Video Article

Generation of Transgenic *Hydra* by Embryo Microinjection

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Abstract

As a member of the phylum Cnidaria, the sister group to all bilaterians, *Hydra* can shed light on fundamental biological processes shared among multicellular animals. *Hydra* is used as a model for the study of regeneration, pattern formation, and stem cells. However, research efforts have been hampered by lack of a reliable method for gene perturbations to study molecular function. The development of transgenic methods has revitalized the study of *Hydra* biology[1]. Transgenic *Hydra* allow for the tracking of live cells, sorting to yield pure cell populations for biochemical analysis, manipulation of gene function by knockdown and over-expression, and analysis of promoter function. Plasmid DNA injected into early stage embryos randomly integrates into the genome early in development. This results in hatchlings that express transgenes in patches of tissue in one or more of the three lineages (ectodermal epithelial, endodermal epithelial, or interstitial). The success rate of obtaining a hatchling with transgenic tissue is between 10% and 20%. Asexual propagation of the transgenic hatchling is used to establish a uniformly transgenic line in a particular lineage. Generating transgenic *Hydra* is surprisingly simple and robust, and here we describe a protocol that can be easily implemented at low cost.

Video Link

The video component of this article can be found at https://www.jove.com/video/51888/

Introduction

Hydra has been used to study regeneration, pattern formation, and stem cells for approximately 250 years[2]. *Hydra* has a simple body plan consisting of three cell lineages: ectodermal epithelial, endodermal epithelial, and interstitial. The tubular body is formed by the ectodermal and endodermal epithelial lineages, each of which is a single cell layer. All of the epithelial cells in the body column are mitotic. When epithelial cells are displaced into the extremities[3], the head (mouth and tentacles) at the oral end or the foot (basal disc) at the aboral end, they arrest in the G2 phase of the cell cycle and change cell fate[4]. The cells of the interstitial lineage reside within the interstices between the epithelial cells. This lineage is supported by multipotent stem cells that are located in the ectodermal epithelial layer of the body column[5]. The interstitial stem cells give rise to three somatic cell types (nerves, gland cells, and nematocytes) and the germ cells[6,7].

As a member of the phylum Cnidaria, the sister group to all bilaterians, *Hydra* can shed light on fundamental biological processes shared among multicellular animals. Until recently, these efforts were impeded by the lack of reliable methods for the perturbation of gene function. However, with the development of transgenic methodology[1], we are now able to take full advantage of *Hydra* to gain a better understanding of the basic mechanisms common to multicellular animals, such as stem cell function, regeneration, and patterning. Transgenic *Hydra* lines are established by injection of plasmid DNA into embryos, which results in random integration and chimeric expression in a substantial frequency of hatchlings. A line with uniform expression in a particular lineage can be established by asexual propagation. The ability to clonally propagate transgenic *Hydra* lines is an advantage over the majority of animal models, which can be propagated only by sexual reproduction. In addition, transgenic cells can be tracked easily *in vivo* due to the transparency of the animal and the absence of endogenous fluorescent proteins [8].

In the seven years since the first transgenic *Hydra* lines were made[1], such lines have been used for a variety of applications. Expression of fluorescent proteins in different cell types has made it possible to track cell movement, observe changes in cell shape, and track cell fates both in wild type conditions and after chemical perturbation[1,5,9-12]. In addition, expression of different fluorescent proteins in the various lineages allows for FACS isolation of specific cell populations. This technique has been used for the sequencing of stem-cell specific mRNAs and lineage-specific small RNAs[13,14]. While the promoter of one of the two *Hydra* actin genes has been most widely used, a few cell-type specific promoters have been identified and used to drive expression of GFP in transgenic *Hydra*[9,11,15,16]. In the future, cell-type specific promoters will allow for the observation and collection of any specific cell type. In addition, a transgenic approach was successfully used to define the cis-acting regulatory elements of the Wnt3 promoter[17].

The development of transgenic methods in *Hydra* provides a robust approach for testing the function of genes by ectopic expression, overexpression, and knockdown. Transgenic animals have been made that express fluorescently-tagged proteins in order to examine both function and cellular localization[18-20]. In addition, the expression of RNA hairpins in the 3'UTR of a GFP transgene leads to knockdown of target genes[21,22]. In these approaches GFP is required to identify and track the transgenic tissue during the creation of the transgenic line. However,

it is likely that in some cases the GFP molecule would interfere with the function of the tagged protein. A recent study demonstrates that *Hydra* genes can be arranged in an operon configuration, *i.e.,* polycistronic transcripts are made, which are then separated by trans-spliced leader addition and translated separately[23]. By placing a gene encoding a protein or an RNA hairpin in the upstream position of an operon and a fluorescent protein gene in the downstream position, one can track transgenic tissue without having to tag the gene encoding the protein or RNA hairpin. This method has been used to express an RNA hairpin in an operon configuration with DsRed2 in order to achieve gene knockdown[14].

Protocol

1. Preparation of Plasmid DNA, Needles, and Injection Dishes

1. Prepare the plasmid DNA using a High Speed Maxi or Midi kit and concentrate the DNA to 2.5 mg/ml using ethanol precipitation. The DNA pellet should be dissolved in nuclease-free deionized water. Store the DNA in 10-20 µl aliquots at -20°C. (See **Supplementary Table 1** for a list of available vectors)
2. Make an injection dish using agarose to construct a trough for holding embryos in a 100 x 15 mm Petri dish.
    1. Place a 75 x 50 glass microscope slide into a 100 x 15 mm Petri dish at an angle. Pour approximately 50 ml of 2% agarose melted in *Hydra* medium[24] into the Petri dish. Alternatively, pour the agarose into the dish first and float a “Microinjection Fish Mold” on top.
    2. After the agarose has solidified, remove the glass slide or the mold. If a glass slide was used, cut away excess agarose with a razor blade to form a wall. Pour *Hydra* medium into the dish and use it immediately or store it at 4°C until needed. Note: Injection dishes can be reused if stored at 4°C between uses.
3. Pull needles from borosilicate glass capillaries with a filament on the micropipette puller using the following conditions: heat 525, pull 75, velocity 100, time 50. Store the needles by pressing them into a narrow strip of clay in a Petri dish such that they are held parallel to the bottom of the dish.
4. To prepare the injection solution, combine 3 µl of 2.5 mg/ml plasmid DNA solution with 2 µl of 10% phenol red. Vortex the solution briefly and then spin it for 10 min at maximum speed in a microcentrifuge to remove any particles that might clog the needle.
5. Under a dissecting microscope, clip the needle a few millimeters from the tip with forceps to create a small opening. Note: There is some flexibility in the appropriate size of the opening because the pressure can be adjusted in step 3.3 to accommodate this variation.
6. Place the Petri dish so that it is holding the needle in a vertical position with the tip down. Load the needle with approximately 0.5 µl of injection solution by pipetting the liquid into the back end of the needle. Allow capillary action to pull the solution into the tip of the needle.

2. Preparation of Embryos for Injection

1. On a daily basis scan the *Hydra* AEP strain culture for polyps producing eggs. Collect these polyps and set them aside in a separate culture dish. Observe these polyps every few hours during the day to monitor the progress of egg formation. When eggs break through the ectoderm and sit on a ring of retracted ectodermal cells they are ready for injection[25]. Place these polyps in a dish with several *Hydra* that have testes for at least 1 hr before injection to allow for fertilization. *Hydra* males will release sperm without any special manipulation.
2. If females are found in the AEP colony with fully-formed eggs, which are approximately 400 µm in diameter[25], or 1- to 8-cell stage embryos, also collect these for injection. Immediately inject embryos that have started cleaving. Note: It is not possible to tell the difference between fully-formed eggs and single-celled zygotes, thus these should be incubated with males prior to injection.
3. Before injection, remove most of the parental tissue above and below the embryo using a scalpel with a #15 blade. Leave only the embryo with a small piece of body column attached, to be used to manipulate the embryo with forceps if necessary. Note: The tissue that is dissected away from the embryo will regenerate and should be saved to ensure that females remain in the *Hydra* colony.
4. Using a Pasteur pipette, move embryos to the injection dish, arranging them parallel to the wall of the agarose trough.

3. Microinjection of Plasmid DNA

1. Mount the microinjector on a magnetic stand that sits on an iron plate. Mount the injection holder, which is the part of the microinjector that holds the needle, on a joystick micromanipulator. Mount the joystick manipulator to the iron plate, with a magnetic stand. Place this entire set-up on the right side of a dissecting microscope and position the injection holder so that it is visible in the observation field.
2. Place the injection dish with embryos under the dissecting microscope, oriented such that the vertical wall of the agarose trough is to the left. Insert the needle into the injection holder and lower the tip of the needle into the *Hydra* medium in the dish.
3. Slowly turn the knob of the syringe in the clockwise direction until mineral oil fills the top of the needle and a steady stream of injection solution is observed exiting the needle into the *Hydra* medium. If the stream is too strong, lower the pressure by turning the knob counterclockwise. Practice this step to routinely obtain a suitable pressure, which depends on the size of the needle opening (*i.e.,* how the needle was clipped in step 1.5). Note: If the stream is too strong, the embryo will not survive the injection.
4. While viewing through the dissecting microscope, move the injection dish so that the first embryo is at the center of the field. Move the needle so that it is touching that embryo. Use the micromanipulator to pierce the embryo with the needle. Note: The vertical wall of the agarose trough will keep the embryo from being pushed aside by the needle.
5. Allow the injection solution to flow into the embryo 1 or 2 sec and then remove the needle quickly. If the embryo has more than one cell, inject each cell individually. Use forceps to reorient the embryo to align the next cell with the tip of the needle.
6. After the first embryo is injected, move the dish so that the next embryo is in the injection position and repeat the procedure; continue until all embryos have been injected.

4. Culturing and Hatching of Embryos

1. Move all of the injected embryos into a dish with a few *Hydra* that have testes (this is to ensure that all embryos are fertilized). Incubate the embryos in an 18 °C incubator while they continue embryogenesis.
2. Once the cuticle stage is reached, move each injected embryo to an individual well of a 24-well plate filled with *Hydra* medium.
3. Incubate the injected embryos for 2 weeks in the dark at 18 °C.
4. After the 2 week incubation period, check each injected embryo under the dissecting microscope. Note any cases in which the cuticle-stage embryo has detached from the small piece of parental tissue and the parental tissue has regenerated. Remove this regenerated polyp immediately so that it is not mistaken for a new hatchling.
5. Move the dish of injected embryos under an aquarium light at RT. Check the plates each day and collect hatchlings. New hatchlings will be white because the typical pink color of *Hydra* polyps comes from the *Artemia* they eat.
6. Observe the hatchlings under a fluorescence microscope to detect transgene expression.

Representative Results

Establishing transgenic *Hydra* lines

Feed transgenic hatchlings every 2-3 days with *Artemia nauplii*. Hatchlings sometimes do not eat for a day or two after hatching. Some new hatchlings will never eat, and thus will not survive. If the hatchling is transgenic in either the ectodermal (**Figure 1A**) or endodermal epithelial tissue, allow the animal to bud and collect the buds that have the most transgenic tissue (**Figure 1B, C**). Continue to do this with the new buds until a transgenic line is established with uniform expression of the transgene in either the ectodermal (**Figure 1D**) or endodermal epithelial lineage. Simultaneously, a second line can be established that does not contain the transgene but is otherwise genetically identical (**Figure 1B**). This line serves as a negative control for future experiments. If the transgenic tissue does not move into a bud, it is sometimes possible to cut the animal and allow it to regenerate such that the transgenic tissue will be in the new budding zone. However, if the transgenic tissue is too close to the extremities it will likely be lost as it is displaced during the normal growth of the animal. Often there is nothing that can be done in this case. In our hands, if the transgene is neutral (*i.e.,* has no impact on biological function) we are able to establish a uniform epithelial line from approximately 30% of *Hydra* that hatch with epithelial transgenic tissue. The remaining 70% either die or the transgenic tissue is lost. An endodermal line is approximately 2-3 times as common as an ectodermal line. If a transgene is being used that disrupts biological function, this may have an impact on the feasibility of establishing a uniform line. In such cases, inducible promoters will be essential additions to the toolkit.

If the hatchling is transgenic in the interstitial lineage, allow the animal to bud and collect the hatchlings with an increasing number of transgenic cells in the interstitial lineage. Be aware that sometimes it is not immediately clear that an animal is transgenic in the interstitial lineage, especially if it is also transgenic in an epithelial lineage. It is highly unlikely that a line completely transgenic in the interstitial lineage will be established simply by budding. If it is required that the interstitial lineage be fully transgenic, this can be accomplished by cloning in aggregates from interstitial stem cell-depleted animals such that the entire lineage is established from a single transgenic stem cell[7].

In cases where a tissue- or cell type-specific promoter is used to drive expression of a fluorescent protein, transgenic animals may not be evident initially. For example if a promoter that is active only in the tentacles is used and the initial patch of transgenic tissue is in the body column, no fluorescent cells will be seen in the hatchling. So all hatchlings need to be propagated to allow transgenic tissue to be displaced into the tentacles and become evident.

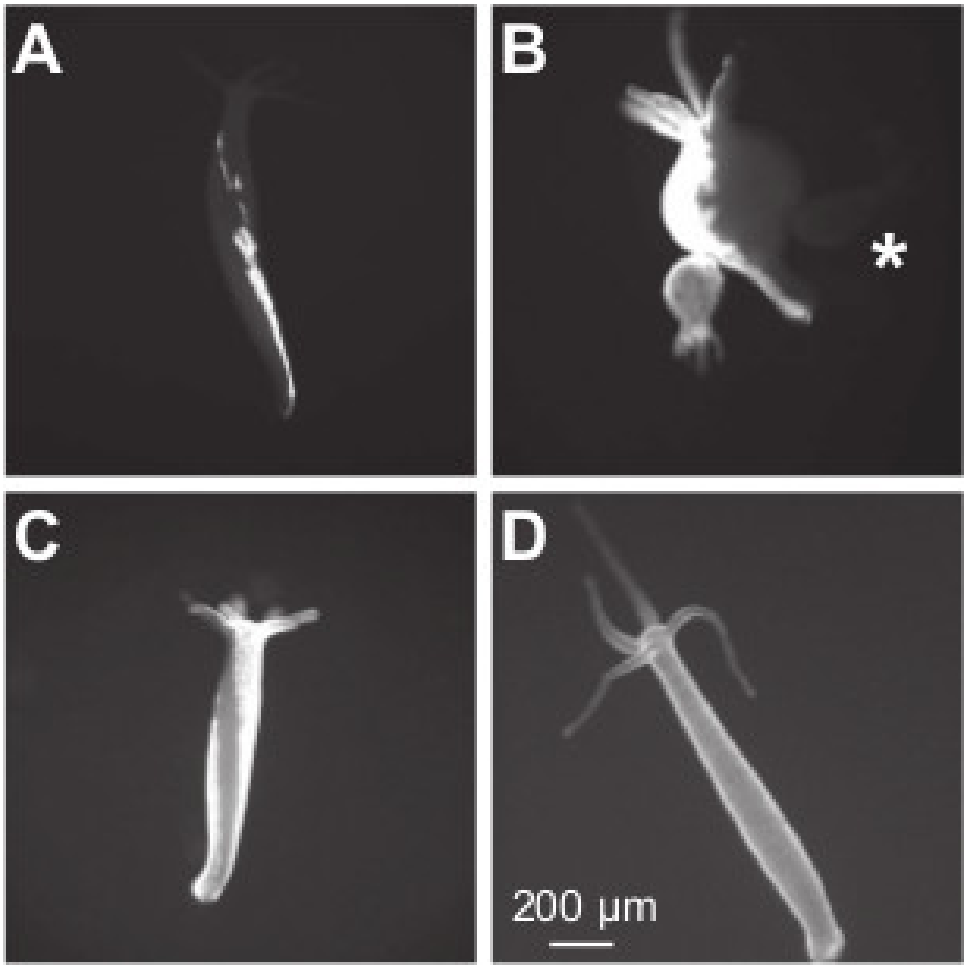

Figure 1. Establishing a transgenic line with uniform ectodermal epithelial expression of DsRed2. (A) A hatchling with chimeric expression of a DsRed2 transgene under control of an actin promoter in a patch of ectodermal epithelial cells. **(B)** A first-generation bud from the hatchling in panel A is now producing two new buds. The bud labeled with an asterisk has no transgenic tissue and was used as the founding animal for a control line that is genetically identical to the transgenic line, except for the presence of the transgene. **(C)** A second-generation bud produced from the *Hydra* in panel B. **(D)** An example of a polyp from the transgenic line that was established with DsRed2 expression throughout the ectoderm. Please click here to view a larger version of this figure.

Discussion

Hydra routinely reproduces asexually, but requires environmental stimuli to begin producing gametes. These stimuli are not well-defined for most *Hydra* species and may differ from strain to strain. A significant hurdle to the production of transgenic *Hydra* is obtaining embryos on a regular basis because it can be difficult in a laboratory setting to induce *Hydra* to become sexual. The AEP strain[25], however, produces gametes readily in the laboratory and this is the only strain that has been used so far for making transgenic lines. The most common method for inducing gamete production is by diet manipulation. As previously described, the animals should be fed daily for three weeks, starved for 5 days, and then fed twice a week, during which time gametes will be produced[1]. It has also been our experience that culturing AEP *Hydra* on vertical plates in an aquarium[26], perhaps simulating a more natural environment, leads to egg production even when animals are fed at regular intervals. In addition, lines obtained from AEP self-crosses sometimes produce gametes more regularly than the parent strain. Thus establishing an F1 line of AEP is another possible method for obtaining a more reliable source of embryos.

The percentage of embryos that survive injection and develop to the cuticle stage depends on the health of the embryos, the amount of solution injected, and the amount of damage done by the injection. With the constant flow injection set-up described in this protocol, it is difficult to control the amount of solution that is injected into the embryo. In our hands, approximately half of the embryos injected as described here successfully complete embryogenesis and form a cuticle. Of these, 50-75% hatch and of those that hatch, approximately 50% will have at least some transgenic tissue. Therefore, 10-20% of the initially injected embryos yield an F1 *Hydra* with transgenic tissue. The amount of solution injected can be precisely controlled with more expensive equipment such as the IM-300 microinjector. The original transgenic lines were made with equipment from Eppendorf, which allows a great deal of precision in manipulating and injecting the embryo. While this level of accuracy may give a higher survival rate of injected embryos, this expense is not necessary for routine establishment of transgenic lines.

The integration of the transgene is random and could potentially occur in multiple locations in the genome or in a tandem array in a single location. Based on Southern blot analysis, the number of integrations was estimated at five in one epithelial transgenic line created previously[1]. In an interstitial lineage transgenic line in which the transgene undergoes germline transmission, it has been demonstrated by genome

sequencing that only a single copy of the transgene was integrated (C. E. Dana and R. E. Steele, unpublished observation). However, aside from these two examples, there is no information available regarding transgene integration sites and copy number. Since it is possible that a transgene could interrupt gene function by insertional mutagenesis, more than one transgenic line should be made when analyzing phenotypes from, for example, RNAi or overexpression constructs. It is also important to note that transgene expression is constitutive when driven by the commonly used actin gene promoter, and thus transgenes that lead to serious or lethal phenotypes when expressed constitutively will not be maintained. For the future, it will be important to develop an inducible system of transgene expression to circumvent this problem.

Disclosures

The authors have nothing to disclose

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