablation of the medfly female and male germ-line. A total number of 2,400 embryos were injected with a 2 µg/µl dsRNA mixture, from which 548 larvae hatched and 216 adults survived (unpublished data). In about 75% of the adults, testes and ovaries appeared to be either under-developed or totally absent (**Figure 2**). Quantification of *innexin-5* transcript abundance in the abdomens of individuals from both sexes confirmed the significantly lower expression of the gene, as compared to the controls.

Generation of transgenic strains with fluorescent spermatozoa.

Medfly transgenic strains with fluorescently labeled sperm were generated by Scolari and colleagues[34] by injecting embryos with plasmid DNA. The mixture contained two plasmids mixed in fixed relative percentages: one, the "Helper plasmid", encoded the *piggyBac* transposase; the other, the "Donor plasmid", contained the artificial transposon and carried two fluorescent markers, one expressed in the soma of males and females, the other specifically in the testes. The promoter of the *beta 2-tubulin* gene, responsible for the testes-specific expression, was fused at the ATG with the coding sequences of the fluorescent proteins turboGFP (construct #1260) or DsRedExpress (construct #1261), respectively. A total number of 821 embryos were injected, from which 205 larvae hatched and 37 adults survived. 9 female and 8 male crossings were set-up, 6 of which gave fluorescent progeny[34] (License to reuse these data obtained from Elsevier - License Number 3796240759880). The successful transformation led to the development of strains with green and red fluorescent testes, respectively (**Figure 3**).

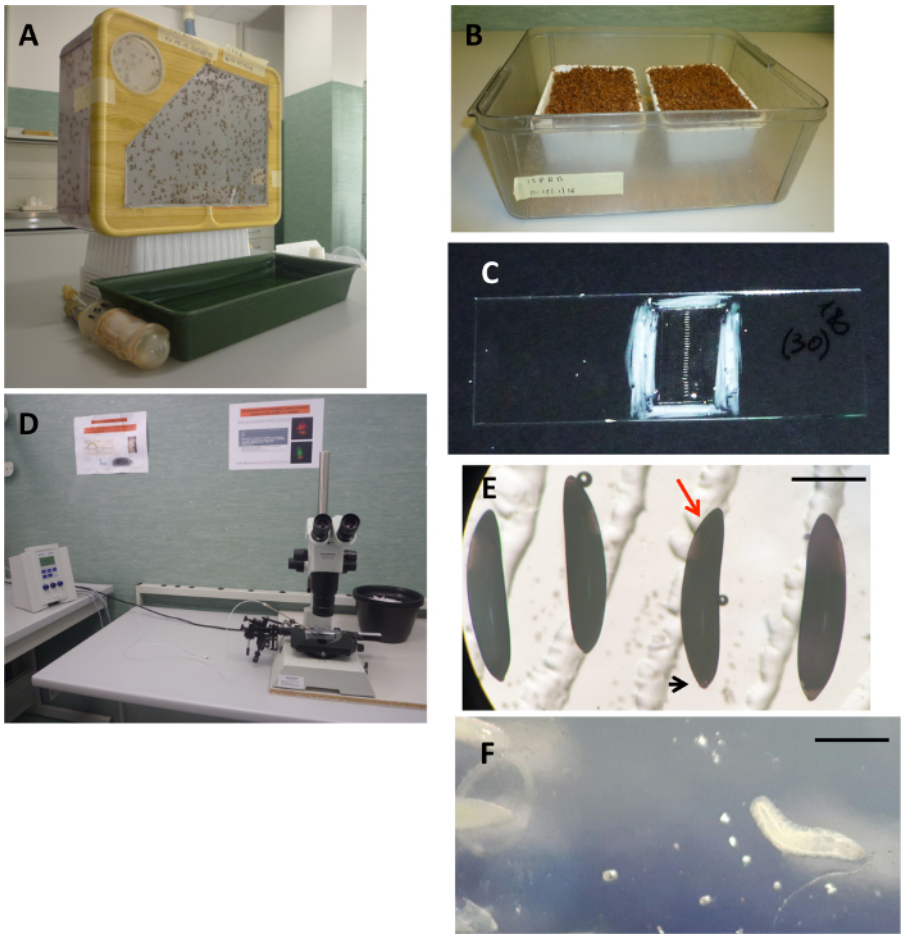

Figure 1. Insectary equipment and embryos. (**A**) Standard rearing cage containing 1,500-2,000 adults. The females lay eggs through the brass mesh at the front of the cage. Eggs are collected in water. On the left side of the cage, the strainer used to filter the eggs is visible. (**B**) Two boxes of standard larval food containing larvae. The boxes are placed in a bigger clear plastic box containing bran to facilitate pupation; the net covering the box has been removed. (**C**) Embryos arranged in rows. The edges of the double-coated tape on the slide have been marked with a white china marker. Eggs aligned on the tape will be covered with chlorotrifluoroethylene oil. (**D**) Microinjection apparatus (left) connected to a stereomicroscope equipped with a micromanipulator (right). (**E**) Embryo poles; the red arrow indicates the posterior pole (the injection site), whereas the black arrow indicates the anterior pole (where the micropyle is located). Scale bar = 500 µm. (**F**) Hatched larva moving in the oil. Scale bar = 500 µm. Please click here to view a larger version of this figure.

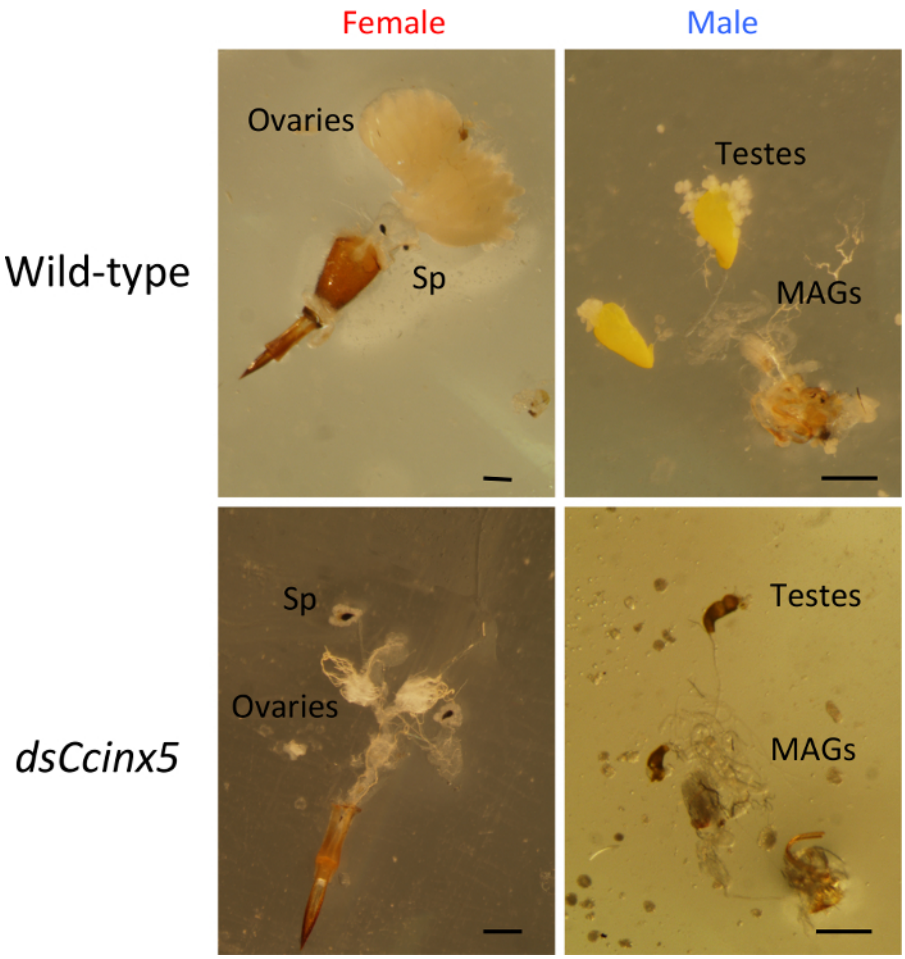

Figure 2. Male and female with under-developed gonads. Female (left) and male (right) dissected reproductive tracts. Above: wild-type individuals with normal gonads. Below: interfered individuals with under-developed gonads. MAGs: Male Accessory Glands; Sp: spermathecae. Scale bar = 500 µm. Please click here to view a larger version of this figure.

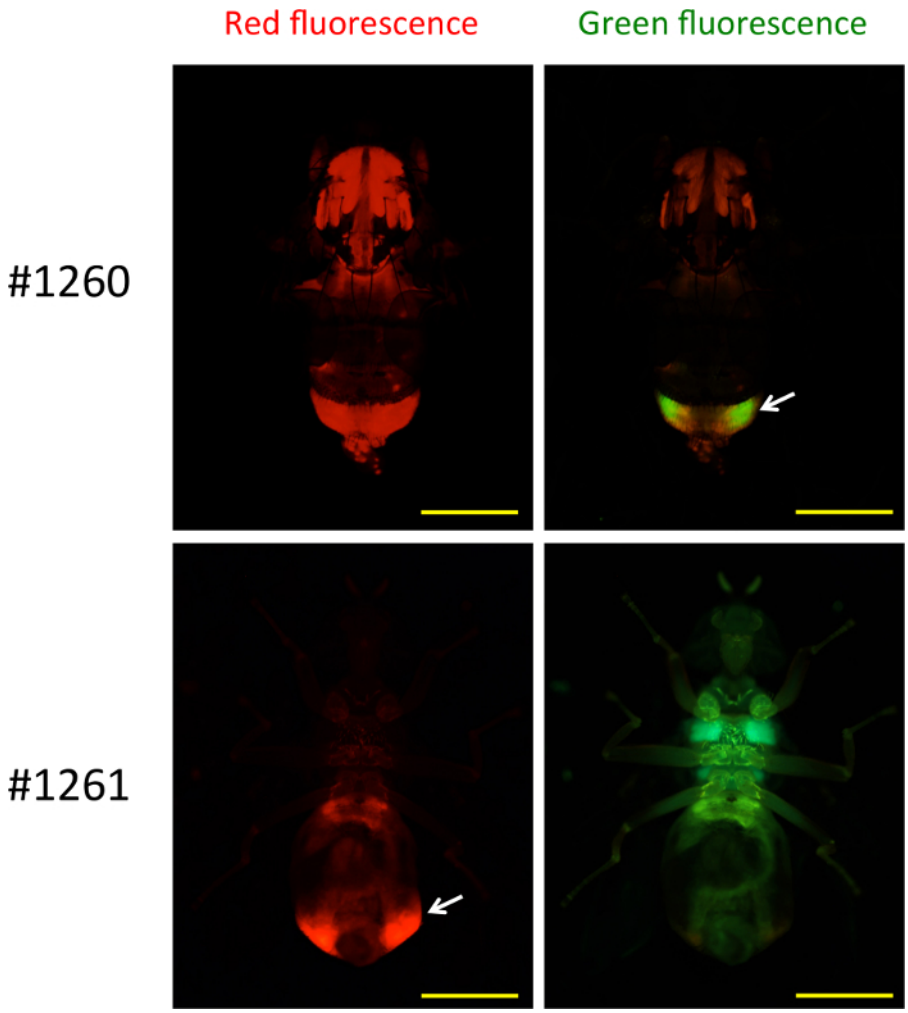

Figure 3. Transgenic males with fluorescent testes and sperm. Adult males transformed with construct #1260 and #1261, respectively. Arrows indicate the fluorescent testes. #1260 males show red body and green testes, whereas #1261 males show red testes and green bodies. Scale bar = 2 mm. Please click here to view a larger version of this figure.

Discussion

Microinjection of nucleic acids in insect embryos is a universal technique that facilitates both the analysis of gene function and biotechnological applications.

The recent publication of the genome sequences from an increasing number of insect species leads to an urgent need for tools for the functional characterization of genes of yet unknown function. RNA-interference has proven to be one of the most valuable methods to infer molecular functions[49] and embryo microinjection facilitates these studies.

Injection of plasmid DNA can be used to modify insect genomes using transposase-mediated gene insertion, and, more recently, genome-editing tools (*e.g.,* TALEN, CRISPR/Cas9, HEGs). These techniques have already allowed the development of strains of multiple insects species that might be used for pest control programs, aiming both at the eradication or at the replacement of wild population with insects with modified biological features. In this protocol, we describe an optimized method for medfly embryo microinjection with either plasmid DNA or dsRNA.

The availability of well-established and cost-effective semi-dry larval medium for medfly rearing, as well as the extensive molecular information achieved over the years by researchers studying the sex determination and cellularization process in this species, greatly facilitate the establishment of a reliable embryo microinjection protocol. In particular, the rearing protocol has been optimized to ensure the maximum fertility and vitality of the embryos. This is important to maximize the probability of obtaining viable adults with the least number of injected embryos possible. Different protocols are available for medfly rearing, such as the carrot-based larval food that we describe to rear the injected larvae. However, this method is more expensive and its preparation is more time-consuming than other media used for routine rearing.

An important barrier to the use of microinjection is the non-specific damage caused by the mechanical manipulation of the embryos. This includes multiple variables influencing survival of embryos, such as the piercing of the embryo membranes with the paintbrush used to orientate the embryos, the injected volumes, the injection site and buffer, and the type of needle used[50]. Some of these parameters have been optimized,

such as the injected volumes and the buffer. In the case of needles, they can also be produced in-house using a puller, and this requires an optimization step to determine the ideal protocol.

Among the major drawbacks in the microinjection procedure is also dechorionation: although this step is essential to make the eggs easier to handler (*i.e.,* less slippery) and to facilitate injection, prolonged exposure to bleach can heavily affect embryonic vitality. For this reason, the protocol we describe here reports the use of a very short dechorionation time (5 sec), which has been established as a good compromise between removal of the chorion and survival rate.

The protocols used to rear the subsequent life stage are also relevant. Larvae hatched from injected embryos have to be removed from the oil as soon as possible. Oil is indeed essential to prevent desiccation of the embryos, but can be noxious to the larvae. The method used to remove larvae, the time spent into the oil, and the type of food used are all important variables that need to be considered since they can compromise the final survival rate.

When screening adult and larvae, the immobilization methods can be also critical. Medfly adults can be immobilized using either ice or CO_2, but prolonged exposure might be deleterious for adult survival. As an alternative to embryo microinjection, oral delivery has proven to be a less-invasive and potentially high-throughput method to perform RNAi assays. It can be particularly effective in species that are not amenable to microinjection, as well as for RNAi-mediated pest control in field populations.

In this paper, two cases have been reported as examples of the possible applications of the protocol described: first, the successful transformation through transposase-mediated gene insertion of the medfly genome, which led to the establishment of multiple strains with fluorescent testes. Using a similar microinjection procedure, it is also possible to inject dsRNA, with the effects of the knockdown being visible till the adult stage.

The establishment of a reliable microinjection protocol for the medfly embryos opened the way to consider genetically-modified strains as a tool to control flies in the wild, as alternative or complementary strategies to classical approaches, including the Sterile Insect Technique. Finally, recent advances in the technology for automated microinjection in zebra fish embryos might potentially help the development of high throughput delivery systems with important relevance for creating transgenic strains of the medfly and other relevant pests[51].

Disclosures

The authors declare that they have no competing financial interests.

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References

1. Diamantidis, A. D., Carey, J. R., Nakas, C. T., & Papadopoulos, N. T. Population-specific demography and invasion potential in medfly. *Ecol. Evol.* **1**, 479-488 (2011).
2. Augustinos, A. A. *et al.* Exploitation of the Medfly Gut Microbiota for the Enhancement of Sterile Insect Technique: Use of Enterobacter sp. in Larval Diet-Based Probiotic Applications. *PLoS ONE.* **10**, e0136459 (2015).
3. Liquido, N., Shinoda, L., & Cunningham, R. Host plants of Mediterranean fruit fly: an annotated World review. *Ann Entomol Soc Am.* **77**, 1-52 (1991).
4. Szyniszewska, A. M., & Tatem, A. J. Global assessment of seasonal potential distribution of Mediterranean fruit fly, Ceratitis capitata (Diptera: Tephritidae). *PLoS ONE.* **9**, e111582 (2014).
5. Malacrida, A. R. *et al.* Globalization and fruitfly invasion and expansion: the medfly paradigm. *Genetica* **131**, 1-9 (2007).
6. Dyck, V. A., Hendrichs, J., & Robinson, A. S. *Sterile Insect Technique: Principles and practice in Area-wide Integrated Pest Management.* Springer (2005).
7. Hagler, J. R., & Jackson, C. G. Methods for marking insects: current techniques and future prospects. *Annu. Rev. Entomol.* **46**, 511-543 (2001).
8. *Genome Sequencing Initiative for Insects and Other Arthropods.* <http://arthropodgenomes.org/wiki/i5K> (2016).
9. Handler, A. *Medfly Genome Annotation Groups.* <https://www.hgsc.bcm.edu/arthropods/medfly-genome-annotation-groups> (2016).
10. Pane, A., Salvemini, M., Delli Bovi, P., Polito, C., & Saccone, G. The transformer gene in Ceratitis capitata provides a genetic basis for selecting and remembering the sexual fate. *Development.* **129**, 3715-3725 (2002).
11. Loukeris, T. G., Livadaras, I., Arcà, B., Zabalou, S., & Savakis, C. Gene transfer into the medfly, Ceratitis capitata, with a *Drosophila hydei* transposable element. *Science.* **270**, 2002-2005 (1995).
12. Rubin, G. M., & Spradling, A. C. Genetic transformation of Drosophila with transposable element vectors. *Science.* **218**, 348-353 10.1126/science.6289436 (1982).
13. Hoy, M. *Insect Molecular Genetics. An Introduction to Principles and Applications.* 3rd edn, 840, Academic Press (2013).
14. Christophides, G. K., Livadaras, I., Savakis, C., & Komitopoulou, K. Two medfly promoters that have originated by recent gene duplication drive distinct sex, tissue and temporal expression patterns. *Genetics.* **156**, 173-182 (2000).

15. Handler, A. M., McCombs, S. D., Fraser, M. J., & Saul, S. H. The lepidopteran transposon vector, piggyback, mediates germ-line transformation in the Mediterranean fruit fly. *Proceedings of the National Academy of Sciences of the United States of America.* **95**, 7520-7525 (1998).
16. Handler, A. M., & Harrell, R. A., 2nd. Transformation of the Caribbean fruit fly, Anastrepha suspensa, with a piggyBac vector marked with polyubiquitin-regulated GFP. *Insect Biochem Mol Biol.* **31**, 199-205 (2001).
17. Koukidou, M. *et al.* Germ line transformation of the olive fly Bactrocera oleae using a versatile transgenesis marker. *Insect Mol Biol* **15**, 95-103 (2006).
18. Condon, K. C. *et al.* Germ-line transformation of the Mexican fruit fly. *Insect Mol Biol* **16**, 573-580 (2007).
19. Raphael, K. A. *et al.* Germ-line transformation of the Queensland fruit fly, Bactrocera tryoni, using a piggyBac vector in the presence of endogenous piggyBac elements. *Genetica* **139**, 91-97 (2011).
20. Meza, J. S., Nirmala, X., Zimowska, G. J., Zepeda-Cisneros, C. S., & Handler, A. M. Development of transgenic strains for the biological control of the Mexican fruit fly, Anastrepha ludens. *Genetica.* **139**, 53-62 (2011).
21. Schetelig, M. F., & Handler, A. M. Strategy for enhanced transgenic strain development for embryonic conditional lethality in Anastrepha suspensa. *Proc. Natl. Acad. Sci. U. S. A.* **109**, 9348-9353 (2012).
22. Catteruccia, F. *et al.* Stable germline transformation of the malaria mosquito Anopheles stephensi. *Nature* **405**, 959-962 (2000).
23. Allen, M. L., O'Brochta, D. A., Atkinson, P. W., & Levesque, C. S. Stable, germ-line transformation of Culex quinquefasciatus (Diptera: Culicidae). *J Med Entomol.* **38**, 701-710 (2001).
24. Handler, A. M. Use of the piggyBac transposon for germ-line transformation of insects. *Insect Biochem Mol Biol.* **32**, 1211-1220 (2002).
25. Nolan, T., Bower, T. M., Brown, A. E., Crisanti, A., & Catteruccia, F. piggyBac-mediated germline transformation of the malaria mosquito Anopheles stephensi using the red fluorescent protein dsRED as a selectable marker. *J Biol Chem.* **277**, 8759-8762 (2002).
26. Rodrigues, F. G., Oliveira, S. B., Rocha, B. C., & Moreira, L. A. Germline transformation of Aedes fluviatilis (Diptera:Culicidae) with the piggyBac transposable element. *Mem Inst Oswaldo Cruz.* **101**, 755-757 (2006).
27. Terenius, O., Juhn, J., & James, A. A. Injection of An. stephensi embryos to generate malaria-resistant mosquitoes. *J Vis Exp.* e216 (2007).
28. Jasinskiene, N., Juhn, J., & James, A. A. Microinjection of A. aegypti embryos to obtain transgenic mosquitoes. *J Vis Exp.* e219 (2007).
29. Concha, C. *et al.* Efficient germ-line transformation of the economically important pest species Lucilia cuprina and Lucilia sericata (Diptera, Calliphoridae). *Insect Biochem Mol Biol* **41**, 70-75 (2011).
30. Takken, W., & Scott, T. W. *Ecological Aspects for Application of Genetically Modified Mosquitoes.* Springer Netherlands (2003).
31. Handler, A. M. in *Insect transgenesis: methods and applications.* (eds A. M. Handler & A. A. James) 3-26, CRC Press (2000).
32. Handler, A. M. A current perspective on insect gene transformation. *Insect Biochem Mol Biol.* **31**, 111-128 (2001).
33. Gong, P. *et al.* A dominant lethal genetic system for autocidal control of the Mediterranean fruitfly. *Nat. Biotechnol.* **23**, 453-456 (2005).
34. Scolari, F. *et al.* Fluorescent sperm marking to improve the fight against the pest insect *Ceratitis capitata* (Wiedemann; Diptera: Tephritidae). *N Biotechnol* **25**, 76-84 (2008).
35. Schetelig, M. F. *et al.* Site-specific recombination for the modification of transgenic strains of the Mediterranean fruit fly Ceratitis capitata. *Proc. Natl. Acad. Sci. U. S. A.* **106**, 18171-18176 (2009).
36. Schetelig, M. F., Caceres, C., Zacharopoulou, A., Franz, G., & Wimmer, E. A. Conditional embryonic lethality to improve the sterile insect technique in Ceratitis capitata (Diptera: Tephritidae). *BMC Biol.* **7**, 4 (2009).
37. Ogaugwu, C. E., Schetelig, M. F., & Wimmer, E. A. Transgenic sexing system for Ceratitis capitata (Diptera: Tephritidae) based on female-specific embryonic lethality. *Insect Biochem Mol Biol.* **43**, 1-8 (2013).
38. Lacroix, R. *et al.* Open field release of genetically engineered sterile male Aedes aegypti in Malaysia. *PLoS ONE.* **7**, e42771 (2012).
39. Harris, A. F. *et al.* Successful suppression of a field mosquito population by sustained release of engineered male mosquitoes. *Nat. Biotechnol.* **30**, 828-830 (2012).
40. Leftwich, P. T. *et al.* Genetic elimination of field-cage populations of Mediterranean fruit flies. *Proc. Biol. Sci.* **281** (2014).
41. Gantz, V. M. *et al.* Highly efficient Cas9-mediated gene drive for population modification of the malaria vector mosquito Anopheles stephensi. *Proc. Natl. Acad. Sci. U. S. A.* **112**, E6736-6743 (2015).
42. Economopoulos, A. P., & Judt, S. Artificial Rearing of the Mediterranean Fruit Fly (Diptera: Tephritidae): Size of Oviposition Holes. *J. Econ. Entomol.* **82**, 668-674 (1989).
43. Thailayil, J., Magnusson, K., Godfray, H. C., Crisanti, A., & Catteruccia, F. Spermless males elicit large-scale female responses to mating in the malaria mosquito Anopheles gambiae. *Proc. Natl. Acad. Sci. U. S. A.* **108**, 13677-13681 (2011).
44. Gilbert, L. *Insect Development. Morphogenesis, Molting and Metamorphosis.* 730, Academic Press (2009).
45. Schetelig, M. F., Horn, C., Handler, A. M., & Wimmer, E. A. in *Area-Wide control of insect pests. From research to field implementation.* (eds M. J. Vreysen, A. Robinson, & J. Hendrichs) 85-93, Springer (2007).
46. Gabrieli, P. *et al.* Sex and the single embryo: early development in the Mediterranean fruit fly, *Ceratitis capitata. BMC Dev Biol.* **10**, 12 (2010).
47. Tazuke, S. I. *et al.* A germline-specific gap junction protein required for survival of differentiating early germ cells. *Development.* **129**, 2529-2539 (2002).
48. Gabrieli, P., Marois, E., & Catteruccia, F. in *Transgenic insects: techniques and applications.* (ed M.Q. Benedict) 188-207, CABI (2014).
49. Scolari, F. *et al.* How functional genomics will impact fruit fly pest control: the example of the Mediterranean fruit fly, Ceratitis capitata. *BMC Genet.* **15 Suppl 2**, S11 (2014).
50. Scott, J. G. *et al.* Towards the elements of successful insect RNAi. *J Insect Physiol* **59**, 1212-1221 (2013).
51. Spaink, H. P. *et al.* Robotic injection of zebrafish embryos for high-throughput screening in disease models. *Methods.* **62**, 246-254 (2013).

