FINAL SCRIPT: APPROVED FOR FILMING

Submission ID #: 69267
Scriptwriter Name: Sulakshana Karkala
Project Page Link: https://review.jove.com/account/file-uploader?src=21126483

Title: The Golden Apple Snail *Pomacea canaliculata*: From Zygotes to Stable Mutant Lines

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Author Questionnaire

1. **Microscopy**: Does your protocol require the use of a dissecting or stereomicroscope for performing a complex dissection, microinjection technique, or something similar? **Yes** all done
2. **Software:** Does the part of your protocol being filmed include step-by-step descriptions of software usage? **No**
3. **Filming location:** Will the filming need to take place in multiple locations? **Yes**

   If **Yes**, how far apart are the locations? One floor with accessible elevator
4. **Testimonials (optional):** Would you be open to filming two short testimonial statements **live during your JoVE shoot**? These will **not appear in your JoVE video** but may be used in JoVE's promotional materials. **Yes**

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Current Protocol Length
Number of Steps: 29
Number of Shots: 58

Introduction

Videographer: Obtain headshots for all authors available at the filming location.

INTRODUCTION:

~~What is the scope of your research? What questions are you trying to answer?~~

1.1. **Alice Accorsi:** The scope of this work is to share the methods we developed to generate apple snail stable mutant lines to study gene function.

1.1.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera.

~~What are the current experimental challenges?~~

1.2. **Alice Accorsi:** The next experimental challenge is to generate transgenic apple snails, through stable DNA insertion in their genome, allowing for lineage tracing experiments.

1.2.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera.

CONCLUSION:

~~What research gap are you addressing with your protocol~~?

1.3. **Alice Accorsi:** Through the adoption of these protocols, we can dissect the molecular pathways involved in development and regeneration of complex sensory organs in apple snails.

1.3.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera.

~~How will your findings advance research in your field?~~

1.4. **Alice Accorsi:** Through our findings we established apple snails as a novel system to answer questions about development, regeneration, evolution and molluscan physiology.

1.4.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera.

~~What new scientific questions have your results paved the way for?~~

1.5. **Alice Accorsi:** Our results highlight the conserved roles of genes between apple snails and vertebrate animals, opening the door to questions about visual system regeneration and evolution.

1.5.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera.

Videographer: Obtain headshots for all authors available at the filming location.

Testimonial Questions (OPTIONAL):

Videographer: Please capture all testimonial shots in a wide-angle format with sufficient headspace, as the final videos will be rendered in a 1:1 aspect ratio. Testimonial statements will be presented live by the authors, sharing their spontaneous perspectives.

How do you think publishing with JoVE will enhance the visibility and impact of your research?

1.6. **Asmita Gattamraju, Junior Specialist in the Accorsi Lab, UC Davis** : (authors will present their testimonial statements live)

1.6.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera.

~~Can you share a specific success story or benefit you've experienced—or expect to experience—after using or publishing with JoVE? (This could include increased collaborations, citations, funding opportunities, streamlined lab procedures, reduced training time, cost savings in the lab, or improved lab productivity.)~~

~~1.7. **Alice Accorsi, Assistant Professor, UC Davis**: (authors will present their testimonial statements live)~~

~~1.7.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera.~~

NOTE: Authors did not film 1.7

Ethics Title Card

This research has been approved by the University of California, Davis, the USDA/APHIS (permit # 526-24-304-32436) and the CDFW (permit # D-0043327935-6)

Protocol

2. Maintenance and Reproductive Handling of Apple Snails

Demonstrator: Asmita Gattamraju ~~and Alice Accorsi~~

2.1. To begin, replace daily 5 to 10 percent of the water in each tank of a recirculation system housing apple snails **[1].** Siphon the bottom of the tanks to remove debris **[2]**.

2.1.1. WIDE: Talent replacing a portion of water from the snail tank.

2.1.2. Shot of the debris in the tank bottom being siphoned.

2.2. Feed the snails three times per week with organic lettuce **[1]**. Collect the egg clutches from the tanks every day in Petri dishes **[2]**.

2.2.1. Talent placing pieces of organic lettuce into the tank.

2.2.2. Talent collecting egg clutches from the tank wall and transferring them into Petri dishes.

2.3. Transfer freshly laid clutches from the tank walls to a dry environment for optimal development **[1]**. Observe the egg clutches as they transition in color from bright pink to pale gray in about 12 days **[2]**.

2.3.1. Talent moving egg clutches from tank wall to a dry dish.

2.3.2. Shot of Petri dishes showing differently colored egg clutches.

2.4. Once the clutches are mostly gray, immerse fingers in system water **[1]** and gently crush them to manually hatch the eggs **[2]**. The outer casing will float while the hatchlings sink **[3]**.

2.4.1. Talent immersing fingers into the system water.

2.4.2. Shot of the gray clutches being crushed with submerged fingers.
AUTHOR'S NOTE: Shots 2.4.1-2.4.2 were shot together

2.4.3. Shot showing hatchlings sinking and casing floating in water.

2.5. For sex determination, place snails aged 1.5 to 2.5 months in ice for 10 minutes to relax their muscles **[1]**. Using a hemostat locking scissor clamp, pull the operculum anteriorly, ventrally, and posteriorly to expose the head of the snail **[2]**.

2.5.1. Talent placing snails on ice.

2.5.2. Talent manipulating the operculum with hemostat clamp to expose the snail head.

2.6. Examine for sex-specific features to determine the sex. The male possesses a small pink

penial bulb, a larger pale orange penis pouch and a large white penis-sheath **[1],** while the female possesses tubular oviduct and rectum **[2]**.

2.6.1. LAB MEDIA: Figure 1 D

2.6.2. LAB MEDIA: Figure 1 E

2.7. Return the sexed snails to system water **[1]**. If the mantle detaches from the shell during the operation, stop and avoid further stretching **[2].**

2.7.1. Talent placing the snail back into system water.

2.7.2. Close-up shot of detached mantle and talent halting manipulation.

3. Preparation and Testing of Perivitelline Fluid Extract (ePVF) in Apple Snail Embryos

3.1. To prepare and test the perivitelline fluid extract or ePVF *(E-P-V-F)*, first clean the workspace and tweezers with 70 percent ethanol **[1]**.

3.1.1. Talent wiping the workspace and tweezers with an ethanol-soaked cloth.

3.2. Transfer clutches aged 1 to 2 days post fertilization into a 50-milliliter tube containing 40 milliliters of 0.8 percent sodium hypochlorite for 3 minutes **[1]**. Then rinse the clutches twice with autoclaved distilled water **[2]**.

3.2.1. Talent placing 1 dpf clutches into sodium hypochlorite solution.

3.2.2. Talent rinsing the clutches with autoclaved distilled water.

3.3. Incubate the clutches in fresh autoclaved distilled water for 2 minutes to remove residual sodium hypochlorite **[1]**. Lay the tube horizontally during the incubations **[2]**.

3.3.1. Talent adding fresh autoclaved distilled water to the tube with the clutches.

3.3.2. Shot of the 50 milliliter tube laid horizontally.

3.4. Remove the water from the 50-milliliter tube **[1]** and transfer the dry clutches to a Petri dish using forceps **[2-TXT]**. Using tweezers, crush all the capsules in the clutch **[3]**.

3.4.1. Talent pouring out the water from the tube.

3.4.2. Talent using forceps to move clutches. **TXT: Keep each clutch seperate**

3.4.3. Talent crushing capsules with tweezers.

3.5. Use curved forceps to transfer the crushed material into 2 milliliter centrifuge tubes **[1]**. Then centrifuge the tubes at 21,000 *g* for 40 minutes at 4 degrees Celsius **[2]**.

3.5.1. Talent transferring material to 2 milliliter centrifuge tubes.

3.5.2. Talent placing tubes into centrifuge.

3.6. Observe three distinct layers after centrifugation **[1]**. Transfer only the top clear pink layer into a fresh 1.5 milliliter tube without combining extracts from different clutches

[2-TXT].

3.6.1. LAB MEDIA: Figure 3 C ~~Shot of tube showing separated layers.~~
NOTE: Authors prefer to use figure for this shot

3.6.2. Talent pipetting top clear layer into fresh tube. **TXT: Store at - 20 °C until needed**

3.7. For collecting wild type embryos, add 40 milliliters of Pc-EM *(P-C-E-M)* to a Petri dish containing sterile 2-day old clutches **[1-TXT]** and use tweezers to mix the broken capsules to dislodge the embryos from the PVF **[2]**.

3.7.1. Talent adding 40 mL PC-EM to a Petri dish.
TXT: Pc-EM:
6 mM KCl
6.6 mM $CaCl_2$
3.3 mM $MgCl_2$
1 M HEPES
33.4 mM NaCl in dH_2O
Filter the entire solution

3.7.2. Shot of broken capsules being mixed with tweezers.

3.8. Under transmitted light on a stereomicroscope, collect the released embryos **[1]**. Then transfer the embryos into a clean Petri dish or 4-well plate pre-filled with 5 percent fetal bovine serum in Pc-EM **[2-TXT]**.

3.8.1. SCOPE: 69267_SCOPE_3.8.1-Collect-embryos-under-stereomicroscope.mp4 00:08-00:18 .

3.8.2. Talent transferring embryos to clean dish with FBS solution. **TXT: Swirl gently and repeat wash 2x**
AND
SCOPE: 69267_SCOPE_3.8.2a-Transfer-embryos-in-FBS-solution.mp4 00:07-00:14
Video Editor: Please play both shots side by side in a split screen

3.9. To test the ePVF, place the lid of a 35-millimeter Petri dish facing up inside a 60-millimeter Petri dish **[1]**. Pipette two 60-microliter drops, each 3 millimeters in diameter from each ePVF aliquot into the lid **[2]**.

3.9.1. Talent placing smaller Petri dish lid inside a larger one.

3.9.2. Talent pipetting equal-sized drops of ePVF into the lid.

3.10. With a P20 *(P-Twenty)* pipette, transfer 3 to 4 healthy embryos into each ePVF drop **[1]**. Then pipette 3 milliliters of paraffin oil to cover the drops, starting between the adjacent drops and then fully covering them **[2-TXT]**.

3.10.1. SCOPE: 69267_SCOPE_3.10.1a-Pick-up-embryos-for-culturing.mp4

00:04-00:07 **AND**
69267_SCOPE_3.10.1b-Transfer-embryos-in-culture.mp4 00:06-00:15 .

3.10.2. Talent gently pouring 3 mL paraffin oil to coat the drops. **TXT: Incubate: 27 °C, in the dark, 8 - 10 days**

3.11. Check daily for embryo development, formation of air bubbles over ePVF drops, and signs of contamination **[1-TXT]**.

3.11.1. SCOPE: 69267_SCOPE_3.11.1-Remove-air-bubbles.mp4 00:05-00:20 . **TXT: Remove bubbles with P20 pipette**

4. Microinjection, Embryo Collection, and Genetic Analysis in Pomacea canaliculata

4.1. For microinjection of the zygotes, first collect freshly laid clutches into a 50-milliliter tube containing freshly prepared L-cysteine solution **[1-TXT].**

4.1.1. Shot of the clutches being transferred into a 50 mL tube with L-cysteine solution. **TXT: L-cysteine: 15 g/L L-cysteine in dH_2O, pH 7.5 with NaOH**

4.2. Pour 20 milliliters of the solution and the capsules into a 100-millimeter Petri dish **[1].** Then add 20 milliliters of Pc-EM to the dish **[2].** With a pair of tweezers and a stereomicroscope set to 10X, open the capsules one by one, tearing the external membrane **[3].**

4.2.1. Talent pipetting 20 mL of the L-cysteine solution and the capsules into a 100 mm Petri dish.

4.2.2. Shot of 20 mL Pc-EM being pipetted into the dish.

4.2.3. SCOPE: 69267_SCOPE_4.2.3-Torn-open-capsules.mp4 00:00-00:16.

4.3. When the PVF is exposed to the L-cysteine solution, gently swirl the contents of the dish **[1].** After letting it settle down for 2 minutes, use a P20 pipette to collect the released embryos under a stereomicroscope **[2-TXT].**

4.3.1. Talent gently swirling the contents of the dish.

4.3.2. SCOPE: 69267_SCOPE_4.3.2-Torn-open-capsules.mp4.mp4 00:00-00:21 . **TXT: Perform multiple rounds of collection, alternating with PVF mixing to gently release embryos**

4.4. Now, using an inverted microscope equipped with a 20X Differential Interphase Contrast objective, micromanipulators, microinjector, pinpoint cell penetrator and a cold plate, open the needle breaking its tip against the holding pipette **[1]**.

4.4.1. SCOPE: 69267_SCOPE_4.4.1-Open-tip-of-needle.mp4 00:00-00:30.

4.5. Place one of the prepared embryo culture dishes at room temperature and the other at 4 degrees Celsius to slow development **[1]**. Add a large drop of 5 percent fetal bovine serum in Pc-EM onto depression slides **[2]**. Then move 10 to 20 embryos from the room

temperature dish to the depression slide **[3]**.

4.5.1. Talent positioning two dishes at different temperatures.

4.5.2. Talent pipetting FBS drop onto depression slide.
Added shot: 4.5.3 : Talent moving embryos into the depression slide
NOTE: Please play this shot before the 4.5.3 SCOPE shot as per the author's request

4.5.3. SCOPE:69267_SCOPE_4.5.3-Trasfer-embryos-to-the-slide.mp4 00:15-00:24.

4.6. After placing embryos on the slide, move the leftover embryo dish to 4 degrees Celsius **[1-TXT].** Secure one embryo at a time using the holding pipette, aligning the needle and embryo membrane in the same focal plane **[2]**.

4.6.1. Talent placing the leftover embryos at 4 °C.
TXT: Simultaneously move cold dish to RT

4.6.2. SCOPE: 69267_SCOPE_4.6.2-Align-needle-and-embryo.mp4 00:07-00:26.

4.7. Inject the embryos by alternating an injection and a pulse until the needle enters the embryo **[1-TXT]**.

4.7.1. SCOPE: 69267_SCOPE_4.7.1-and-4.8.2-Inejct-embryo.mp4 00:01-00:10, 00:19-00:24 . **TXT: Watch for membrane stretching**
AND
Shot of the alternating food pedals being pushed
Videographer: Please capture this shot
Video Editor:Please show this shot as a split screen with the SCOPE

4.8. If the needle is too dull, the embryo may die after penetration. If too sharp, the embryo may lyse on contact **[1]**. Withdraw the needle quickly and firmly without stalling inside the embryo **[2]**.

4.8.1. SCOPE: 69267_SCOPE_4.8.1-Lysed-embryo.mp4 00:00-00:17.

4.8.2. SCOPE: 69267_SCOPE_4.7.1-and-4.8.2-Inejct-embryo.mp4 00:11-00:18

4.9. Before the end of the day, culture *ex ovo* the embryos that are both dividing and fluorescent using high-quality ePVF **[1]**. After approximately 11 to 13 days, when embryos are too large for the drops, pipette them out of the culture **[2]**.

4.9.1. LAB MEDIA: 69267_SCOPE_4.9.1-Fluorescent-embryos.png

4.9.2. Talent removing large embryos from culture and place them in Pc-Hatchling media.
AND
SCOPE: 69267_SCOPE_4.9.2a-11-13-days-embryos-in-culture.mp4.mp4 00:00-00:04
69267_SCOPE_4.9.2b-Remove-large-embryos-from-culture.mp4 00:04-00:08

69267_SCOPE_4.9.2c-Place-large-embryos-in-media.mp4 00:03-00:11

Video Editor: Please play the SCOPE files side by side in a split screen with the regular shot. Also please play the scope files one after the other

4.10. After 3 to 5 days, feed the hatchlings small pieces of lettuce **[1]**.

4.10.1. Talent adding lettuce to the hatchlings.

4.11. Once F1 juveniles reach at least 6 millimeters in diameter, wait for tentacle exposure, then cut the tip of one tentacle with micro-scissors **[1]**. Transfer the snail to a 6-well plate and the tentacle in 50 microliters of Lysis Buffer in a PCR tube before sample processing **[2-TXT]**.

4.11.1. Shot of tentacles being cut when exposed.

4.11.2. Talent transferring snail into a 6-well plate and tentacle being pipetted into a PCR tube with 50 µL Lysis buffer. **TXT: Process sample at 55 °C for 3 h and at 98 °C for 10 min and run PCR or qPCR**

Results

5. Results

5.1. Formation of air bubbles on top of the ePVF droplets was observed during the first 48 hours of ex ovo culture **[1]**. By 9 days post fertilization, embryos displayed normal development with shell, foot, eyes, and tentacles, and were seen actively moving in the droplet **[2]**.

5.1.1. LAB MEDIA: Figure 5C and D. *Video editor: Highlight the single round air bubble labelled "air bubble".*

5.1.2. LAB MEDIA: Figure 5A *Video editor: Highlight the embryo labelled "good development" showing shell, foot, eyes, and tentacles in A*

5.2. Contaminants and debris were observed grouped into one mass by the embryos at 9 days post fertilization, and contamination remained under control at this stage **[1].**

5.2.1. LAB MEDIA: Figure 5B. *Video editor: Highlight the clumped dark material labelled "contamination under control".*

5.3. Partial or complete drying of the ePVF showed to impair proper embryo development **[1]**, particularly when the paraffin oil overlay was improperly applied or air bubbles were not removed **[2]**.

5.3.1. LAB MEDIA: Figure 5E. *Video editor: Highlight the droplet labelled "partially dried".*

5.3.2. LAB MEDIA: Figure 5E. *Video editor: Highlight the droplet labelled "fully dried".*

5.4. Contaminated droplets showed darker, irregular areas **[1]**, and embryos in these droplets stopped growing and eventually died if not transferred promptly **[2].**

5.4.1. LAB MEDIA: Figure 5F. *Video editor: Highlight the large dark spot labelled "contamination" in contrast with the visible embryo.*

5.4.2. LAB MEDIA: Figure 5G. *Video editor: Highlight the widespread, uneven dark area labelled "contamination".*

5.5. During microinjection, ideal outcomes were achieved when the needle penetrated the membrane without resistance, and embryos maintained a spherical morphology with no leakage **[1]**. Embryos that retained a spherical morphology had a higher survival rate **[2]**, whereas damaged embryos showed deformation or loss of cytoplasmic content **[3].**

5.5.1. LAB MEDIA: Figure 6J and K

5.5.2. LAB MEDIA: Figure 6G. *Video editor: Highlight the spherical embryo labelled "healthy".*

5.5.3. LAB MEDIA: Figure 6H and I. *Video editor: Highlight the misshaped embryo*

labelled "damaged".

Pronunciation Guide:

 Pomacea
Pronunciation link: https://www.howtopronounce.com/pomacea
IPA: /poʊˈmeɪ.si.ə/
Phonetic Spelling: poh-MAY-see-uh
 canaliculata
Pronunciation link: https://www.howtopronounce.com/canaliculata
IPA: /ˌkæn.ə.lɪk.jəˈlɑː.tə/
Phonetic Spelling: kan-uh-lik-yuh-LAH-tuh
 Zygote
Pronunciation link: https://www.merriam-webster.com/dictionary/zygote
IPA: /ˈzaɪˌgoʊt/
Phonetic Spelling: zye-goht
 Transgenic
Pronunciation link: https://www.merriam-webster.com/dictionary/transgenic
IPA: /trænzˈdʒɛnɪk/
Phonetic Spelling: tranz-JEN-ik
 Molluscan
Pronunciation link: https://www.merriam-webster.com/dictionary/molluscan
IPA: /məˈlʌs.kən/
Phonetic Spelling: muh-LUSS-kuhn
 Operculum
Pronunciation link: https://www.merriam-webster.com/dictionary/operculum
IPA: /oʊˈpɝː.kjə.ləm/
Phonetic Spelling: oh-PUR-kyuh-luhm
 Hemostat
Pronunciation link: https://www.merriam-webster.com/dictionary/hemostat
IPA: /ˈhiː.məˌstæt/
Phonetic Spelling: HEE-muh-stat
 Perivitelline
Pronunciation link: https://www.howtopronounce.com/perivitelline
IPA: /ˌpɛr.ɪ.vɪˈtɛl.iːn/
Phonetic Spelling: pair-ih-vih-TEL-een
 Hypochlorite
Pronunciation link: https://www.merriam-webster.com/dictionary/hypochlorite
IPA: /ˌhaɪ.poʊˈklɔː.raɪt/
Phonetic Spelling: high-poh-KLOR-ite
 Autoclaved
Pronunciation link: https://www.merriam-webster.com/dictionary/autoclave
IPA: /ˈɔː.təˌkleɪvd/
Phonetic Spelling: AW-tuh-klayvd

 Centrifuge
Pronunciation link: https://www.merriam-webster.com/dictionary/centrifuge
IPA: /ˈsɛn.trəˌfjuːdʒ/
Phonetic Spelling: SEN-truh-fyooj
 Stereomicroscope
Pronunciation link: https://www.merriam-webster.com/dictionary/stereomicroscope
IPA: /ˌstɛr.i.oʊˈmaɪ.krəˌskoʊp/
Phonetic Spelling: stair-ee-oh-MY-kruh-skohp
 Paraffin
Pronunciation link: https://www.merriam-webster.com/dictionary/paraffin
IPA: /ˈpær.ə.fɪn/
Phonetic Spelling: PAIR-uh-fin
 Microinjection
Pronunciation link: https://www.merriam-webster.com/dictionary/microinjection
IPA: /ˌmaɪ.kroʊ.ɪnˈdʒɛk.ʃən/
Phonetic Spelling: my-kroh-in-JEK-shuhn
 Micromanipulator
Pronunciation link: https://www.merriam-webster.com/dictionary/micromanipulator
IPA: /ˌmaɪ.kroʊ.məˈnɪp.jəˌleɪ.tɚ/
Phonetic Spelling: my-kroh-muh-NIP-yuh-lay-ter
 Differential Interference Contrast
Pronunciation link: No confirmed link found
IPA: /ˌdɪf.əˈrɛn.ʃəl ˌɪn.tɚˈfɪr.əns ˈkɑːn.træst/
Phonetic Spelling: dif-uh-REN-shuhl in-ter-FEER-uhns KON-trast
 Fluorescent
Pronunciation link: https://www.merriam-webster.com/dictionary/fluorescent
IPA: /flʊˈrɛs.ənt/
Phonetic Spelling: floor-ESS-uhnt
 Ex ovo
Pronunciation link: No confirmed link found
IPA: /ˌɛks ˈoʊ.voʊ/
Phonetic Spelling: eks-OH-voh
 Cytoplasmic
Pronunciation link: https://www.merriam-webster.com/dictionary/cytoplasmic
IPA: /ˌsaɪ.təˈplæz.mɪk/
Phonetic Spelling: sigh-tuh-PLAZ-mik
 Canaliculate
Pronunciation link: https://www.merriam-webster.com/dictionary/canaliculate
IPA: /kəˈnæl.ɪ.kjə.lət/
Phonetic Spelling: kuh-NAL-ih-kyuh-luht