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Chapter 26

Random Integration Transgenesis in a Free-Living Regenerative Flatworm *Macrostomum lignano*

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Abstract

Regeneration-capable flatworms are highly informative research models to study the mechanisms of stem cell regulation, regeneration, and tissue patterning. Transgenesis is a powerful research tool for investigating gene function, but until recently, a transgenesis method was missing in flatworms, hampering their wider adoption in biomedical research. Here we describe a detailed protocol to create stable transgenic lines of the flatworm *M. lignano* using random integration of DNA constructs through microinjection into single-cell stage embryos.

Key words *Macrostomum lignano*, Flatworms, Regeneration, Transgenesis, Microinjection, Random integration, Irradiation

1 Introduction

Macrostomum lignano is a free-living marine flatworm capable of regeneration anterior to the brain and posterior to the pharynx [1]. During the last decade, the interest in this research model steadily increased [2]. Similar to other flatworms, regeneration in *M. lignano* is fueled by stem cells called neoblasts [3]. It is a small and transparent animal that is easy to culture in laboratory conditions. *M. lignano* is a non-self-fertilizing hermaphrodite with a short generation time of 2–3 weeks [4, 5]. When cultured in standard laboratory conditions, animals lay approximately one egg per day. Embryonic development takes 5 days, and hatchlings reach adulthood in about two weeks. The laid eggs are fertilized, relatively large (100 μm) and follow the archoophoran mode of development [4, 5], i.e., they have a large, and yolk-rich oocyte instead of a small oocyte supplied by dedicated yolk cells. These features, together with the recently reported genome and transcriptome assemblies [6–8], make *M. lignano* a versatile model organism for

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research on stem cells and regeneration [2, 9]. In addition, the availability of transgenic techniques renders this flatworm a unique research model among Platyhelminthes [8]. Here we present a method for transgenesis in *M. lignano* using microinjection of different components into single-cell stage embryos. The method includes preparation and maintenance of animal cultures, design of transgenic constructs, microinjection procedures, and selection of transgenic animals.

2 Materials

A typical *M. lignano* transgenesis work space is similar to configurations used for transgenesis in other animals, where DNA is delivered by microinjection into cells. It includes instruments for preparation of microinjection needles (a micropipette puller and a microforge), a stereomicroscope and an inverted microscope equipped with micromanipulators and a microinjector (Fig. 1).

1. *M. lignano* line suitable for laboratory culture (*see* **Note 1**).
2. Unicellular diatom *Nitzschia curvilineata* (Heterokontophyta, Bacillariophyceae). It is the main and only source of food for the flatworm.
3. Artificial sea water (ASW): 32 g/L commercially available sea salt (e.g., Red Sea) in an autoclaved and rinsed bottle of dH_2O. Shake until almost all salts are dissolved, autoclave, and cool down.
4. f/2 salt solutions: 3.58 g $MnCl_2 \cdot 4H_2O$, 0.44 g $ZnSO_4 \cdot 7H_2O$, 0.20 g $CoCl_2 \cdot 6H_2O$, 0.20 g $CuSO_4 \cdot 5H_2O$, 0.12 g $NaMoO_4$. Prepare each separately in 20 mL dH_2O.
5. f/2 medium stock solution I: 15 g $NaNO_3$ in 100 mL dH_2O. Autoclave for at least 20 min at 120 °C. Store in a cool and dark place. Use within 6 months. Discard if there are changes in transparency, color or if a precipitate occurs.
6. f/2 medium stock solution II: 1 g $NaH_2PO_4 \cdot H_2O$ in 100 mL dH_2O. Handle and store as stock solution I.
7. f/2 medium stock solution III: 3 g $Na_2SiO_3 \cdot 9H_2O$ in 100 mL dH_2O. Handle and store as stock solution I.
8. f/2 medium stock solution IV: 0.88 g Na_2–EDTA, 0.63 g $FeCl_3 \cdot 6H_2O$, 0.2 mL of each of five f/2 salt solutions. Handle and store as stock solution I.
9. f/2 medium vitamin solution: 50 mg thiamine–HCl (B1), 200 μg biotin, 200 μg cobalamin (B12) in 100 mL dH_2O.

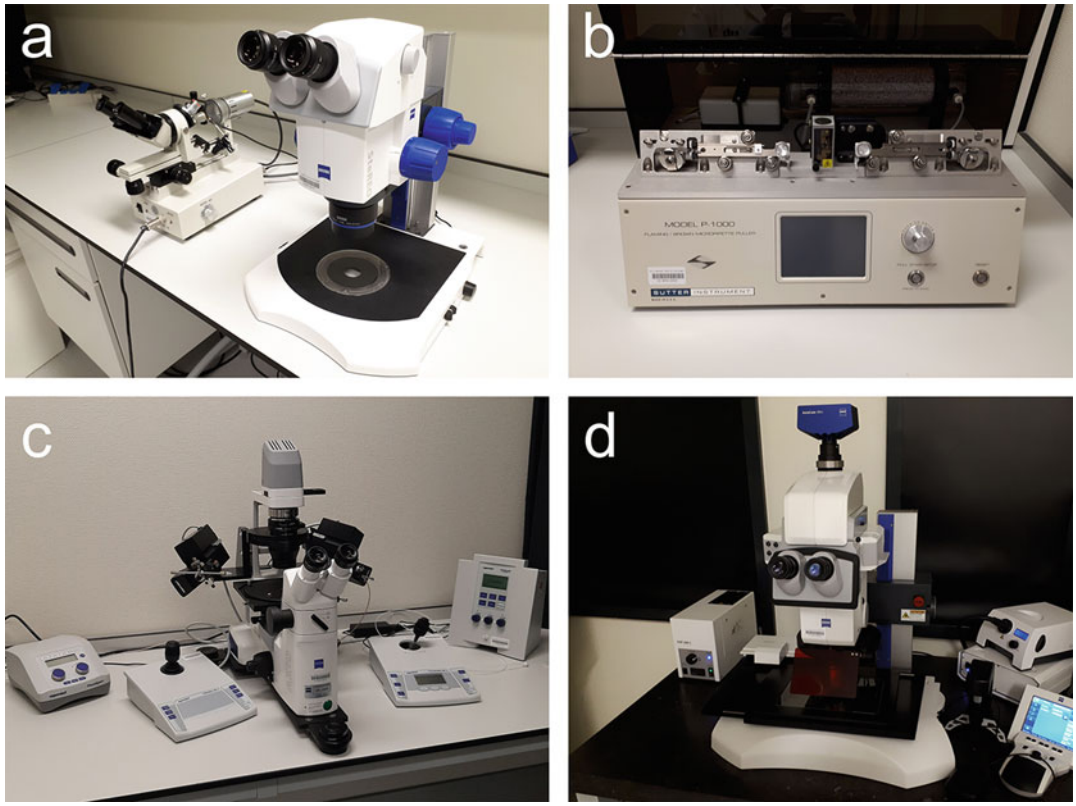

Fig. 1 Typical microinjection working station equipment. (**a**) On the right: a stereomicroscope for worm transferring and egg picking. On the left: a microforge for fine preparation of pulled microcapillaries into holders and/or needle opening. (**b**) A micropipette puller. (**c**) An inverted microscope equipped with micromanipulators and a microinjector. (**d**) A fluorescence stereomicroscope for the selection of eggs and worms positive for a transgene expression

10. Nutrient enriched ASW (Guillard's f/2 medium): 2 mL of each of the stock solutions (I-IV), 1 mL of the vitamin solution in 4 L ASW. Filter using a 0.22-μm filter.
11. Plastic petri dishes.
12. Climate chamber with possibility to use the following settings: 20 °C and 25 °C with constant aeration, a 14/10 h day/night cycle.
13. Food source: diatom grown on petri dishes with f/2 medium at 20 °C with constant aeration, and 14/10 h day/night cycle for 10–20 days to <100% confluency.
14. 30-mm round glass cover slides.
15. Plastic six-well plates.
16. Plastic 24-well plates.
17. Micropipette puller.

18. Aluminosilicate glass capillaries with filament.
19. Borosilicate glass capillaries without a filament.
20. Microforge (e.g., MF2).
21. Bunsen burner or spirit lamp.
22. Gamma-ray source.
23. Gel/PCR silica column-based DNA purification kit.
24. Stereomicroscope.
25. Inverted microscope.
26. Fluorescence stereomicroscope.
27. Micromanipulator to position the holding pipette.
28. Micromanipulator to position the injection pipette.
29. Microinjector (e.g., FemtoJet Express).
30. Piezo drill.
31. Microvolume spectrophotometer.
32. Microloader micropipette tips.
33. Low retention micropipette tips.

3 Methods

3.1 Setting Up Egg Producing Worm Cultures

1. Use a large population of worms (over 2000 in total) split into 4–6 separate petri dishes.
2. Transfer the worms to fresh food source using a 200-μL micropipette loaded with low retention tips (*see* **Note 2**).
3. Incubate at 25 °C for 2 days in the climate chamber. The worms should lay a substantial number (usually more than 4000) of eggs.
4. Transfer the worms to new petri dishes with fresh food (*see* **Note 3**) and incubate the plates with the laid eggs at 25 °C for an additional week to hatch the eggs.
5. Transfer between 300 and 500 hatchlings on a petri dish with fresh food and make a microinjection set consisting of six such petri dishes (*see* **Note 4**).
6. Incubate the hatchlings for an additional week at 25 °C.
7. Transfer to fresh food and keep at 20 °C, transferring to fresh food with intervals no longer than 4 days (*see* **Note 5**).

3.2 Preparing Single Wild-Type Worms for Crosses

Macrostomum lignano cannot self-fertilize and therefore requires a crossing partner. It is useful to have a number of worms dedicated for this purpose prepared in advance, so that crosses can be started as soon as transgenic founders are generated.

1. Select single wild-type hatchlings and put them separately in single wells in a 24-well plate with diatom.
2. Keep the worms at 20 °C until needed.
3. Transfer to a 24-well plate with fresh food every 2 weeks.

3.3 Preparation of Plastic Pickers for Egg Collection

M. lignano eggs are covered with a sticky mucus, which helps to fix the eggs on a surface. Most of the time, eggs are laid closely to each other and form clumps. Plastic pickers are used to separate eggs in the clumps. Additionally, the mucus around the eggs adheres to the tip of the picker, which helps to transfer the eggs from a petri dish to a microinjection slide and then attach them to the slide surface. A second picker is usually necessary to assist the release of the egg from the first picker. After that, the eggs can be easily manipulated to the desired location on the microinjection slide by gentle touching with the tip of the picker.

1. Take two plastic tips (preferably a microloader tip, because of their diameter; however, standard p10 pipette tips will work as well).
2. Set flame on a Bunsen burner or a spirit lamp.
3. Melt one of the tips by putting it on fire.
4. Extinguish the fire by blowing it off; the tip should be hot and melted at this moment.
5. Take the second tip and touch the melted fragment, it should melt as well.
6. Slowly start separating both the tips by pulling the second one away from the first. It should elongate into a thin plastic thread.
7. Check the size and shape of the picker; you can adjust it by cutting or bending using forceps or with your fingers. You can reuse the first tip for melting when pulling more than one picker (*see* **Note 6**). *See* Fig. 2a, b for a typical picker example.

3.4 Preparation of the Holders

1. Glass holders are pulled from borosilicate glass capillaries without a filament using the following settings (for a P-1000, Sutter Instrument, USA): Heat = ramp+18, Pull = 0, Velocity = 150, Time = 115, Pressure = 190.
2. Break the pulled glass capillary using a microforge to create a tip of approximately 140 μm outer diameter and 50 μm inner diameter.
3. Heat-polish the pipette tip to create smooth edges using the glass bead on the microforge filament.
4. Using the microforge, bend the tip to a ~20° angle. To do so, rotate the tip vertically and apply heat close to the point where the bend is needed. Do not touch the heat source to prevent

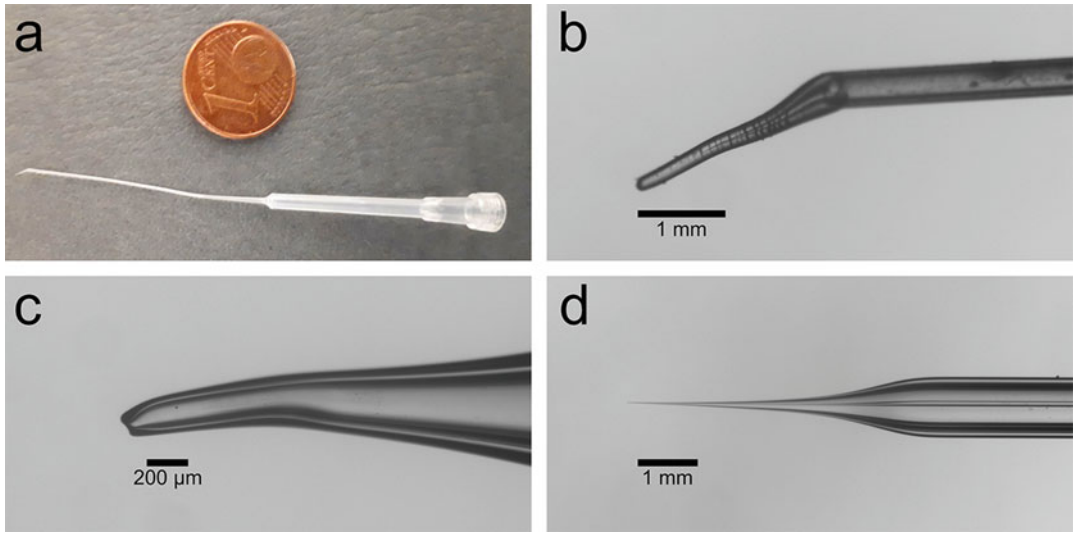

Fig. 2 Typical microinstruments used to manipulate and inject *M. lignano* eggs. (**a**) A plastic picker for egg collection made from a microloader tip. (**b**) A close up on the plastic picker tip. (**c**) A close up on the tip of a holder. (**d**) A close up on the taper and the tip of a microinjection needle. Note the filament inside

the glass from melting into the microforge. *See* Fig. 2c for a typical holder example.

3.5 Preparation of the Microinjection Needles

1. Use aluminosilicate glass capillaries with filament to prepare microinjection needles.
2. Pull the capillary using the following settings (for a P-1000, Sutter Instrument, USA): Heat = Ramp-17, Pull = 60, Velocity = 60, Time = 250, Pressure = 500, to produce two needles.
3. The tip of the freshly pulled needle is sealed. Use a microforge to break the tip of the needle and sharpen it by scratching against the glass bead on the microforge filament. Keep in mind that aluminosilicate glass breaks rather easily so not much force is required for this step (*see* **Note 7**). *See* Fig. 2d for a typical needle example.

3.6 Design of Transgenic Constructs

There are four necessary components that must be present in any transgenic construct planned for random integration: a promoter of the gene you want to study/use, a 5′ untranslated region (UTR), a protein-coding DNA sequence (CDS), and a 3′ UTR. Here we show an example of how to design a transgenic construct to study the expression pattern of the promoter of a gene of interest using the reporter green fluorescent protein (GFP) as the CDS.

1. Locate your gene of interest in the genome assembly using the *M. lignano* genome browser (http://gb.macgenome.org) either by entering the corresponding transcript ID in the

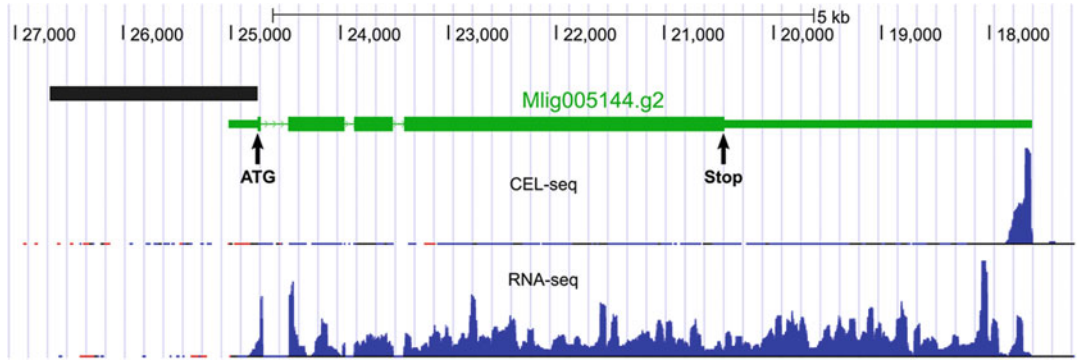

Fig. 3 Selection of the promoter for a gene of interest using *M. lignano* genome browser. A genomic region encompassing *Mlig005144.g2* gene (*APOB* homolog) shows the structure of the gene, and RNA-seq and CEL-seq tracks. Region selected for the promoter cloning is annotated as a black rectangular block upstream of the ATG

genome browser search field or by searching its sequence using the BLAT tool (*see* **Note 8**).

2. Identify the start and the end of the gene CDS, 5′ UTR, and 3′ UTR parts by looking at the Gene track in the genome browser (Fig. 3). Although the exact promoter boundaries can vary, the rule of the thumb is to start with a 1.5 kb region just upstream of the start codon of the CDS (*see* **Notes 9–11**).
3. Select two pairs of primers to PCR amplify this region and the 3′ UTR for later cloning into the plasmid vector with your preferred cloning strategy upstream and downstream of the GFP, respectively (*see* **Note 12**). GFP sequence itself can be directly cloned or PCR amplified from any existing vector, or ordered as a gene block with a codon optimized sequence for enhanced translation efficiency (*see* **Note 13**).

3.7 Microinjection Mix: DNA for Random Integration

The chosen DNA can be used in three different forms: as a circular plasmid, as a linearized plasmid, or as a PCR product. In the first case, a plasmid suspension in DNase/RNase-free water or TE-buffer with concentration of 150–300 ng/μL is recommended.

To prepare the cut plasmid:

1. Use the restriction sites flanking the desired region and digest approximately 3 μg of plasmid with the appropriate restriction enzyme(s).
2. Run the digested plasmid on an agarose gel and isolate the correct DNA fragment using gel purification silica-based column kit.
3. Estimate the concentration of the cut plasmid using the spectrophotometer. For microinjections, it should be around 50 ng/μL.

To prepare the PCR product:

1. Run a standard PCR of 200 μL.
2. Run a 1–5 μL aliquot on an agarose gel to check the size and integrity of the PCR product.
3. Clean the remaining PCR using silica-based column purification kit (*see* **Note 14**).
4. Estimate the concentration of the PCR product using the spectrophotometer. For microinjections, it should be around 50 ng/μL.

3.8 Microinjection Procedure

1. Transfer the worm culture to petri dishes with ASW (without diatom). The standard density of a microinjection plate is between 600 and 1000 worms per 6 cm petri dish. Fewer worms do not produce enough eggs and high number of worms seems not to start egg production at all (*see* **Note 15**).
2. Keep the transferred worms overnight at 20 °C to slightly starve them.
3. In the morning on the following day, transfer the worms again to fresh ASW and put them in the dark at 20 °C for approximately 2–3 h (can be in a shelf or a drawer at room temperature).
4. Move the worms into light (they can be returned to the incubator) and keep them there for 30 min.
5. Once the first eggs are laid, the egg collecting step can be started using a stereomicroscope.
6. Put a drop (150–200 μL) of ASW on a 30-mm non-treated round glass cover slide or any other glass slide that fits into a well of a six-well plate.
7. Use the plastic pickers to collect the laid eggs and transfer them to the drop of ASW (*see* **Notes 16–18**).
8. Put the slide with the eggs on the microinjection stage and focus the inverted microscope on the first egg using low magnification (5× or 10× objective) (Fig. 4a).
9. Mount the holder capillary on the micromanipulator and position it near the egg (Fig. 4a).
10. Load the microinjection needle with 1 μL of your choice of material-to-be-injected. There are no differences in the microinjection procedure in regard to the material used for injections (*see* **Note 19**). The needle can be loaded using a microloader tip or by capillary force by applying material to its back.
11. Mount the loaded needle on the micromanipulator and connect the pressure tube to the pressure supply unit of the microinjector.

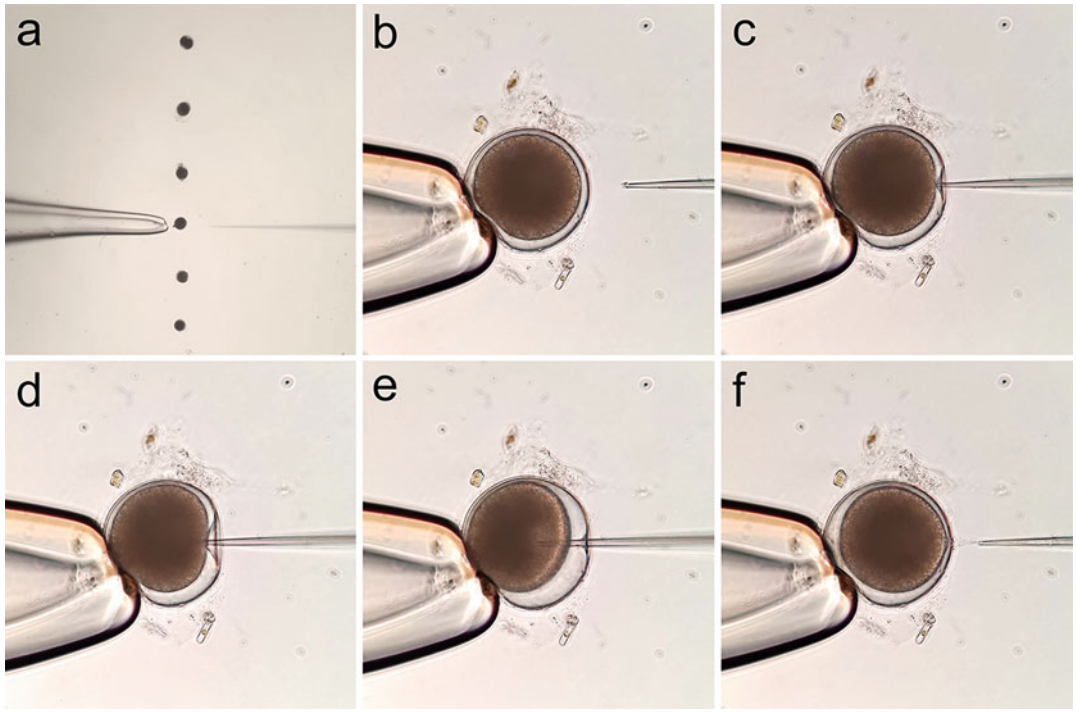

Fig. 4 Highlights of a typical microinjection procedure into the *M. lignano* single-cell eggs. (**a**) Positioning of the eggs, a loaded needle, and a holder under 5× objective of the inverted microscope. (**b**) The holder touching the edge of the egg, and the needle in the position to "clean" before the injection (40× objective). (**c**) The needle touching the egg shell before puncturing. (**d**) The needle puncturing through the egg shell. (**e**) The moment of injection is seen as a burst inside the egg. (**f**) The needle is removed from the successfully injected egg. Scale reference: *M. lignano* egg size ~100 μm

12. Make sure that the needle is opened, and there are no air bubbles in the tip. Use the microinjector's clean button if available (*see* **Note 20**).
13. Position the needle in the proximity of the egg (Fig. 4a).
14. Change the magnification to 40× objective and bring the egg, the holder, and the needle to focus (Fig. 4b) (*see* **Note 21**).
15. Position the needle so that it touches the edge of the egg (Fig. 4c) (*see* **Note 22**).
16. Pierce the egg shell with the needle, this moment should be clearly visible (Fig. 4d).
17. Push the needle deeper into the egg to pierce through the cell membrane (*see* **Note 23**).
18. Press the injection button and make sure you see a burst of the injected material appearing in the cell (*see* **Note 24)** (Fig. 4e).
19. Slowly remove the needle from the egg (*see* **Note 25**) (Fig. 4f).
20. Move the holder away from the egg.

21. Use the stage to position the next egg between the needle and the holder.
22. Repeat **steps 12–19** until all the eggs from the slide are injected.
23. Remove the slide from the microinjection stage and put it into one of the wells in a six-well plate filled with 3 mL of ASW.
24. Proceed to the next slide.
25. Repeat **steps 13–24** to process all slides.

3.9 Irradiation of Injected Eggs

Eggs can be exposed to gamma-ray radiation after injection of the desired construct to stimulate non-homologous recombination and increase integration rates [8]. Irradiate the six-well plate containing the injected eggs in ASW at a dose of 2.5 Gy of gamma-ray. This procedure should be carried out within 1-h post-injection, as long as the eggs are kept on ice until irradiation.

3.10 Transgenic Eggs Maintenance and Selection of Homozygous Lines

1. Incubate the injected eggs in an incubator at 25 °C until hatched (*see* **Note 26**).
2. Check the injected eggs for markers of positive injections (*see* **Note 27**) (Fig. 5a).
3. Use a glass pipette or a metal needle and kill all the negative eggs by pressing down the tip into the egg (*see* **Note 28**).
4. On the third day of incubation, add food to each of the wells containing a slide with injected eggs (*see* **Note 29**).
5. Worms will usually hatch between the fourth and fifth day. However, sometimes they need an additional day or two. The delay is caused by the damage inflicted during the injections.
6. Select the positive hatchlings and transfer them to single wells in a 24-well plate with diatom.
7. Cross the positive hatchlings with single wild-type worms (*see* Subheading 3.2).
8. Incubate at 25 °C until the worms start laying eggs (*see* **Note 30**).
9. Select the positive progeny and transfer to single wells in a 24-well plate (*see* **Note 30**).
10. Cross again with single wild-type worms.
11. Incubate at 25 °C until hatchlings appear (*see* **Note 31**).
12. Check the hatchlings. Homozygous worms will produce only positive offspring.
13. Select the homozygous worms and transfer them to single wells in a 24-well plate with diatom.
14. Incubate at 25 °C until the worms stop producing eggs (*see* **Note 32**).

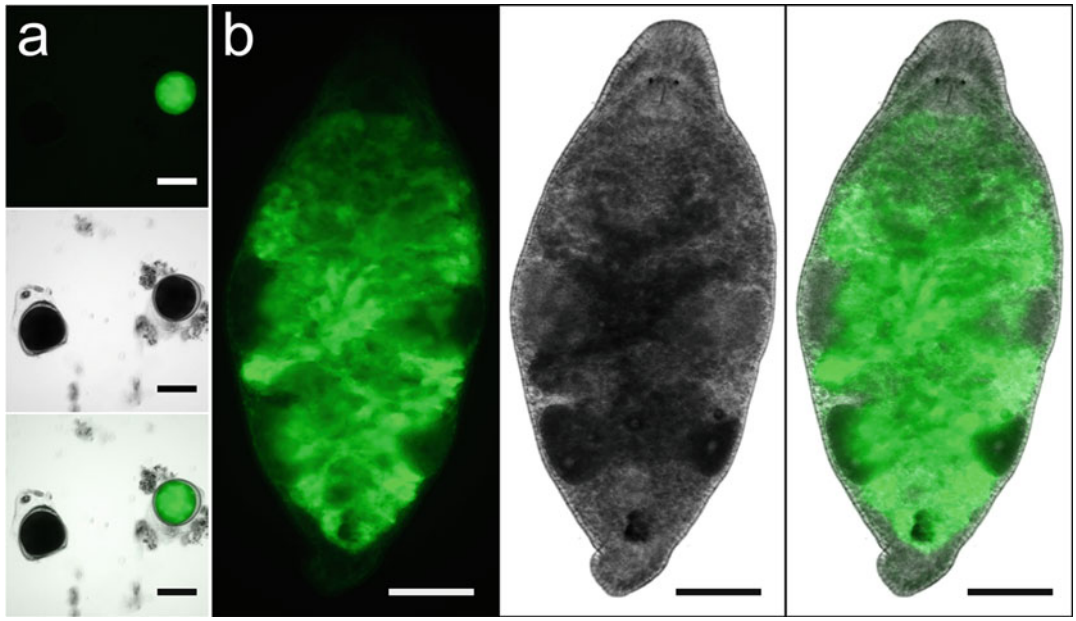

Fig. 5 An overview of an APOB::GFP::Ef1a_3′UTR transgene expression in *M. lignano* eggs and in the whole worm. (**a**) Comparison of positive and negative eggs under fluorescent stereomicroscope 1 day after the injection with the PCR DNA fragment encoding the transgene. From top to bottom: FITC channel, bright-field, and merged. Scale bars: 100 μm. (**b**) Promoter of the *M. lignano APOB* homolog (Mlig005144.g2) exhibits gut-specific expression pattern in the worm. From left to right: FITC channel, bright-field, and merged. Scale bars: 100 μm

15. Put the homozygous positive transgenic worms together in a six-well plate with food and incubate to produce offspring.
16. The line should reach the density of 100 worms within 2 months (*see* **Note 33**).
17. Use the transgenic line according to the needs (*see* **Note 34**) (Fig. 5b).

4 Notes

1. Any appropriate *M. lignano* line that can generate sufficient number of eggs can be used. For experiments in wild-type animals, we recommend NL12 line, which can be obtained from the Berezikov laboratory.
2. Regular micropipette tips should be avoided to prevent the worms from adhering to the inside walls of the tip, which can lead to the loss of animals. Worms that are adhering to the surface of the petri dish can be detached by discharging the liquid from the pipette close to the worm.

3. Put the transferred worms to 20 °C. They will not be used for microinjection but can be used for any other experiments.
4. Several microinjection sets can be maintained in parallel to ensure steady daily egg production.
5. The worms need a recovery period of 2 weeks after they are being starved to produce eggs for microinjections, keep that in mind when establishing a microinjection culture. If the culture is not dense enough, it will result in insufficient number of eggs laid, in which case you need to have more worms. If the culture is too dense, for example, if there are over 800 worms per petri dish, they will eat all the food very quickly, which will cause starvation. If the worms are not transferred regularly to fresh food, a new population of worms will hatch on the same plate. The hatchlings lower the overall egg production by competing with the adults for food and space. Keep the plates on food, regularly transferring the worms until the hatchlings become adults.
6. We use plastic pickers as they cause the least damage to the cells, are relatively cheap, easy, and fast to prepare. We strongly discourage using glass-based pickers, because of the damage they cause during the egg transfer.
7. The needle tips can also be broken using the holder when both the loaded needle and the holder are already mounted on the microinjection stage (*see* **steps 9–11** in Subheading 3.8 for mounting instructions).
8. Orientation of the transcript on the scaffold can be determined by the color of the mapped RNA-Seq reads indicated in the RNA-seq track: red—forward and blue—reverse. The position of the 3′ end can also be readily found by looking at the peak on the CEL-seq track of the genome browser.
9. If the selected 1.5 kb candidate promoter region spans another upstream gene or a large repeat element, it usually can be shortened by selecting the sequence between the end of this gene/repeat and the CDS start. The actual functional promoter sequences can be established in subsequent experiments by further truncating the initial promoter region that generated the expected expression pattern.
10. A substantial fraction of genes in *M. lignano* are trans-spliced [7, 10]. Whether the gene is trans-spliced can be determined by looking at the SL track in the genome browser, which reflects the mapping of RNA-seq reads containing SL sequences. Trans-spliced genes contain an SL peak at the beginning of their transcripts. The best strategy for promoter selection for trans-spliced genes remains to be investigated.

11. In many cases, the 3′UTR of *Ef1a* gene can be used instead of the native 3′UTR, but care should be taken in the interpretation of the resulting transgene expression patterns.
12. We find that monomeric fluorescent proteins mNeonGreen [11] and mScarlet-I [12] work well for the most of purposes.
13. The effect of codon optimization on transgene expression in *M. lignano* was not extensively investigated but an approach used for codon optimization in *Caenorhabditis elegans* is implemented and available at http://www.macgenome.org/codons.
14. If no size selection of the PCR product is required, we avoid the gel purification step, since gel-purified DNA tends to clog microinjection needles more. Instead, the PCR product can be cleaned-up directly on the columns following the manufacturer's protocol.
15. Try to avoid non-adult worms in microinjection cultures as they seem to lower the egg yield from microinjection cultures.
16. When transferring the eggs, we arrange them in a straight line to speed up the injection process and avoid double injection of the same egg.
17. When collecting the eggs, use the pickers to disperse worms that are forming ball-shaped clumps, usually there will be some freshly laid eggs inside.
18. Once picked, slides with eggs can be stored on ice or in a fridge to prevent them from dividing before starting the injections. In the meantime, you can continue picking eggs for new slides. Eggs in a fridge can be stored at least for 2 months without noticeable anomalies and can then be used right away for injections when necessary [5].
19. If the needle is clogged. The needle can be clogged by the viscosity of the injected material or by the sticky mucus surrounding the egg. If the injected material is expected to be viscous (e.g., proteins or mRNA), remember to spin down the mix. If this does not help, breaking the tip of the needle to create a larger opening might solve the problem, as well as applying higher pressure during the injections. If the mucus causes the clogging, using piezo pulses is the best solution. If the clog cannot be removed, the needle needs to be changed for a fresh one.
20. We use injection pressure (pi) of approximately 600–650 hPa and compensation pressure (pc) of 50–60 hPa. However, the settings are adjusted based on the amount of mucus and debris surrounding the eggs.
21. If the egg you want to inject is divided, chances of correctly delivering the material into a single cell decrease, and the

probability of generating mosaic animals increases. Thus, inject only an egg when it is in a single-cell stage.

22. If there is too much debris on the collected eggs, the best possible way to clean the eggs is to starve the worms. However, piezo pulses are also helpful in removing any sticky material from the needle.
23. This step is crucial. The needle needs to go through the cell membrane. You can use piezo pulses to assist this process.
24. We use manual control over the injection time. The usual value is around 0.1 s. However, it may vary. Use pedal or button for the desired time of injection, until you see a burst. If needed, repeat procedure to make sure material is delivered in the egg. Injection button corresponds to the function of foot or hand control. Foot control is used to speed up the injection procedure as both hands can be manipulating both needle and holder while the injection pulse occurs.
25. In order to prevent egg leakage, briefly stop at the point where needle leaves the egg shell.
26. Elevation of incubation temperature from 20 °C to 25 °C significantly speeds up the development of the eggs, as well as growth and maturation of the worms [5].
27. This depends on the material injected. Usually, first fluorescent signal appears after an overnight incubation, but it may take longer.
28. An egg is considered killed when the brown dark cell content is replaced by a light brown color (meaning that the cell membrane burst), or when the content of the egg clearly expelled outside the egg shell. Empty egg shells will not interfere with the subsequent procedures and can be left on the slide.
29. Use one petri dish with fresh diatom. Scratch quarter of the plate's surface using a pipette tip and resuspend the attached diatom in the ASW. Add equal volume of ASW to dilute the floating diatom. 50 μL of the diatom prepared in this way is sufficient for a single well.
30. Transfer to fresh food once a week, but keep the old plate in case of any eggs that were laid there.
31. If a heterozygous line is sufficient for further use, this step can be modified and all the positive progeny can be put together starting a line.
32. Transfer to fresh food once a week, the old plates can be discarded, as the offspring from the cross with the wild type animals will be heterozygous. This step can take up to 2 months.

33. When transferring to fresh food, keep the old plates with food, in order to incubate the laid eggs and select the hatchlings, adding them to the main culture.
34. *Macrostomum* eggs can be stored at 4 °C for at least 2 months [5]. Longer storage is possible but may affect the hatching rate of the stored eggs.

Acknowledgments

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